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This book is dedicated to Khayot Ulmasov, Ph.D., for his pioneer field studies of heat shock response in desert animals.

Preface

The authors are well suited to write such a book, having studied large natural populations at the organismal and molecular levels of the heat shock response. Even during the heyday of the molecular biology of heat shock genes and proteins using model organisms, our authors recognized the value of comparative analyses of evolutionarily closely related and more distant wild species from thermally different environments. In the Introduction, they included Susan Lindquist's prescient statement from 1986, "in order to master the control of thermoresistance of a living organism, it is necessary to understand how nature did it in the course of evolution." Why are model laboratory organisms insufficient for this purpose? Model organisms are inbred genetically to the point where one can truly speak of the genome of a particular inbred stain. Such a unique set of genetic alleles may be a minority genome or not exist at all in a natural population of the organism. Given what we know now about allelic interactions and their effects on the control of gene expression and therefore the physiological diversity of wild populations, this matters a great deal in trying to understand a state such as acquired thermotolerance or stress conditioning as it is sometimes called. Our knowledge of organismal thermoresistance based on model laboratory strains is certain to be incomplete at best and misleading at worst. Understanding the deployment of inducible defenses in natural populations will be key to evaluating the effects of deepening environmental stress such as the threat of global warming to natural populations.

The Chapters begin with a historical chapter on the discovery of the heat shock response and up-to-date descriptions, classifications, and nomenclature of the major groups of heat shock genes and proteins. The molecular functions of heat shock proteins are discussed in Chap. 2. The color illustrations in this chapter of molecular pathways and interactions are particularly clear. Investigators newly trained in the methods of modern molecular biology and genetics began an intensive study of heat shock genes and their encoded proteins shortly after the discovery of these proteins by Alfred Tissières and coworkers in 1974. It is important to note that just as field studies of wild populations will have an expanding role in future studies of their integrated responses to environmental stress, model organisms with useful genetic systems and accessible biochemistry have been invaluable to the dramatic progress

made in understanding the basic science of the heat shock genes and proteins along with the rise of molecular chaperones. The regulation of heat shock gene expression is covered in Chap. 3. This is another area in which progress has been dramatic with the discovery of additional members of the HSF family, new accessory factors, and interactions among a variety of cellular pathways and HSFs to form integrated response networks. In addition, important post-transcriptional regulatory points are described. With Chap. 4, readers will move on to the environmental studies beginning with a summary of the roles of heat shock proteins in adaptation to variable and extreme environments. The authors go beyond heat shock proteins to bring up additional molecules that provide protection such as thermoresistance and the variety of mechanisms used by different organisms for cellular defense. Here, they begin a presentation of their comprehensive studies of lizard species from the deserts of Turkmenistan and from temperate climates. Most if not all of the major comparative studies of organisms in a variety of different environments including parasites and hibernating animals have been included. A very helpful summary of the various adaptations in different organisms is presented at the end of this chapter. Different trends in the evolution of heat shock genes are discussed in Chap. 5. These differences appear to provide optimum responses to changing environments. Chap. 6 covers the role of mobile genetic elements in the evolution and function of heat shock protein systems. The authors have many years of experience studying Drosophila mobile genetic elements. Not surprisingly, ancient genes like the heat shock genes have collected over evolutionary time their fair share of mobile genetic elements along with interactions with other ancient defense mechanisms such as RNA interference. Near the end of the book (Chap. 7), the authors tie together some loose ends by describing the details of fine-tuning of promoters of heat shock genes that occurred during the divergence of species and taxa along with their adaptation to rapidly changing thermal conditions. Heat shock regulatory regions have been the darlings of evolutionary tinkering in contrast to the well-conserved coding sequences. But even these vary considerably in their rates of evolutionary change even within the same organism. In the final chapter, the authors survey mutational approaches to studying the function of individual heat shock genes and proteins in thermal adaptation and stress resistance mostly in yeast and Drosophila.

An important purpose behind the writing of this book according to the authors in their own words is "to give credit to our deceased co-workers and dear friends Dr. Khayot Ulmasov and Dr. Vladimir Lyashko who actively participated in the project from the very beginning and whose enthusiasm made possible the collection of various animal species and their analysis in the field under extremely unfavorable conditions of Turkmenistan deserts." Indeed, this book is a fitting tribute.

It is recommended for researchers at the level of postdoctoral fellows and graduate students in the fields of ecology, biogeography, evolution, molecular biology, and genetics.

Storrs, CT Larry Hightower

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List of Abbreviations and Basic Nomenclature

a. a.	Amino acids
bps	Base pairs
ER	Endoplasmic reticulum
hnRNPs	Heterogeneous nuclear ribonucleoprotein
HS	Heat shock
HSP	Heat shock proteins
HSR	Heat shock response
kbs	Kilobases
kD	Kilodalton
ME	Mobile element
MYA	Millions years ago
ORF	Open reading frame
RNAP II	RNA polymerase II
TNF	Tumor necrosois factor
UPS	Ubiquitin-proteasome system
UPR	Unfolded protein response
UTR	Untranslated region

Basic Nomenclature of Genes and Proteins (As Examples)

dnaK	Bacterial genes
DnaK	Bacterial proteins
Hsp	Eukaryotic nonmammalian genes
Hsp	Eukaryotic nonmammalian proteins
HSP	Human and other mammalian genes
HSP	Human and other mammalian proteins

Contents

Introduction

After the discovery of heat shock proteins (Hsps) and correspondent genes in *Drosophila* by two independent groups of researchers (Ashburner and Bonnert 1979; Lewis et al. 1975; reviewed by Lindquist 1986) numerous papers appeared describing molecular functions and structure of this highly conserved system in various organisms and the peculiarities of its regulation in model species such as yeast, mice and flies. At that time we initiated large scale experiments with the goal to investigate heat shock response (HSR) in the field at the molecular level studying natural populations of close and distant model (laboratory) and non-model species from thermally contrasting environments (Evgen'ev et al. 1978, 1987; Lyashko et al. 1994; Ulmasov et al. 1988, 1993).

As Suzan Lindquist, justly noted "in order to master the control of thermoresistance of a living organism, it is necessary to understand how the nature did it in the course of evolution" (Lindquist 1986).

The original idea was to monitor HSR in non-model forms that thrive in extreme or xeric conditions for many millions years and compare them with related organisms from moderate climate in an attempt to link biogeography to physiology and adaptation. We were very lucky from the very beginning when compared cell cultures of two moth species and demonstrated that heat-resistant species (silkworm *Bombyx mori*) is able to synthesize Hsps at much higher temperatures in comparison with the species (*Lymantria dispar*) from temperate climatic zone. The observed characteristic differences in threshold of Hsps induction in these thermally contrasting species impelled us to carry out similar comparative studies of HSR in various animals from deserts of Turkmenistan (Middle Asia) and related forms from temperate climatic regions. These broad-scale studies exploring various species of lizards, leishmania, flies, moths, and mammals including humans clearly established that the magnitude, kinetics, threshold and diversity of Hsps expression are correlated with the prevailing levels of stress that species naturally undergo. The analysis of such broad spectrum of organisms from different taxa enabled us to reveal a few features of HSR common to most of the studied species. Thus, we demonstrated that species from high-temperature climates as a rule have higher constitutive levels of Hsp70 (the major heat shock protein) than related species from more moderate

climates. Our field studies were not restricted to the deserts of Turkmenistan. Thus, subsequently we included several species of *Stratiomyidae* family (Diptera) into our analysis. This family comprises species from hot and mineralized volcanic springs as well as highly eurithermal and stenothermal species. Besides, special investigation has been performed to describe HSR in Baikal Lake amphipods strikingly different in terms of their microclimate conditions.

During all these 30 years of investigation we obtained multiple genomic libraries from many organisms including *Drosophila* species, *Stratiomyidae* species, camel etc. Genes responsible for major heat-induced stress protein (Hsp70) were cloned and sequenced from all these libraries. The comparative analysis of the sequenced *Hsp70* genes enabled us to describe peculiarities of their general arrangement in the genomes of species compared including copy number and the structure of regulatory regions. At the next step exploring *in vitro* systems we directly measured the "strength" of the obtained *Hsp* genes promoters and possible role of various transcription factors underlying the observed differences. Finally, we developed *D. melanogaster* transgenic strains containing *Hsps* genes from distant thermoresistant species in an attempt to obtain forms more tolerant to stress.

In the last decade multiple studies appeared implicating Hsps and in particular Hsp70 (the major heat shock protein) in protection against inflammation, various neurodegenerative diseases, aging and cancer. We are well aware of this field of studies and actually carried out experiments with human exogenous Hsp70 which turned out to be a potent therapeutic agent in several models of sepsis and Alzheimer's disease (Bobkova et al. 2014; Kustanova et al. 2006; Rozhkova et al. 2010; Vinokurov et al. 2012). However, in this book we purposely decided not to include our own and similar studies on neuroprotective role of Hsps because they are definitely beyond its scope and, besides, there are excellent reviews covering this topic (e.g. *Heat Shock Proteins and the Brain: Implications for Neurodegenerative Diseases and Neuroprotection*, Editors Asea and Brown 2010).

In fact, it was not easy to restrict ourselves to certain spectrum of heat shock related problems herein because the number of existing and appearing heat shock related publications is immense. Thus, on purpose we included almost nothing about plants and bacteria HSR because these objects deserve special attention and a lot of space. We also apologize for not including numerous related papers in the reference list due to space restrictions.

In fact, when designing this book we used as a guide line the story of our own field and laboratory investigations and besides included into the MS pertinent papers and generally accepted knowledge concerning structure and function of heat shock system in eukaryotic organisms (predominantly animals).

This knowledge includes classification of heat shock proteins and correspondent genes (Chap. 1), their functions (Chap. 2), regulation of Hsps expression (Chap. 3) and possible role of Hsps in adaptation to extreme and rapidly changing environments (Chap. 4). Besides, we summarized various data including our own results concerning different trends in evolution of *Hsp* genes (Chap. 5) and the role of mobile elements in reorganization and modifying of *Hsp* genes functions (Chap. 6), including recent results on complex interaction between Hsps and RNAi systems. These studies enabled us to merge two avenues of research independently developing

in our laboratory for many years, namely, heat shock-related work and investigation of mobile elements in *Drosophila*. In Chap. 7 we described the details of fine tuning of *Hsp* genes promoters activity occuring in the course of species and taxa divergence and adaptation to rapidly changing thermal conditions. Finally, we briefly review a few experimental approaches intended to investigate the participation of individual Hsps in thermal adaptation and modify the stress resistance in various organisms (Chap. 8).

An important purpose of this book is to give credit to our deceased co-workers and dear friends Dr. Khayot Ulmasov and Dr. Vladimir Lyashko who actively participated in the project from the very beginning and whose enthusiasm made possible the collection of various animal species and their analysis in the field under extremely unfavorable conditions of Turkmenistan deserts.

The book is recommended for researchers at the level of postdoctoral fellows and graduate students in the fields of ecology, biogeography, evolution, molecular biology and genetics.

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Chapter 1
The Discovery of Heat Shock Response System and Major Groups of Heat Shock Proteins

Drastic changes in the genes expression pattern in response to heat stress were originally demonstrated in *Drosophila busckii* and *D. melanogaster* (Ritossa 1962, 1963). Initially, it was shown that in the both species a few new large puffs in the salivary gland chromosomes were formed immediately after heat shock treatment. Specifically, in *D. melanogaster* the puffs were observed at several chromosomal loci in all large autosomes (33B, 63B, 64B, 67B, 70A, 87A, 87B, 93D and 95C) (Ritossa 1963). These changes in chromosome morphology can be easily seen under a light microscope due to giant size of polythene chromosomes in the larval salivary glands of *Drosophila* (Fig. 1.1). Only one decade later the main groups of proteins corresponding to the individual heat-induced puffs were identified and called "heat shock proteins" (Hsps) (Ashburner and Bonnert 1979; Lewis et al. 1975; Lewis and Pelham 1985; Tissières et al. 1974).

After the discovery of heat shock proteins in *Drosophila* active studies began into the structure and functions of proteins of this class, which were found in practically all studied species: from *E. coli* to man (Schlesinger et al. 1982). It was demonstrated in numerous studies exploring various organisms that *Hsps* genes and in particular members of *Hsp70* family are exceptionally conservative like many house-keeping genes. For example, the comparison of human, *Drosophila* and *Escherichia coli* Hsp70 amino acid sequences showed that human HSP70 is 73 % identical to *Drosophila* and 47 % identical to orthologous *E. coli* Hsp70 (DnaK). Surprisingly, at the level of nucleotide sequences the human and *Drosophila* genes are 72 % identical while human and *E. coli* genes are 50 % identical, which is more highly conserved than expected given the degeneracy of the genetic code. The lack of accumulated silent nucleotide substitutions enables to speculate that there may be additional information in the nucleotide sequence of the *Hsp70* genes or the corresponding mRNAs that precludes certain level of divergence allowed in the silent codon positions (Hunt and Morimoto 1985).

Expression of the genes encoding some Hsps is maintained in most organisms under normal physiological conditions at a certain level or is altogether absent and is strongly induced in the presence of different stress factors. In addition to heat

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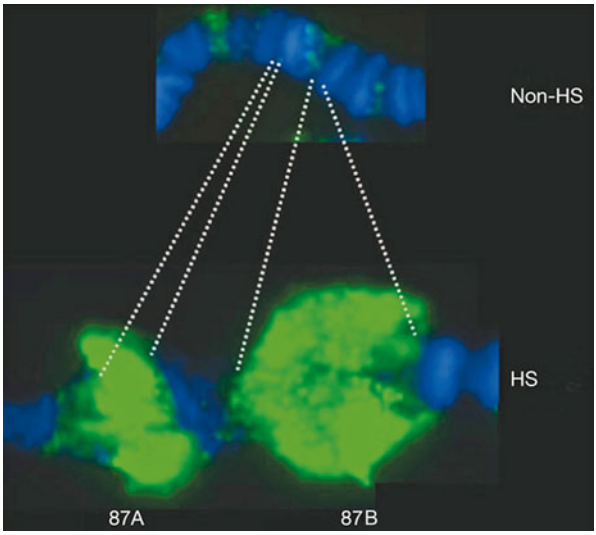

Fig. 1.1 A small segment of *D. melanogaster* fixed chromosome 3R before (*top*) and after (*bottom*) heat shock. Chromosomes are stained for DNA (Hoeschst dye; *blue*) and for RNA Polymerase II (fluorescent labeled antibodies; *green*). 87A and 87B-mark huge puffs containing *Hsp70* genes. *HS* heat shock (From Lis 2007 with permission)

shock, synthesis of HS proteins (Hsps) can be induced by a number of agents, including hypoxia, ultraviolet light, tissue damage, various chemicals including heavy metals, viruses, aging and other forms of stressful stimuli (Hunt and Morimoto 1985; Lindquist 1986; Lozovskaya and Evgen'ev 1984; Hightower 1991; Margulis and Guzhova 2000; Feder and Hofmann 1999). In this regard Hsps may be called "stress proteins". At present, Hsps are classified according to their molecular weights, amino acid sequences and functions and form several distinct groups (Guzhova and Margulis 2006; Hartl and Hayer-Hartl 2002; Lindquist 1986; Lozovskaya and Evgen'ev 1984; Sorensen et al. 2003).

Basing on electrophoretic separation of labeled proteins it has been established that several fractions of proteins with various molecular masses are synthesized in the cells of different organisms after temperature elevation (Feder and Hofmann 1999; Parsell and Lindquist 1993). Moreover, basically the same distinct in size classes of Hsps are synthesized in highly diverged organisms from flies to humans (Lindquist 1986; Lindquist and Craig 1988). Hence, at the present time Hsps are routinely classified basing on their molecular masses into the following major groups: small heat shock proteins (sHsps) family which comprises proteins with molecular masses from 10 to 30 kD; Hsp40 family (40 kD); Hsp60 (or chaperonins) with molecular mass close to 60 kD; Hsp70 family (70 kD); Hsp90 family (83–90 kD); and, finally Hsp100/110 family with molecular masses of members exceeding or equal to 100 kD (Kampinga et al. 2009; Mayer 2010; Parsell et al. 1994). Each of the above mentioned families may include multiple members (homologues) with similar or slightly different functions which may significantly differ by molecular masses (up to 10 kD).

Besides classification based on molecular mass all Hsps may be divided basing on the expression pattern in two large classes, namely, inducible (Hsp) and constitutive or cognate proteins (Hsc). Inducible Hsps are usually synthesized at a very low level or are completely silent under normal temperature conditions. Thus, transcription of

Table 1.1 Classification of canonical eukaryotic heat shock proteins and their major functions

Family	Some of members	Localization	The most important functions in the cell
Small Hsps	Hsp22	Mitochondria	Protein aggregation preventing, microfilament remodeling
	Hsp23	Cytosol	
	Hsp26		
	Hsp27	Cytosol/nucleus	
Hsp40	DnaJA1	Cytosol	Co-chaperones of Hsp70, binding with protein substrates
	DnaJC1	ER	
	DnaJA3	Mitochondria	
Chaperonins	GroEL	Mitochondria	Direct protein folding
	GroES		
	CCT	Cytosol	Direct protein folding, cytoskeleton assembly
Hsp70	HSPA1A and HSPA1B	Cytosol	Direct protein folding, translocation of nascent proteins into organelles
	HSPA5 (GRP78 or BiP)	ER	
	HSPA9 (GRP75 or mortalin)	Mitochondria	
Hsp90	HSPC1	Cytosol	Modulation of activity of major regulatory proteins
	HSPC4 (GRP94)	ER	
	HSPC5 (TRAP1)	Mitochondria	
Hsp110	HSPH1	Cytosol	Nucleotide exchange factor for Hsp70, preventing protein aggregation
	HSPH4 (GRP170)	ER	

Localization of small Hsps is given for *Drosophila*, because correspondent data regarding cellular representation of certain human sHSPs is fragmentary. About heat shock protein functions see Chap. 2 for details

Hsp70 genes in *D. melanogaster* under physiological conditions can be detected by only highly sensitive RT-PCR technique, while temperature elevation leads to hundreds fold induction of Hsp70 synthesis within a few minutes after HS (Lakhotia and Prasanth 2002; Pardue et al. 1980). Cognate *Hsps* genes often representing products of reverse transcription of corresponding mRNA of heat-inducible genes exhibit 50–80 % homology with inducible members of the same family and may be either silent or play house-keeping functions in the cells under normal temperature conditions (Kampinga et al. 2009). Some of Hsp families got the name of molecular chaperones in accordance with their functions as companions in folding of other proteins (see Chap. 2 for details).

Each family may comprise several inducible and several constitutive members with different functions (see Table 1.1). Below we shall provide modern classification of Hsps families described in humans and *Drosophila melanogaster* as an example. Nomenclature of human HSPs is given basing on Kampinga et al. (2009).

Small heat shock proteins family (sHsps) are characterized by the presence of crystalline domain flanked with variable N- and/or C-terminal regions. Hsp27 and other proteins of this family are constitutively abundant and ubiquitously present in many organisms. In humans there are 11 genes encoding proteins of this family comprising *HSPB* group. Accordingly, these proteins are designated from HSPB1 to HSPB11.

HSPB1 or HSP27 is the best studied member of this family in humans as well as HSPB4 and HSPB5 that correspond to αA- and αB-crystallins respectively (Kampinga et al. 2009).

In *Drosophila melanogaster* the major proteins of this family (sHsps) are encoded by seven genes with various orientations in the genome located in the same locus 67B which forms large puff in salivary gland polytene chromosomes after HS (Ayme and Tissieres 1985). According to the modern view major protective functions after HS in flies are performed by the following four small heat shock proteins: Hsp22, Hsp23, Hsp26 and Hsp27. All these genes are strongly induced by HS, they are highly homologous and have different intracellulal localization. Thus, Hsp22 is found in mitochondrial matrix, Hsp23 and Hsp26 in cytosol and Hsp27 have predominantly intranuclear localization (Morrow et al. 2000; Marin and Tanguay 1996). Totally there are 12 ORFs in the genome of *D. melanogaster* that contain α-crystallin domain (Morrow et al. 2006).

Hsp40 family probably represents the most abundant family of Hsps described so far. The members of this family characteristically contain the so-called "J-domain" exhibiting homology with well-known *E. coli* DnaJ protein. J-domain containing proteins serve as co-factors (co-chaperones) of Hsp70 family members, by stimulation ATP-activity of the latter group of proteins (Hennessy et al. 2005). There are more than 50 genes encoding J-domain containing proteins in the human genome (Kampinga et al. 2009). These proteins are divided into three sub-groups basing on the degree of homology with DnaJ designated DNAJA, DNAJB and DNAJC. DNAJA subgroup includes four proteins (DNAJA1 – DNAJA4) with highly homologous J-domain located at the N-terminus of the molecule while C-terminus is variable. Subgroup DNAJB consists of 14 homologues and includes the most heat-inducible human DNAJ member, DNAJB1. Finally, human genome contains DNAJC subgroup also containing J-domain which, however, is not necessarily found at the N-terminal end of the molecule. Besides, computer search in various data bases exploring NCBI and InterPro provided at least eight hypothetical ORFs that may encode proteins with significant homology to DnaJ of *E. coli*. It is of note that in the course of computer analysis multiple pseudogenes apparently belonging to *hsp40* family were detected (Kampinga et al. 2009). In contrast to humans, the genome of *D. melanogaster* harbors only five members of *Hsp40* family, containing J-domain, however, this number may be increased after more thorough search is performed.

Chaperonins form another distinct class of stress proteins that are found in both prokaryotes and eukaryotes. This class is subdivided into two subfamilies. The first group is represented by Hsp60 and Hsp10 that are homologous to bacterial GroEL and and its cofactor GroES that help prokaryotes to survive sever stress (Lund 1995). In humans there are two single genes (*HSPD* and *HSPE*) belonging to this family, which correspond to bacterial *groEL* and *groES* and coding proteins located in mitochondria (Kampinga et al. 2009). The Berkley *Drosophila* Genome Project has revealed four *Hsp60* genes in *D. melanogaster*, which have been named *Hsp60A*, *Hsp60B*, *Hsp60C* and *Hsp60D* (Sarkar and Lakhotia 2005).

The second group of chaperonins designated CCT (chaperonins containing t-complex polypeptide 1) or TRiC (TCP1 Ring Complex) functions in archaea and in cytosol of different eukaryotic organisms. Thus, proteins belonging to this group were described in *Arabidopsis* and other plants (Hill and Hemmingsen 2001). In eukaryotes, proteins of this family form oligomers consisting of eight subunits encoded by different genes (Kampinga et al. 2009). Eukaryotic CCT/TRiC is a 16 subunits complex composed of two back-to-back stacked rings, each containing eight different subunits of approximately 60 kD (α, β, γ, δ, ε, ζ-1, η and θ). These subunits are 30 % homologous and exhibit 15–20 % similarity with GroEL suggesting that they have origin in common (Brackley and Grantham 2009).

The Hsp70 (DnaK in bacteria) family is the most thoroughly studied group of stress proteins in many organisms. This family is very diverse and includes many constitutive as well as stress-inducible proteins with multiple functions.

In humans there are 13 genes encoding the proteins of this family named HSPA with different numbers. After temperature elevation maximal induction has been demonstrated for *HSPA1A*, *HSPA1B* and *HSPA6* genes and correspondent proteins move into nuclei after HS and bind chromatin. On the other hand, HSPA1L and HSPA2 are tissue-specific proteins and are intensively synthesized in testis and a few other tissues. HSPA5 also named GRP78 or BiP (Immunoglobulin heavy chain Binding Protein) is localized in the endoplasmic reticulum and is necessary for correct folding of proteins entering endoplasmic reticulum including immunoglobulins. *HSPA7* was considered to be a pseudogene for a long time but basing on the recent data probably it is a functional gene sharing high homology with *HSPA6* (Kampinga et al. 2009). HSPA8, previously named HSC70 or HSP73, represents the major house-keeping protein highly abundant in human cells. This protein is localized in cytosol and actively participates in cotranslational folding of various proteins and their transport through membranes into different cellular compartments. HSPA9 is a mitochondrial protein previously named "mortalin" (or mtHSP70/GRP75). There are two highly homologous isoforms of mortalin (mot-1 and mot-2) in mouse (Kaul et al. 2007). HSPA13 is localized in microsomes and may be yet another compartment-specific HSPA member with house-keeping functions. HSP12A, HSP12B and HSP14 represent more evolutionary diverse forms of HSP70 family in human with not yet defined functions (Kampinga et al. 2009). *HSPA1A*, *HSPA1B* and *HSPA1L* genes form a cluster in human genome and in the genomes of other mammals while other family genes are scattered in the genome (Brocchieri et al. 2008; Kampinga et al. 2009).

D. melanogaster genome contains 5–6 copies of highly homologous copies preserved by gene-conversion and responsible for the synthesis of inducible Hsp70 after HS (Bettencourt and Feder 2001; Maside et al. 2002). These copies are found in two clusters localized at 87A and 87B loci of polytene chromosomes. Heat-inducible *Hsp68* gene located at 95D locus also belongs to Hsp70 family and has slightly different functions after stress (Holmgren et al. 1979; Kellett and McKechnie 2005). Besides, the inducible members in *D. melanogaster* there are seven genes belonging to the same family but encoding constitutive proteins (Hsc70)

that are expressed under normal temperature conditions namely, *Hsc70-1*, *Hsc70-2*, *Hsc70-3*, *Hsc70-4*, *Hsc70-5*, *Hsc70-6* и *Hsc70Cb*. The functions of most of these cognate proteins are known, thus, *Hsc70-1* and *Hsc70-4* genes encode proteins located in cytosol that are 82 % homologous and have similar molecular mass (70 kD). *Hsc70-3* encodes the protein with molecular mass equal to 72 kD localized in endoplasmic reticulum, while *Hsc70-5* encodes 74 kD protein which contains signal sequence for transport to mitochondria. This particular protein shares 64 % homology with yeast mitochondrial Hsp70, and exhibits only 50 % similarity with the rest *Drosophila* Hsc70 members. Generally speaking, different members of *Hsp70* family genes often exhibit higher homology with orthologs from other unrelated organisms than with other endogenous members of the same family (Rubin et al. 1993). This feature suggests early amplification of different sub-families of *Hsp70* family in the course of species divergent evolution.

Likewise, baker's yeast *Saccharomyces cerevisiae* genome contains eight individual genes belonging to the *Hsp70* superfamily that are divided into a few different groups according with their evolutionary relations and intracellular localization. Thus, the Ssa proteins group consists of four members, designated "Ssa1 – Ssa4", with cytosolic localization. The Ssb group has two members, Ssb1 and Ssb2, which are also located in the cytosol. Both *S. cerevisiae* mitochondria and endoplasmic reticulum contain single Hsp70 member, Ssc1 and Kar2, respectively (Boorstein et al. 1994).

Another group of Hsps (Hsp110) with molecular mass varying from 100 to 170 kD, and exhibits significant homology with Hsp70 and probably diverged from it at the early stages of evolution of higher eukaryotes (Lee-Yoon et al. 1995). Proteins belonging to this family are found in different organisms (Kampinga et al. 2009). In humans there are four genes belonging to this family named *HSPH1 – HSPH4*. The proteins encoded by the *HSPH1 – 3* members of the family are found in cytosol, while HSPH4 (GRP170) is localized in endoplasmic reticulum (Kampinga et al. 2009).

In *D. melanogaster* two genes, *Hsp70Cb* and *CG2918*, were preliminarily described as belonging to this group but they are not yet thoroughly characterized at the present time (Easton et al. 2000). However, accordingly to their amino acid sequence one may conclude that Hsp70Cb is a homolog of the human cytosolic protein HSPH1, while CG2918 has a strong homology with HSPH4 (GRP170).

Hsp90 represents another important family of heat shock proteins that are usually constitutively active but may be strongly induced by stressful stimuli (Kampinga et al. 2009). Hsp90 family is distinct from other molecular chaperones in that most proteins of this family display chaperoning functions predominantly for multiple unstable signal transducers to keep them poised for activation and thus represent important components of cellular networks under normal temperature conditions and after stress. In humans this family is represented by five members, named HSPC1 – 5 with different localization. *HSPC1*, *HSPC2* and *HSPC3* encode cytosolic proteins while HSPC4 (previously designated GRP94) is endoplasmic protein, and HSPC5 (old designations TRAP1, HSP90L) is localized in mitochondria (Kampinga et al. 2009). Genome of *D. melanogaster* contains a single gene (*Hsp83*)

which encodes cytosolic protein with molecular weight 83 kD; there are also genes belonging to this family encoding endoplasmic and mitochondrial proteins (Felts et al. 2000; Sorger and Pelham 1987). Correspondent bacterial protein with molecular weight 90 kD is designated HtpG (Chen et al. 2006).

Several of the above mentioned Hsps may be separated into a distinct group of the so-called glucose regulated proteins (GRP) because the proteins of this family were first discovered when the polypeptides synthesized in cells deprived of glucose were studied in details. This group includes certain members of Hsp70, Hsp90 and Hsp110 families designated as GRP78, GRP94 and GRP170 or HSPA5, HSPC4 and HSPH4, respectively, using modern nomenclature (Kampinga et al. 2009). These proteins are localized in endoplasmic reticulum (ER) while function of GRP75 (HSPA9) is restricted to mitochondria. All these glucose-regulated proteins share the same type of regulation named unfolded protein response (UPR) triggered by ER proteins misfolding. Thus, expression of all GRP is induced by the disturbances of normal proteins folding in endoplasmic reticulum which may be induced by heat shock, heavy metals, inhibitors of N-glycosylation and several other factors (Chen and Brandizzi 2013).

Another special chaperones belong to "AAA+" family (ATPases associated with a wide variety of cellular activities). In yeast and bacteria these chaperones have molecular weight near 100 kD and take part in protein aggregates resolubilization and proteolysis (Parsell et al. 1994). In eukaryotes this family includes many highly diverse proteins taking part in refolding and degradation of proteins often exploring ubiquitin-dependent pathway (Ciechanover and Stanhill 2014; Mayer 2010).

It is of note, that not all proteins induced by HS and other forms of stress are chaperones *per se*. There are multiple proteins such as collagenase, heme oxygenase, many regulatory proteins (e.g. eIF2α) and many others that are induced by stress and participate in many cellular events in a chaperone independent manner. In this respect ubiquitin represents the most illustrative example of "non-standard" heat-induced proteins. Thus, it has been demonstrated that temperature elevation (HS) leads to five to six fold increase in ubiquitin mRNA increase because large quantities of ubiquitin is require to utilize high cellular proteins damaged by HS. Importantly, promoter regions of all heat-induced genes usually contain heat shock elements (HSEs) necessary for binding of heat shock factor (HSF1) which induces *in cis* heat shock genes (Fornace et al. 1989).

In parallel with the induction of *Hsp* genes transcription and rapid accumulation of correspondent proteins, HS and other stimuli strongly induces transcription of certain genes that produce non-coding RNAs as functional end product. Thus, the *hsr*-ω gene of *D. melanogaster* is developmentally active in many tissues and is one of the most strongly induced genes following temperature elevation. The 10 kbs nucleus limited transcripts of this gene are associated with different hnRNAs and participate in the formation of the nucleoplasmic omega speckles. It was suggested that the omega speckles play important role in regulation of nuclear trafficking and availability of hnRNPs and other RNA binding proteins in the cell (Lakhotia 2012). Moreover, recently, various elegant experiments of Lakhotia's group implicated *hsr*-ω transcripts in apoptosis (Mallik and Lakhotia 2011).

1.1 Conclusions

After the discovery of heat shock genes in *Drosophila* a great number of papers appeared describing similar batteries of genes in various organisms from yeast to humans. Amazingly, genomes of all organisms studied contain virtually the same classes of *Hsp* genes exhibiting high similarity even between phylogenetically very distant organisms suggesting early appearance of heat shock genes in eukaryotes which apparently predated their divergence and specialization.

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Chapter 2
Molecular Functions of Heat Shock Proteins

It has been established long time ago that among many changes in cellular activity the most remarkable event in stressed cells of all know organisms is massive production of highly conserved set of heat shock proteins (Schlesinger et al. 1982). Soon after their discovery heat shock proteins (Hsps) have been implicated in thermotolerance based on the ability to recover heat-induced denatured proteins to their native state (Maloyan et al. 1999).

It has been demonstrated by many groups that Hsps help cells and organism as a whole to adapt to elevated temperatures and other forms of stress. Thus, preliminary treatment of culture cells or individuals (e.g. flies) with moderate temperature enables them to survive the consequent severe heat shock treatment which would have been otherwise lethal. This phenomenon demonstrated in various organisms was termed "induced thermotolerance" (see Feder and Hofmann 1999 for review). There are abundant data suggesting that accumulation of Hsps after mild heat shock is accounted for the induced thermotolerance phenomenon. Along these lines, it was shown that artificial increase of Hsp70 concentration after transfection of the cells with correspondent constitutive expressed plasmids also led to the resistance against thermal, oxidative and other stresses (Angelidis et al. 1991; Chong et al. 1998; Li et al. 1992; Park et al. 2000). Transgenic organisms with an increased *Hsp70* gene copy number are more thermoresistant compared with wild-type (Feder et al. 1996). On the contrary, inhibition of protein synthesis in the cells after HS as well as knockout of certain heat shock genes results in reduced thermoresistance and prevents functional recovery of cellular genes after stress (Johnson and Kucey 1988). Furthermore, mutations in individual *Hsp* genes or their deletions as well as mutations in the gene encoding correspondent transcription factor (HSF) ablate induced thermotolerance (Craig and Jacobsen 1984; Gong and Golic 2006; Jedlicka et al. 1997). In the following years it was well established that cellular heat shock protection mechanism is dependent on the ability of Hsps to prevent misfolded proteins to form aggregates and effectively regulate degradation and translocation of various proteins to cellular compartments (Feder and Hofmann 1999; Hightower 1991; Hartl and Hayer-Hartl 2002; Pelham 1986).

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After HS, hypoxia, various chemicals, including heavy metals and many other forms of stressful stimuli cellular proteins undergo denaturation which often results in the exposure of hydrophobic regions of polypeptide chains normally packed inside protein three-dimensional structure to cytosolic hydrophylic environment. Because of such exposure proteins begin "to stick" to each other by their hydrophobic regions and, hence, form insoluble sedimenting aggregates highly toxic for the cells (Szalay et al. 2007). Besides, denatured proteins lose their ability to exercise normal activity in the cells which leads to the irreversible damage of most housekeeping systems and eventual death of the cells. It was demonstrated that Hsps may non-specifically interact with hydrophobic regions of denatured proteins and thus facilitate the restoration of their native tertiary structure and prevent insoluble aggregates formation (Fig. 2.1a). Other representatives of Hsps do not directly interact with misfolded proteins but may work as cofactors (or co-chaperones) with other Hsps. Thus, dissociation of Hsp70 and protein substrate requires ATP hydrolysis which takes place with participation of Hsp40. The product of this reaction (ADP) is subsequently exchanged for ATP with the involvement of co-chaperones (see below).

Besides Hsp70, several other groups of Hsps including small Hsps, Hsp60, Hsp90 and Hsp110 protect cellular proteins from misfolding and aggregation. While prevention of aggregates formation is definitely the major function of various Hsps, certain Hsps are able to dissolve already formed aggregates (Mayer 2010). Furthermore, chaperones together with ubiquitin-proteasome system (UPS) are involved in degradation of many cellular proteins (Bercovich et al. 1997; Hartl et al. 2011). Major pathways of proteins homeostasis maintained in the cells with Hsps involvement are depicted in Fig. 2.1b.

Hsps and especially certain members of Hsp70 family do not only prevent aggregates formation after HS or other stress but under normal conditions may bind newly synthesized proteins in parallel with their translation processing. They assist translocation of these proteins to different cellular compartments and help to maintain the right conformation. Hsp70 and other proteins capable to non-specifically bind with highly diverse polypeptides and help them to maintain native structure and be transported to their destination in the cell are named "chaperones" after French word "chaperone" – companion or duenna (Fig. 2.2). However, as we mentioned above while not all Hsps have this specific chaperone function part of them are named co-chaperones because they are also induced by stress and assist other proteins to bind peptides and translocate them to various cellular compartments.

Fig. 2.1 (**a**) Fluctuations of free energy in the course of proteins folding and aggregates formation. Molecular chaperones stimulate the formation of normal tertiary structures and prevent aggregation process. (**b**) The proteostasis network. Molecular chaperones participate in the folding and transport of synthesizing proteins, restoration of misfolfed peptides and disaggregation of denatured proteins. While prevention of aggregates formation definitely represents the major role of chaperones, representatives of AAA+family (e.g. yeast Hsp104) are able to dissolve already formed aggregates and facilitate their degradation by UPS (ubiquitin proteasome system) pathway (Modified from Hartl et al. 2011 with permission)

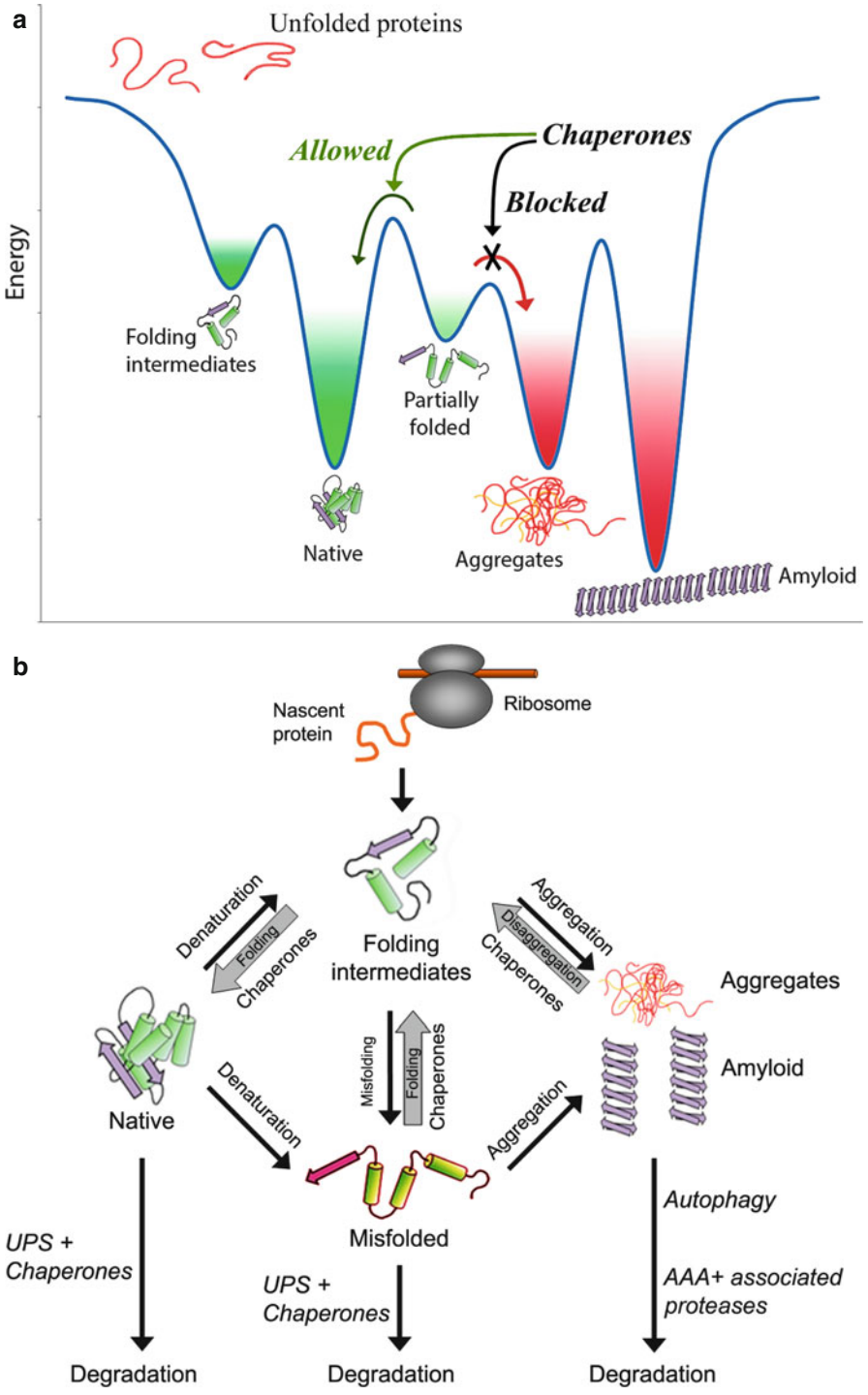
a
Unfolded proteins
Allowed
Chaperones
Blocked
Energy
Folding intermediates
Partially folded
Native
Aggregates
Amyloid
b
Nascent protein
Ribosome
Folding intermediates
Denaturation
Folding
Chaperones
Aggregation
Disaggregation
Chaperones
Aggregates
Native
Misfolding
Folding
Chaperones
Amyloid
Denaturation
Aggregation
Misfolded
Autophagy
UPS + Chaperones
AAA+ associated proteases
UPS + Chaperones
Degradation
Degradation
Degradation

Fig. 2.2 "The Chaperone", the painting of Herman ten Kate. The chaperone is a lady second from *left*

Below we shall briefly describe the major functions of different heat shock proteins belonging to basic families mentioned above.

All small stress proteins (sHsps) contain α-crystallin domain 80–100 amino acids in length located at the C-terminal domain of the proteins. The homology of this domain within the family may vary from 20 to 60 % depending on the compared shps relationship. Additionally, some of small Hsp family members at the N-terminal end contain a short conserved region with phosphorylation sites implicated in the regulation of small Hsps activity (Kampinga et al. 2009).

The most remarkable feature of all sHsps is the ability to form oligomeric complexes: thus nine β-sheet structures comprising α-crystallin domain form stable intermolecular contacts with other small Hsps. Furthermore, both homo- and heterooligomeric complexes with molecular weights varying from 200 to 400 kD may be formed (de Jong et al. 1998). Such large complexes exist in the cells under normal physiological conditions and serve as a depot where small heat shock proteins are stored. The temperature elevation and other forms of stress result in dissociation of these preexisting complexes and fast release of small Hsps. Increased expression of sHsps during HS response correlates with better survival from cytotoxic stress. Small Hsps in the form of dimers interact with denatured cellular proteins in ATP-independent manner producing large granules. In the course of such interaction hydrophobic regions of denatured proteins become shielded from each other and loss the ability to form insoluble aggregates. At the next step the restoration of native tertiary structure of stress-damaged proteins is accomplished by Hsp70 and Hsp60 family members (Carver et al. 1995; Fernando and Heikkila 2000).

Besides chaperoning function small Hsps display other diverse roles. For example in response to growth factors stimulation they regulate the formation of actin microfilaments and stabilize actin network damaged by stress (Gusev et al. 2002; Khlebodarova 2002). αB-crystallin, a small Hsp family member closely related to Hsp27 is constitutively expressed and is especially abundant in eye where it is involved in lens formation (Horwitz 1976; Ingolia and Craig 1982). Moreover,

members of small Hsp family and especially Hsp27 regulate apoptosis exploring several pathways. It was demonstrated, that Hsp27 negatively regulates the activation of procaspase-9 and can block release of cytochrome c from mitocondria in cells exposed to various pro-apoptotic factors (Arrigo et al. 1998; Mehlen et al. 1996; Kamradt et al. 2001). Besides, small Hsps may inhibit apoptosis induced by various other factors such as activators of receptors sFAS/APO-1 (Khlebodarova 2002). In addition, ubiquitously present Hsp27 modulates TNF-induced apoptosis by inhibiting IkB degradation (Kammanadiminti and Chadee 2006).

Recent experiments with *D. melanogaster* strains clearly demonstrated that enhanced expression of small Hsps significantly increases the resistance of flies to different forms of stress and extends life span. High concentration of Hsp22 was detected in mitochondria which are very sensitive to reactive oxygen species (ROS). The flies with suppressed synthesis of Hsp22 were more sensitive to moderate stress and were characterized by reduced lifetime (Morrow et al. 2006). When studying chaperoning activity measured as the ability to restore thermally denatured luciferase structure characteristic differences were revealed between individual members of small Hsps family. According to this assay, at the presence of Hsp22 luciferase activity was restored by 50 %, in the case of Hsp23 and Hsp26 by 30 % and in the case of Hsp27 by 40 %. Characteristically, Hsp22 localized in mitochondria which exhibit high sensitivity to oxidative stress manifested maximal restoration activity in these tests (Marcillat et al. 1989; Li et al. 2002). However, it should be noted that the observed differences may be partially attributed to different specificity of individual sHsps to substrate (luciferase in this assay) (Morrow et al. 2006).

Small Hsps may be abundant in certain tissues or organs at specific stages of development under normal physiological conditions. Thus in mammals small heat shock proteins are constitutively expressed in heart and muscles where they are probably involved in the preservation of microfilament network. It was also shown that gonads and heads of the newly hatched young flies are enriched with Hsp23 and Hsp26 while concentration of these small proteins is drastically decreased in these organs in aged flies (Morrow and Tanguay 2003). Furthermore, Hsp23 is actively synthesized at certain stages of ontogenesis in the brain of *Drosophila* in neuronal cells and in glia (Michaud and Tanguay 2003). Characteristically, the most drastic changes in Hsp22 accumulation take place in flies in the course of aging. Concentration of Hsp22 is increased in the heads and in the thoraxes of aged flies 60 and 20 folds respectively (King and Tower 1999; Wheeler et al. 1995). It was also demonstrated that Hsp22 plays an important role in preserving mitochondria from the consequences of oxidative stress occurring in aged flies (Morrow et al. 2004).

Hsp40 family comprises another group of heat shock proteins belonging to the so-called “J-proteins” family named basing on their similarity with bacterial *DnaJ* protein described in *E. coli* (Hartl and Hayer-Hartl 2002). Since the major function of Hsp40 is to serve as co-factor of Hsp70, all representatives of this highly diverse group contain J-domain necessary for interaction with Hsp70 family members. It was demonstrated that Hsp40 stimulates ATP-ase activity of Hsp70 by means of interaction between J-domain of Hsp40 with ATP-ase domain of Hsp70 and stabilizes complexes between Hsp70 and protein substrates (Fan 2003). According

to the recent model, Hsp40 also recognizes and binds misfolded proteins before their association with the general chaperone Hsp70 (Summers et al. 2009). Different J-proteins function in cytosol, endoplasmic reticulum or mitochondria and serve as cofactors for cytosolic HSP70 and HSC70, or endoplasmic HSPA5/BiP and mitochondrial HSPA9/GRP75 in mammals (Christis et al. 2008).

The Hsp70 family is probably the most thoroughly studied group of stress proteins. According to x-ray analysis the molecule of canonical human HSPA1A consist of highly concerved 450 a. a. domain at the N-terminal end and rather variable 200 a. a. C-terminal domain. The N-terminal domain manifests ATP-binding activity (behave as ATPase in the presence of Hsp40 cofactor) and in terms of tertiary structure resembles actin ATP-binding domain while variable C-terminal domain represents substrate-binding region of Hsp70 (Flaherty et al. 1990). High affinity of Hsp70 members to ATP enables to easily isolate proteins of this family using ATP-Sepharose for laboratory and practical uses (Welch and Feramisco 1985).

Members of Hsp70 family execute various functions in the cell under stressful or normal conditions mainly related to their well-known chaperoning activity.

The molecular basis of Hsp70 chaperoning activity is based on the ability of these proteins to effectively bind misfolded cellular proteins and, hence, to prevent their aggregation (Nollen and Morimoto 2002). By binding the denatured proteins Hsp70 stabilizes them in partially unfolded state (Hartl and Hayer-Hartl 2002). The ability of Hsp70 proteins to recognize and bind to unfolded polypeptides is defined by peculiarities of their structure: C-terminal domen of Hsp70 contains hydrophobic "pocket" reminiscent in structure of peptide-binding region of the major histocompatibility complex (MHC) proteins, and in particular MHCII. It is of note, however, that peptide-binding site in Hsp70 has more open configuration making possible to interact with much longer peptides (Flajnik et al. 1991; Rippmann et al. 1991). Due to its specific structure Hsp70 recognizes polypeptides enriched with hydrophobic amino acids. Under physiological conditions such hydrophobic motifs are hidden inside normally folded protein and, hence, the appearance of such a. a. sequences on the surface of the protein molecule is usually a landmark of misfolded (or newly synthesized) proteins. It was demonstrated that hydrophobic amino acid sequences recognized by Hsp70 are found in polypeptides on the average every 40 a.a. (Frydman 2001). The interaction with Hsp70 leads to stabilization of protein substrates in unfolded state and, hence, prevents their aggregation.

At the next stage the restoration of native conformation may occur either with direct involvement of Hsp70 and its co-chaperone Hsp40 or partially restored proteins may be transported to chaperonins or Hsp90 (for certain proteins) for complete restoration of their native structure (see below).

In fact the detailed interaction of Hsp70 with substrate was initially described in *E. coli* for DnaK (Liberek et al. 1991) and later in eukaryotes (Summers et al. 2009). As a rule, folding of proteins with participation of Hsp70 or DnaK (in *E. coli*) requires several repeated cycles of association-dissociation accompanied by ATP hydrolysis. At the first stage Hsp70 interacts with complex of unfolded protein substrate and Hsp40 (DnaJ) in ATP-bound form (see Fig. 2.3). At the second stage DnaJ stimulates ATP hydrolysis and stabilizes the formed Hsp70-substrate

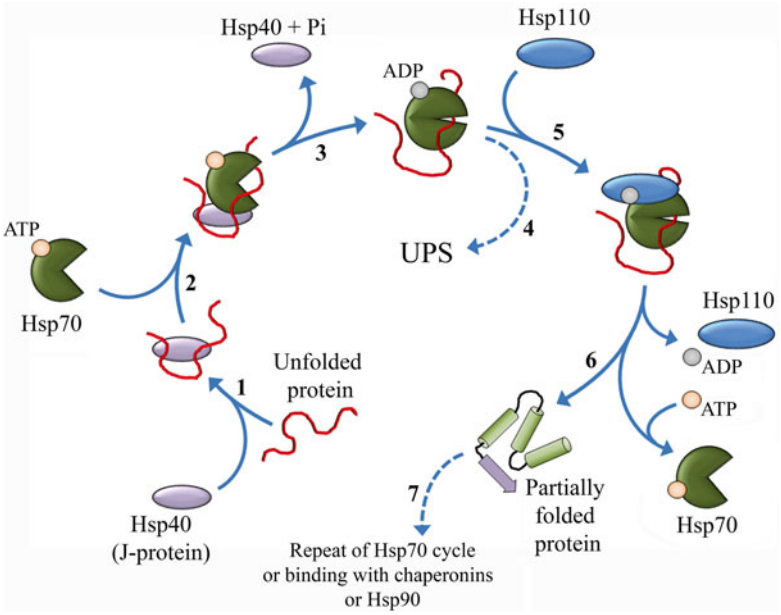

Fig. 2.3 Hsp70-substrate interaction cycle. *1* Initially, a non-native polypeptide is bound by Hsp40. *2* Hsp40-substrate complex binds with Hsp70 via J-domen of hsp40. *3* J-domain-stimulated ATP hydrolysis in the nucleotide-binding domain induces a conformational shift in the Hsp70 substrate-binding domain, increasing affinity for the non-native polypeptide that is released from the Hsp40. *4* In the case of polyubiquitinated protein substrate Hsp70 can direct them into proteasome for subsequent degradation. *5* and *6* – nucleotide exchange factors (NEFs) such as the BAG1 or Hsp110 replace the ADP with ATP, and the polypeptide releases from Hsp70. *7* If the polypeptide remains in a non-native conformation, the cycle can be repeated until folding is complete. *UPS* ubiquitin-proteasome pathway, *ATP* adenosine triphosphate, *ADP* adenosine diphosphate and *Pi* phosphate (From Summers et al. 2009 with modification)

complex. The affinity of Hsp70 to the product of reaction – ADP – is significantly higher than to ATP and, thus, dissociation of ADP requires cochaperone nucleotide exchange factor – GrpE in *E. coli.* After the dissociation of Hsp70-ADP complex the substrate is released and Hsp70 binds a new ATP molecule. Interestingly, in eukaryotic cells the interaction of Hsp70 with protein substrate also requires J-protein for hydrolysis of ATP, while dissociation of ADP is stimulated by cochaperones HspBP1 (Hsp70 binding protein 1) and BAG-1, and, according to the last data, Hsp110 (Summers et al. 2009). Furthermore, binding of Hsp70 with ATP and substrate is promoted by special protein Hap (Hsp70- and Hsc70-associating protein) (Gebauer et al. 1998; Houry 2001). Certain proteins e.g. steroid hormone receptors and many transcriptional factors after interaction with Hsp70 form stable complexes with Hsp90. The subsequent dissociation of these complexes requires interaction with a specific ligand or phosphorylation (see below). In some cases misfolded proteins bound with Hsp70 do not restore its native state but undergo degradation by UPS (Fig. 2.3). Furthermore, members of Hsp70 family are sometimes involved

in degradation of misfolded proteins under normal physiological conditions. For instance, constitutively abundant Hsc70 participates in ubiquitin-dependent degradation of many cellular proteins such as actin, α-crystallins, histone 2A and many others (Bercovich et al. 1997). Therefore, constitutive members of Hsp70 family play important roles under physiological conditions and provide normal folding of newly synthesized proteins (Nelson et al. 1992). Practically all house-keeping proteins at some point during their translation process and release from the polysomes interact with Hsp70 family members (John et al. 1992; Ku et al. 1995). Chaperones and in particular certain Hsp70 family members participate in translocation of cellular proteins to endoplasmic reticulum and mitochondria. Thus, in cytosol HSPA1L and HSPA2 recognize and bind to newly synthesized proteins and during the translocation of such proteins through mitochondrial membrane dissociation coupled with ATP hydrolysis occurs. In cytosol HSPA1L and HSPA2 maintain polypeptides in unfolded state thus prevent formation of tertiary structure which inhibits the translocation through the membrane. It was shown that translocation of proteins denatured by urea across the mitochondrial membrane does not require ATP. In the course of translocation through the mitochondrial membrane and cleavage of N-terminal signal sequence, matrix Hsp70 (HSPA9 or GRP75 associated with mitochondrial Hsp40) binds to polypeptide chain. At the next step polypeptide is released with ATP hydrolysis and got caught by mitochondrial Hsp60 which facilitates its final folding and/or oligomerization (Neupert et al. 1990; Voos 2009, 2013).

Furthermore, BiP in cooperation with GRP94 is involved in the assembly of secreted immunoglobulins (Melnick and Argon 1995). Complexes formed by BiP and GRP94 with antibodies molecules are structurally similar to the complexes formed by Hsp70 and Hsp90 with glucocorticoid receptors and protein kinases in cytosol. Correct folding of MHCI and MHCII molecules also requires chaperones participation. Characteristically, while BiP interacts with both MHCI and MHCII, GRP94 is specific to MHCII and does not interact with MHCI. Furthermore, GRP94 interacts with Toll-like receptors, receptor of insulin-like growth factor and integrins. It is of note that five specific J-proteins (Hsp40 family) function in ER in cooperation with BiP (Christis et al. 2008). In addition, ER-resident molecular chaperones form heterocomplexes with collagen type IV chains and participate in presentation of antigenic peptides by MHCI (Binder et al. 2001; Ferreira et al. 1996). Major stages of proteins folding and transport with participation of Hsp70 and other components of chaperonic machinery are depicted in Fig. 2.4.

Proteins of another family of chaperones (HSP110 or HSPH) are rather similar to Hsp70 in general structure. The major difference is larger size of C-terminal peptide-binding domain and linker region between N-terminal ATP-binding domain and C-terminal domains in HSP110 (Kampinga et al. 2009; Lee-Yoon et al. 1995). Recent evidence shows that HSPH members play a role of nucleotide exchange factors for the HSPA family (Dragovic et al. 2006; Raviol et al. 2006). Futhermore, they also show chaperone activity of their own. Unlike Hsp70, Hsp110 lacks the ability to assist in protein folding; however, they exhibit high activity in preventing aggregation of denatured proteins for which is even more efficient than that of Hsp70. For this reason, Hsp110s are called “holdases” but not “foldases”, the term used for Hsp70 family (Xu et al. 2012).

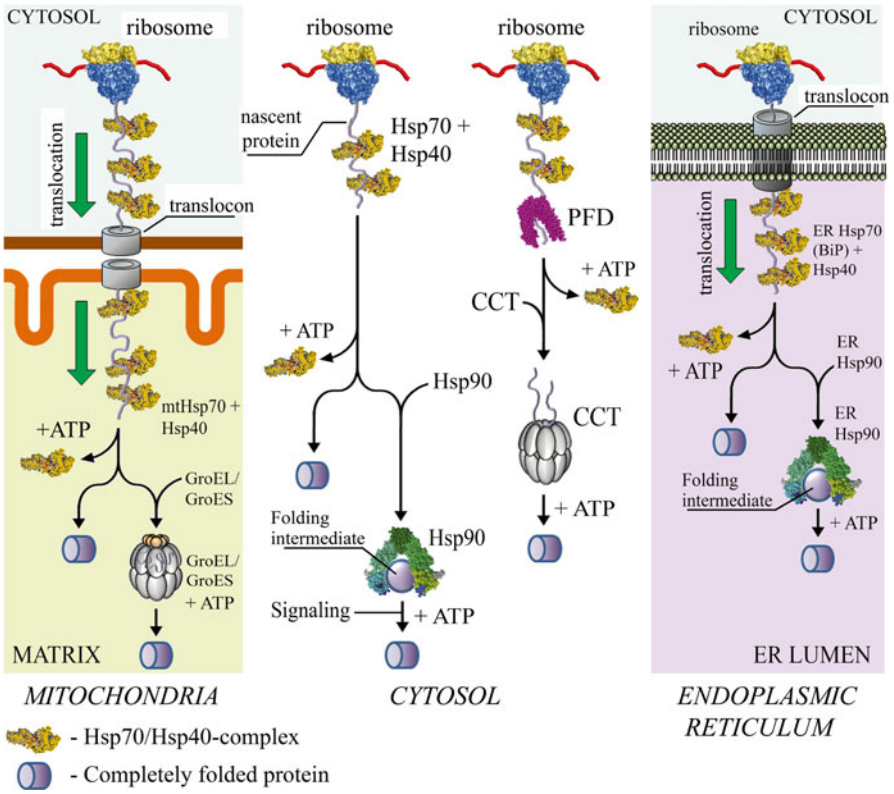

Fig. 2.4 General protein folding pathways in mitochondria, cytosol and endoplasmic reticulum of eukaryotic cell. During translocation across the mitochondrial membrane nascent polypeptides interact with cytosolic Hsp70 before and with mitochondrial Hsp70 (*mtHsp70*) after the transfer through the translocon. After mtHsp70 action, polypeptides may take native conformation immediately, or can interact with GroEL-GroES complex for subsequent folding. Similarly, in cytosol, nascent proteins may fold immediately after interaction with Hsp70, or interact with subsequent Hsp machinery, such as Hsp90 or cytosolic chaperonin CCT, after Hsp70 dissociation. In this case, before transfer to CCT, nascent polypeptide must be marked by prefoldin (*PFD*). In endoplasmic reticulum, translocated proteins bind with BiP, the ER homolog of Hsp70, and then dissociate with complete folding or interact with ER Hsp90. Therefore, it is evident that chaperonin component of the folding machine is absent in the ER, while substrates of the mitochondrial Hsp90 (Trap1) are unknown. *ATP* adenosine triphosphate, *CCT* chaperonins containing t-complex polypeptide 1, *ER* endoplasmic reticulum (Hartl et al. 2011; Voos 2013)

Chaperonins represent another very peculiar family of stress proteins. They include bacterial GroEL and its mitochondrial homologue in eukaryotes (HSPD or Hsp60), as well as eukaryotic cytosolic protein CCT (TRiC). CCT may function without any cochaperones while GroEL requires cofactor (GroES also named Hsp10). Hsp60 of archebacteria is homologous to eukaryotic CCT and also functions without cochaperones (Ditzel et al. 1998). The main feature which differentiates chaperonins from chaperones is their ability to form complex structures resembling

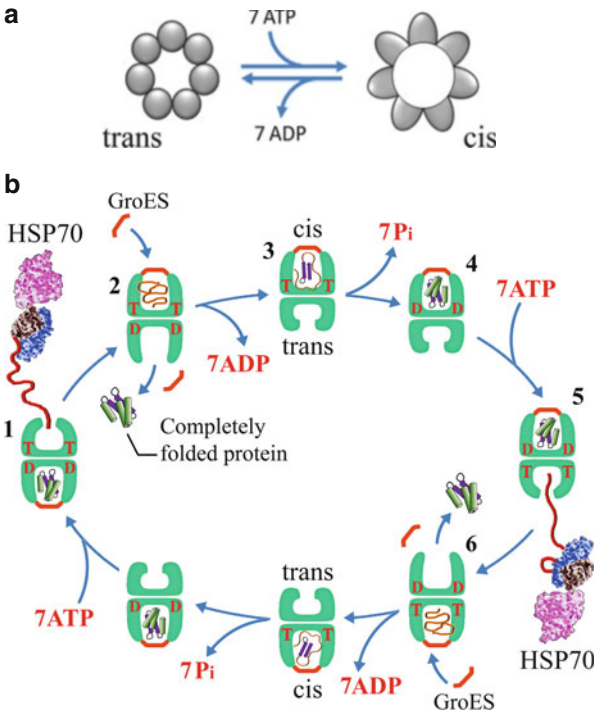

Fig. 2.5 Proteins folding with participation of GroEL/GroES chaperonins system. (**a**) *Top view* of GroEL complex. GroEL represents as an asymmetric stack of two heptameric rings, one of which is situated in the *cis*-conformation (ADP/ATP and substrate bound), and the other in the *trans-conformation*. Seven ATP molecules bind anticooperatively between rings. (**b**) The cycle of Gro-EL/GroES action. Chaperonins bind with protein substrates after its interactions with Hsp70 which can be accompanied by local substrate unfolding. *1* ATP-bound GroEL ring interacts with protein substrate (red squiggle). *2* GroES associates with substrate-bound complex and dissociation of GroES and folded protein takes place from the opposite ring. *3* Folding stage. GroES and protein substrate are bound with the apical GroEL domain, substrate escape is prevented. *4* ATP hydrolysis in *cis*-complex permits entry of ATP into the *trans*-complex. *5* and *6* ATP binding is an allosteric signal to the *cis*-complex for GroES, protein substrate and ADP dissociation

a stack consisting of two rings. Each ring consists either of seven like in the case of GroEL or eight like in the case of CCT monomers. Characteristically, GroEL oligomers are formed by identical monomers while CCT complex consist of eight heterosubunits encoded by different genes. Co-chaperone GroES also forms ring-like homooligomeres which are complementary to GroEL complex. In the center of each ring between monomers there is a cavity where folding of protein-substrates takes place (Fig. 2.5). The sequences of each monomer contain several hydrophobic amino acid residues, hidden inside the pore. Protein substrates interact with these residues. Folding of the proteins requires ATP hydrolysis like in the case of typical chaperones. In the case of GroEL the capture of protein substrate inside the pore is accompanied with GroES binding and subsequent ATP hydrolysis. GroES binds the pole of GroEL

oligomer and closes the cavity as a lid (Fig. 2.5). Therefore, folding takes place in the environment separated from the cytosol. After this GroES dissociates from the complex and release of protein-substrate takes place. Such mechanism is very efficient and able to completely isolate protein-substrate from cellular environment (Hartl et al. 2011). It is of note, that final folding of many proteins after interaction with members of Hsp70 family requires the involvement of chaperonins (Fig. 2.4).

It has been recently demonstrated that over-expression of GroEL and GroES in bacteria helps to maintain mutations which lead to the decrease of protein stability. Apparently chaperonins maintain mutant proteins in active form and prevent aggregation of misfolded proteins. As a result such mutants may survive for some time until compensating mutation occurs which may restore proteins stability. Therefore, chaperonins may have pronounced evolutionary impact promoting the formation of proteins with quite novel activities and characteristics (Tokuriki and Tawfik 2009; Wyganowski et al. 2013).

While GroEL-GroES system functions in bacteria and mitochondria, CCT was found in archea and in eukaryotic cytosol. In eukaryotes this system participates in the folding of major components of cytoskeleton, actin, tubulin and several proteins involved in cell cycle regulation (Brackley and Grantham 2009). Before CCT machinery binding a protein substrate should interact with a special protein named "prefoldin" which marks the substrates for CCT (Vainberg et al. 1998). In contrast to GroEL, CCT activity does not requires co-factors such as GroES. The closing of central cavity in the case of octameric CCT complex occurs through the conformational changes of apical parts of CCT subunits themselves (Mayer 2010).

The Hsp90 family includes abundant constitutive proteins which can be also induced by heat shock and other forms of stress. In contrast to Hsp70 family Hsp90 interacts with substrates proteins in the form of V-like dimers. Typical molecule of Hsp90 consists of three domains: N-terminal domain executes ATP hydrolysis and resembles in general structure DNA-binding sites of topoisomerases and DNA-girases of bacteria; the central domain provides the interactions with protein substrates; and C-terminal domain is required for dimerization (Freeman and Yamamoto 2002; Harris et al. 2004).

It was shown that Hsp90 does not bind newly synthesized proteins and does not participate in the process of denatured proteins refolding. However, under stressful conditions Hsp90 prevents aggregation of denatured proteins and helps to transport them to Hsp70 for subsequent 3D structure restoration (Nollen and Morimoto 2002).

Hsp90 displays a central chaperoning role in the folding, activation and transport of various regulatory proteins which play a key role in signal transduction pathways including normal function of cell cycle. All protein substrates of Hsp90 share the same characteristic feature: they may exist and function in several alternative conformations (Johnson 2012). Association of protein partners with Hsp90 prevents their aggregation and help to maintain the conformation optimal for their interaction with other proteins or low molecular weight ligands. Binding and release of proteins from Hsp90 complexes requires ATP and co-chaperones Hop and p23 (Nollen and Morimoto 2002). Specifically Hsp90 in complex with Hsp70 and several co-chaperones regulates signal protein kinases, NO synthases, telomerase, aminoacyl

tRNA synthetases, and multiple transcription factors containing helix-turn-helix motif such as HIF-1, HSF, p53 etc. (Abravaya et al. 1992; Kosano et al. 1998; Sato et al. 2000; Whitesell et al. 1998; Xu and Lindquist 1993). In this respect it is necessary to emphasize an important role playing by Hsp90 in the regulation of major heat shock transcription factor (HSF1) which induces intensive transcription of all heat shock genes after temperature elevation and other stresses (see Chap. 3). The major results regarding interaction between Hsp90 and protein-targets were accumulated in studies of progesterone and glucocorticoid receptors.

It was shown that activation of progesterone receptor begins with its recognition by Hsp70 and Hsp40 with subsequent transfer by Hop protein onto Hsp90 accompanied by ATP hydrolysis. The final result of these events contains dimer of Hsp90, p23 and immunofilins providing optimal conformation of the receptor with the ligand (Nollen and Morimoto 2002). In similar way Hsp90 maintains the optimal conformation of multiple other proteins that participate in signal cascades.

In certain cases Hsp90 may have oncogenic potential and promote malignant transformation of cells. Carcinogenesis may result from the mutations in a few regulatory proteins, such as signal protein kinases, p53 and other regulatory factors that are involved in interaction with Hsp90.

Normally Hsp90 interacts with c-Src protein kinase, which participates in signal transduction which stimulates cell division. Oncogenic variant of SRC contains a few mutations that disturb its normal function. Hsp90 may interact with oncogenic forms of protein kinases and transcription factors involved in cell division and, stabilizing them, promote oncogenesis (Xu and Lindquist 1993). Furthermore, Hsp90 and Hsp70 participate in folding of p53 and by forming stable complexes with mutant variants of p53 prevent its degradation (Nagata et al. 1999). It was shown that p53 may form immunoprecipitates with Hsp70 and Hsp90 after hyperthermia (Nagata et al. 1999; Whitesell et al. 1998). It has been also demonstrated that in certain tumors the level of Hsp90 is increased by two to ten folds in comparison with surrounding normal tissues (Schwartz et al. 2003). Basing on such data Hsp90 is considered to be a perspective target for developing antitumor drugs for the purposes of chemotherapy. The suppression of Hsp90 synthesis leads to inactivation and degradation of signal proteins that promote carcinogenesis and may either restore the normal phenotype of the cell or induce apoptosis. Moreover, Hsp90 inhibition was shown to promote tau protein degradation in a mouse model of tauopathy (Dickey et al. 2007). It was shown that cochaperones CHIP, Hop and Hsp40 are constituents of the Hsp90 chaperone complex that promotes tau degradation (Cook and Petrucelli 2013; Dickey et al. 2007). At the present time there exists a variety of hsp90 inhibitors both natural and synthetic. Several of them are now passing different stages of preclinical or clinical trials and some were approved by FDA. Cisplatin is one of the Hsp90-blocking agents already widely used at the present time for chemotherapy (Rosenhagen et al. 2003). Unfortunately, high cytotoxity common for various Hsp90 inhibitors represents a serious obstacle for their use in clinics (Cook and Petrucelli 2013; Garcia-Carbonero et al. 2013; Patki and Pawar 2013). The other disadvantage of many Hsp90 binders, even such as geldanamycin passing the 3-d stage of clinical trials, is that they often induce the synthesis of Hsp70

probably as compensation for the Hsp90 inhibition and this synthesis causes the enhancement of cell tolerance to a variety of anti-cancer remedies.

It was demonstrated by Suzan Lindquist and other groups studying *D. melanogaster* and *Arabidopsis thaliana* that, surprisingly, inhibition of Hsp90 synthesis by rafamycin or geldanamycin leads to the expression of multiple new phenotypes (Rutherford and Lindquist 1998; Queitsch et al. 2002). Moreover, the patterns of expressed phenotypes are strain-specific. The observed phenomenon may be explained by interaction of Hsp90 with mutant regulatory proteins of various signal pathways maintaining them in inactive form. After stress Hsp90 begins to interact with multiple denatured proteins appearing in the cell in high concentration which leads to the release of previous protein-partners including mutant variants that were associated with Hsp90.

This process may result in the increase of possible signal pathways development and their subsequent fixation in the genome (Rutherford and Lindquist 1998; Queitsch et al. 2002; Sangster et al. 2008a, b). Further selection may lead to the appearance of stable phenotypes that will be expressed independently on stress and Hsp90 level in the cell (Rutherford and Lindquist 1998). Therefore, Hsp90 apparently may play important role in the evolution by preserving and accumulating new genetic variants (Cowen and Lindquist 2005).

Later it was shown that Hsp90 prevents phenotypic variation by suppressing transposon-induced mutagenesis through piRNAs. It was demonstrated that deficit in Hsp90 activity reduces piRNA expression, activates TE transposition and causes genotype variation (Specchia et al. 2010).

Furthermore, it was demonstrated that Hsp90 forms a complex with Piwi protein and regulates its phosphorylation. It was proposed that post-translational regulation of Piwi by Hsp90 may allow Piwi to form active complexes with piRNAs and/or epigenetic factors to promote epigenetic and transposon silencing, leading to canalization of certain signaling pathways (Gangaraju et al. 2011).

Hsp104/ClpA/ClpB proteins comprise a separate group characterized by the presence of AAA+domain (ATPases associated with a wide variety of cellular activities). These proteins were designated “unfoldases”, due to their ability to unfold the tertiary structures of other proteins. Bacteria, fungi, protozoa, chromista and plants all harbor homologues of Hsp104, a AAA+ATPase that collaborates with Hsp70 and Hsp40 to promote protein disaggregation and reactivation. Curiously, however, metazoa do not possess an Hsp104 homologue. In mammals, slowly dissolving of protein aggregates may be realized by complexes of Hsp110, Hsp70 and Hsp40 (Shorter 2011).

Hsp104 functions in the form of ring-like hexamers with six active centers localized inside the pore which is formed in the center of hexamer. Polypeptides substrates in un-folded state are pulled through the pore like a thread through the eye of the needle (Fig. 2.6). Certain representatives of Hsp104 family (ClpA and ClpX in *E. coli*) function in complex with proteases by means of unfolding of the substrate proteins and translocating them into protease complexes ClpP. Other members of Hsp104 family (ClpB in bacteria and Hsp104 in yeast) dissolve protein aggregates in association with Hsp70 and Hsp40 (Mayer 2010; Zolkiewski et al. 2012).

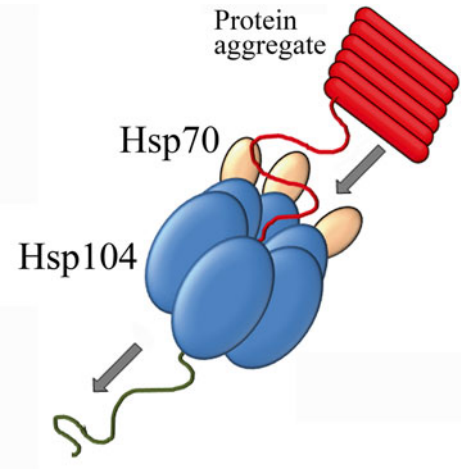

Fig. 2.6 The participation of Hsp70-Hsp104 (AAA + family member) complex in protein aggregates dissociation. Usually, protein substrates after Hsp104-assistant unfolding enter different proteolysis complexes

Importantly, yeast Hsp104 plays a significant role in the adaptation to extreme environmental conditions, based on the ability to recover splicing after HS. Thus, *S. cerevisiae Hsp104* mutants do not differ in terms of growth kinetics from the wild type cells, while under normal conditions, after acute heat shock or ethanol treatment the mutants die 100–1,000 fold more frequently (Lindquist and Kim 1996; Vogel et al. 1995; Schirmer et al. 1996). AAA + family includes 19S proteasome complex, mitochondrial Hsp78 and MCX1, that work in cooperation with proteases Pim1/LON and ClpP respectively, and many other different eukaryotic proteins (Ciechanover and Stanhill 2014; Voos 2009).

Along with the described above "classical" Hsps, to molecular chaperones can be classified certain enzymes, such as disulfide isomerases (PDIs) involved in the formation of disulfide bonds during protein folding processes, and prolyl-peptidyl isomerases (PPIases), which interconverts the *cis* and *trans* isomers of peptide bonds formed by the amino acid proline. These enzymes are found both in prokaryotes and eukaryotes, and are essential for different pathways of the cell physiology (Christis et al. 2008).

It is evident, therefore, that representatives of different groups of Hsps usually function in the cells in cooperation with members of other Hsps families as well as with multiple cofactors. At the present time there are more than 180 components of chaperone system including cofactors that regulate activity of various Hsps groups (Hartl et al. 2011). In other words, it is clear nowadays that enhanced synthesis of a single Hsp group cannot account for thermoresistance of the cell and organism as a whole. Apparently thermotolerance is provided by complex interaction of multiple proteins and each of them plays a specific role in protecting cells from harmful effects of stress.

Apart from their chaperoning functions, Hsps are involved in recovery of normal genome activity after HS and other forms of stress. It was demonstrated exploring different approaches that normal genetic activity fails to recover if protein synthesis after HS is inhibited. It was shown along these lines that following thermal stress

Hsp90 is accumulated in the cytoplasm and enters the nuclei in large quantities where it binds histones and performs other protective functions (Prodromou et al. 1997). If the translocation of Hsps into the nuclei after HS is blocked, the chromatin structure is disturbed due to histones aggregation and inactivation of DNA topoisomerases. As a consequence, unwinding of DNA necessary for normal transcription does not occur after stress termination. Hsp70 also plays an important role in recovery of normal genome functioning after stress. Immunofluorescent analysis revealed the transport of Hsp70 from cytoplasm into nuclei, which is especially pronounced after DNA damage by free radicals and adriamycine (Abe et al. 1995; Knowlton et al. 2000). It was demonstrated that after translocation Hsp70 interacts with proteins of nuclear matrix and is predominantly accumulated in the nucleolus (Abe et al. 1995).

Apart from their direct chaperoning functions, majority of the constitutive as well as stress induced heat shock proteins interact with members of the apoptotic cascades since pro-apoptotic stimuli frequently induce Hsps (Li et al. 1999).

At the present time abundant material is accumulated indicating important anti-apoptotic role of certain mammalian HSPs and in particular HSP70 and HSP27. Importantly, such effect depends on the cell line and particular apoptotic-signaling pathway (Ahn et al. 1999; Brar et al. 1999; Lasunskaia et al. 1997; Takano et al. 1998; Wagstaff et al. 1999). Increased expression of HSP27 during stress response correlates with better survival from cytotoxic stress induced by different stimuli. It was shown that HSP27 can efficiently block release of cytochrome c from mitochondria in cells exposed to staurosporine or cytochalasin D (Paul et al. 2002).

On the other hand, HSP70 is able to efficiently block p53-induced apoptosis but is not effective in the case of Fas-mediated apoptosis (Schett et al. 1999). Heat shock-induced HSP70 significantly inhibits the activation of stress kinases of SAPK/JNK family. These kinases are involved in phosphorylation of p53 and antiapoptotic protein bcl-2 and, hence, initiate apoptosis. The latter protein (bcl-2) controls the release of cytochrome c from mitochondria and serves as one of the major inhibitors of apoptosis. Overexpression of HSP70 inhibits JNK activity and prevents translocation of pro-apoptotic protein Bax from cytoplasm to mitochondria where it triggers release of various death factors (Mosser et al. 1997; Stankiewicz et al. 2005). It is of note, that anti-apoptotic effect of HSP70 is realized in this case not by direct inhibiting of JNK substrates phosphorylation but rather by blockade of early stages of its activation by controlling the activity of certain phosphatases that use JNK as a substrate (Gabai et al. 1998; Kumar and Tatu 2003). HSP70 may regulate apoptosis through interaction with BAG-1 protein which activates proapoptotic protein Bax (Mosser et al. 1997). HSP70 may also directly interact with Apaf-1 and, thus, inhibit formation of functional apoptosome complex and activation of initiator caspase-9 (Mosser et al. 2000). Therefore, it was demonstrated that HSP70 function as anti-apoptotic agent at the very early stages of apoptosis which may explain its high protective potential. There are data suggesting that HSP70 may exercise its anti-apoptotic functions at later stages of apoptosis as well. Thus Mosser with coauthors demonstrated that constitutive expression of HSP70 decreases proteolysis of caspase-3 substrates while *in vitro* HSP70 can not modulate the function of activated caspase-3. Probably HSP70 is able to bind caspases substrates and thus protects them from proteolysis. The ability of HSP70 to protect cells from death due

to expression of active caspase-3 corroborates this hypothesis (Jaattela et al. 1998; Komarova et al. 2004a, b; Mosser et al. 1997).

Various death-inducing stimuli such as TNF, may cause apoptosis via activation of transcription factors of NF-kB family. Heat-induced HSP70 interacts with p65 and c-Rel proteins belonging to this family and prevents their nuclear translocation thus hampering the TNF-induced apoptosis in U-937 cells. The protective effect is independent on the presence of NF-kB inhibitor (IkB), because IkB was not detected in complexes of HSP70 with p65 and c-Rel (Komarova et al. 2004a, b).

Furthermore, HSP27 protects fibrocarcinoma cells from apoptosis induced by Fas receptor activation and protein kinase C inhibitor staurosporin and provides resistance to adriamycine to the breast tumor cells. The essential protective anti-apoptotic action of Hsp27 is based on its two important characters: first, HSP27 is able to decrease solubility of multiple transcription factors including p53, thus modulating their activity and transport in the cell. Second, HSP27 is able to efficiently bind cytochrome c released from mitochondria and, thus, prevents its interaction with Apaf-1 and inhibits caspase-9 activity (Arrigo et al. 1998; Kamradt et al. 2001; Mehlen et al. 1996).

The above brief survey clearly shows that various HSPs may modulate apoptosis at different stages and interact with various components of cell death program realization. This may explain high efficiency of anti-apoptotic action of HSPs and strain-specific pattern of their modulating effects. If the intensity of stress does not exceed certain threshold rapid switching on of the battery of heat shock genes and resulted inhibition of apoptosis enables the cell to survive the challenge. However, in the case of a heavy and prolonged stress HSPs are not able to inhibit or block apoptosis and in this instance the cell eventually dies. The cell death is executed due to rapid activation of protein kinases p38 and JNK, that phosphorylate HSF1 at the regulatory domain and, this in turn, blocks its activity and prevents HSPs accumulation after shock (Anckar and Sistonen 2007; Chu et al. 1996; He et al. 1998).

Basing on the data mentioned above it is possible to assume that involvement of HSPs in apoptosis may play two different roles for the cells and organisms. On one hand, the demonstrated anti-apoptotic effects of HSPs in the case of brain or heart ischemia enable to consider HSPs as one of the most effective protective systems of an organism. On the other hand, the observed inhibition of apoptosis in many types of malignant cells by HSPs allows to consider them as powerful promoters of carcinogenesis. It is well-known that most cancer cells are characterized by high constitutive levels of HSP70 and HSP27 which render them to be highly resistant to many apoptosis-inducing drugs as well as to high temperature (hyperthermia) or hypoxia (Jaattela 1999). We are not going herein to discuss in detail the involvement of different HSPs in carcinogenesis because it is beyond the scope of the book and there are excellent reviews on the subject (Ciocca et al. 2013; Cohen et al. 2010; Murphy 2013; Rappa et al. 2012). However, it is of note that Hsp70 expressing in great quantities in certain cancer or virus-infected cells may be released to culture medium or blood and carry tumor or viral antigens serving as modulator of both innate immunity and adaptive activity and exercise multiple other functions (Calderwood and Ciocca 2008; Didelot et al. 2007; Joly et al. 2010).

It is necessary to mention in this context that for more than two decades after the initial discovery of heat shock proteins including various members of Hsp70, all Hsps were considered to be intracellular proteins and it was generally believed that a protein the size of Hsp70 or more could not pass through the cell membranes without the involvement of a special membrane transporter or a pore. However, several reports appeared indicating apparent release and cell-to-cell transfer of the Hsp70 protein (Hightower and Guidon 1989; Tytell 2005; Tytell et al. 1986, 2010). Later with the discovery that Hsp70 (exogenous Hsp70 or eHsp70) was detectable in the intercellular circulation of humans (Pockley et al. 1998, 2002), a lot of publications appeared demonstrating the presence of Hsp70 in many other extracellular fluids including cerebrospinal fluid (Asea 2007; Tytell et al. 2010). Originally elevated basal levels of eHsp72 were reported in people suffering from a variety of diseases including hypertension, artherosclerosis etc (Reviewed by Johnson and Fleshner 2006; Asea 2007).

Not long after these reports, it was shown by several groups that organisms in the absence of clinical disease may also rapidly respond to acute physical or psychological stressors by pronounced increase in the eHsp70 concentration in the blood (Asea 2007; Johnson and Fleshner 2006; Tytell et al. 2010).

These results enabled to conclude that stress-induced release of eHsp70 into the blood represents quite common feature of the normal stress response exhibited by various organisms including humans that encounter adverse environmental conditions such as high temperature or various xenobiotics. There is no doubt at the present time that eHsp70 has functional significance for both immune system and tolerance to different forms of metabolic stress.

Interestingly, benzene-poisoned workers showed a high incidence of antibodies against Hsp72 which was associated with a decrease in white blood cells and high frequency of lymphocyte DNA damage. These data suggest that antibodies against Hsps can potentially be useful biomonitors to assess if people have experienced abnormal xenobiotic-induced stress within their living or working environment (Wu et al. 1998).

There are several potential release mechanisms for Hsp70 and other Hsps that lack the signal peptide targeting them to secretory vesicles. First of all Hsps may be released from cells whose membranes were damaged by trauma or necrosis induced by various means. Besides such unregulated leakage which may happen in all cells and tissues, regulated release of Hsps and specifically Hsp70 may take place exploring membrane-bounded vesicles called “exosomes” that can be produced in a wide variety of cells (Fevrier and Raposo 2004; Johnson and Fleshner 2006). Though exosomes may account for Hsp70 release by various cells in many cases, recent investigations demonstrated that secretion via conventional secretory vesicles is also an option and can not be excluded (Tytell et al. 2010). Once released, Hsps can interact with the membranes of various cells, be involved in cell-to-cell interactions and enter the cytoplasm of target cells through the membrane by different not yet completely understood mechanisms (Ekimova et al. 2010). It became clear that Hsp72 in human cells apparently has unique releasing signals and immunomodulatory functions when expressed in an extracellular context on the cell surface or in the circulation in blood or other extracellular fluids.

While protective therapeutic role of intracellular Hsp72 was clearly demonstrated by many groups in the treatments of multiple neurodegenerative disorders such as Parkinson's disease, Huntington's disease, Alzheimer's disease and other proteinopathies the ability of eHsp70 to inhibit chronic inflammation and/or exacerbates inflammatory diseases such as Alzheimer's inflammatory bowel disease or artherosclerosis have only recently been investigated in detail (Bobkova et al. 2013; Ekimova et al. 2010; Tytell et al. 2010; Vinokurov et al. 2012).

As a result of numerous papers describing pronounced anti-inflammatory effects of endogenous intacellular and extracellular heat shock protein 72 exploring various mammalian models of sepsis and neurodegeneration several years ago first attempts were made to apply recombinant Hsp70 as a therapeutic drug and monitor its neuroprotective effects exploring various methods of Hsp70 administration (Bobkova et al. 2014; Ekimova et al. 2010). Although these studies demonstrated that recombinant eHsp70 may be a practical therapeutic agent for treatment of neurodegenerative diseases associated with abnormal protein biogenesis and cognitive disturbances, such as AD, the molecular mechanisms underlying the observed neuroprotection remain unknown.

2.1 Conclusions

Molecular chaperones are highly conserved proteins, providing non-covalent folding, assembling and transport of a wide range of cellular proteins. Expression of many chaperones is stress-induced, and, hence, these proteins protect cells from various forms of metabolic stress, by restoring the conformation of denatured proteins and eliminating of irreversibly damaged proteins. Other constitutively expressed chaperones perform folding and intracellular transport of newly synthesized proteins under normal physiological conditions. In addition, chaperone network is involved in regulation of a large number of cellular signal transduction pathways and cytoskeleton assembling. Different classes of molecular chaperones and their protein co-factors (co-chaperones) work in tight cooperation providing complex house-keeping and anti-stress system, which plays a key role in protein homeostasis in a cell. Recombinant Hsps and in particular Hsp70 represent promising therapeutic agents for treatment of various neurodegenerative diseases associated with abnormal protein biogenesis.

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Chapter 3
Regulation of Heat Shock Genes Expression

Cellular stresses modulate intracellular signaling pathways that control almost all aspects of cell physiology and metabolism. Heat shock genes system activation in response to various forms of stress is extremely rapid and, hence, represents an excellent model for investigation of gene regulation at all levels. In *Drosophila* it is possible to detect local chromatin decondensation leading to puffs formation in salivary glands polytene chromosomes within the first 1–2 min after HS challenge. Fast activation of *Hsp* genes is apparently necessary for cell survival under stress conditions and for cross-protection against unrelated stresses. Activation of HS system is rapidly initiated at all stages of genetic information realization including transcription, export of mRNA and translation.

In *E. coli* and other prokaryotes regulation of genes activity depends on RNA polymerase σ-factors that are able to recognize specific nucleotide sequences and direct RNA-polymerase to specific promoters. In *E. coli* under normal temperature conditions low level transcription of HS genes *groES* and *groEL* is maintained by major vegetative factor σ70. After temperature elevation complex of RNA polymerase with σ70 becomes destabilized and is substituted by a complex with recruited σ32. The σ32 is a product of *rpoH* gene, and it recognizes a specific motif in the promoters of heat-regulated genes. The level of *Hsp* genes expression in *E. coli* positively correlates with intercellular concentration of σ32. In the absence of stress σ32 is not able to induce significant transcription primarily because its half-life is less than 30 s due to effective proteolysis of σ32 with the involvement of proteases HflB and ClpP (Segal and Ron 1998). Under stressful conditions a pronounced increase in the quantity of misfolded and damaged proteins occurs. Such abnormal proteins produced after stress serve as target for the different intracellular proteases. As a result HflB and ClpP proteases switch to other substrates and half-life of σ32 is significantly increased. Besides, HS leads to significant increase in σ32 translation efficacy. Elevated temperature induces melting of hairpin structure located in σ32-factor 5′-mRNA and, thus, intensifies the translation process (Morita et al. 1999). Therefore, the regulation of σ32 concentration in the prokaryotic cells is realized at transcriptional and post-transcriptional levels.

M.B. Evgen'ev et al., *Heat Shock Proteins and Whole Body Adaptation to Extreme Environments*, DOI 10.1007/978-94-017-9235-6_3

In *Hsp* genes of all eukaryotes at the 5′-regulatory region upstream of TATA box there are specific motifs, the so call "heat shock elements" (HSEs). The optimal context of such sequences represents 5′-nnGAAnnTTCnnGAAnn-3′, where n – any nucleotide (Amin et al. 1988). The major functional unit in this sequence is either GAA or TTC motif. Besides this typical HSE there are so-called "gap-type HSEs" consisting of two inverted units separated from a third unit by a 5-bps gap (nTTCnnGAAn(5 bps)nGAAn), and "step-type HSEs" consisting of direct repeats of nGAAn or nTTCn motifs separated by 5 bps (nGAAn(5 bps)nGAAn(5 bps) nGAAn) (Hashikawa et al. 2006; Yamamoto et al. 2005). In addition to HSEs, *Hsp* genes frequently contain TATA-box and certain other species-specific elements. Besides HSEs, in most cases *HSP70* genes contain Sp1 binding sites and CCAAT motif in the human *HSP70* genes or GAGA motifs in *Drosophila* species. While one copy of the complete HSE sequence (see above) is sufficient for heat shock induction most of the investigated promoters of *HSP70* genes contain several HSEs located at different distances from the transcription start. As an example, *Hsp70* genes of *D. melanogaster* contain four HSEs (Amin et al. 1987; Tian et al. 2010). Various lines of evidence demonstrated that the increase in HSEs copy number in the promoters of *Hsp* genes significantly enhance their transcription (Amin et al. 1988, 1994; Lerman and Feder 2005). The insertion of HSE at the 5′-end of any gene may render it heat-inducible (Bienz and Pelham 1986). In fact there are multiple genes in *Drosophila* genomes that contain HSEs and can be induced by HS (Sørensen et al. 2005).

Almost simultaneously in *Drosophila* and in humans a special heat shock transcription factor (HSF) which specifically binds to HSEs after temperature elevation was described. Characteristically, after HS HSF binds predominantly HSE motifs localized in the regions with landmarks of active chromatin which includes histone acetylation, H3K4 trimethylation, the presence of RNA Polymerase II, and coactivators (Guertin and Lis 2010).

There are at least five representatives of HSF family in vertebrates: HSF1, 2, 3, 4 and HSFY (Åkerfelt et al. 2010; Kinoshita et al. 2006; Morimoto 1998; Wu 1995). HSF2 is mainly involved in constitutive Hsps expression and the synthesis of Hsps in embryogenesis and cell differentiation (Åkerfelt et al. 2010; Loones et al. 1997; Wu 1995). The obtained data show that HSF2 and HSF1 can form heterotrimers activating transcription in response to different types of stress and signals during development (Ostling et al. 2007). HSF3 has been originally described in birds and similarly to HSF1 is heat-induced, but only in the case of severe heat shock (Åkerfelt et al. 2010).

Later another transcription factor also designated "HSF3" was described in mice (Fujimoto et al. 2010). However, in mice HSF3 is not able to induce *HSP70* transcription and participates in activation of several "non-canonical" stress-induced genes. HSF4 was cloned from the genomes of mice, rats and humans (Morimoto 1998; Wu 1995). HSF4 is a mammalian factor characterized by its lack of a suppression domain that modulates formation of DNA-binding homotrimer. It was shown that HSF4 gene generates both an activator and a repressor of heat shock genes by alternative splicing. Although both mouse HSF4a and HSF4b form

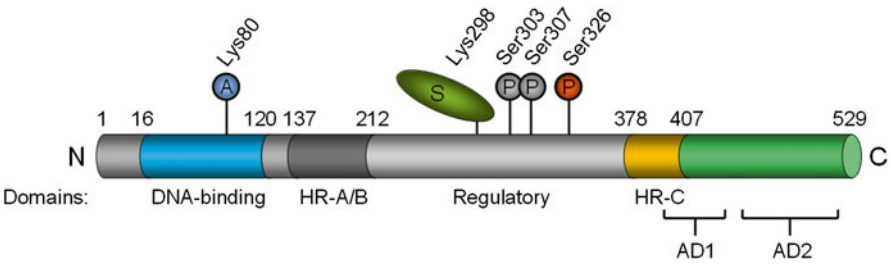

Fig. 3.1 The structure of mammalian HSF1. The relative positions of major structural domains and sites of post-translational modifications are indicated. *AD* activation domains involved in the transcription induction of target genes. HR-A/B and HR-C – hydrophobic repeats responsible for trimerization and downregulation, respectively. Ser303 and Ser307 are phosphorylated under normal non-stressful conditions and participate in downregulation after HS. Lys298 – sumoylation site (S). Phosphorylation of Ser326 plays a key role in activation of HSF1 after trimerization and DNA-binding. Lys80 – undergoes acetylation in the course of HSF1 inactivation (Modified from Åkerfelt et al. 2010 with permission)

trimers in the absence of stress, these two isoforms exhibit different transcriptional activity; HSF4a acts as an inhibitor of the constitutive expression of heat shock genes, and hHSF4b acts as a transcriptional activator. Moreover, heat shock and other stresses stimulate transcription of target genes by HSF4b in mammalian cells (Tanabe et al. 1999).

The fifth mammalian HSF, HSFY, is encoded by gene located in Y chromosome and expresses predominantly in testis. There are data suggesting that deletion of HSFY results in azoospermia syndrome (Shinka et al. 2004).

Interestingly, in *D. melanogaster* only one representative of *hsf* gene family was detected. This gene produces four differently spliced transcripts and participates in the induction of all *Drosophila Hsp* genes (Zimarino et al. 1990). Baker's yeast *Saccharimyces cerevisiae* also comprises only one copy of *hsf* gene (Hahn and Thiele 2004).

Despite low amino acid sequence similarity (usually less than 40 %), heat shock factors of different species share highly conserved secondary and tertiary structures (Morimoto 1998). The structure of mammalian HSF1 is depicted in Fig. 3.1.

DNA-binding domain which recognizes HSE sequences within promoters of heat-inducible genes is located at N-terminal part of HSF. C-terminal end of HSF1 molecule comprises of two domains AD1 and AD2 that modulate the activity of transcription complex (Shi et al. 1995). The 212–407 a. a. region of mammalian HSF1 includes elements responsible for negative regulation of this transcription factor under normal physiological conditions (Fig. 3.1). Specifically, a region between amino acids residues 137 and 212 includes three hydrophobic heptapeptide repeats (designated HR-A/B – hydrophobic repeats A and B) that form leucine zipper motifs responsible for trimerization of HSF1 monomers. The additional leucine zipper is localized within 378–407 a. a. region (HR-C). This fourth leucine zipper (HR-C) is involved in negative regulation of HSF1 by means of intramolecular interaction with the HR-A/B repeats in the absence of stress. Deletion of this

region leads to constitutive trimerization of HSF1 and its transition into active DNA-binding form under normal conditions (Orosz et al. 1996; Rabindran et al. 1993; Westwood and Wu 1993; Zou et al. 1998). Another element which participates in negative regulation of HSF1 is localized within 203–227 region and under normal conditions is able to effectively inhibit activity of transactivation domain (395–503 a. a.) (Morimoto 1998; Rabindran et al. 1993). A region between 300 and 310 a. a. is also involved in negative regulation of HSF1 being a target for constitutive phosphorylation.

The pattern of activation of HSF1 after HS necessary for the initiation of transcription of *Hsp* genes is common for different organisms. The system of heat shock response is in general highly conserved in various phyla. Thus, *D. melanogaster* HSF is able to induce transcription of *HSP* genes in mouse cells where following exposure to stress recruitment and activation of HSF1 occurs (Clos et al. 1993). Under normal conditions HSF in flies and HSF1 in mammals are present in the form of inactive monomers. In mammals inactive HSF1 located mostly in cytosol, while in *Drosophila* HSF at normal conditions reside predominantly in nuclei (Orosz et al. 1996; Wang et al. 2004a; Yao et al. 2006). Inactive HSF1 exists in complex with multiple Hsps as well as many other transcriptional factors regulated by these proteins (see Chap. 2). After temperature elevation these complexes rapidly dissociate and conversion of monomeric form into active trimeric conformation with high-affinity DNA binding capacity occurs in all eukaryotic organisms.

The trimerization results from intermolecular interaction between HR-A/B leucine zippers. Afterwards, in the form of trimers HSF1 is translocated to correspondent chromosomal loci where it binds HSEs (Zimarino et al. 1990; Zou et al. 1998). HSF1 and HSF2 may form heterotrimers that are able to activate transcription in response to variety of stresses and/or at certain stages of development (Ostling et al. 2007). Next, HSF1 undergoes phosphorylation at multiple serine or treonine sites and becomes competent to activate transcription. Interestingly, in baker's yeast *S. cerevisiae* HSF constitutively binds HSEs in the form of trimer under normal conditions due to lack of HR-C leucine zipper. Thus, in *S. cerevisiae* activation of HSF is realized by phosphorylation and interaction with other transcription modulators, because binding of HSF to DNA is required but is not sufficient to activate transcription of heat-induced genes (Bonner et al. 2000; Gallo et al. 1991). It was speculated that *Drosophila* HSF and mammalian HSF1 may itself play a role of thermosensor i.e. its conformational changes leading to trimerization occurs directly as a result of temperature increase. Along these lines, it has been demonstrated that *Drosophila* HSF is constitutively active in human HeLa cells (Clos et al. 1993). It is well known that *Drosophila* dwells at 20–25 °C and, hence, the cultivation temperature of mammalian cells (37 °C) definitely represents stress temperature for the flies cells which should induce *Drosophila* HSF activation and Hsps synthesis. Indeed, it was subsequently demonstrated that xenotransplantation of *D. melanogaster* neuronal cells into mouse brain induces a pronounced synthesis of Hsp70 (Korochkin et al. 2002). On the other hand, the trimerization temperature of human HSF1 in *Drosophila* cells is ten degrees lower than in endogenous (human) cells. It is evident that in the latter case some other factors besides temperature are involved in HSF1 activation.

It was shown that HSF1 trimerization may be triggered at normal temperature by introduction of denatured proteins e.g. BSA into the cell (Ananthan et al. 1986). Besides, activation of HSF1 may be achieved using proteasome inhibitors such as MG132 and clasto-lactacystin β-lactone, leading to dramatic accumulation of poly-ubiquitinated proteins (Pirkkala et al. 2000). According to the modern view under normal (non-stress) conditions HSF1 in human cells exists in complex with Hsp90 dimer, co-chaperone p23 and immunophilin FKBP4 (designated "thermosensitive multichaperoning complex"). Under normal conditions Hsp90 preserves HSF1 in inactive conformation due to intramolecular hydrophobic interactions between leucine zippers HR-A/B and C. After accumulation of denatured proteins in the cytosol Hsp90 begins to interact with them as more highly affinity substrates and, hence, the dissociation of thermosensitive complex and release of HSF1 occurs (Zou et al. 1998). In monomeric state HSF1 is able to rapidly form trimers and bind DNA. Trimerization of HSF1 and transition into DNA-binding form may be also achieved by antibiotic geldanamycin which specifically inhibits Hsp90 activity and induces rapid dissociation of various Hsp90-containing complexes.

Important role of Hsp70 in the negative regulation of HSF under normal conditions was demonstrated in *Drosophila melanogaster* (Solomon et al. 1991). Later physical interaction between *Drosophila* HSF and DroJ (Hsp40) was also shown. Moreover, the DroJ depletion by RNAi technique leads to HSF activation in *Drosophila* cells under normal conditions. Co-depletion of DroJ together with Hsp70/Hsc70 or with Hsp90 leads to the full level induction of the heat shock response. This findings support a model in which synergistic interactions between DroJ1 and Hsp70/Hsc70 and Hsp90 chaperones modulate HSF activity in the course of heat shock response by the negative feed-back interaction (Marchler and Wu 2001).

Thus, in *Drosophila* after temperature elevation Hsp70 is found in all HS puffs in polytene chromosomes where it colocalizes with HSF. It was demonstrated that Hsp70 in cooperation with Hsp40 forms complexes with HSF1 both *in vivo* and *in vitro* (Marchler and Wu 2001). Characteristically, these complexes dissociate after ATP administration. It was further shown that the C-terminal domain of HSF1 is responsible for interaction with Hsp70. Over-expression of Hsp70 placed under the control of constitutive promoter significantly decreases HSF1 activity and inhibits transcription from HSE-containing promoters. Interestingly, in this case HSF1 remains in trimeric form bound to DNA. Therefore, Hsp70 and Hsp40 apparently interfere with the integration of HSF1 into transcription machinery (Baler et al. 1996).

At the stage of recovery after HS, HSF1 interacts with Hsp90 which leads to monomerization and refolding due to intramolecular interaction of leucine zippers A/B and C (Shi et al. 1998). Therefore, accumulation of Hsps after temperature elevation or other challenge inhibits transcription of *Hsp* genes and prevents their own extra accumulation which may be toxic to the cell.

After trimerization HSF1 usually undergoes stress-induced phosphorylation. Several protein kinases including Ca^{2+}/calmodulin-dependent protein kinase (CaMKII), protein kinase C (PKC), DNA-dependent protein kinase (DNA-PK), and possibly cAMP-dependent protein kinase (PKA) are involved in this process.

It was shown along these lines, that CaMKII activation protects cardiomyocytes from HS-induced apoptosis and hypoxia by means of phosphorylation of HSF1 and Hsp70 induction (Peng et al. 2010). Similarly, the application of CaMKII inhibitors such as staurosporine significantly decreases HSF1 activity and heat-inducible accumulation of *HSP70* and *HSP27* mRNA in human glioblastoma cells. On the other hand, the application of PKC agonists such as PMA or ionomycin results in incorporation of (^{32}P) into HSF1 and its hyperactivation (Ding et al. 1997). In yeast protein kinase Snf1 is responsible for inducible HSF phosphorylation under glucose starvation conditions (Hahn and Thiele 2004).

Protein kinases involved in positive regulation of HSF1 activity may be induced by HS due to the increase in concentration of ceramide, cAMP and release of Ca^{2+} ions from endoplasmic reticulum. It is possible that different protein kinases are involved in HSF1 phosphorylation after different kinds of stressful stimuli and the type of protein kinase involved may depend on cell type. There are 12 potential phosphorylation sites within HSF1 molecule located at the following positions: Ser121, Ser230, Ser292, Ser303, Ser307, Ser314, Ser319, Ser326, Ser344, Ser363, Ser419 and Ser444. Phosphorylation of Ser326 probably plays the key role in HSF1 activation after HS and chemical stress (Guettouche et al. 2005; Holmberg et al. 2001; Kiang et al. 1998).

Furthermore, it was demonstrated that different forms of stress activate various phospholipases including neutral and acid sphingomyelinase, phospholipase C, phospholipase A_2 and phospholipase D. Such activation leads to the increase in ceramide C2 concentration due to hydrolysis of sphingomyelin (Nikolova-Karakashian and Rozenova 2010). In *Saccharomices cerevisiae* HS also induces the increase of ceramide level, but in this case, in contrast to mammals, ceramide is synthesized de novo from sphingosine (Wells et al. 1998). The increase of ceramide level may activate SAPK/JNK family of protein kinases and stimulate PKC activity. The increase in ceramide concentration *per se* is not sufficient to induce trimerization of HSF1 or enhance its transcription induction activity. However, elevated level of ceramide significantly extends heat-induced HSF-HSE binding which is necessary for transcription initiation (Nikolova-Karakashian and Rozenova 2010). It is possible to assume that ceramide is one of the most important players at the early stages of HSF1 activation cascade and, hence, while it does not influence the trimerization process it is definitely involved in modulation of phosphorylation system. Therefore, there are two types of early messengers leading to activation of cellular response to different forms of stress. First type represents the appearance of high concentration of denatured proteins resulted in dissociation of HSF1 complexes with chaperones and HSF1 trimerization; second type of messengers includes the increase in concentration of low molecular weight agents such as cermide or cAMP and calcium release from endoplasmic reticulum to the cytosol. These substances trigger protein kinase activation and switching on various regulatory cascades (Nikolova-Karakashian and Rozenova 2010). Low molecular weight mediators, including cAMP serve as important links in many signal pathways including hormone-inducible and, hence, their participation in stress response is not surprising. It is of note, that phosphorylation of different serine residues may have opposite

effect on HSF1 activity. Therefore, while it is known that activation of HSF1 requires phosphorylation of Ser326, phosphorylation of Ser303 and Ser307 leads to inactivation of HSF1 and represents an obligatory step of negative regulation of heat shock response. Furthermore, phosphorylation at Ser303 and Ser307 residues takes place in the course of recovery process after HS and is accomplished by mitogen-activated/extracellular signal regulated protein kinases (MAPK/ERK), p38/JNK (c-jun N-terminal kinase) and glycogen synthase kinase-3β (GSK-3). Stress-induced protein kinases p38 and JNK activity may lead to the death of cells by apoptosis after severe stress by means of HSF1 inactivation and blocking *Hsp* genes induction (Anckar and Sistonen 2007; Chu et al. 1996; He et al. 1998).

It is of note that there is strict hierarchy in phosphorylation of different serine residues within HSF1 molecule. Thus, phosphorylation of Ser303 occurs only after phosphorylation of Ser307. Negative influence of Ser303 phosphorylation on HSF1 activity is based on two mechanisms. First, HSF1 phosphorylated at Ser303 position binds with protein 14-3-3epsilon which facilitates its transport from nucleus to cytoplasm (Wang et al. 2004b). Second, phosphorylation at Ser303 residue serves as a signal for sumoylation of HSF1 at lysine-298.

Proteins belonging to SUMO family (SUMO1 and SUMO2/3) are also involved in HSF1 regulation. SUMO family (Small Ubiquitin-related Modifier) comprised several ubiquitin-like proteins that interact with their substrates by means of isopeptide bonds and are involved in the regulation of multiple transcriptional factors. It was shown that SUMO1 binding effectively inhibits transcriptional activity of both HSF1 and HSF2 (Anckar and Sistonen 2007). Similarly, SUMO2/3 proteins are also able to inhibit HSF1 activity. Interestingly, modulation of HSF1 activity by means of SUMO2/3 requires HSP27 which, thus, may modulate the expression of its own genes by negative feedback after excessive accumulation in the cell (Simioni et al. 2009).

In addition to 14-3-3 epsilon and SUMO2/3 oligomeric form of HSF1 specifically binds to HSBP1 (HSF-binding protein 1) which interacts with oligomerization domain of HSF1 (leucine zippers A/B) and inhibits its activity. Thus, over-expression of HSBP1 in C. *elegans* cells renders them less resistant to HS and arsenite (Cotto and Morimoto 1999). Apart from phosphorylation, HSF1 activity is regulated via acetylation/deacetylation of DNA-binding domain. In fact, acetylation of lysine-80 results in the loss of DNA-binding ability of HSF1 which represents one of the important steps in negative regulation of HSF1 activity in the course of heat shock response (Westerheide et al. 2009). General scheme of HSF1 regulation is depicted in Fig. 3.2.

Studies performed on *Drosophila* contributed significantly to the understanding of mechanisms underlying eukaryotic *Hsp* genes and in particular *Hsp70* activation. It was shown that the basic transcriptional machinery is pre-assembled at the stress-responsive genes under non-stress conditions, and it is the binding of HSF1 and its phosphorylation at the promoters that leads to rapid induction of stress-inducible genes expression. Specifically, in *D. melanogaster* cells RNAP II is constitutively bound to the promoters regions of *Hsp70* and *shsps* genes at −12…+65 bp position in relation to the transcription start (Belikov and Karpov 1996; Lis 2007).

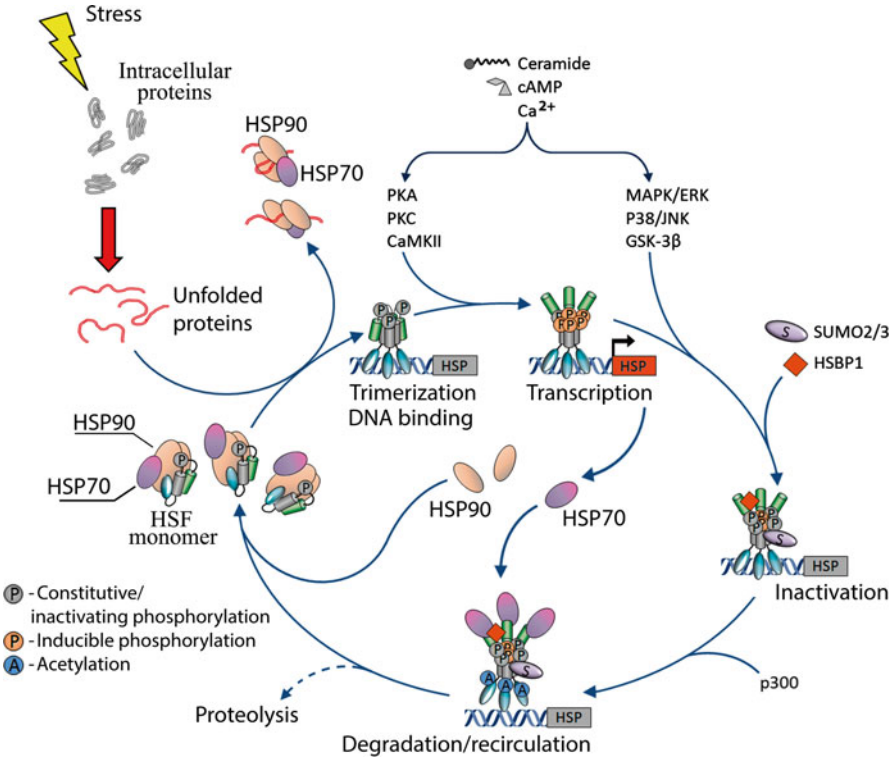

Fig. 3.2 Activation and downregulation cycle of HSF1. Under non-stress conditions HSF1 monomers form complexes with Hsp90 and (as was shown in *Drosophila*) Hsp70. The appearance of large quantities of denatured proteins leads to the dissociation of HSF1 complexes with Hsp90 and its trimerization. Such trimers bind to DNA, but do not induce transcription. At the next stage HSF1 is activated by phosphorylation which renders it effective transcription inducer of the heat shock genes. In turn, phosphorylation at Ser303 and Ser307 residues, triggers sumoylation and as a result subsequent inactivation of HSF1. Stress-induced protein kinases involved in HSF1 phosphorylation are activated by elevated cytosolic levels of ceramide, cAMP and Ca^{2+} ions. HSF1 also interacts with HSBP1 protein, which inhibits its activity. After this HSF1 form complex with Hsp70 which does not influences its DNA-binding activity but effectively excludes HSF1 from transcription machinery. Finally, HSF1 loses its DNA-binding activity due to acetylation and binds to Hsp90 acquiring monomeric inactive form. In certain cases inactivated HSF1 may be subjected to proteolysis (Åkerfelt et al. 2010; Anckar and Sistonen 2007; Belikov and Karpov 1996; Lee et al. 1992; Peng et al. 2010; Zhang et al. 1998, 1999)

The packaging of DNA into nucleosomes affects all phases of the transcription cycle. Specifically, recruitment of chromatin remodeling factors to *hsp* genes plays a key role in their rapid induction by providing transcription factor accessibility at stress-responsive genes promoters (Guertin and Lis 2010; 2013). It was demonstrated that 5′-end of *Drosophila Hsp70* genes is devoid of histone H1 under non-stress conditions and contains a nucleosome-free region that extends further downstream (Karpov et al. 1984; Shopland et al. 1995; Tsukiyama et al. 1994). In response to HS chromatin architecture throughout *D. melanogaster Hsp70* genes

undergoes an initial dramatic change, a change that does not depend on transcription, followed by a second disruption of nucleosome structure that is transcription-dependent (Petesch and Lis 2008).

The widespread changes in chromatin structure at *Hsp70*-containing region following HS also critically dependent on poly (ADP-ribose) polymerase (PARP). PARP has been previously identified as a critical factor for polytene chromosome puffing in response to HS. Catalytic inhibition of PARP results in the failure of *Hsp70* to undergo rapid transcription-independent loss of the chromatin structure following HS (Petesch and Lis 2008). These data suggest that production of long nucleic acid-like poly(ADP-ribose) (PAR) molecules may result in a histone loss and chromatin decondensation throughout the whole length of transcribed DNA, the event that facilitates the movement of RNAP II along DNA (Guertin et al. 2010; Petesch and Lis 2012).

These peculiarities of regulatory regions facilitate extremely rapid response of *Hsp* genes transcription machinery to HS and other forms of stress. Thus, *Hsp* mRNA in *Drosophila* cells is detectable within 15–30 s after HS. In *D. melanogaster* the product of *trl* gene plays important role in supporting the open chromatin structure and RNAP II positioning on the *Hsp70* and certain other *Hsp* promoters. This protein designated GAGA-binding factor (GAF) is able to bind with GA/CT repeats (Omelina et al. 2011). *Drosophila Hsp70* promoters contain several regularly spaced GAF-binding sites within 150 bps upstream of transcription start site in direct (GAGAGAG) and inverse (CTCTCTC) orientations (Wilkins and Lis 1997; Georgel 2005). Binding GAF to *Hsp70* promoter triggers modification of histone H3 throughout the whole *Hsp70* genes transcribed region and results in chromatin decompactization. It is assumed that insulators scs (specialized chromatin structures) and scs' represent boundaries of DNA decompactization in *Hsp70* gene cluster in *Drosophila* (Hart et al. 1997; Petesch and Lis 2008). More details concerning the functions of these insulators are provided in Chap. 5.

Apart from chromatin modification, GAF is required for interaction of RNAP II with transcription initiation region in complex with general transcription factors (GTFs) such as TBP (TATA-box binding protein). Interestingly, other general transcription factors from TFII family are present in the promoters of *Hsp70* genes under non-stress conditions but dissociate after temperature elevation (Lebedeva et al. 2005). In the case of *Drosophila* in non-stressed cell *Hsp* genes are already bound by RNAP II, but the polymerase is paused after transcribing 20–40 nucleotides. The paused polymerase is associated with NELF (negative elongation factor) and Spt4/5 (or DRB sensitivity inducing factor, DSIF), that effectively inhibit transcription elongation. Following temperature elevation or other challenge, escape of the paused polymerase requires recruitment and activation of HSF resulting in dissociation of NELF. At the next stage the large subunit of RNAP II is phosphorylated at C-terminal domain (CTD) by positive transcription elongation factor PTEFb (Lee et al. 2008a, b; Wu et al. 2003) to activate transcription. PTEFb also phosphorylates negative elongation factor (NELF) and transcription elongation factor Spt5, thus releasing polymerase into productive transcription elongation (Fig. 3.3). Interestingly, HSF does not directly interact with RNAP II rather acts through the

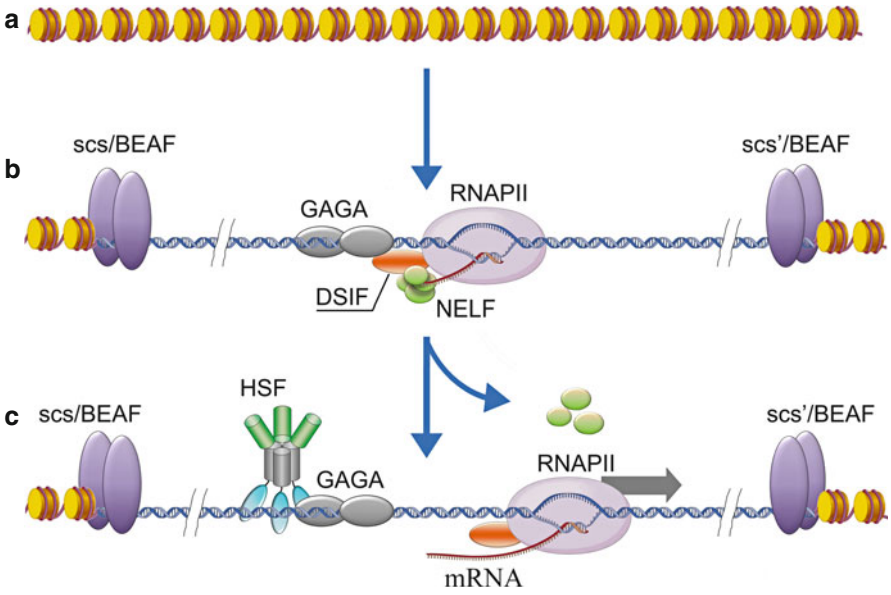

Fig. 3.3 Role of GAGA-factor (GAF) in transcription activation of *D. melanogaster Hsp70* genes. Interaction of GAF with promoters results in chromatin decondensation in *Hsp70* genes region providing conditions for the formation and positioning of RNAP II preinitiation complex. In non-stressed cells after initiation RNAP II interacts with DSIF and NELF factors and is paused after transcribing 20–40 nucleotides (**a**, **b**). Afterwards, RNAP II bound with short transcript and DSIF and NELF factors remains in the transcriptional pausing state until binding of HSF takes place. Interaction of HSF with transcriptional complex results in dissociation of NELF and transition of RNAP II into elongation stage (**c**). scs/scs′ elements bound with BEAF32 protein serve as boundaries of decondensed active chromatin (Modified from Hart et al. 1997; Petesch and Lis 2008; Wu et al. 2003)

mediator complex (Park et al. 2001). It is of note, that the described mechanism of *Hsp70* genes activation is not universal even for different *Drosophila* heat shock genes. For instance, promoters of *Hsp68* и *Hsp83* genes do not contain GAGA motifs and, hence, do not interact with GAF. However, the transcription of these genes is strongly induced by HS and they form large puffs in polytene chromosomes after temperature elevation and other forms of stress.

In mammals under normal temperature conditions CHBF protein (constitutive HSE-binding factor) or Ku-autoantigen is bound to the promoter of *HSP70* (Kim et al. 1995; Tang et al. 2001; Turturici et al. 2009; Yang et al. 1996). This protein exists in heterodimeric form consisting of two subunits with molecular masses 70 and 86 kD, respectively. Ku-protein was originally described as transcriptional factor for RNA Polymerase III involved in tRNA synthesis. Additionally, CHBF (Ku-protein) plays important role in DNA reparation system and in the process of V(D)J-recombination (Nussenzweig et al. 1996). In the case of *HSP70* regulation, Ku interacts with Sp1 and GAGA-binding protein, and may play a significant role in the *HSP70* constitutive expression (Turturici et al. 2009). In *Drosophila* YPF1

(Yolk protein factor 1) is orthologues to the mammalian Ku-protein and binds specifically to *Hsp70* promoters in non-stress conditions (Jacoby and Wensink 1994). Heat shock alleviates this interaction and CHBF is partially substituted by activated HSF1. The reverse process occurs during recovery from HS.

Certain chemical agents such as arsenite or kadmium chlorid may induce the transcription of constitutive member of *Hsp70* family, namely *Hsc70*. However, after such treatment inducible Hsp70 may not be expressed and CHBF may remain bound to HSE. Similarly, while mild HS (41 °C) in mammalian cells induces HSF1 activation the expression of Hsp70 is not increased and CHBF remains bound to the promoter. Only after acute HS the expression of *HSP70* genes is dramatically activated and CHBF is released from promoter and superseded from transcriptional complex by HSF1 (Yang et al. 1996).

In 2005 a new factor termed "menin" involved in *D. melanogaster hsp* genes regulation has been described (Papaconstantinou et al. 2005). It was shown that menin belongs to stress-inducible factors and binds to *Hsp* genes promoters after HS. Inactivation of menin results in the time decrease of *Hsp70* and *Hsp23* transcription after HS, while over-expression of menin significantly prolonged *Hsp* transcription period after HS and during the recovery period.

As we mentioned above, in different organisms there are other factors involved in *Hsp* genes transcription regulation besides HSF1. Thus, small heat shock genes in *Drosophila* may be induced by molting hormone ecdysone (Cheney and Shearn 1983; Thomas and Lengyel 1986). In mammalian cells Sp1 protein as well as CBP (CCAAT-binding protein) and CTF (CCAAT-box-binding transcription factor) binds to promoters of *HSP70* genes providing their constitutive transcription (Bevilacqua et al. 1997; Morgan et al. 1987). Interestingly in mammals promoters of *HSP70A1A*, *HSP70A1B* and *HSP90* genes contain recognition sites for NF-kB and STAT-3. These proteins can regulate heat-shock genes in response to cytokine (TNF and interferon-γ) stimulation. Furthermore, NF-IL6 and HSF1 interacts with each other as antagonists (Stephanou et al. 1999; Tang et al. 2001).

Recently group of Evgeny Nudler described in mammalian cells long non-coding RNA termed "HSR1" (heat shock RNA-1) which according to their experiments serves as thermosensor and represents another key determinant in this process (Shamovsky et al. 2006; Shamovsky and Nudler 2009). They showed that *in vitro* HSR1 forms a complex with eEF1A (eukaryotic elongation factor 1A), which is required for HSF1 trimerization and its subsequent DNA binding (reviewed by Place and Noonan 2014). Unfortunately, HSR1 gene was not found in the genome of any mammalian species yet.

The system comprised of HSF and associated factors responsible for activation of "classical" heat shock genes is triggered predominantly by increased concentration of denatured proteins in the cytosol. Besides temperature elevation and other non-specific stressful stimuli influencing all cellular systems, there are other forms of stress (e.g. administration of inhibitors of N-glycosylation) that act selectively at the level of protein folding in endoplasmic reticulum (ER). Therefore, cells need to specifically control the state of proteins in endoplasmic reticulum. Indeed, such system designated "UPR" (unfolded protein response) exists and effectively

functions (Chen and Brandizzi 2013). UPR system includes several chaperones (ER-chaperones) translocated to endoplasmic reticulum after HS. The synthesis of this class of chaperones represents adaptive reaction at the cellular level in response to abnormal protein folding in the ER.

On the other hand activation of UPR system may induce cell death by apoptosis in the case of severe stress which cannot be compensated by the chaperones protective activity (Chen and Brandizzi 2013).

Like HSF-regulated response, UPR system is rather conserved. Ire1 protein (inositol-requiring enzyme 1), represents the basic element of this system. In different organisms including yeasts, mammals and plants Ire1 serves as receptor able to sensor stressful stimuli at the level of endoplasmic reticulum and activate transcription of the target genes. Ire1 is a typical transmembrane protein localized in the endoplasmic reticulum membrane and comprised of three functional domains: stress-sensory intraluminal domain which serves as receptor and two catalytic domains of cytosolic localization, responsible for protein kinase and endoribonuclease activities, respectively (Fig. 3.4). In mammals there are two isoforms of Ire1 – IRE1α and IRE1β. Under non-stress conditions IRE1α is blocked by interaction of intraluminal domain with HSPA5/BiP. After stress IRE1α and BiP dissociate from the complex because misfolded or denatured proteins that appear in endoplasmic reticulum have higher affinity to BiP.

Activation of HSF and UPR-systems in mammalian cells shares several important features. In both cases chaperones play a role of negative regulators under non-stress conditions and form stress-sensory complex with major transcription factor (HSF1 or Ire1). This complex readily responds to the accumulation of denatured proteins. On the other hand in the case of Ire1 in yeast and IRE1β in mammals activation is driven by direct interaction with denatured proteins, and is independent on BiP dissociation from the complex (Chen and Brandizzi 2013). Following activation, Ire1 oligomerizes and undergoes autophosphorylation leading to drastic conformation change and activation of endoribonuclease domain. Subsequently, endoribonuclease performs noncanonical splicing of the mRNA encoding factor, responsible for transcription of UPR target genes, described in yeast, plants, *Drosophila* and mammals, as Hac1, bZIP60 and Xbp-1, respectively. Under normal conditions mRNA of these genes is localized in cytosol in inactive state. After UPR-system activation the intron containing translation attenuator is cleaved out from the target mRNA by Ire1 endoribonuclease (Mori et al. 1996, 1998). Therefore, after splicing Hac1/bZIP60/Xbp-1 mRNA may be efficiently translated. Synthesized protein is then translocated into the nucleus and binds to regulatory regions of target genes drastically enhancing their transcription efficiency (Fig. 3.4). In yeast Hac1 recognizes specific nucleotide motif designated UPRE (unfolded protein response element). The sequence represents imperfect palindrome with 1 bp spacer: CAGCGTC (Mori et al. 1998). In yeast UPRE-like elements are involved in regulation of seven genes including *Kar2*, *Lhs1* and *Cer1p* that belong to *Hsp70* family. These genes (e.g. *Kar2*) may contain HSE motifs within promoter region and, hence, be regulated by HSF (Foti et al. 1999). On the other hand, yeast HSF as well as Hac1 may be activated through phosphorylation by protein kinase Snf1 in

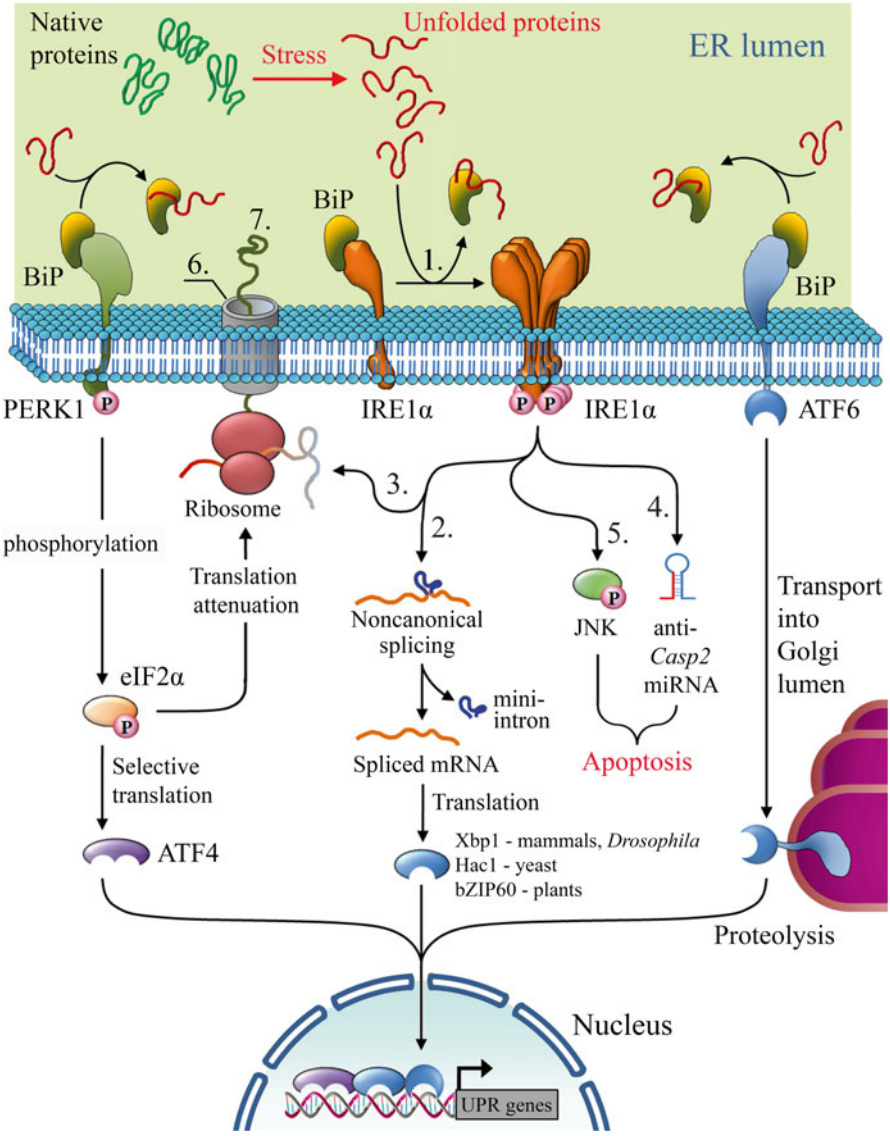

Fig. 3.4 General scheme of UPR regulation. *1* Appearance of unfolded proteins as a result of the ER stress leads to the Ire1α-BiP complex dissociation and Ire1α oligomerization. After this Ire1α autophosphorylation and activation occurs, and Ire1α induces a few signal cascades: *2* noncanonical splicing of mRNAs coding Xbp1/Hac1 transcriptional factors; *3* ER-proteins mRNAs cleavage leads to the block of the translocation of certain proteins into ER and subsequent apoptosis in the case of severe stress; *4* and *5* proapoptotic signalling through antiapoptotic miRNA cleavage and phosphorylation and activation of JNK. Translocone and nascent protein translocated into ER are denoted in this scheme as *6* and *7* respectively. *ER* endoplasmic reticulum, *BiP* binding immunoglobulin protein, *JNK* c-jun N-terminal kinase (Modified from Lee 2001; Chen and Brandizzi 2013)

response to ER-stress stimuli, e.g. glucose deprivation (Hahn and Thiele 2004). In mammals, besides, Xbp-1 there are several other transcriptional factors such as ATF4 and ATF6, as well as NF-Y, YY1 and YB-1 involved in regulation of ER-chaperones (Li et al. 1997; Roy et al. 1996). In order to respond to UPR activation promoters of target genes in mammals should contain two alternative sequences designated ERSE and ERSE-II (ER stress element). ERSE represents three structure unit 5′-CCAAT(N_9)CCACG-3′, where N_9 – GC-rich region with consensus CGGCGGCGG (Foti et al. 1999; Lee 2001; Roy and Lee 1999). In humans promoter of *HSPA5* gene contains three ERSE elements spaced at −126, −94 and −61 bps upstream of TATA-box. Promoter of *GRP94* gene contains two ERSE in inverse orientation located at −195 and −137 bps upstream the transcription start site and the third ERSE at -72 bps. ERSE-II (ATTGG(N)CCACG) was found in the promoter of *Herp* and several other genes (Lee 2001).

Apart from splicing of Xbp-1 mRNA in *Drosophila* and mammals Ire1 cleaves various RNA preventing the translation of certain proteins transported into endoplasmic reticulum by means of recognizing consensus sequences responsible for endoplasmic localization of the proteins (Regulated IRE1-Dependent Decay, or RIDD). Thus, Ire1 activity blocks the translocation of certain proteins into ER until the conditions necessary for normal folding of the proteins in ER are restored. Extremely severe stress instead of adaptive response may induce apoptosis due to cleavage by Ire1 of a few anti-apoptotic pre-mRNAs involved in suppression of caspase-2 (Chen and Brandizzi 2013).

In contrast to yeast, in mammals besides Ire1 two other factors (PERK and ATF6) are involved in regulation of UPR-system (Fig. 3.4). Similarly to Ire1, they represent typical transmembrane proteins activated by stress factors interfering with normal function of endoplasmatic reticulum. PERK (RNA-dependent protein kinase-like ER kinase) is a protein kinase which attenuates translation by phosphorylation of eIF2α, one of the key translation initiation factors. The decline of translation level as well as cleavage of certain mRNAs by Ire1 leads to general decrease in the amount of proteins transported to ER subjected to denaturation and aggregation and, hence, facilitate the functioning of chaperonic machinery under stress conditions. On the other hand, phosphorylated eIF2α initiates translation of a small specific subpopulation of mRNAs containing small upstream open reading frames (suORFs) in its 5′-leader region including ATF4 (activating transcription factor 4) which in turn activates the transcription of certain UPR target-genes (Harding et al. 2000). The third ER-stress response factor is ATF6 (activating transcription factor 6). When ATF6 is activated it is translocated into Golgi complex where it undergoes specific proteolisis leading to cleavage of cytosolic domain. This domain is transported into the nuclei, where it binds regulatory sequences in dimeric form and activates the target genes (Fig. 3.4) (Chen and Brandizzi 2013). It is important to note that PERK and ATF6 are activated only after their dissociation from complex with BiP (de la Cadena et al. 2013).

After heat shock and several other forms of stress in parallel with dramatic activation of *Hsp* genes battery, the repression of virtually all other genes takes place. This is clearly illustrated by a rapid regression of all non-heat shock inducible

puffs in *Drosophila* polytene chromosomes after HS. Ribosomal and histone genes remain transcriptionally active after HS (although their splicing is inhibited) as well as tRNA loci and genes encoded by mitochondrial genome (Bonner and Pardue 1977; Bonner et al. 1978). Temperature elevation results in rapid disruption of old polysomes and formation of new ones where Hsps are predominantly synthesized. The disruption of old polysomes occurs not as a result of old and newly synthesized mRNA competition. Thus, if simultaneously with the temperature increase α-amanitin or actinomycin D is added to the cells, new mRNAs would not be synthesized, but the old polysomes, however, would be mostly destroyed. Some pre-HS polysomes are stored in the cytoplasm but their translation is blocked. When the temperature returns to normal the synthesis of normal cellular proteins resumes on these preexisting polysomes (Kruger and Benecke 1981).

Taken together, temperature elevation and other forms of stress not only represses the transcription of most genetic loci but also blocks the translation of the mRNAs already present in cytoplasm, decreasing the amount of proteins that may be damaged by stressful stimuli. On the other hand strong induction of Hsps synthesis is definitely of protective nature and serves to protect the genetic apparatus of the cell from harmful stress consequences.

Selective transcription of *HSP* genes in mammalian cells after stress is provided by means of drastic changes in phosphorylation kinetics of the C-terminal domain (CTD) of the large subunit of RNAP II. It is known that during transcription RNAP II CTD phosphorylation occurs at serine and treonine residues. Furthermore, dephosphorylated form of CTD is involved in the formation of transcription initiation complex while the transition to elongation stage requires phosphorylation of CTD. Several CTD-specific protein kinases including DNA-PK and MAPK as well as CTD-specific phosphatase FCP-1 are involved in the dynamic process of CTD phosphorylation/dephosphorylation. It was demonstrated in HeLa cells that specific stress-CTD-kinases (e.g. DNA-PK and MAPK) are responsible for CTD hiperphosphorylation during the HS. It is of note, that CTD-kinases that function in the cells under non-stress conditions are inactivated by temperature elevation and other forms of stress. Simultaneously, inactivation and denaturation of FCP-1 takes place (Dubois et al. 1999; Venetianer et al. 1995). It is likely that hyperphosphorylation of CTD results in the loss of RNAP II affinity to the promoters of non-heat shock genes and, hence, leads to drastic increase of *Hsp* genes transcription.

The second mechanism by which heat shock suppresses gene transcription is through the upregulation of inhibitory non-coding RNAs that block general RNAP II activity. Two non-coding RNA species including B2 RNA in mice and Alu RNA in humans are transcribed by RNA polymerase III from short interspersed elements (SINEs). During normal cellular growth, these RNA species accumulate at relatively low levels; however, their abundance transiently increases by as much as 40-fold under stress conditions (i.e. heat shock). There is no shared sequence homology between B2 and Alu RNAs. However, their biological functions are considered to be very similar if not identical. Following stress, B2 or Alu RNAs directly bind to the active site of RNAP II, disrupting its interaction with promoter DNA and interfering with CTD phosphorylation (Place and Noonan 2014).

It was found in *Drosophila* that after HS activated HSF also binds to promoters of a large set of genes that are not transcriptionally induced by temperature elevation. Characteristically, the promoters of these genes are usually enriched with the insulator protein, BEAF prior to HS (Gonsalves et al. 2011). It was hypothesized that *Drosophila* HSF might play a role in the transcriptional repression of certain genes that are known to be inactivated during HS (Westwood et al. 1991). HSF binding sites were also found in introns of certain genes involved in developmental processes and reproduction. For example, HSF during HS binds to introns of the three major ecdysone-inducible genes, and HSF binding with these genes is coincident with their repression. Therefore, HSF may play a direct role in the repression of these genes (Gonsalves et al. 2011).

Along these lines, it was demonstrated that in mammalian cells HSF1 may repress the transcription of TNFα gene by binding to its 5'-UTR region (Singh et al. 2002).

Importantly, HS effectively inhibits splicing of normal cellular pre-mRNAs. It is of note, that inducible *Hsp* genes in most organisms are intronless and, hence, do not require splicing for normal function. It was shown that high molecular weight Hsps such as Hsp90 and Hsp70 in cooperation with Hsp40 are involved in restoration of normal splicing process after stress termination (Vogel et al. 1995). Interestingly, in organisms with intron-exon arrangement of *Hsp* genes (e.g. *Trypanosoma*, *C. elegans*), splicing of *Hsp70* mRNA occurs with maximal efficacy after moderate HS while splicing of "normal" cellular genes such as tubulin is concomitantly blocked by not yet described mechanism (Huang and Van der Ploeg 1991).

Furthermore, HS and certain viral infections severely inhibit the translation of most cellular mRNAs due to drastic changes in the translation initiation complex. The mechanisms underlying translation inhibition by HS and during viral infection share many features. As a result, in both cases strong activation of double stranded RNA-activated protein kinase (PKR) is usually observed. Furthermore, hypoxia and HS leads to activation of protein kinase HRI (heme regulated inhibitor of translation), while the disturbance of ER functions by stress activate protein kinase PERK. Both protein kinases phosphorylate α-subunit of eukaryotic translation initiation factor eIF2 at serine-51 (Sheikh and Fornace 1999). After phosphorylation eIF2 is incapable to perform the GTP-GDP exchange in complex with eIF2B factor, and, hence, the blockade of translation initiation occurs. The degree of eIF2α phosphorylation is increased two to three folds after temperature elevation (Duncan et al. 1995; Gallie et al. 1997; Menon and Thomason 1995).

Dephosphorylation of eIF4E-bound proteins including eIF4E-BP1, eIF4E-BP2 and eIF4E-BP3 represents the second mechanism of translation inhibition after stress including HS. Dephosphorylation blocks the interaction of cap-recognizing factor eIF4E with eIF4G (but not with cap itself). Dephosphorylated form of eIF4E-BP exhibits high affinity to eIF4E and its binding results in general decrease of total translation level in the cell (Vries et al. 1997). Heat shock and viral infection induce dephosphorylation of eIF4E, decreasing its affinity to mRNA 5′-cap. It was also demonstrated that Hsp27 inhibits the activity of eIF4G. *In vitro* and *in vivo* experiments revealed their direct interaction. It was shown that Hsp27 binds to

eIF4G and transforms it into insoluble state. In contrast, Hsp70 initiates the reverse process leading to the restoration of eIF4G activity (Cuesta et al. 2000).

After HS or viral infection concomitant to substantial drop in translation efficiency (up to ten fold) in the affected cells selective translation of viral or *Hsp* mRNAs proceeds. Such selectivity is provided (among other reasons) due to the presence of specific motifs within the sequences of viral or *Hsp* RNAs. The characteristic differences at the 5′-UTRs of mRNAs of heat shock genes and "normal" cellular genes transcribed under non-stress conditions were detected soon after the discovery of HS response at the molecular level. Thus, in most organisms including *Drosophila* species and various mammals, *hsp70* mRNA contains 250 bps 5′-leader consisting predominantly of purines. Different lines of evidence suggest that translation of *HSP70* mRNA in humans after HS resembles translation of picornaviruses or late adenoviruses mRNAs. It is generally assumed that after HS *HSP70* mRNA is translated exploring mechanism designated by different author as "jumping", "shunting" or "hopping" (Yueh and Schneider 2000). According to the assumed scheme, in the process of scanning of the 5′-leader, ribosomal 40S subunit "jumps" over certain region. Such a mechanism is determined by the secondary structure of 5′-UTR leader of *HSP70* mRNA. Thus, under normal conditions 40S ribosome may scan the whole length of 5′-leader while after HS or viral infection the translation of specific mRNAs containing certain structure in their leaders occurs by "jumping" mechanism. This mechanism does not require a complete assembly of translation initiation complex, hence, enabling *HSP70* or adenovirus mRNA to be selectively translated. 5′-UTR of adenoviral mRNA (Ad5) contains three regions (C1, C2 and C3, respectively) exhibiting strict complementation with two regions of the hairpin located at the 3′-end of 18S RNA from 40S ribosome subunit. The deletion of both C2 and C3 regions results in 20 fold drop in translation efficacy corroborating their direct involvement in the process. Similarly, 5′-UTR of mammalian *HSP70* mRNA contains a region complementary to 3′-hairpin of 18S rRNA and the deletion of this particular region decreased the translation after 44 °C HS more than three times. Molecular mechanism underlying initiation in the case of *HSP70* and adenoviral mRNAs is not fully understood yet, but possibly resembles that of prokaryotes which requires Shine-Dalgarno sequence (Yueh and Schneider 2000).

According to other authors (Hernández et al. 2004; Rubtsova et al. 2003) *HSP70* mRNA is translated through a well-known mechanism described for many viral mRNAs. This mechanism is based on internal initiation due to the presence of IRES sequences (Internal Ribosome Entry Site). IRES binds to preinitiation complex together with 40S ribosome subunit and eIF2 and eIF3 factors and positioned mRNA start codon exploring cap-independent mechanism missing preliminary scanning of 5′-UTRs with involvement of eIF4G and eIF4A factors. Interestingly, the addition of *HSP70* 5′-UTR to a reporter construct enhances its translation efficiency more than 100 fold which is close to the effect of classical picornavirus IRES (Rubtsova et al. 2003).

In this way, heat shock causes inactivation of the cap-binding initiation factor eIF4E and drastically reduces the abundance of the both components and assembled translational initiation complexes. Under these conditions, rapid inhibition of

normal cellular mRNAs translation occurs, while *Hsp70* mRNA is selectively translated by cap-independent mechanism.

Recently it was found using mouse and human cells that severe heat stress triggers global pausing of translation elongation of most cellular mRNAs at around 65 codon. It was also demonstrated that severe HS reduces HSC70/HSP70 association with ribosomes and alters interactions with the translational machinery. Preferably pausing occurs on nascent peptides with hydrophobicity of the N-termini which binds with Hsp70. In the absence of ribosome-associated Hsp70 during heat stress, nascent peptides with stronger Hsp70 binding motifs might have a greater tendency to misfold or aggregate, potentially impacting the efficiency of translation. Hsp70 overexpression or mild preliminary heat shock protects cells from heat shock-induced elongation pausing. Elongation pausing represents an important component of cellular stress responses (Shalgi et al. 2013).

It is of note, that although the details of heat shock genes activation and functioning attracted a lot of attention (see above) the whole picture is far from being clear. Thus, recently Prof. Nudler and his colleagues demonstrated that in mammals translation elongation factor eEF1A1 which has a well-defined role in the protein-synthesis machinery also functions as an essential regulator of human HSPs. This factor was shown to participate in each major step of Hsps induction. Upon stress, nuclear pool of eEF1A1 increased dramatically and the protein activates transcription by recruiting HSF1 to *Hsp* promoters. Subsequently, this factor associates with 3′UTRs of HSP mRNAs, stabilizing them and facilitating their transfer from the nucleus to the ribosomes. Interestingly, prior to stress, eEF1A1 is required for *Hsp* gene silencing (Vera et al. 2014).

3.1 Conclusions

Heat shock response system is highly conserved in all eukaryotes and comprises the same regulatory features practically in all organisms studied. Different forms of stress cause accumulation of misfolded proteins and a few specific low-molecular messengers (such as ceramide) in the cell. Misfolded proteins interact with molecular chaperones as high-specific substrates which results in dissociation of the temperature sensitive complex between certain Hsps and HSF, the major stress transcription factor. After that HSF forms homotrimers, which specifically recognize heat shock regulatory elements (HSEs) within promoters of *Hsp* genes and activate transcription. After Hsps accumulation following heat shock they bind and inactivate HSF by negative feed-back mechanism. Besides general transcription factors there are multiple coactivators of HSR that participate in modifications of histones and subsequent decompactization and remodeling of chromatin landscape which determines HSF binding at the regions of heat-induced transcription. Besides HSF, there are many other proteins involved in heat-induced transcription and translation of *Hsp* genes (e.g. eEF1A1). Thus, HS response represents a complex multilevel system, very flexible in terms of the ability to rapidly react to a variety of environmental and metabolic stresses.

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Chapter 4
Heat Shock Proteins and Adaptation to Variable and Extreme Environments

4.1 General Response to Heat Shock and Other Forms of Stress

Eukaryotic organisms evolved sophisticated sensing mechanisms and generic responses at the cellular level to various forms of mild, chronic or acute stresses including HS. Exposure to elevated temperature as well as many other stressful factors disturbs normal folding of proteins while preexisting proteins may undergo denaturation and aggregation which is harmful for the cell (Hightower 1991; Szalay et al. 2007). Gene expression changes are a major component of various stress responses, along with alterations in protein-lipids ratio, rate of metabolism, fragmentation of Golgi complex and endoplasmic reticulum and other multiple disturbances (Kruuv et al. 1983; Vigh et al. 2007; Welch and Suhan 1985). Drastic changes in cytoskeleton structure (Toivola et al. 2010; Welch and Suhan 1986) and drop in ATP level in the cell represent important landmarks of stress response observed in different organisms (Lambowitz et al. 1983; Patriarca and Maresca 1990). Specifically, HS inhibits process of splicing of cellular mRNAs and may lead to complete blockade of cell cycle (Yost and Lindquist 1986). Acute HS usually resulted in the accumulation of high concentration of denatured proteins may also induce cell death by apoptosis or necrosis. Notably, adaptive responses to stress definitely depend on the organism and the natural environments in which it has been evolved and underwent selection.

Live organisms exhibit amazing plasticity in the regulation of genes involved in response to different forms of stress. Up- and down regulation of the expression of multiple genes in response to stress play central role in the adaptation of organisms to both short-term and long-term pronounced changes of environment. The regulation of adaptive response may be realized at the level of transcription induction, translation efficacy and/or stability of resulted mRNAs or proteins.

Stress response usually represents a choice between two antagonistic programs such as induction of adaptogenes including various Hsps or switching on the genes

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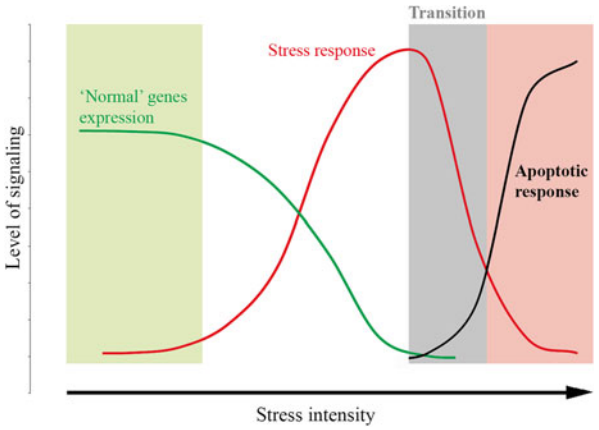

Fig. 4.1 Stress response in terms of "normal" cell genes expression changes and apoptosis process depending on stress severity

underlying cells death by apoptosis (Anckar and Sistonen 2007; López-Maury et al. 2008; He et al. 1998; Chen and Brandizzi 2013; Chu et al. 1996). Moderate heat shock predominantly leads to the induction of heat shock genes playing chaperoning function to prevent proteins misfolding and degradation and, hence, provide adaptive response. More severe heat stress inhibits the expression of *Hsp* genes and activate the expression of genes responsible for apoptosis (Fig. 4.1) (Chen and Brandizzi 2013; Murray et al. 2004).

At the present time during genomic era, new approaches including High-throughput Sequencing and DNA microchip technologies have been developed and used to monitor the consequences of heat shock and other forms of stress at the genome level. These studies performed in yeast, fruit flies and mammals including humans enabled one to describe complete transcriptome after various forms of stress including HS. The large-scale transcriptomic study of many species showed that organisms respond to drastic temperature changes with a multitude of transcriptional changes: up to 40 % of genes showed an altered expression after temperature exposure experiments depending on the species. High temperature has usually stronger effects on gene expression than low temperature, reflected by the higher number of temperature-responsive genes. Such studies help to gain insights into molecular processes underlying induced thermotolerance and acclimation and allowed to describe a lot of genes besides "classical" *Hsp* genes that dramatically changed their expression after temperature elevation in yeast (Gasch et al. 2000; Lyne et al. 2003), *Drosophila* (Girardot et al. 2004), human cells (Murray et al. 2004), *E. coli* (Riehle et al. 2005), fishes (Buckley et al. 2006) etc. Such studies revealed hundreds of stress-related genes and demonstrated that the level of transcription and translation modulation depends on the severity and duration of stress applied. It is of note that phylogenetically distant forms express functionally similar groups of genes including heat shock genes, antioxidative genes, genes involved in the metabolism of hydrocarbons, and genes involved in ubiquitin-dependent degradation of proteins (Buckley et al. 2006). The role of many genes with temperature-dependent expression is not clear yet. For example, it was shown in *Drosophila* that heat shock leads to significant induction of transcription of genes *shark* and *TotA*

(from *turandot* family), however, functions of these genes in the cells after HS were not revealed (Ekengren and Hultmark 2001).

We are well aware that Hsps discussed in this review is not the only protective molecules providing heat-resistance in various organisms. Thus, in other strains and species, mechanisms of basal thermotolerance may include synthesis of thermoprotective osmolites such as trehalose in the yeast (*Saccharomyces cerevisiae*), (Hottiger et al. 1987) and homeoviscious adaptation (i.e. adjustment in the lipid constituents of cells). Besides, thermotolerance may be due to enhanced stability of cellular proteins through modification of their primary structure and regulated depression of normal house-keeping cellular functions to moderate energy requirements under stressful conditions. Thus, in yeast the production of the disaccharide trehalose is induced by temperature elevation more that 20-fold (Hottiger et al. 1987). Transgenic strain of *D. melanogaster,* transformed with overexpressed trehalose-6-phosphate synthase gene (*tps1*), exhibits high tolerance to hypoxia. It was shown that the increase of trehalose concentration in the cells prevents aggregation of various proteins such as Na^+/K^+-ATPase and tubulin and participates in proteins folding in cooperation with chaperones (Chen et al. 2002; Diamant et al. 2001). Generally speaking, adaptive stress responses may be divided into generic responses shared by many stresses and specific responses evolved to cope with a particular challenge (Feder 2007; Lindquist 1986; Nadal et al. 2011). Below we shall discuss in detail the presumptive adaptive roles of heat shock proteins synthesized in response to HS and many other stresses in various eukaryotic organisms, although we clearly understand that stress tolerance results from numerous physiological and molecular mechanisms, of which Hsps are collectively only one.

All proteins and other compounds synthesized in response to temperature elevation may be roughly divided into the following major groups:

1. Molecular chaperones comprising Hsps *per se* and their co-factors involved in restoration of misfolded cellular proteins and, hence, prevention of harmful aggregates formation (Ellis et al. 1989; Lindquist 1986).
2. Components of ubiquitin-proteasome system (UPS) responsible for degradation of irreversibly damaged and denatured proteins (Raboy et al. 1991).
3. RNA- and DNA-modifying enzymes necessary for DNA reparation and restoration of normal RNA-processing mechanisms (Bügl et al. 2000; Jantschitsch and Trautinger 2003).
4. Carbohydrates metabolism enzymes (Buckley et al. 2006; Malmendal et al. 2006; Voit and Radivoyevitch 2000).
5. Regulatory proteins including transcriptional factors and protein kinases.
6. Transport and antioxidant proteins (e.g. glutathion S-transferase) as well as enzymes involved in toxin inactivation (cytochrome p450 etc.).
7. Proteins involved in cellular membranes stabilization (Welker et al. 2010).
8. Non-coding RNAs, e.g. *hsr-ω* (Arya et al. 2007; Jolly and Lakhotia 2006).

Therefore, rapid and abundant synthesis of various Hsps represents the most generic cellular response after temperature elevation, hypoxia, high concentrations of heavy metals, oxidative stress and other multiple forms of stresses. The synthesis of similar but not identical groups of Hsps has been repeatedly demonstrated for

organisms from different unrelated phyla including yeast (*S. cerevisiae*), mollusks (*Mytilus*), insects (*Cataglyphis, Drosophila, Bombyx mori*), fishes (*Gillichthys*), amphibians (*Xenopus*), reptiles (*Phrynocephalus, Gymnodactylus, Lacerta*) etc. (reviewed by Feder and Hofmann 1999). Although it was shown by different groups that Hsps are actively synthesized after stress in the laboratory, it was necessary to prove by field work that their synthesis represents adaptive response evolved in nature to maximize cell and whole organism survival in response to drastic changes in the environment that put them at risk. It was also important to dissect the mechanisms underlying general HS response and define specific roles of individual Hsps families in ecological context in buffering extracellular challenges to minimize intracellular damage.

In general, all organisms may be divided into two groups: stenothermal and eurythermal species. Stenotherms inhabit areas with narrow temperature ranges and may be further subdivided into thermophilic and cold-adapted organisms. Endoparasites of mammalians represent good examples of thermophilic stenothermal organisms. On the other hand, Antarctic animals that have evolved for many millions of years under highly stable, cold temperature represent extreme cold-adapted stenotherms (Somero 2005). Eurythermal species dwell in habitats with highly variable temperature and intertidal organisms that experience every day fluctuations represent an excellent example of this particular group. Notably, from the very beginning Hsp70 was the main object of the investigations on adaptive role of Hsps in aggressive and highly variable environments. Such attention is quite understandable taking into account the key role of Hsp70 family members in proteins folding and transport under normal conditions and after stress (see Chap. 2). Besides, this family of Hsps is highly conserved and abundant synthesis of Hsp70 is easy to detect after HS in various organisms from yeast to humans and flies by Western blotting technique even using heterologous anti-bodies (see Craig et al. 1983; Schlesinger 1990; Feder and Hofmann 1999 for reviews). Therefore, many attempts were performed to correlate the synthesis of Hsp70 with adaptation of various organisms to fluctuating or extreme environmental conditions (Hightower 1991; Hoffmann 2010; Lyashko et al. 1994; Tomanek and Somero 2000; Ulmasov et al. 1993). However, it becomes evident that the unambiguous attribution of stress resistance to Hsp70 or other Hsps groups requires more than correlate evidence (Feder 2007; Jensen et al. 2010; Somero 2005).

4.2 Role of Hsps in Adaptation to Fluctuating Environmental Conditions of Terrestrial Organisms

4.2.1 Interspecific Comparisons (Distant Taxa)

The modes of thermal adaptations certainly greatly depend on the present state of environment and the evolution the species underwent. Many organisms successfully cope with extreme environmental conditions including heat shock by multiple

behavioral adaptations such as hiding and escaping heat stress while others live in habitats with comparatively constant temperature regimes (Feder 2007; Feder and Hofmann 1999; Somero 2005).

Sometimes, different life stages e.g. larvae and adults routinely encounter strikingly different environmental conditions in terms of stress. Thus, while larvae and pupae of *Drosophila* and many other insect species are often heated to stressful temperatures in their natural medium (e.g. rotten fruits) the adults may easily escape high temperature areas and rapidly move to microhabitats with temperatures close to the physiological ones (Feder 1997). Thus, Hamada et al. (2008) identified a thermal sensing pathway in *D. melanogaster* that is tuned on to avoid non-preferred temperatures. The ion channel of the transient receptor potential (TRP) family dTrpA1 functions as a molecular sensor of temperature and activates a small set of anterior cell neurons, the function of which is crucial for selection of preferred temperatures.

Besides, many species from various taxonomic groups including insects and mammals evolved diapauses, hibernation or occasionally desiccation to survive adverse periods of life (Gusev et al. 2011; King et al. 2013; Rinehart et al. 2006).

The comparison of Hsps levels in geographical populations of the same species or different species inhabiting environments with contrasting temperature regimes represents the most widely approach to implicate expression of various Hsps and in particular Hsp70 in adaptation. Such integral ecological approach exploring field studies of non-model organisms may not only shed light on physiological functions of individual Hsps but also permits to evaluate their role in evolution of adaptations to adverse environmental conditions.

Historically, this ecology-based approach with the goal to investigate Hsps specifically in thermally adapted organisms was originally applied in 1980s by several groups including ourselves (Evgen'ev et al. 1987; Feder and Hofmann 1999; Kee and Nobel 1986; Steinert and Pickwell, 1988).

For our large-scale analysis lasted for more than 30 years we used different organisms from leishmania and lizards to mammalian species including camel and human tribes inhabiting thermally contrasting landscapes such as deserts and cold or temperate climatic zones and active at different time during the day.

Thus, we began our studies by comparing cell lines of silk worm (*Bombyx mori*) and gypsy moth (*Lymantria dispar*) (Evgen'ev et al. 1987). It is believed that silk worm (*B. mori*) originated from warm regions of South-East Asia while gypsy moth (*L. dispar*) dwells in cold and moderate climate areas. To our surprise the cells of *B. mori* survive and synthesize all groups of Hsps at extreme temperatures up to 49 °C, while *L. dispar* cells abolish all protein synthesis and die at temperatures exceeding 40 °C. In other words, they behave exactly as *Drosophila* cells and most of the investigated insect species (Dehghani et al. 2011; Feder et al. 1996; Feder and Hofmann 1999; Lindquist 1986; Lozovskaya and Evgen'ev 1984). Subsequently, we performed a comparative analysis of the larval and adult forms of the two above-mentioned moth species and corroborated our results obtained using cell lines. In all such experiments *B. mori* specimens or isolated organs actively synthesized Hsps at higher temperatures and exhibited much higher thermotolerance in comparison with

L. dispar. After this discovery we decided to check whether high thermotolerance coupled with the ability to synthesize Hsps at extreme temperatures represents a common feature of organisms from warm climate zones and deserts.

In the frame of these large-scale studies we compared heat-shock protein expression in several lizard species from Middle Asia sand deserts (Turkmenistan) and common lizard (*Zootoca vivipara* by modern nomenclature or *Lacerta vivipara* in old papers), a species inhabiting temperate areas up to the latitude of the Polar Circle and higher. In the deserts of Turkmenistan the day temperature in summer may exceed 45 °C, while the sand surface is heated up to 70 °C. However, even most resistant lizards (e.g. toad headed sand lizards *Phrynocephalus interscapularis*) cannot survive such extreme temperatures and try to escape over-heating by digging into the sand or by other behavioral adaptations.

We assumed that xeric species inhabiting for a long time extremely hot and dry desert environments should have evolved adaptive behavioral and physiological mechanisms to cope with such adverse conditions and possibly peculiarities of Hsps synthesis represent an essential component of these adaptations. Therefore, we investigated in detail heat shock response (HSR) in nine lizard species from thermally contrasting climate zones including deserts, and these studies in general corroborated our earlier conclusions made when studying Lepidoptera species (Evgen'ev et al. 1987; Ulmasov et al. 1992). Thus, we demonstrated that desert lizard species are able to synthesize Hsps at much higher temperatures in comparison with the species from cold or moderate areas. In our investigation we used an extremely thermotolerant xeric species Chinese toad-headed sand lizard (*P. interscapularis*) active at the day time, a desert species Caspian gecko (*Caspian gecko*) which is active at night, and temperate climate common lizard (*L. vivipara*). The comparison of ^{35}S-methionine incorporation into proteins of the compared species indicated that in highly eurythermal Chinese toad-headed sand lizard (*P. interscapularis*) proteins are still synthesized at 50 °C and higher while in the common lizard *L. vivipara* inhabiting cold and temperate latitudes all protein synthesis is abolished at temperatures higher than 42 °C and the animals rapidly die. Furthermore, there was a clear-cut positive correlation between the constitutive level of Hsp70 in the cells and thermotolerance of all studied species. The analysis of constitutive level of Hsp70 detected in the cells of the above species indicated that toad-headed lizards (*P. interscapularis*) exhibits a maximum concentration of Hsp70, Caspian gecko also exhibits a lower but significant level of Hsp70, while in common lizard the presence of Hsp70 under normal physiological conditions is hardly detectable (Fig. 4.2).

The presence of two isoforms of Hsp70 family that are synthesized at different temperature regimes in *P. interscapularis* represents another important observation made in this study (Fig. 4.3). We designated these proteins Hsp68(+) and Hsp68(−) and demonstrated that while the first protein is synthesized predominantly in response to moderate HS, the latter form Hsp68(−) is constitutively synthesized under normal temperature conditions and after acute HS when the synthesis of all other cellular proteins is completely inhibited (Ulmasov et al. 1992). Subsequently, differential synthesis of various Hsp70 family members depending on the severity of HS was corroborated in many other studies (Feder and Hofmann 1999; Mizrahi et al. 2012; Tomanek and Somero 1999; Tomanek 2005).

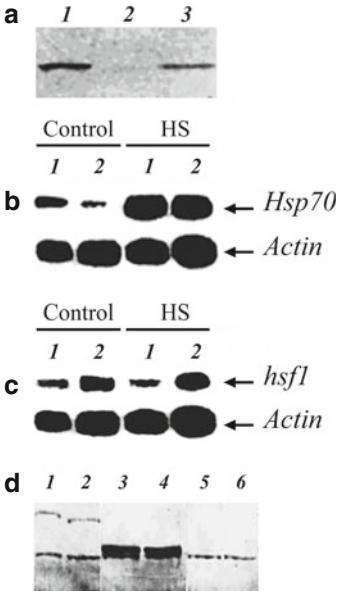

Fig. 4.2 Heat shock response in lizards from contrasting thermal habitats. (**a**) Western blot analysis with antibodies recognizing inducible member of Hsp70 family in different lizard species under normal non-stress conditions. (*1*) *P. intercapsularis*; (*2*) *L. vivipara*; (*3*) *G. caspius*. It is evident that both xeric thermophylic species (*1* and *3*) contain significant amounts of Hsp70 while this protein is not detectable in common lizard from temperate climate (*2*). (**b**, **c**) Northern blot analysis of mRNA present in the cells of *P. interscapularis* (*1*) and *L. vivipara* (*2*) at normal temperature (25 °C) and after HS for 1 h at 42 °C. The membrane was probed with lizard (*L. vivipara*) *hsf1* gene (**b**) and *Xenopus laevis Hsp70* gene (**c**), and reprobed with actin gene to ensure equal loading. (**d**) Western blot analysis of HSF1 levels. *G. caspius* (lanes *1* and *2*); *L. vivipara* (lanes *3* and *4*); and *P. interscapularis* (lanes *5* and *6*) at normal temperature (lanes *1*, *3* and *5*) and after 1 h at 42 °C (lanes *2*, *4* and *6*) (From Zatsepina et al. (2000) with permission)

It is known that all desert animals regularly encounter drastic fluctuations in temperature and, hence, the body temperature of these poikilothermal animals should also change significantly depending on these fluctuations. Along these lines, Cherlin and Muzichenko (1983) demonstrated that daily fluctuations of the body temperature of Middle Asia desert lizard range from 20 to 44 °C.

It was interesting to find out whether the observed fluctuations in the environmental and body temperature, somehow, influence the synthesis of Hsps and specifically Hsp70 in the body of the animal subjected to such drastic changes of the temperature in the field conditions. In the course of these field studies the lizards (*P. interscapularis*) were collected in the desert throughout the day starting from 6 a. m. and *in vivo* injected with ^{35}S-methionine. The labeled proteins were isolated from the livers of injected animals at different time intervals, run on two-dimensional (2D) gels, and labeled Hsp70 members, hsp68(+) and Hsp68(−), were excised and quantified. These experiments demonstrated clear-cut positive correlation between the body (and soil) temperature observed during the day and the levels of Hsp70 synthesis in the cells of *P. interscapularis* (Fig. 4.4). Notably, comparatively

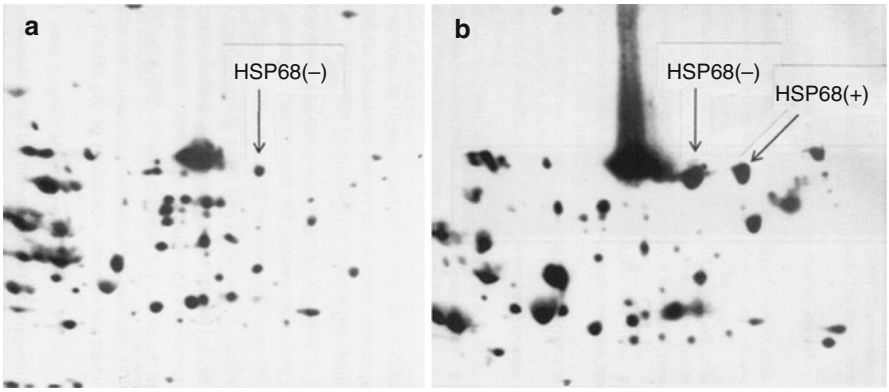

Fig. 4.3 Two dimensional electrophoresis of total proteins labeled *in vivo* by ^{35}S-methionine isolated from toad-headed lizard. (**a**) Normal physiological temperature; (**b**) heat shock. The induced two Hsp70 (Hsp68 in lizards) family isoforms are indicated by arrows (From Ulmasov et al. (1999) with permission)

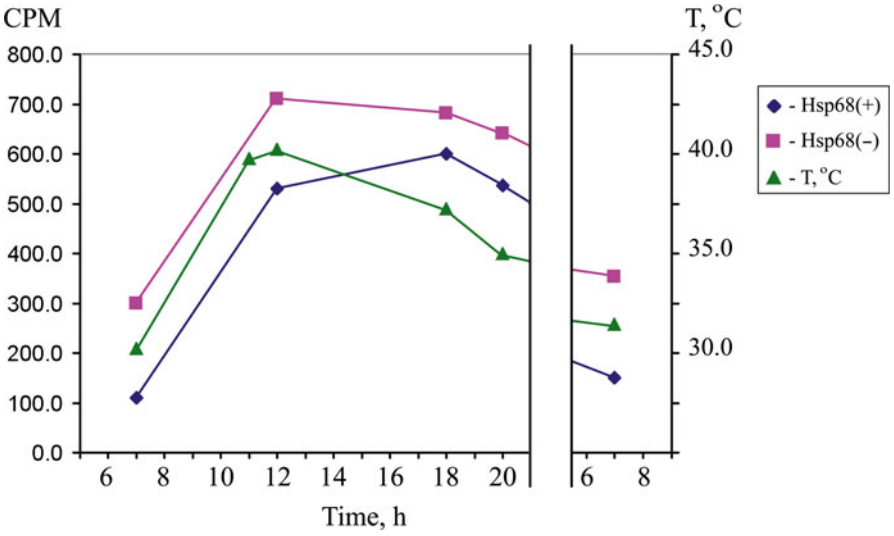

Fig. 4.4 The daily dynamics of body temperature fluctuations and levels of Hsp68(+) and Hsp68(–) in Chinese toad-headed lizard (*P. interscapularis*). Clear-cut correlation is evident (From Ulmasov et al. (1999) with permission)

high level of Hsps synthesis continues for some time after the body temperature drops to 30–35 °C (Ulmasov et al. 1999).

Therefore, it was suggested that high constitutive level of Hsp70 observed in desert lizard species probably represents the major adaptation of xeric organisms in general to environmental challenges such as drastic fluctuation in temperature regime and provides extremely high thermotolerance characteristic for various xeric animals.

High content of Hsp70 and other chaperones in the cells under normal physiological conditions apparently represents "preparative defense strategy" which enables xeric organisms to normally function in the broad range of environmental temperatures without *de novo* induction of stress proteins and switching off the synthesis of normal cellular house-keeping genes.

Thus, when comparing ^{35}S-methionine incorporation into proteins of thermotolerant desert species Caspian gecko and common lizard (*L. vivipara*) we demonstrated that the intensity of proteins synthesis after heat shock (39 and 42 °C) is significantly higher in the latter species. Moreover, the temperature of Hsp70 synthesis induction (T-ON) in *L. vivipara* is significantly lower in comparison with gecko. In other words, as a rule in Northern species the threshold of Hsps induction is lower while the intensity of Hsps synthesis is significantly higher after moderate HS in comparison with desert thermoresistant species with high constitutive levels of Hsps.

The observed high thermoresistance of desert lizard species may be in principle explained by either high stability of Hsps present in their cells after they encounter stressful conditions or by constitutive presence of high concentration of correspondent mRNA. Northern blot analysis demonstrated that the observed differences in the constitutive levels of Hsp70 between *P. interscapularis* and other xeric lizard species and common lizard (*L. vivipara*) are most likely regulated at the level of transcription, and thermoresistant species are usually characterized by significantly higher levels of *Hsp70* mRNAs under non-stress conditions (Ulmasov et al. 1992) (Fig. 4.2).

Later, similar differences in HSR were revealed when the *Cataglyphis* ant, which is one of the most thermotolerant land insect known, was compared with a few other insect species. The two species of *Cataglyphis* used in the study forages at body temperature above 50 °C and their critical thermal maxima are equal to 54–55 °C (Gehring and Wehner 1995). The synthesis and accumulation of heat shock proteins were analyzed in these highly thermotolerant ants and compared with another ant species *Formica polyctena*, an ant living in more temperate climates and to two *Drosophila* species. Similar to *B. mori* and desert lizards (*P. interscapularis*) protein synthesis in *Cataglyphis* ant species continues at temperatures higher than 45 °C as compared to 39 °C determined for *Formica* ants and studied *Drosophila* species. In contrast to *Drosophila* species, desert ant species (*Cataglyphis*) were characterized by significant constitutive levels of Hsps in their cells prior to HS. These findings were interpreted by the authors as preadaptation to extreme temperatures encountered by this species (Gehring and Wehner 1995).

The observed pronounced differences in the constitutive levels of Hsp70 between species from habitats with contrasting thermal regimes may be also explained by different quantity or/and structure of HSF1 in the compared organisms. In order to explore this possibility we investigated by Northern and Western blot analysis the concentration of HSF1 and correspondent mRNA in the cells of *P. interscapularis* and *L. vivipara* (Zatsepina et al. 2000). The experiments clearly demonstrated that the level of HSF1 and correspondent mRNA is significantly higher in the cells of *L. vivipara* in comparison with *P. interscapularis*. Therefore, there is a clear-cut

negative correlation between the concentration of Hsp70 and HSF1 in the cells of the species studied. Southern heat-adapted species are characterized by high level of Hsp70 and low level of HSF1 in the cells under normal conditions while Northern species (*L. vivipara*) exhibits the reverse relationship between these two values (Fig. 4.2). It is plausible to speculate that high constitutive concentration of Hsps in the cells of xeric species allows them to cope with temperature fluctuations without additional induction of Hsps. On the other hand, Northern species rarely exposed to HS normally do not contain high concentration of Hsps in the cells but are able to rapidly respond to temperature increase by intensive synthesis of Hsps due to high constitutive content of HSF1 in the cells. In fact the observed negative correlation between Hsps and HSF1 in the compared lizard species perfectly corresponds to the so called "cellular thermometer" model (see the Chap. 3).

Furthermore, the intensity of *Hsp* genes transcription strongly depends on the efficiency of transcription factors (e.g. HSF1) binding with promoters of stress genes which may include competition between negative and positive regulators of transcription. We performed *in vitro* binding experiments to monitor HSF1 binding efficiency in the studied lizard species and demonstrated that thermotolerant species characteristically differ from the more high-latitude species *L. vivipara* by the presence of certain amount of activated HSF1 bound to *Hsp* genes promoters under normal physiological conditions.

On the other hand, activation (trimerization) of HSF1 and its detectable binding with HSEs in common lizards occurs at 34 °C, and only at 39 °C in *P. interscapularis.* Interestingly, dissociation of HSF1 from DNA in the course of recovery after HS is much longer in *L. vivipara* in comparison with xeric *P. interscapularis* (6 and 1 h, respectively). In addition, competition experiments demonstrated significantly higher binding affinity of HSF1 with *Hsp* promoters of xeric lizard species in comparison with that of *L. vivipara* (Zatsepina et al. 2000).

Therefore, high constitutive level of Hsp70 and other Hsps in the cells of desert lizards is likely controlled at the transcription level and may be due to the presence of certain amount of active HSF1 at their *Hsp* genes promoters providing their "leakage" under normal temperature conditions (Zatsepina et al. 2000). Likewise, subsequently, it was shown in goby (*Gillichthys mirabilis*) that the temperature of HSF1 activation positively correlated with acclimation temperature, which suggests that apparent plasticity in HSF1 activation may play an important role in the heat shock response in various eurythermal organisms and, hence, participate in general environmental control of *Hsps* gene expression (Buckley and Hofmann 2002).

Generally speaking, in the case of phylogenetically distant forms discussed above the observed variability in Hsps levels may resemble their different evolutionary history, while the differences in Hsps constitutive synthesis and induction patterns after HS detected in geographical populations of the same species or phylogenetically close forms from contrasting ecological conditions may rather resemble their adaptation to specific thermal conditions including extreme ones.

In fact, any comparison of phylogenetically distant forms dwelling in contrasting environments and looking for correlations is apt to severe critics because unrelated species should be by definition quite different in many respects due to the divergence

process (Garland and Adolph 1994). On the other hand, the investigation of multiple species from contrasting habitats (e.g. multiple species of lizards from different taxa) may provide some useful information which should be, however, accepted with caution. Therefore, the most direct way to detect correlation between the manifestation of certain trait (e.g. Hsp70 levels) and adaptation to adverse conditions is to study closely related species or, ideally, several geographical populations of the same species from contrasting thermal environments.

4.2.2 The Comparative Analysis of Closely Related Forms

The comparison of close species belonging to the same group or genus but differing by distribution, latitude and consequently average temperature of their habitats represents another and probably more reliable and widely used approach to look for correlation between thermal adaptation and pattern of Hsps synthesis.

Along these lines, during the last two decades thermotolerance was studied in a wide spectrum of *Drosophila* species and strains originating from different climatic zones and considerably differing from one another in the ambient temperature of their habitats. The species that lived in hot climate as expected usually have a higher thermotolerance but exhibited various patterns of HSR (Dehghani et al. 2011; Garbuz et al. 2008; Feder and Hofmann 1999; Feder and Krebs 1997; Lindquist 1986; Somero 2005; Ulmasov et al. 1992).

It was shown, that in *Drosophila Hsp70* genes are practically not active in most tissues under normal temperature conditions but can be rapidly induced by temperature elevation or other forms of stress producing huge amounts of chaperones which can reach about 1 % from total amount of cellular proteins (Velazquez et al. 1983). Notably, under non-stress conditions certain copies of *Hsp70* family are characterized by low tissue-specific expression in spermatogonia of second instar larvae and prepupa (Lakhotia and Prasanth 2002). In *Drosophila*, constitutive members of *Hsp70* family (e.g. Hsc70) apparently serve as major chaperones at normal temperature while the induction of *Hsp70* genes likely serves as last defense line under critic and subcritic conditions.

Therefore, in this respect *Drosophila* species differ drastically from most other insect species studied so far where usually significant constitutive levels of Hsps are observed under normal temperature conditions (Dehghani et al. 2011; Garbuz et al. 2008; Gehring and Wehner 1995).

In our studies we explored several closely related *Drosophila* species belonging to the *virilis* group and several other species of the same genus as outgroups.

D. virilis is the most karyotipically primitive species of the *virilis* phylad and is probably ancestral to it if not to the entire *virilis* group (Patterson and Stone 1952). *D. virilis* is distributed throughout the Northern Hemisphere primarily South to 40°N latitude. *D. lummei,* considered the closest relative of *D. virilis*, occurs from just above 40°N to just above 65°N latitude and from Sweden West to the Pacific coast of Asia (Fig. 4.5). These species are separated by at least five

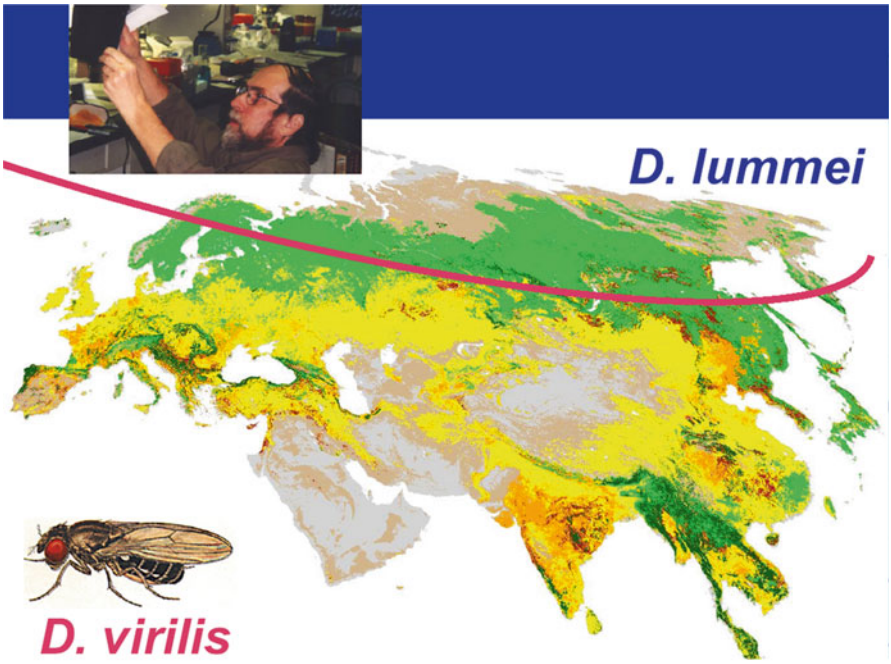

Fig. 4.5 Approximate distribution of *D. lummei* and *D. virilis* throughout Euroasia (*red line*) and one of the authors (M.E.) working with the flies

to six millions years of divergence and can be even crossed in the laboratory to provide fertile progeny (Morales-Hojas et al. 2006; Patterson and Stone 1952; Spicer and Bell 2002). Many aspects of the thermal phenotypes of the species under study exhibit countergradient variation similar to that in other taxa (Feder and Hofmann 1999; Ulmasov et al. 1992; Zatsepina et al. 2001); i.e. the magnitude and threshold for traits correspond to the thermal environments in which the species occur. Thus, the lower-latitude species *D. virilis* exceeds the higher-latitude species *D. lummei* in basal thermotolerance (see also Garbuz et al. 2003), and temperature threshold for HSF activation. Amazingly, we failed to observe induced thermotolerance in *D. lummei* and pretreatment with mild temperatures even decreased the rate of survival after acute HS (Fig. 4.6). As also shown previously (Garbuz et al. 2003), *D. virilis* and *D. lummei* express similar amounts of heat-shock mRNA and protein at low to moderate heat-shock temperatures. On the other hand intense heat shock (e.g. 40–41 °C) almost abolishes heat-shock mRNA and protein expression in *D. lummei*, whereas considerable expression persists after such heat shock in *D. virilis* (Fig. 4.7). For example, Hsp70 concentration is similar in *D. virilis* and *D. lummei* after 37.5 °C heat shock but is markedly greater in *D. virilis* than in *D. lummei* after more severe heat shocks (Fig. 4.7). Notably, in *D. lummei* the reduction in protein level after acute heat shock is even greater for the small Hsps and Hsp40 than for Hsp70. Northern hybridization data

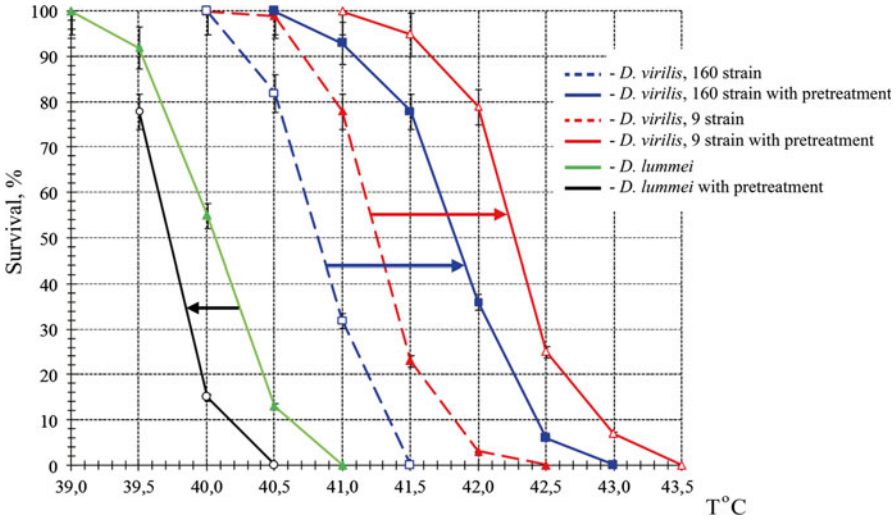

Fig. 4.6 Basal and inducible thermotolerance in *D. virilis* and *D. lummei*

with total RNA demonstrated that in *D. lummei Hsp70* genes transcription is completely inhibited at 40 °C and restored only after 1 h of recovery at normal temperature. In the latter species the Hsp70 accumulation after acute HS is detectable only after 3 h recovery period. On the other hand, HSF in *D. lummei* efficiently binds with promoter DNA as revealed *in vitro* assay even at 41 °C which implies that acute HS (40 °C and higher) does not block HSF activation in this species but apparently interferes with other components of transcription machinery. In contrast, in *D. virilis* significant synthesis of *Hsp70* mRNA efficiently occurs at 40° and even 41 °C. Most species of the *virilis* group studied exhibited positive correlation between the Hsp70 accumulation after heat exposure and thermotolerance. Thus, *D. montana* species from comparatively cold climatic zones was significantly less thermotolerant and was able to synthesized Hsps only after moderate HS in contrast to xeric *D. novamexicana* species belonging to the same species group (Garbuz et al. 2003).

Different levels of Hsp70 after HS in species of the *virilis* group are correlated with the structure of *Hsp70* cluster and *Hsp70* copy number comprising the cluster (see Chap. 5 for the details). Thus, 2D electrophoresis revealed more Hsp70 isoforms in *D. virilis* geographical strains with higher *Hsp70* copy number (Garbuz et al. 2003; Evgen'ev et al. 2004).

However, while the relationship between species thermoresistance and Hsp70 synthesis was evident when species and strains of the *virilis* species group were compared, this correlation was lacking when a species from other *Drosophila* groups were included into the analysis (Fig. 4.7). Thus, adults of *D. melanogaster* (Oregon R strain) found the most thermosensitive strain in our studies synthesize maximal quantity of Hsp70 at 36–37 °C exceeding in this parameters all other *Drosophila* strains and species taken for comparison (Fig. 4.7a) (Krebs 1999).

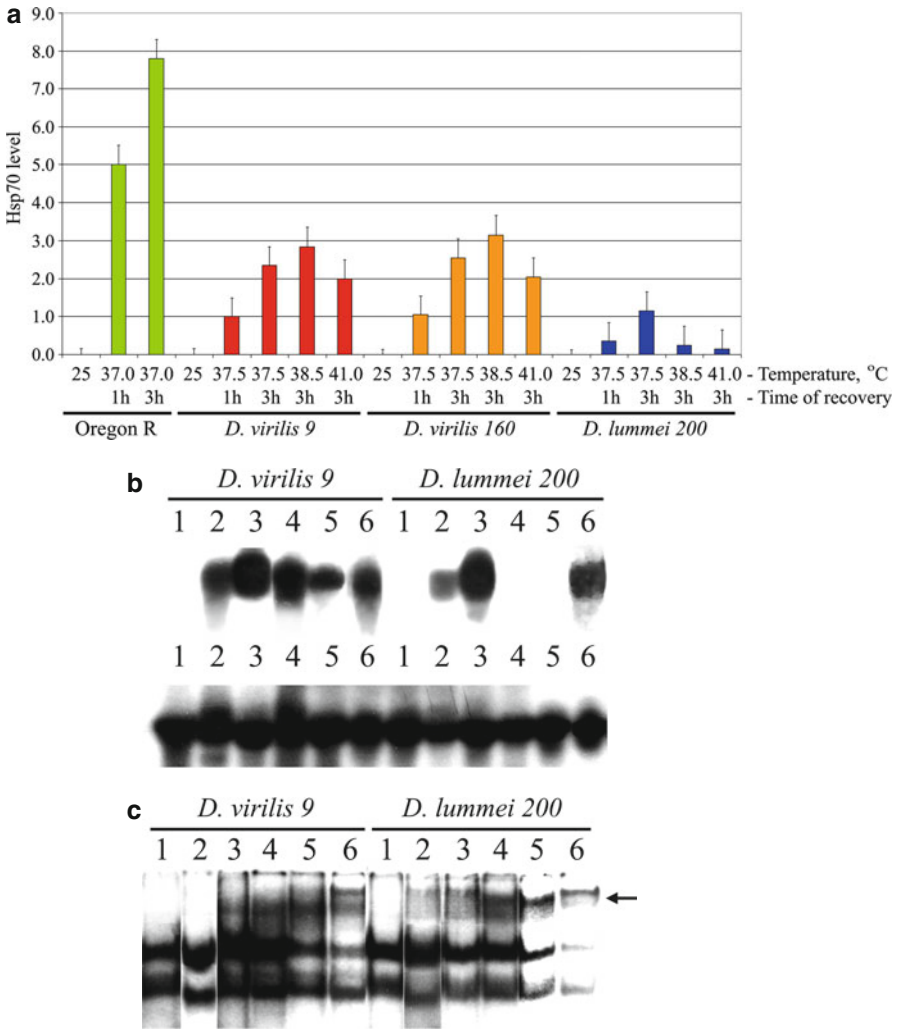

Fig. 4.7 Heat shock response at different levels in *Drosophila* species. (**a**) Hsp70 levels in *Drosophila* strains and species under normal conditions, after HS treatments and after different periods of recovery (adult flies were used). (**b**) Northern blot hybridization of mRNA isolated from *D. virilis* and *D. lummei* subjected to different HS treatments (all 30 min) with labeled *Hsp70* probe. (**c**) HSF-HSE complex in *D. virilis* and *D. lummei* after different heat treatments (marked by *arrow*). 1–25, 2–31.5, 3–37.5, 4–40.0, 5–41.0, 6–40.0 °C and 1 h of recovery at 25 °C (From Garbuz et al. (2003) with permission)

Likewise, *D. mojavensis*, a highly eurythermal species from *repleta* group, with critic temperature equal to 43.5 °C synthesize significantly less Hsp70 after mild HS in comparison with *D. melanogaster* flies (Krebs 1999). Probably in desert flies that often encounter temperature fluctuations induction of intensive Hsps synthesis after mild HS will be deleterious for their growth and development (Feder and Krebs 1997; Zatsepina et al. 2001).

It is of note that all xeric *Drosophila* species used in our studies (e.g. *D. virilis*, *D. novamexicana* and *D. mojavensis*) were characterized by comparatively high temperature for maximal Hsp70 induction (38.5 °C) which was 1 °C higher than that of *D. lummei* and *D. montana* and 1.5 °C higher than in *D. melanogaster* (Garbuz et al. 2002, 2003). Remarkably, at higher temperatures after severe HS all studied thermotolerant species accumulate significantly more Hsp70 in comparison with *D. melanogaster* and other relatively thermosensitive species (Garbuz et al. 2002).

It is noteworthy that according to diverse genetic and biochemical evidence, the inducible Hsp70 proteins play key roles in basic and inducible tolerance specifically to extreme temperatures close to critic ones for the given species. Both the magnitude and threshold of inducible Hsp70 expression are correlated with the natural thermal regime in many species in many taxa (Feder and Hofmann 1999; Norris and Hightower 2000). Our results and data accumulated by other groups corroborated this conclusion showing that in *Drosophila* species inducible members of Hsp70 family play important protective role particularly in the organisms from extreme environments or after acute HS (Garbuz et al. 2003). The investigation of *D. melanogaster* strain with deleted *Hsp70* copies which is not able to survive acute HS (Bettencourt et al. 2008; Gong and Golic 2006) provided independent proof for this statement (see Chap. 8 for details).

Since it is known that Hsp70 is not the only Hsps responsible for thermotolerance the patterns of induction of other Hsps families after heat exposure in a wide spectrum of *Drosophila* species were also compared. Thus, significantly higher thermotolerance observed in *D. virilis* and other xeric species of the group coupled with lower levels of Hsp70 accumulation in comparison with *D. melanogaster* strains after moderate HS suggests possible involvement of other chaperones providing *D. virilis* thermotolerant phenotype. It was demonstrated that *D. virilis* and xeric *Drosophila mojavensis* from *repleta* group are characterized by higher levels of Hsp40 and small Hsps synthesis after moderate and acute HS in comparison to *D. lummei*, and certain strains of *D. melanogaster* (Garbuz et al. 2003; Shilova et al. 2006). It is known that sHsps prevent denatured proteins from aggregation (Morrow et al. 2006), while Hsp40 belonging to J-proteins family serves as a cofactor for Hsp70 (See Chap. 2). Therefore, in principle, significant accumulation of Hsp40 may enhance the Hsp70 efficiency without the increase in Hsp70 concentration. The results obtained suggest that the Hsp40 and small heat shock proteins may play a significant role in thermotolerance and adaptation to temperature fluctuations in thermophilic *Drosophila* species.

It is of note that there are a few exceptions from the general pattern implying the important role of Hsps in thermotolerance in desert animals. Thus, when comparing Hsp expression in two closely related snail species of the genus *Sphincterochila*, a desert species *S. zonata* and a Mediterranean species *S. cariosa*, it was demonstrated that adaptation to different habitats results in the development of distinct strategies of Hsp expression in response to stress, namely the reduced expression of Hsps in desert-inhabiting species (*S. zonata*). The authors speculated that the observed different strategies reflect the difference in heat and humidity encountered in the natural habitats, and that the desert species *S. zonata* relies on

mechanisms and adaptations other than Hsps induction and constitutive Hsp70 synthesis thus avoiding the fitness consequences of continuous Hsp upregulation (Mizrahi et al. 2012).

Differential induction of individual Hsps in snail organs represent another HSR peculiarity described by this group, thus, in *S. cariosa,* heat stress caused rapid induction of Hsp70 and Hsp90 in the foot and kidney tissues, whereas the desert-inhabiting xeric species *S. zonata* displayed delayed induction of Hsp70 proteins in the foot and upregulation of Hsp90 alone in the kidney. Interestingly, similarly to highly eurythermal lizard *P. interscapularis* the investigated snail species contain two isoforms of Hsp70 that are synthesized with different kinetics and in response to different forms of HS (Mizrahi et al. 2012).

Another pertinent investigation has been recently performed using a well-studied model object, migratory locust (*Locusta migratoria*), which is native to the semiarid regions of the World and excellent for study adaptation to fluctuating temperature conditions. In this organism previous exposure to sublethal high temperatures effectively protects neuronal function against future hyperthermia, but unlike many other organisms described above, the deep physiological adaptations are not accompanied by a robust increase of *Hsp70* transcript or protein in the nervous system. It was supposed that the observed lack of Hsp70 induction after HS may be explained by very high level of the constitutive level of Hsps in the tissues and the thermoprotective effect of pre-HS on the nervous system might be mediated not by Hsp70 induction but rather by post-translational modifications or protein trafficking (Dehghani et al. 2011).

In contrast to flies, locusts and other highly mobile organisms that may rapidly escape high temperature zones (Feder and Hofmann 1999), many sessile organisms, as well certain ontogenetic stages such as eggs, larvae and pupae dwelling in periodically heated substrate are subject to daily or seasonal variations in temperature and water availability and, hence, apparently use Hsps synthesis as major component of their survival strategy.

4.2.3 Intraspecific Comparison

Although multiple interspecific comparisons in ecological aspect provided rather reliable correlations between the levels and patterns of Hsps synthesis and species thermotolerance in ecological context (Ulmasov et al. 1992; Feder and Hofmann 1999; Tomanek 2005), in the case of intraspecific studies the situation is not so unequivocal.

In *D. melanogaster*, a number of physiological traits related to thermal extremes including adult cold resistance and heat resistance show clinal patterns (reviewed in Hoffmann et al. 2003). There is also clinal variation in this species for the incidence of reproductive diapause, thorax length and cold and heat resistance (Hoffmann and Weeks 2007; Mitrovski and Hoffmann 2001; Schmidt et al. 2005) that correlates with levels of resistance to thermal extremes (Schmidt and Paaby 2008). In India,

highland populations of *D. melanogaster* have higher levels of desiccation resistance and melanism compared with lowland populations, which matches the hotter and drier conditions experienced in highlands (Parkash et al. 2008a, b).

However, within *D. virilis* and *D. lummei* geographical strains, little if any countergradient in terms of thermotolerance and Hsps synthesis variation is evident (Figs. 4.6 and 4.7). Possibly, the extensive gene flow among populations swamps incipient adaptation to local conditions, a situation that may not be universal in *Drosophila* species (Michalak et al. 2001). Likewise, the investigation of latitudinal clines for stress resistance in *D. simulans* from eastern Australia did not reveal any clinal pattern for desiccation or heat resistance (Arthur et al. 2008).

Furthermore, as in the case of *D. melanogaster* (Krebs et al. 1996), adaptation to laboratory conditions of *D. virilis* wild type strains caught in nature does not seem to significantly contribute to the observed intraspecific HSR patterns. Thus, two strains caught at the same location near Tashkent, Uzbekistan (T53 and T61) exhibit identical patterns of thermotolerance and Hsp70 induction despite the fact that T61 was captured recently and the T53 more than 30 years ago. The only conspicuous departure from this pattern is for *D. virilis* strain 160, a marker strain with at least one known recessive mutation on each autosome. The basal thermotolerance for this strain is considerably lower than for all other *D. virilis* strains studied so far probably due to multiple mutations present (Garbuz et al. 2003).

Likewise, Jensen et al. (2010) were not able to correlate heat-induced Hsp70 synthesis and several measurements of adult heat tolerance in three independent populations of *D. melanogaster,* estimated independently in three laboratories and using slightly different protocols. Although these exceptionally thorough studies revealed substantial within population variation in both Hsp70 and heat tolerance, the authors were not able to detect any significant correlation between these parameters in adults. It was concluded that variation in Hsp70 expression is only likely to explain a small proportion of the observed variation in adult thermoresistance (Jensen et al. 2010).

Probably highly coordinated heat-induced synthesis of Hsp70 encoded by several conserved copies comprising a cluster in *Drosophila* species provides an optimal response to different microclimatic conditions within the species distribution range and the observed intraspecific variations in adult heat tolerance are not directly connected with Hsps system.

However, constant pressure of natural selection is able to modulate HSR within the species.

"Evolution Canyons" intensively studied in recent years represents a perfect example of such natural selection in *D. melanogaster* (Korol et al. 2006; Rashkovetsky et al. 2006). Micropopulations of this species occur on North- and South-facing slopes of the canyon with greatly differing climatic regimes and in the intervening region; the slopes are 400 m apart at the top and 100 m apart at the bottom. Although adult *Drosophila* can traverse several kilometers in a single day, populations on each slope have diverged in body size, heat and desiccation tolerance, thermal preference for oviposition, fluctuating asymmetry in morphological traits, rates of mutation and recombination, and mate preference (Hübner et al. 2013;

Rashkovetsky et al. 2006). The investigators monitored the divergence of the slopes using neutral microsatellite markers and a candidate gene (*Hsp70*) strongly linked to resistance to environmental stress. The frequencies of putatively neutral and non-neutral (*Hsp70Ba*, a heat shock gene) markers in *D. melanogaster* collected from the middle elevation of each slope were monitored. Luckily it was demonstrated that *D. melanogaster* from the Evolution Canyon in Israel are polymorphic for 1.2-kb *P* element insertion that interrupts a regulatory region of one of *Hsp70* genes and, hence, should interfere with transcription and decrease thermotolerance and resistance to other stresses. The analysis performed demonstrated that there are remarkable differences in the frequencies of both microsatellites and *P* element bearing *Hsp70Ba* allele in micropopulations inhabiting the two slopes (Michalak et al. 2001). Characteristically, flies dwelling in the Southern slope and often exposed to heat contain many folds less frequently the *Hsp70Ba* allele with disrupted promoter.

Notably, when the authors directly investigated the role of small Hsps and Hsp40 in thermoadaptation of flies collected from both slopes they were able to demonstrate more pronounced expression of Hsp40 in the flies collected from the Southern slope after mild HS implying a significant contribution of Hsp40 to thermoadaptation under local microclimatic conditions (Carmel et al. 2011). Therefore, it was possible to conclude that the observed divergence in thermoresistance and local adaptation is consistent with certain degree of genetic isolation despite the contiguity of the *D. melanogaster* populations in the canyon (Hübner et al. 2013; Korol et al. 2006; Rashkovetsky et al. 2006).

D. melanogaster flies (T strain) collected in sub-equatorial semiarid tropical zone of Africa in the 1970s (J.R. David, personal communication 1978) represents another example of pronounced differences in heat shock response observed in geographical populations of the same species. T strain was remarkably tolerant of sustained laboratory culture above 30 °C previously considered to be the limit for continuous culture of this species (Parsons 1973). Furthermore, the basal thermotolerance of T strain adults significantly exceeded that of standard wild-type (Oregon R) strain used as a control. In this exceptional ability T strain outperformed other *D. melanogaster* strains and compares favorably with many cactophilic desert *Drosophila* species (Krebs and Loeschcke 1999; Stratman and Markow 1998). However, surprisingly, induced thermotolerance of high temperatures, which in *D. melanogaster* is due in part to the inducible molecular chaperone Hsp70, is only modest in T strain. Expression of Hsp70 protein and *Hsp70* mRNA is likewise reduced and has slower kinetics in this strain (T) than in a standard wild-type Oregon R strain. T strain has higher critic temperature (i.e. the temperature of heat shock when less than 1 % of treated flies survive) in comparison with Oregon R flies (41 °C vs. 40 °C). Notably, maximal level of Hsp70 synthesis was observed in both compared strains at 37 °C but in the case of T strain the level of Hsp70 accumulation was two to threefold lower (Zatsepina et al. 2001). On the other hand, at higher temperatures close to critic value (e.g. 39 °C) flies of T strain still synthesized Hsp70 while in Oregon R flies the synthesis of this chaperone was completely inhibited although *Hsp70* mRNA was detected in both strains. This observation

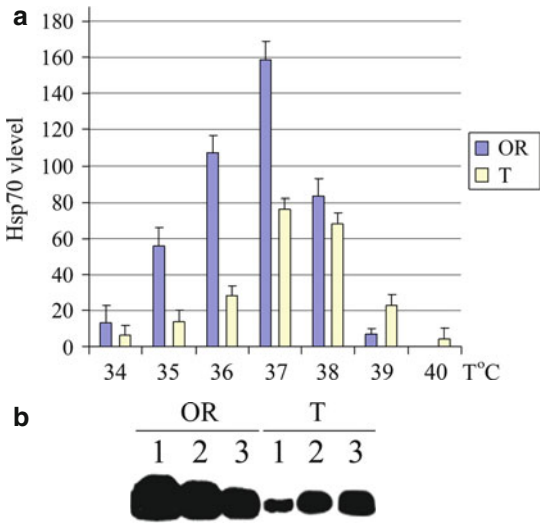

Fig. 4.8 (**a**) Hsp70 levels in Oregon R (OR) and T strains after 30 min HS at different temperatures. (**b**) *Hsp70* mRNA level in both strains after 30 min HS at different temperatures. 1–35, 2–37, 3–39 °C (From Zatsepina et al. (2001) with permission)

suggests higher thermostability of translational machinery in strain T (Fig. 4.8). The compared strains also differ in a similar way in the patterns of constitutive and heat-inducible levels of other molecular chaperones. Thus, T strain is characterized by higher levels of Hsp40 and small heat shock proteins after HS in comparison with *D. melanogaster* Oregon R strain (Zatsepina et al. 2001).

It was shown that the lower Hsps expression in the T strain after moderate HS apparently has no basis in the compromised activation of the heat-shock transcription factor (HSF), which is similar in T and Oregon R flies. Subsequently, it was demonstrated that the observed reduced expression of Hsp70 likely stems from insertion of two transposable elements, *H.M.S. Beagle* in the intergenic region of the 87A7 *Hsp70* gene cluster and *Jockey* in the *Hsp70Ba* gene promoter (Zatsepina et al. 2001) (see Chap. 6 on the role of transposable elements in HSR).

The data accumulated when studying *D. melanogaster* micropopulations from the "Evolution Canyon" and T strain versus Oregon R strain imply strong variation within a single species. Evidently, adaptation via natural selection is sufficiently strong to overcome even the immense phylogenetic inertia of the heat shock response.

4.2.4 Adaptive Role of Hsps in Homothermal Thermophilic Organisms

Most of the studies on the possible role of Hsps in thermoresistance were performed using insects and other poikilothermal organisms, because homothermal animals preserve constant body temperature under various temperature regimes and may only slightly modulate it predominantly in skin or other external tissues subjected to

temperature challenge. However, in some special cases significant changes in external tissues subjected to environmental challenges were observed, thus, in camels often experiencing high temperature in arid areas the skin may reach 40 °C and even more. The camel (*Camelus dromedarius*) represents a homothermal organism perfectly adapted to extreme conditions of arid zones and is capable of tolerating extreme heat accompanied by a significant elevation (up to 41 °C) of the whole body temperature (Schmidt-Nilsen 1972). Western blot analysis demonstrated that camel lymphocytes constitutively express Hsp73 protein which can be significantly induced by heat shock (Ulmasov et al. 1993). Surprisingly, another member of Hsp70 family (Hsp72) is not induced in lymphocytes but can be induced in skin fibroblasts. Probably skin fibroblasts of this animal, which are often exposed to an extremely high temperature challenge, required the synthesis of additional protective protein (Hsp72). Notably, based on labeled methionine incorporation analysis, it was concluded that the general synthesis of proteins in camel cells is more resistant to heat than that seen in human cells (Ulmasov et al. 1993). Recently, inducible and constitutive camel *Hsp70* genes have been sequenced and characteristic differences in the structure of human and camel *Hsp70* regulatory regions were detected (Garbuz et al. 2011a).

Similarly, when two ethnic human groups inhabiting for many generations contrasting thermal environments (Russians vs Turkmens, inhabiting desert Turkmenistan, Middle Asia) were investigated it was demonstrated that while fibroblasts isolated from Turkmens after severe heat shock exhibited intensive synthesis of all Hsps in parallel with synthesis of many other cellular proteins, only trace synthesis of Hsps was observed in the second group, Russians (Fig. 4.9) (Lyashko et al. 1994).

When survival of fibroblasts after severe heat shock treatment was assessed by colony formation assay, the cells of the first group (Russians) exhibited significantly lower survival rates (Lyashko et al. 1994).

Another way to explore the role of Hsps in whole body adaptation in mammals is to monitor the allele frequency of specific SNPs in different heat shock genes in different populations of the same breed. It was shown by several authors that nucleotide changes occurring naturally in the flanking regions of the *HSPA1A* gene might affect the inducibility, level of expression, and stability of *HSP70* mRNA and, hence, contribute to stress tolerance. Thus, in human peripheral mononuclear cells, polymorphisms in the coding region of *HSPA1A* gene were associated with an increased ability to respond to heat stress (Singh et al. 2006). In pigs, an *HSP70* gene polymorphism located in the promoter and 3'-UTR was associated with mRNA stability and stress response (Schwerin et al. 2002). Recently haplotype analysis of the eight SNPs of the *HSPA1A* gene revealed their involvement in heat resistance in Chinese Holstein cattle (heat sensitive breed of cattle that originated in Europe). The analysis demonstrated the presence of significant differences between individuals carrying different haplotypes for most of the heat-tolerance traits studied (Xiong et al. 2013). Such SNPs may be useful in the future as molecular markers to assist selection for heat tolerance in various domestic animals and maybe used as genetic tools to experimentally improve tolerance to high temperature and other forms of stress.

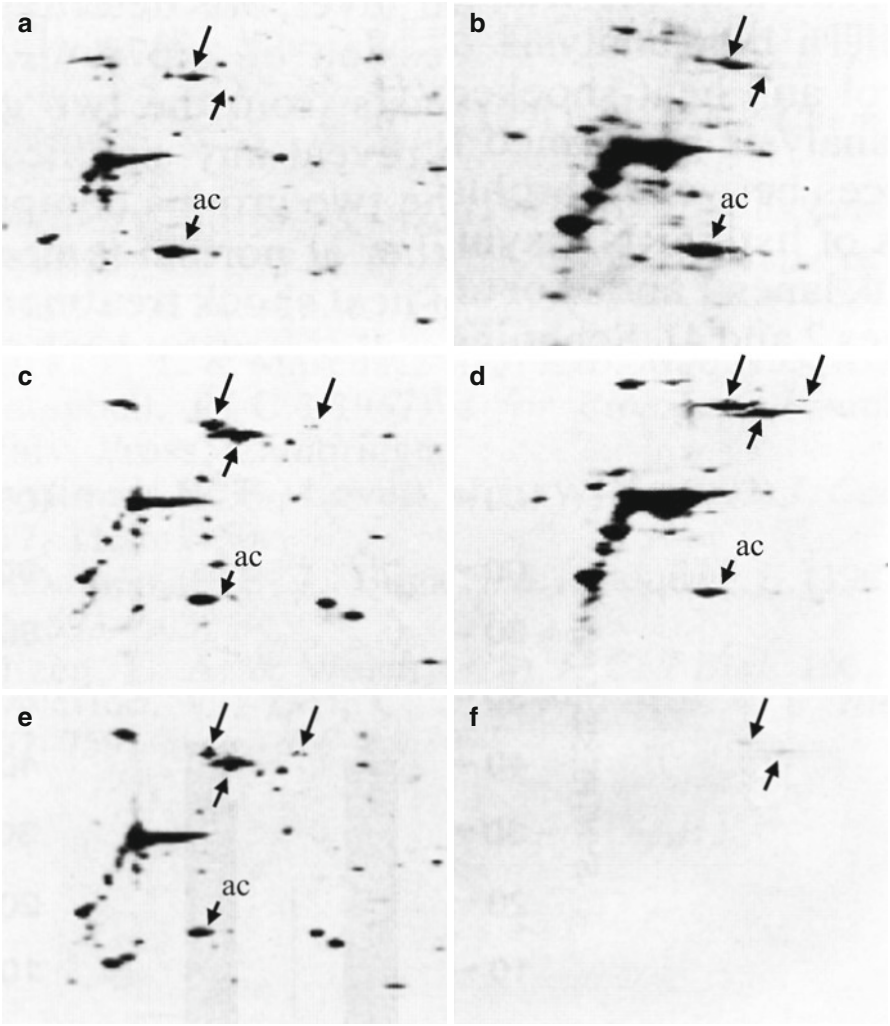

Fig. 4.9 Effect of heat shock duration on protein synthesis in human skin fibroblasts of different origin, observed by two-dimensional electrophoresis of radioactively labeled cellular proteins (**a**, **c**, and **e** cells of Turkmen origin; **b**, **d**, **f** cells of Russian origin). Skin fibroblasts were labeled with ^{14}C-amino acids after incubation at 37 °C (**a**, **b**), after 42.5 °C, 2-h exposure (**c**, **d**), and after 42.5 °C, 6-h exposure (**e**, **f**). Stress proteins of the Hsp70 family are indicated by *arrows*. *Ac* actin (From Lyashko et al. (1994). Copyright (1994) National Academy of Sciences, U.S.A)

4.3 Comparative Data on HSR in Aquatic Organisms

It is evident that aquatic species due to physical properties of water which includes high thermal conductivity should have body temperatures close to that of their surrounding which, however, can change very rapidly in the intertidal zone or in shallow water, which is heated by the sun.

Various intertidal and littoral aquatic (mostly marine) organisms represent another good object for investigation of diverse behavioral and molecular mechanisms, including Hsps synthesis, evolved to cope with fluctuating temperature conditions.

One of the most variable and unpredictable habitats on Earth is the marine rocky intertidal zone located at the boundary between the terrestrial and marine environments. Mussels dominate rocky intertidal habitats throughout the world and, being sessile, endure wide variations in temperature, salinity, oxygen, and food availability due to daily, tidal, seasonal and climatic cycles. Analysis of gene-expression changes in the mussels, fishes and crustaceans species dwelling in different intertidal habitats were summarised in numerous publications (Buckley et al. 2001, 2006; Dong et al. 2008; Podrabsky and Somero 2007; Somero 2005; Stillman and Somero 2000; Tomanek 2008).

In general, when studying various marine organisms from contrasting environments it was demonstrated that similar to terrestrial organisms, threshold of HSF activation and, hence, Hsps induction in the cold-adapted species is significantly lower that in more thermophilic forms. Thus cold-adapted clams *Mytilus trossulus* begin to synthesize Hsp70 at lower temperature than a more Southern species *M. galloprovincialis* (Buckley et al. 2001). In a study of Hsp70 in a genus of outbred subtropical fish, a direct correlation between Hsp70 levels and survival at elevated temperature as a test of phenotypic diversity in acquired thermotolerance was found (Norris and Hightower 2000).

Moreover, the threshold of Hsps induction in snails, mussels, crabs and fishes characteristically varies depending on the climatic cycle and in summer the temperature of Hsps induction is significantly higher than in winter (Buckley and Hofmann 2002; Dong et al. 2008; Tomanek 2008).

Characteristically, as a rule acclimation procedure increases the threshold of Hsps induction in relatively thermophylic or eurythermic species to less extent in comparison with cold-adapted organisms and the latter can usually survive larger temperature increase in comparisons with their physiological range than thermophylic related species. Thus, the investigation of consequences of acclimation process in snails of the genus *Tegula* demonstrated that a more thermoresistant species *T. funebralis* exhibits lower ability to respond to acclimation in terms of modulation of Hsps threshold temperature etc. in comparison with closely-related cold-adapted species *T. brunnei* and *T. montereyi* (Tomanek 2005; Tomanek and Somero 2000). As a rule, species subjected to daily temperature changes are characterized by maximal thermoresistance. Multiple experiments exploring various thermally contrasting intertidal species of mussels and other marine organisms enable one to conclude that acclimation procedure may be highly efficient and can enhance thermoresistance of organisms occupying moderately variable thermal environments (range <10 °C), like the subtidal zone. In such organisms acclimation may activate the HSR at temperatures significantly above those they normally experience in their habitats. In contrast, species from highly variable thermal environments (range <20 °C) or certain eurythermal species have a limited acclimatory plasticity because such species probably “exhausted” genetic components of their thermal adaptation.

Thus, they may live close to their thermal limits and any further increase in temperature is probably going to push them beyond those limits (Tomanek 2005; Tomanek and Somero 1999).

It is evident that the ability to acclimate to variable environmental conditions may affect the biogeographic range of species, their success in colonizing new habitats, and their likelihood of surviving rapid anthropogenic climate change. The responses to temperature acclimation (4 weeks at 7, 13 and 19 °C) in gill tissue of the warm-adapted intertidal blue mussel *Mytilus galloprovincialis*, an invasive species in the Northeastern Pacific, and the cold-adapted *M. trossulus*, the native congener in the region, were compared (Tomanek 2005). Using two-dimensional gel electrophoresis and tandem mass spectrometry, the authors demonstrated that the cold-adapted *M. trossulus* showed increased abundances of molecular chaperones after acclimation at 19 °C, but *M. galloprovincialis* did not, suggesting that the two species differ in their long-term upper thermal limits. In contrast, the warm-adapted *M. galloprovincialis* showed a stronger response to cold acclimation than *M. trossulus*, including changes in abundance in higher number of proteins and differing protein expression profiles between 7 and 13 °C, a pattern absent in *M. trossulus*. It is necessary to keep in mind that consequences of the acclimation process by far are not restricted by the increase of certain Hsps synthesis but lead to profound changes in the general transcription pattern. Thus, acute heat shock exposure (24, 28 and 32 °C) after acclimation to 13 °C for 4 weeks enabled to identify 47 and 61 distinct proteins that changed abundance in *M. galloprovincialis* and *M. trossulus*, respectively. As expected the onset temperatures of greater abundance of some members of the heat shock protein Hsp70 and small Hsps families were lower in the cold-adapted *M. trossulus*.

In the work of Hamer et al. (2004) seasonal changes of the Hsp70 level in mussels were registered. Maximal levels of Hsp72 and Hsp70 were observed in mussels after summer (September), and minimal levels in winter (December). The observed small changes in sea salinity could not cause significant Hsp70 proteins induction.

Overall, these results help to explain why *M. galloprovincialis* has replaced *M. trossulus* in Southern California over the last century, but also suggest that *M. trossulus* may maintain a competitive advantage at colder temperatures. It was also speculated that anthropogenic global warming may reinforce the advantage *M. galloprovincialis* has over *M. trossulus* in the warmer parts of the latter's historical range (Tomanek 2005).

In general, intertidal organisms exhibit patterns of Hsps synthesis similar to those of lizard species inhabiting thermally contrasting biotopes (see above).

Thus, four limpet species of the genus *Lottia*, exhibiting a broad vertical distribution on wave-exposed rocky shores and subjected to dramatic temperature changes during the low tide apparently have distinct strategy of Hsp70 expression depending on the height and orientation in the rocky intertidal zone. In the high-intertidal zone, *L. scabra* typically occupies horizontal surfaces fully exposed to the sun, *L. austrodigitalis* primarily occupies vertical or overhanging surfaces, while *L. pelta* and *L. scutum* are restricted to the low- and mid-intertidal zones. Western blot analysis demonstrated that field-collected as well as acclimated specimens of

the two high-intertidal species had significantly higher constitutive levels of Hsp70 than the low- and mid-intertidal species. Therefore, their cells are preadapted to dramatic temperature changes occurring in these microhabitat conditions. Species dwelling in moderate and low-intertidal zones do not normally experience such temperature gaps and begin to actively synthesize Hsps only after temperature elevation exactly like was observed in *Lacerta* lizard (Dong et al. 2008).

Very similar pattern of Hsps synthesis was demonstrated for three species of snails in the genus *Tegula*. In these species daily temperature fluctuations for relatively thermophylic and eurythermic *T. funebralis* range from 13 to 36 °C, while in cold-adapted species *T. brunnei* and *T. montereyi* inhabiting low-tide zone the temperature varies from 13 to 19 °C and these organisms are rarely subjected to HS in their natural habitats. As expected, the Hsps synthesis in these cold-adapted species is strictly inducible in contrast to *T. funebris* specimens which constitutively synthesize Hsp70 under normal non-stress conditions exhibiting preparative defense strategy (Tomanek 2005; Tomanek and Somero 2000). Interestingly, the development rate of the latter species is significantly lower in comparison with that of congeneric cold-adapted species possibly as a trade off for high constitutive concentration of Hsp70 which was shown to hamper the development in *Drosophila* (Krebs and Feder 1997).

Although most studies of HS and acclimation in aquatic organisms were conducted using marine organisms virtually the same regularities were determined in freshwater species. Thus, the investigation of HSR in two congeneric species of littoral endemic amphipods (*Eulimnogammarus cyaneus* and *E. verrucosus*) from Lake Baikal (Fig. 4.10) that strikingly differ in their vertical distribution and thermal tolerance was performed.

E. verrucosus is a stenobiotic species that preferred temperature 5–6 °C (Timofeyev and Shatilina 2007). *E. cyaneus* is a representative species of the upper littoral zone, the most thermally fluctuating environment in the lake. *E. cyaneus* is significantly more thermoresistant than *E. verrucosus*, and its preferred temperatures are 11–12 °C (Timofeyev and Kirichenko 2004). It was demonstrated that the basal level of Hsp70 synthesis is five times higher in *E. cyaneus* inhabiting the upper littoral compared to the thermosensitive *E. verrucosus* from the more thermally stable lower littoral habitat. The high basal level of Hsp70 likely contributes to the thermotolerant phenotype of *E. cyaneus* and is possibly evolved in response to drastic temperature fluctuations characteristic for the upper littoral zone of Lake Baikal (Votintsev 1961). In addition to the high constitutive expression of Hsp70, *E. cyaneus* also showed a relatively high temperature threshold necessary for Hsp70 induction (27 °C) and relatively low level of Hsp70 accumulation in response to acute HS (only two-fold). Thus, high basal level of Hsp70 obviously allows *E. cyaneus* to overcome temperature fluctuations without additional synthesis, since both short-term acute HS (25 °C) and the mild stress (16 °C) with following recovery does not induce a significant increase in the level of Hsp70 in this species (Bedulina et al. 2013).

In contrast, the more thermosensitive *E. verrucosus* is characterized by several times lower basal level of Hsp70, and much more pronounced induction of Hsp70 concentration in response to the acute HS, HS with recovery, mild HS. Thus, significant Hsp70 induction in *E. verrucosus* already takes place at 13 °C, which is only

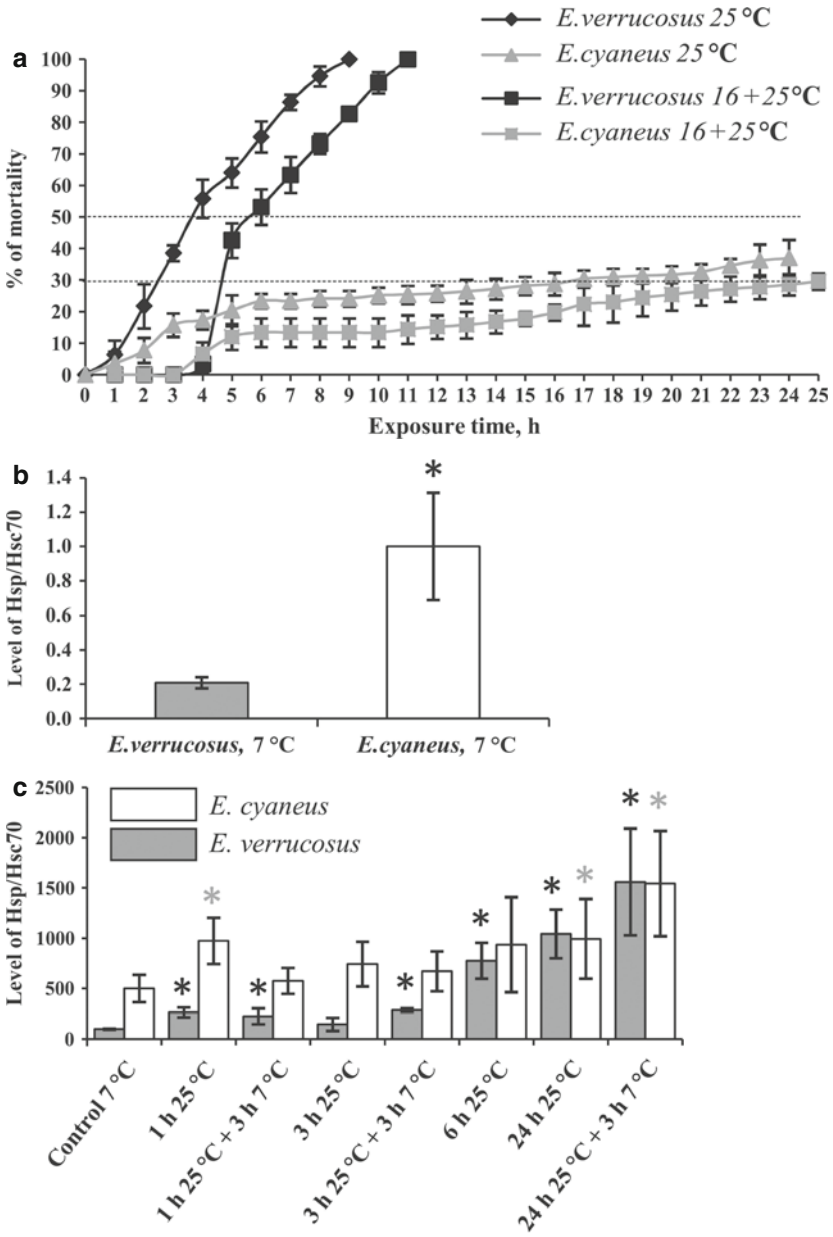

Fig. 4.10 Thermotolerance and Hsp70 synthesis in the two amphipod species. (**a**) Mortality of the amphipods *E. verrucosus* and *E. cyaneus* subjected to 25 °C during 25 h with and without previous exposure to a mild-temperature stress (16 °C for 3 h) followed by 3 h of recovery at 7 °C. The data are expressed as the means and standard deviations. The 50 and 30 % mortality levels are marked by dashed lines. (**b**) The basal levels of Hsp70/Hsc70 normalized to the actin level under control (7 °C) conditions in the studied amphipod species. (**c**) The level of Hsp/Hsc70 in the amphipods under control (7 °C) conditions and under the following heat shock (25 °C) and recovery. The recoveries were 3 h at 7 °C after 3 h of continuous acute HS or after 24 h of continuous HS (From Bedulina et al. (2013))

6 °C above the acclimation temperature, and Hsp70 expression reaches higher levels (eight-fold) within 24 h after the acute HS. Along these lines, studies of the Baltic Sea amphipods also showed that thermally resistant semi-terrestrial intertidal species *Orchestia gammarellus* has a higher basal level of Hsp70 compared to the thermosensitive subtidal *Gammarus oceanicus* as well as less pronounced Hsps induction in response to HS (Bedulina et al. 2010).

4.4 Cold-Adapted Stenothermal Organisms

In the previous section we report about high constitutive concentrations of several Hsps in several aquatic species which often experience dramatic changes in the temperature of their habitats. It is evident that the aquatic environment can be extremely stressful to its sessile inhabitants and to other organisms which cannot rapidly escape the stressful conditions.

In this respect, stenothermal marine high-latitude (in particular, Antarctic) animals that thrive at temperatures down to −1.9 °C are of special interest for understanding molecular mechanisms underlying resistance to stable and extreme conditions. Usually seawater temperatures at 15 m depth range from −1.8 to 1.8 °C. The species dwelling under such conditions are usually highly endemic, they have been geographically isolated in a constantly cold environment for around 25–22 MYA (reviewed in Clarke and Johnston 1996) and are extremely stenothermal (Somero and DeVries 1967) The investigation of heat shock response of Antarctic marine species is very important, because the Southern Ocean has a very stable and narrow temperature range, where sudden temperature rises of 5 °C have not occurred for millions of years. Antarctic species do not survive even modest temperature rise and die even after short temperature exposure at 5–10 °C (Somero and DeVries 1967). In Antarctic notothenioids as well as several invertebrate species the loss of the heat-shock response was discovered (Carpenter and Hofmann 2000; Clark and Peck 2009; Hofmann et al. 2000). Thus, in the notothenioid fish *Trematomus bernacchii*, acute heat stress and cadmium exposure failed to induce Hsp synthesis and increase transcription of any mRNAs for Hsps (except for the cochaperone Hsp40) (Hofmann et al. 2000; Buckley and Somero 2009). Surprisingly, closely related forms sharing the same ancestor and dwelling in cold waters (8–12 °C) of the New Zealand area are able to effectively synthesize Hsps after the temperature elevation (Petricorena and Somero 2007). To elucidate the mechanism responsible for the lack of Hsps induction in *T. bernacchii*, HSF1 activity, Hsp70 mRNA production and protein synthesis patterns, hepatocytes of this species were examined (Buckley et al. 2004). Interestingly, in the course of these studies the presence of activated HSF and Hsp70 mRNA were detected in this organism in non-stress conditions. However, HS and chemical inducers of the heat shock response failed to increase *Hsp70* mRNA levels, HSF1 activity or the levels of any Hsp.

It was also shown that inducible Hsp70 is expressed permanently in the cold-adapted *T. bernacchii* and related Antarctic species, *Pagothenia borchgrevinki* (Nototheniidae), *Harpagifer antarcticus* (Harpagiferidae) and *Lycodichthys*

dearborni (Zoarcidae) (Buckley et al. 2004; Clark et al. 2008; Place and Hofmann 2005). It is likely that certain level of Hsp70 in the cells under non-stress conditions may be necessary to cope with aggregated and misfolded proteins occurring under extremely low temperature (Privalov 1990).

Sequence analysis of the 5′-region of *Hsp70* gene of the Antarctic fish *E. focardii,* which also lack the heat shock response, revealed the presence of potentially functional HSEs and also StREs (Stress response elements), both of which confer the ability to respond to stress (La Terza et al. 2001). One may speculate that because of the presence of permanently active HSF and resulted constitutive Hsps synthesis, the further HSF activation after heat treatment is complicated or requires the induction temperature higher than lethal temperature for the examined Antarctic organisms.

Indeed, investigation of Hsp70 induction in clam (*Laternula elliptica*) revealed up-regulation of two isoforms of *Hsp70* genes at 10–15 °C in digestive gland and gill tissue (Clark et al. 2008). The induction temperature for these genes was between 8 and 10 °C, while permanent expression of Hsp70 and Hsp78 isoforms was detected in this species.

The investigation of the limpet (*Nacella concinna*) also revealed constitutive expression of all members of Hsp70 family. The induction temperature was 15 °C higher than this species can experience in Antarctic waters (seawater temperatures at 15 m depth range from −1.8 to 1.8 °C). Furthermore, three other investigated Antarctic species, a sea star (*Odontaster validus*), a gammarid (*Paraceradocus gibber*) (Clark et al. 2008) and a ciliate (*Euplotes focardii*) (La Terza et al. 2001) completely lost heat shock response. In the case of the sea star and the gammarid several *Hsp70* gene family members were identified in genomes, but they did not show either inducible or significant permanent expression. Larvae of a terrestrial Antarctic flightless midge species *Belgica antarctica* (Diptera, Chironomidae), are also not able to synthesize Hsps after temperature elevation (Rinehart et al. 2006). Interestingly, this midge species also exhibits high level of constitutive Hsps which can be further increased by UV-radiation. Conversely, adults of this species exhibit no constitutive up-regulation of their Hsps synthesis while Hsps induction can be thermally activated (Rinehart et al. 2006). Another well-known model species *Hydra oligactis*, dwelling in extremely cold and stable aquatic conditions also lost the ability to synthesize Hsps in response to temperature elevation despite the presence of correspondent genes in the genome. However, in this particular case the absence of Hsp70 after HS results from very low stability of correspondent mRNA (Brennecke et al. 1998). Surprisingly, close Hydra species (*H. vulgaris*) characterized by the virtual absence of senescence is able in response to HS synthesize Hsps that are implicated in this extraordinary phenomenon (Martínez and Bridge 2012).

In the previous section we described a few species which are not able to respond to temperature elevation by Hsps synthesis induction. Therefore, stenothermal organisms (mostly aquatic) inhabiting stable cold environments for many million years which practically never encounter any significant fluctuations in temperature, salinity and other challenges may lose to different degree their ability to respond to various forms of stress by Hsps induction. The enhanced lipid membrane densities (e.g. higher concentrations of mitochondria), characteristic changes in enzyme

kinetic properties and, even more so, loss of genetic information (e.g. myoglobin and hemoglobin in notothenoid fishes) reflect the specialization of Antarctic organisms to constant low temperature conditions (Pörtner et al. 2007).

Generally speaking, at all levels analyzed, the functional specialization to permanently low temperatures evolved in aquatic organisms implies reduced tolerance to high temperatures, as a trade-off (Petricorena and Somero 2007; Pörtner et al. 2007). Probably high constitutive synthesis of Hsp70 and other chaperones common for such stenothermal forms represents the major compensatory molecular adaptation to deal with elevated levels of protein damage constantly occurring at low temperature conditions which are far from optimum for normal protein folding (Fraser et al. 2007; Fraser and Rogers 2007; Privalov 1990; Place et al. 2004; Todgham et al. 2007). The upregulation of Hsps demonstrated in many Antarctic organisms suggested that low-temperature conditions may be significantly denaturing to cellular proteins, an observation that was supported by elevated levels of ubiquitin-conjugated protein detected in Antarctic notothenoid fish (Place et al. 2004).

4.5 Organisms from High Temperature and Salinity Areas (Extremophiles) and Related Forms

Along with described above desert organisms and aquatic (mostly marine) species that evolved to cope with thermally highly variable environments, there are animal species thriving in highly aggressive extreme areas under conditions of constant high temperature and/or salinity.

During the expedition to Kunashir (Kuril Islands) in 2005 insect larvae of different sizes were collected in the hot mineralized sulphur volcanic spring Fig. 4.11.

The larvae were determined to belong to Diptera species *Stratiomys japonica* van der Wulp (Diptera: Stratiomyidae), common name "soldier flies". The collected larvae were consequently compared with a few other species belonging to the same family (Stratiomyidae). The recent phylogenetic analysis of Stratiomyidae based on molecular characters was performed (Brammer and von Dohlen 2007).

The larvae of Stratiomyomorpha thriving in the hot springs are collectors-gatherers of fine organic particles. They have strong larval cuticle characteristically shagreened and encrusted with plates or "warts" of $CaCO_3$, which is unique among the Diptera (Rozkošný 1982, 1997; Woodley 1989). These features might facilitate colonization of extreme semiaquatic habitats such as hot volcanic springs of Kunashir Island (the Kuril Islands).

Stratiomyid larvae belonging to different lineages are known to inhabit extreme aquatic conditions, such as hypersaline and thermal waters, and demonstrate a remarkable resistance to different chemicals (reviewed in McFadden 1967 and Rozkošný 1982, 1997).

The aim of our study was to compare and try to correlate thermotolerance and the patterns of Hsps expression in (semi)aquatic larvae of several stratiomyid species

Fig. 4.11 (**a**) Major outlet of Stolbovskoi spring on Kunashir Island, general view; (**b**) last instar larva in water; (**c**) adult *S. japonica* (male); (**d**) dead larvae of various instars in an area with local temperature exceeding 50 °C. (**e**) One of the authors (D.G.) during collection trip to Kunashir Island in 2006 (The photos were taken by D. Garbuz and A. Przhiboro)

belonging to different evolutionary lineages and inhabiting extreme habitats with contrasting temperatures and chemistry.

In this work we have investigated four stratiomyids: *Stratiomys japonica* from Stolbovskoi hot spring on Kunashir Island; two species, *Stratiomys singularior* and *Nemotelus bipunctatus* from hypersaline lakes in the Crimea; and *Oxycera pardalina* from a cold spring near St.-Petersburg.

S. singularior is a very common and widely-distributed Transpalaearctic typical eurythermal species. In the Crimea, the larvae of this species were abundant in shallow pools saturated with H_2S, with mineralization approximately 80 g/l. *Nemotelus bipunctatus* was also collected in Crimea in the water margin zone of coastal lagoon-derived Lake Koyashskoe where mineralization of water near the shore was about 280 g/l (Golubkov et al. 2007). The measured temperature in the habitats of the two Crimean stratiomyids used in our studies varied from 15 to 30 °C, but apparently it can be higher in summer and much lower in winter.

For comparison with the above described three thermoresistant species we took cold-adapted species *O. pardalina* from the same family which is widely distributed in Western and Central Europe. The larvae of *O. pardalina* are confined to clear-water cold carbonate springs near St.-Petersburg with a year-round stable low water temperature (5.5–7.5 °C) and low level of total mineralization (0.38–0.42 g/l).

The larvae of the four species were exposed to different temperatures and assessed for survival and Hsps synthesis. The LT_{50} and critical temperature (i.e. the temperature of HS treatment resulting in less than 1 % of survival) were also determined in their larvae.

The LT_{50} and critical temperatures of *S. japonica* larvae did not depend on the temperature of maintaining in the laboratory (31 or 37 °C). Apparently, maintenance of these larvae at different temperatures before HS did not significantly affect their thermotolerance. Therefore, the acclimation procedure was not effective in the studied species. This observation is not surprising because it was previously demonstrated in other highly eurythermal organisms which also did not respond to acclimation procedure (Tomanek 2005; Somero 2005). It was speculated that such forms exhausted genetic components of their thermal adaptation and, hence, live close to their thermal limit. Due to this characteristic feature in the HSR, species from extreme and highly variable environments are likely to be more affected by climate change than species from moderately variable environments.

It is of note, that *D. lummei* flies (see above) were also characterized by the absence of induced thermotolerance probably because in this case the representatives of this cold-adapted species having a diapause rarely (if ever) encounter acute HS in nature.

In general, our experimental data corroborate our field observations on Kunashir Island, in which *S. japonica* larvae died within a few minutes after being swept or transferred into areas of the hot spring where water temperature exceeded 50 °C (Fig. 4.11). For the two Crimean species, LT_{50} for a 30-min heat shock were 45.5 °C for *S. singularior* 46.5 °C for *N. bipunctatus*, while critical temperatures for these species were 47 and 48 °C, respectively (Fig. 4.5). For *O. pardalina* larvae from the cold spring, critical temperature was 43 °C, while LT_{50} was 41.2 °C. Although

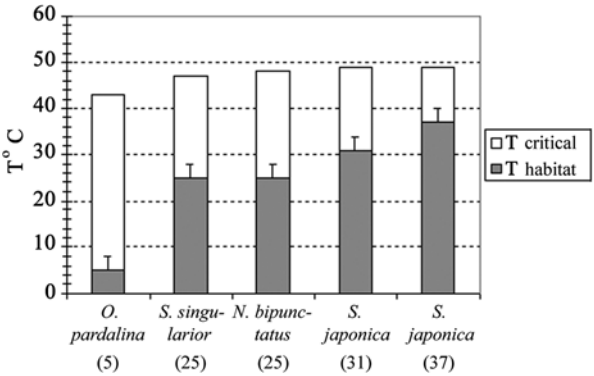

Fig. 4.12 The ratio between control (habitat) temperature and critical temperature for Stratiomyidae species. Numbers in the brackets indicate the temperatures at which the larvae have been maintained before heat-shock treatments. Basically, they are close to the average habitat temperatures of the species studied (From Garbuz et al. (2008) with permission)

heat tolerances were in general correlated with the typical habitat temperatures of these larvae, most surprising is the huge gap (38 °C) between the natural habitat temperatures (6–10 °C) and critical temperatures for *O. pardalina* larvae and in this respect *O. pardalina* apparently greatly exceeds the other three highly eurythermal Stratiomyidae species (Fig. 4.12).

Exploring antibodies recognizing inducible members of Hsp70 family we performed broad-scale analysis of HSR in the Stratiomyidae species studied after different HS treatments. High levels of Hsp70 were present in the larvae of all four species at the temperatures of their habitats, and also at much lower temperatures (ca. 25 °C in the case of *S. japonica* larvae kept several weeks at this temperature in the laboratory). The constitutive concentrations of Hsp70 were approximately the same irrespective of the habitat temperatures of the studied species in nature. A 30-min heat shock treatment increased Hsp70 synthesis two to three-fold in *S. japonica,* maximally after 43 °C treatment. Accompanying the Hsp70 induction in this species are pronounced increases in small Hsps and Hsp68, and a small but significant increase in hsc70 concentration. By contrast, Hsp induction immediately after heat shock treatment is comparatively less in the two Crimean species, and hardly detectable in *O. pardalina* (Fig. 4.13). Notably, the accumulation of Hsp70 continues in the cells of the treated larvae for many hours after heat shock and plateaus approximately 24–36 h after the treatment.

Therefore, the pattern of Hsps in all studied Stratiomyidae species drastically differs from that of *D. melanogaster* and other *Drosophila* species where virtually no inducible Hsp70 is present at normal temperature while even mild HS induced dramatic increase in the synthesis of all groups of Hsps. Furthermore, the synthesis of all Hsps in *Stratiomydae* species in contrast to *Drosophila* species, lasts for many hours and reaches plateaus only after 24–36 h following HS. Intriguely, trypsin proteolysis and subsequent mass spectrometry showed that *Stratiomyidae* Hsp70 is much closer to the correspondent protein from the thermotolerant mosquito species *Aedes aegypti* than to Hsp70 from known species of *Drosophila* (Garbuz et al. 2008). Surprisingly, experiments exploring (^{35}S)L-methionine labeling failed to detect significant synthesis of Hsps in the control untreated larvae of the species studied.

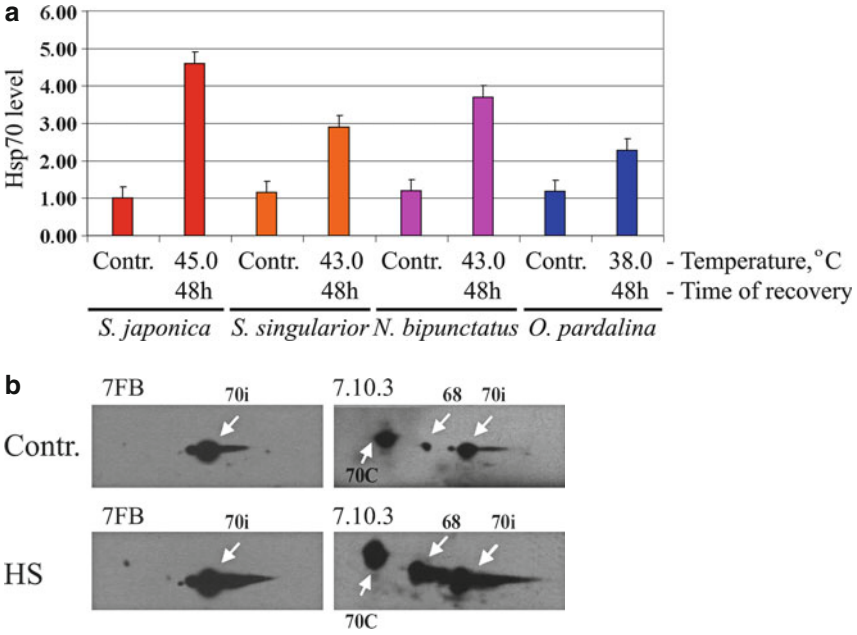

Fig. 4.13 (**a**) The levels of Hsp70 in different representatives of Stratiomyidae family under normal temperature (Contr.) and after heat shock (HS, 30 min). (**b**) The pattern of *Stratiomys japonica* proteins as revealed by two-dimensional electrophoresis and Western blotting of 2D blots sections containing Hsp70 family using 7FB and 7.10.3 antibodies. 7FB recognize predominantly inducible Hsp70 member, while 7.10.3 recognize all Hsp70 family members. Arrows indicate 70i – Hsp70 inducible member; 70C – constitutive member of Hsp70 family; 68 – Hsp68, an inducible member of Hsp70 family (From Garbuz et al. (2008) with permission)

Northern blot hybridization surprisingly failed to reveal any significant levels of either *Hsp70* mRNA or small *Hsps* transcripts under control conditions in any Stratiomyidae species studied. Only RT-PCR studies were able to reveal weak leakage of *Hsp70* genes in *S. japonica* under control conditions (Fig. 4.14). Heat shock, however, resulted in strong and rapid induction of corresponding mRNAs, with a maximum at 43–45 °C and an apparent drop of transcription at 47 °C. The latter temperature represents the LT_{50} for this highly thermoresistant species and probably damages the transcription machinery. The *Hsp70* mRNA synthesized in response to heat shock persists in the cells for many hours and is still detectable in trace amounts even 48 h after HS.

As indicated by electrophoretic mobility-shift assays, in contrast to certain desert lizards mentioned above (e.g. *P. interscapularis*), the active form of HSF is not detectable under control conditions in *Stratiomys* species studied. In response to temperature elevation, however, HSF as expected binds specifically to heat-shock element (HSE) and is able to transactivate the *Hsp* genes. Therefore, the surprisingly high concentration of Hsps observed in the cells of Stratiomyidae species without heat shock is not accompanied by significant transcription of corresponding

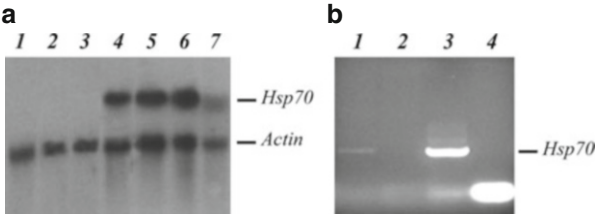

Fig. 4.14 (**a**) Northern blot hybridization of total RNA from *S. japonica* larvae with ^{32}P-labelled *Hsp70* probe in controls and in response to heat-shock treatments. *1*, *2* and *3*–25, 31 and 37 °C respectively (a range of normal conditions in a field), *4*–42, *5*–43, *6*–45 and *7*–47 °C. As a control for equal loading, blots were stripped and rehybridized with actin probe. (**b**) RT-PCR analysis. Lane *1*, *S. japonica* nonheated control larvae kept at 31 °C; lane *2*, *S. japonica*, control reaction mix without reverse transcriptase (larvae kept at 31 °C); lane *3*, *S. japonica* larvae heat-shock treated (43 °C, 30 min); lane *4*, *S. japonica* larvae heat shock treated (43 °C) without reverse transcriptase. The fragment of expected size (661 bps) is indicated. Transfer RNA used as a carrier forms thick bands with lower molecular mass (From Garbuz et al. (2008) with permission)

genes under non-stress conditions. The phenomenon may be probably explained by very low but continuous expression of heat shock genes demonstrated by RT-PCR analysis or, less probably, by the synthesis of Hsps at some earlier stages of larval development when the larvae have been subjected to more severe shock. The last speculation seems less probable since it implies extremely high stability of Hsps in Stratiomyidae species. Furthermore, high concentration of Hsps has been detected in Stratiomyidae larvae kept for a month in the laboratory at 25, 31 or 37 °C.

Apparently, critical temperatures for (semi)aquatic larvae of this family are close to or slightly lower than 50 °C, and the larvae are unable to tolerate higher temperatures. According to Pritchard (1991), no insects were proved to live in hot springs above 50 °C, and very few, mostly Diptera, above 40 °C. Our results confirm this general conclusion.

The data obtained in our studies indicate that the inducible member of the Hsp70 family and other Hsps are continuously present at high concentrations in the larvae of four species of Stratiomyidae family, even in the absence of heat shock.

Characteristically, in contrast to the larvae, in the adult flies of the species investigated, immunoblotting revealed only trace amounts of Hsp70 under non-heat shock conditions.

The magnitude of thermotolerance observed in the investigated Stratiomydae species, in fact, exceeds that of most insect species studied, with *Bombyx mori* being a prominent exception (Evgen'ev et al. 1987). This comparative uniformity in the Stratiomydae species is exhibited despite differing natural thermal regimes, and includes LT_{50} and critical temperatures. Mild heat shock (37 °C) pretreatment did not significantly improve tolerance for extreme temperatures in *S. japonica* larvae which, hence, seem not to have induced thermotolerance.

It is evident that, molecular mechanisms underlying up-regulation of heat shock proteins may differ characteristically depending on the organism. Thus, in desert lizards *Phrynocephalus interscapularis*, high constitutive levels of Hsps and correspondent

mRNA are correlated with activation of HSF under control conditions, and may be further dramatically induced by heat shock treatment (Zatsepina et al. 2000). Apparently animals constantly subject to extreme temperatures have elaborated molecular mechanisms allowing them to maintain a relatively high Hsps level under normal conditions. In this way, they are preadapted for life under the conditions of constant stress without drastic changes in the house-keeping and *Hsp* genes activity.

The observed constitutive expression of Hsps in the cells of the Stratiomyidae larvae may help them to survive the diapause period spent at the larval stage.

Interestingly, the one Stratiomyidae species investigated that naturally occurs in a stable albeit cold thermal environment, *O. pardalina*, although exhibits significant increase in heat shock RNAs synthesis after temperature elevation, synthesized less Hsps than other thermotolerant species after high temperature challenge. The constitutive Hsps synthesis observed in this particular species suggests that extremely cold habitats, may be as challenging for proteins synthesis and homeostasis as in the case of cellular proteins functioning in aggressive extreme conditions of hot volcanic springs (see above Sect. 4.4).

In contrast to the larvae, adults (flies) of all Stratiomyidae species in our study which are able to escape from high temperature areas and, hence, are not subject to the severe conditions of hot or highly mineralized aquatic environments, and their survival apparently does not depend on maintaining high continuous levels of chaperones. The difference in the environment microhabitats probably accounts for the apparent dichotomy between larvae and adults in Hsp70 expression. It is of note, that such dichotomy in the synthesis of chaperones is even more pronounced in the cold-adapted Antarctic animals where larvae in contrast to adults completely lost the ability to activate their Hsps (see Sect. 4.4).

Regardless of the exact mechanism, the functionally extended presence of Hsps in the larvae of all Stratiomyidae species investigated may meet the increased chaperoning needs that these animals experience in response to adverse conditions of hot volcanic streams, highly mineralized Crimean lakes or cold springs in the environs of St.-Petersburg. This maintenance prevents cell injury and irreversible protein aggregation that may occur in response to these types of stress.

Interspecific comparison is a common approach in physiological ecology and other related fields of biology. However, several authors justly underlined many logical and statistical problems associated with using interspecific and in particular two-species comparisons for studying adaptations (Garland and Adolph 1994). In the case of Stratiomyidae species used in the present investigation we cannot state with certainty that exceptionally high thermotolerance and developmental dichotomy in terms of Hsps synthesis represent genetic adaptations in response to natural selection operated in the species studied (high and low temperatures and salt concentration). It is much more likely for this particular group that the observed adaptations to extreme conditions may be a characteristic feature of the ancestor of the whole Stratiomyidae family enabling the descendants successfully colonize various inhospitable environments. There are data suggesting that Mezozoic stratiomyids were already preadapted to colonize different types of extreme habitats, and such

colonization occurred repeatedly in different genera (Rasnitsyn and Quicke 2002; Rozkošný 1997; Whalley and Jarzembowski 1985).

The performed investigation of a broader spectrum of Stratiomyidae and other Diptera species helped to check this preferred hypothesis.

For our analysis Diptera species belonging to different families and inhabiting highly variable environments were taken into analysis. Besides additional Stratiomyidae species i.e. *Oplodonta viridula*, *Beris chalybata* and *Odontomia sp.*, we used two species of horse flies (*Tabanidae*) *Haematopota pluvialis* and *Chrysops relictus* and three species of biting midges belonging to *Ceratopogonidae* family (*Dasyhelea modesta*, *Dasyhelea flavoscutellata* and *Dasyhelea sp. ex. gr. cincta*). The investigation of HSR response at the molecular level including synthesis and stability of Hsps and corresponding mRNAs in several Diptera species enables to describe features probably common to all Diptera as well as molecular adaptations restricted to certain families.

Oplodonta viridula, *Beris chalybata*, *Chrysops relictus* and *Dasyhelea flavoscutellata* belong to typical cold-adapted stenothermal species, *Odontomia sp.* and *Haematopota pluvialis* were collected in the Crimea in the same place as *S. singularior* and *N. bipunctatus*, while biting midges (*Dasyhelea modesta* and *Dasyhelea sp. ex. gr. cincta*) dwell at the littoral area of Northern freshwater lakes. It was shown that all studied species are characterized by significant constitutive level of Hsp70 under physiological conditions of their correspondent niches. Characteristically, the horse flies (*C. relictus*) differ from the investigated representatives of Stratiomyidae and *Drosophila* by significantly higher stability of *Hsp70* mRNA (Garbuz, Przhiboro, personal communication 2014).

Therefore, numerous *Drosophila* species characterized by completely repressed expression of inducible Hsps under normal conditions and strong activation of HSPs synthesis after temperature elevation rather represent exceptions when compared with other Diptera.

Notably, constitutive or chronic expression of Hsps, is not uniformly beneficial. For example, experiments on transgenic *D. melanogaster* strains with extra copies of *Hsp70* genes (see Chap. 8) have demonstrated that overexpression of Hsp70 could be harmful and may increase lethality during development (Krebs and Feder 1997). Evidently the expression level of Hsps in each species and geographical population is a balance between benefits (high thermotolerance) and costs (e.g. negative impact of Hsps overexpression on growth, fertility and other characteristics). It is necessary to keep in mind that *Drosophila* species and other Diptera species used for comparison e.g. Stratiomyidae species greatly (many folds) differ by duration of life-span and this dramatic difference may be the major factor in the observed contrasting patterns of their heat shock response and apparent different sensibility to the constitutive presence of high concentration of Hsp70 in the cells.

In the studies exploring several phylogenetically distant Diptera species we described different patterns of Hsps expression in the families belonging to the order. Thus, while in all *Drosophila* species, the level of Hsps is not detectable under normal physiological conditions and increased dramatically as temperature

Table 4.1 Various levels of *Hsp70* regulation in different Diptera species

	Hsp70 presence under normal conditions	Hsp70 stability	*Hsp70* mRNA presence under normal conditions	*Hsp70* mRNA induction after HS	*Hsp70* mRNA stability
Drosophila	–	–	–	+++	–
Stratiomyidae	+++	+++	–	+++	–
Ceratopogonidae	++	+	+	++	+/–
Tabanidae	+/–	+	–	+++	+++
Chironomidae	+/–	+	+++	+/–	+/–

increased, in the Stratiomyidae species studied we detected high constitutive level of inducible member of Hsp70 in the cells at normal temperature and HS treatment was able to induce only moderate increase in concentration of Hsps including Hsp70. Surprisingly, horse-flies (*C. relictus*) are characterized by high stability of *Hsp70* mRNA after HS.

Finally, larvae of two species of the Chironomidae family dwelling in Antarctic regions and in North Russia exhibit very peculiar pattern of Hsp70 expression. Thus, in contrast to all species of other Diptera families mentioned above, in these species high constitutive level of *Hsp70* mRNA was detected in the larvae under normal physiological conditions and HS treatment was ineffective in the induction of Hsp70 at either transcription or translation levels (Rinehart et al. 2006; Garbuz, Przhiboro personal communication 2014). Therefore, even within the same order (Diptera) different species have strikingly different patterns of heat shock response summarized in Table 4.1.

4.6 Special Cases

4.6.1 Stress Proteins in the Hibernating and Desiccating Organisms

Numerous studies demonstrated important protective role of various Hsps in preserving the integrity of transcription and translation machinery under stressful conditions including extreme ones. Naturally, soon after the discovery of Hsps their concentrations were monitored in "special cases" of adaptations, where organisms enter a diapause, anoxia or severe desiccation. Indeed, various groups of Hsps in many cases dramatically up-regulated in such organisms, at certain stages of their life-cycle associated with long-term anoxia or severe desiccation. Thus, cells of encysted embryos of the brine shrimp (*Artemia franciscana*) survive continuous anoxia for periods of years, during which their metabolic rate is undetectable (King et al. 2013). It was shown that abundant electron-dense granules consisting of the small heat shock/α-crystallin protein (p26) are present in the nuclei of anoxic embryos. Diapause-destined embryos of *Artemia* accumulate massive amounts

of Hsp26 (up to 15 % of total cellular proteins) during the periods of continuous anoxia and this chaperone is not detected or induced in any other life-cycle stages (Beristain et al. 2010). Contrary to expectation, proteins of Hsp70 and Hsp90 families did not undergo anoxia-induced nuclear translocation in the cells of *Artemia* encysted embryos, an unusual result since induction of these Hsps and such translocations were often observed in cells from a variety of organisms experiencing adverse, stressful conditions. For example, Atlantic copepods of genus *Calanus* (*Calanus finmarchicus*) accumulate high quantities of small Hsps during diapause (Aruda et al. 2011). Furthermore, Tardigrade species ("slow steppers") which have unique stress-adaptations that allow them to survive extreme heat, cold and radiation accumulate Hsps including several isoforms of Hsp70 together with glycogen, glycolipids and other specific agents during desiccation (Schill et al. 2004). It is known that low molecular Hsps at high concentration are able to convert the cytoplasm into gel-like matter which effectively preserves water and prevents proteins aggregation.

Furthermore, embryos of annual killifish *Austrofundulus limnaeus*, dwelling in the ephemeral lakes of Northern regions of South America accumulate large quantities of Hsp70 before entering the diapause during the periods of drying of their habitats. These diapausing embryos are highly resistant to a number of environmental insults such as high temperature, dehydration, anoxia, and increased salinity (Podrabsky et al. 2007). Interestingly, in this species embryo-specific form of inducible Hsp70 is expressed during embryonic development and is elevated during diapause. It was suggested that constitutive expression of Hsp70 during development may afford these embryos protection from environmental stresses during development more quickly than relying on the induction of a classic heat shock response.

The sleeping chironomid *Polypedilum vanderplanki*, inhabiting temporary water pools in semi-arid regions of Africa represents another example of possible involvement of Hsps in surviving severe water loss (Fig. 4.15). This chironomid species one of very few insects able to survive prolonged complete desiccation at larval

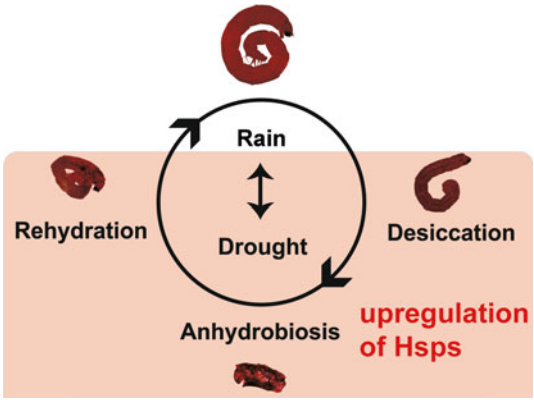

Fig. 4.15 Life cycle of chironomid *Polypedilum vanderplanki* living in Africa droughty regions. Hsp70 elevation takes place at the stage of desiccation and rehydration after dry period (The scheme provided by Oleg Gusev, PhD)

stage, entering a state called anhydrobiosis. Surprisingly, even after years in a dry state, larvae are able to revive within a short period of time, completely restoring metabolism. In order to survive such extreme environments, organisms need to develop special adaptations both at physiological and molecular levels. The analysis performed demonstrated quite different regulation of Hsps in the course of dehydration and rehydration in the larvae. Heat-shock-responsive Hsp70 and Hsp60 genes of this organism showed a two-peak expression in dehydrating and rehydrating larvae. Both small alpha-crystallin heat shock proteins (sHsps) transcripts were accumulated in the desiccated larvae, but showed different expression profiles. Transcripts of *Hsp23* was limited to the late stages of desiccation, suggesting possible involvement of this protein in the glass-state formation observed in anhydrobiotic larvae (Gusev et al. 2011). Furthermore, analysis of the sleeping chironomid genome showed intensive clustering-associated increase in the number of sHsps-coding genes associated with anhydrobiosis. The expression of the sHsps in these anhydrobiotic chironomid-specific clusters are tightly linked to the larvae stage and highly induced by desiccation (Gusev, personal communications).

Similarly, the important role of Hsps in maintaining cellular integrity and enzyme activity during desiccation and rehydration processes has been postulated in the flesh fly (*Sarcophaga crassipalpis*) (Hayward et al. 2004). The patterns of the observed Hsps induction characteristically differed in diapausing and nondiapausing pupae. Thus, in nondiapausing pupae, the expression of inducible Hsp23 and Hsp70 was upregulated by desiccation, while in diapausing pupae, the transcripts of these chaperones are already highly expressed and not further upregulated by desiccation.

The obtained results indicate distinct role for the different Hsps during desiccation stress and rehydration recovery (Hayward et al. 2004).

Up-regulation of various Hsps in response to hypothermy and various forms of oxidative stress usually observed at different stages of hibernation is not restricted to arthropods but was also detected in vertebrates (Sills et al. 1998). The investigation of the heat shock response in anoxia-tolerant turtle (*Trachemys scripta elegans*) demonstrated a strong fivefold increase in the amount of HSF1 under anoxic conditions and upregulation of five Hsps including Hsp70. The data demonstrate organ-specific regulation of Hsps induction during anoxia exposure and aerobic recovery in this turtle species which is probably represents an important aspect of cytoprotection with regard to underwater hibernation of these turtles in cold water (Krivoruchko and Story, 2010).

Mammalian species also explore modulation in the intensity of heat shock proteins synthesis in different phases of hibernation. Thus, compared with summer-active ground squirrels (*Spermophilus tridecemlineatus*), levels of the mitochondrial stress protein GRP75 were consistently higher in certain organs (e.g. intestinal mucosa) of hibernators in each hibernation state (Carey et al. 2000). Furthermore, ground squirrels increase Hsp70 family members as well as ubiquitin-protein conjugates during hibernation (Sills et al. 1998; Feder and Hofmann 1999). Interestingly, different organs of hibernating animal may exhibit a peculiar pattern of Hsps synthesis. Thus it was demonstrated that in the bat (*Murina leucogaster*)

certain muscles did not show any sign of atrophy or tension reduction and this phenomenon correlates with elevated level of Hsp70 in the muscles of the hibernating bats (Lee et al. 2008).

4.6.2 The Role of Hsps in the Life-Cycle of Parasites

Parasites which represent another "special case" may synthesize various Hsps as cellular defense mechanism to survive drastic temperature changes they encounter during their life-cycle. Induction of certain groups of Hsps usually accompanies the infection of mammalian or avian hosts from ectothermal hosts such as insects or by free-living stages. In the case of free-living stages of parasites that do not involve an animal vector sometimes induction of Hsps accompanies the transition from environment to the homothermal host.

The transition from environment with low or temperate temperatures to homothermal host with internal temperature of 37 °C or above represents a characteristic life history feature of many parasitic organisms.

Multiple studies demonstrated developmentally regulated synthesis of Hsps during the life-cycle of parasites which may respond to profound temperature changes by synthesizing different quantities of certain Hsps depending on the variations in their host temperature and life stage. It was shown that different species of intracellular parasites in the genus *Eimeria* (Apicomplexa: Coccidiida) synthesize Hsp70 in the process of infection of mammalian host (mouse) (Clark et al. 1996). The levels of Hsp70 expression in sporozoites of a wild-type parent strain and two precocious lines of *Eimeria tenella*, were compared to investigate the relationship between the heat shock proteins expressed by the parasite and virulence of the strain. The synthesis of Hsp70, which was observed in the entire sporozoites of the wild strain, was drastically reduced to the anterior portion in the precocious lines. The level of Hsps may be dramatically increased in the course of host change, thus in *Trypanosoma brucei* transcription of *Hsp70* and *Hsp83* homologues is enhanced more than 100-fold after the parasite leaves the tsetse fly and enters the mammalian host likely due to cluster organization of *Hsp70* genes providing rapid and high induction of these genes in the species (See Chap. 5) (Muhich and Boothroyd 1989).

Thus, *Schistosoma mansoni* actively synthesized Hsp70 after entering human skin from the water (Neumann et al. 1993). It was also shown that phagocytosis induce Hsp70 expression in *Toxoplasma gondii*. Interestingly, the induction of this chaperone is observed only in virulent strains of this parasite while avirulent forms either do not synthesize Hsp70 in the course of phagocytosis or synthesize low amounts of this protein (Lyons and Johnson 1995).

The malarial organism *Plasmodium falciparum* begins to synthesize Hsp70 at 39 °C, which correlates with periodical body temperature changes usually observed in the malaria patients (Biswas and Sharma 1994). Sometimes the pattern of the Hsps in parasite is organ- or tissue-specific. Thus, when the helminth *Trichinella spiralis* infects rodents, an enhanced expression of Hsp25 and Hsp60

and of these plus Hsp70 was observed at certain, yet different, time-points during infection in rat spleen and rat brain, respectively (Martinez et al. 1999). Multiple examples of developmentally regulated expression of different classes of Hsps in parasitic organisms during the transition to their hosts include parasitic nematodes, protists, cestode parasites and many other species (Biswas and Sharma 1994; Neumann et al. 1993).

It is evident that heat stress severity experienced by the parasites infected poikilothermal organisms such as reptiles or fish or insects should predominantly depend on the temperature of the host body which can vary dramatically.

Along these lines, it was shown that *Leishmania* species dwelling in the blood of thermoresistant desert toad-headed agama actively synthesize Hsps at 40 °C while in the nocturnal gecko all protein synthesis in the parasite cells is completely inhabited at 38 °C (Ul'masov et al. 1988). These experiments exploring different lizard species from thermally contrasting niches for the first time revealed coevolution of parasite-host system with respect to such vital trait as molecular response to temperature elevation (HS).

Notably, large quantities of Hsps usually produced by bacteria and parasites in the host cells after infection often elicit an immune response and may be useful in generating vaccines. Along these lines, it was shown that *Leishmania infantum* deletion mutant, lacking both *Hsp70-II* alleles (*ΔHsp70-II*), provided protection against *Leishmania* infection in the *L. major*-BALB/c infection model and can be a safe live vaccine as immunodeficient SCID mice, and hamsters, infected with mutant parasites did not develop any sign of pathology (Carrión et al. 2011; Folgueira et al. 2008). It was shown that tumour necrosis factor-alpha and phagocytosis induces expression of stress proteins in virulent promastigotes of *Leishmania donovani* (Salotra et al. 1995).

In general, parasites evolved to respond to temperature changes either by high constitutive synthesis of certain Hsps or by their rapid induction following the infection.

Likewise, the heat-induced disaggregase Hsp104 of *Candida albicans* plays a role in biofilm formation and pathogenicity in a worm infection model. Biofilm formation by cells lacking *Hsp104* gene proved to be defective in different *in vitro* models. Thus, biofilms formed by the wild-type strains showed patterns of intertwined hyphae in the extracellular matrix. In contrast, biofilm formed by the *Hsp104* mutant showed multiple structural defects and appeared patchy and loose. Apparently these defects result in decreased virulence of the *Hsp104* mutant observed in the *C. elegans* infection model, hence, providing an indication of a role for Hsp104 in *C. albicans* virulence, in addition to its key role in the thermotolerance (Fiori et al. 2012).

Pathogenic bacteria such as *Salmonella typhimurium* or *Listeria monocytogenes* also actively synthesize various chaperones such as GroEL, GroES, DnaK and HtpG in response to phagocytosis and reactive oxygen species and certain stress proteins e.g. GroEL or DnaK may reach high concentration (Hanawa et al. 1999; Morgan et al. 1986; Wiesgigl and Clos 2001). Similarly, certain bacterial endosymbionts that infect various insect species such as bacterium *Buchnera* constitutively

express a protein, symbionin belonging to Hsp60 (GroEL) family at very high levels as well as several other bacterial chaperones (Sato and Ishikawa 1997).

On the other hand, high conservatism exhibited by Hsps even between very distant phyla sometimes results in cross-reaction of the anti-bodies produced against bacterial Hsps with epitopes of orthologous host's Hsps which may lead to severe autoimmune diseases such as systemic lupus erythematosus (SLE) or rheumatoid arthritis (Tsoulfa et al. 1989; Panchapakesan et al. 1992).

4.6.3 Heat Shock Proteins in Defense

It is known that giant hornets (*Vespa mandarinia japonica*) represent natural enemies of the honeybees and specifically Japanese honeybees (*Apis cerana japonica*). Giant hornets often attack the hives, kill the bees and consume honey stored in the honeycomb. The bees are practically defenseless when attacked by hornets because they cannot pierce their thick chitin. However, the Japanese bees developed an effective defensive strategy for such cases. When the first hornets ("scouts") appear, numerous bees (more than 500) form a ball around the intruder (Fig. 4.16). The temperature inside the bee balls reaches 46 °C and the giant hornets are killed in less than 10 min when they are trapped in a bee ball created by the Japanese honeybees. It was found that the CO_2 concentration inside the bee ball also reaches a maximum in the initial 0–5 min phase after bee ball formation. The lethal temperature of the bees is significantly higher than that of hornets (up to 49–50 °C) and it was concluded that CO_2 produced inside the bee ball by honeybees is a major factor together with the temperature involved in defense against giant hornets (Ono et al. 1995; Sugahara and Sakamoto 2009).

In experimental ecologically relevant episodes of hyperthermia between 33 and 50 °C, Hsp70 expression and Hsp70/Hsc70 activity in brains of nonflying laboratory-held bees increased by only two to three times at temperatures 46–50 °C. In this

Fig. 4.16 Defense heating bee ball around giant hornet attacking the hive (Photo by Masato Ono, Tamagawa University, Tokyo. http://www.hymenoptera.de/html/gallery/Hornissen/exoten/riesenhornisse/Thermal+Defense+by+Acj_Photo+by+Masato+Ono_.JPG.html)

feature honey bee resembles other heat-resistant organisms with low heat shock mediated Hsp70 induction relatively to the basic Hsp70 level. Moreover, Hsp70 induction after such HS was practically undetectable in thoracic-flight muscles. These data suggest that certain honey bee tissues, especially flight muscles, are extremely thermotolerant. Furthermore, Hsp70 expression in the thoraxes of flight-capable bees is probably flight-induced by oxidative and mechanical damage to flight muscle proteins rather than temperature (Elekonich 2009).

A unique case of exploring high temperature for defense was described by Wehner et al. (1992) for desert ants *Cataglyphis* that are able to tolerate body temperature of 50 °C for at least 10 min. These ants presumably voluntarily undergo body temperature of 50 °C and more, probably to escape predators (Wehner et al. 1992).

Furthermore, it was shown in goldfish (*Carassius auratus*) that the expression of Hsp70 can be induced by the presence of a predator. Remarkably, when the fishes were exposed to a potential predator, bluegills (*Lepomos macrochirus*), they experienced an increase in Hsp70 mRNA level in the brain. When goldfish individuals were placed in a transparent tank around which bluegills were swimming, Hsp70 mRNA expression was significantly increased after 6 and 12 h. Notably, goldfish exposed to water circulating through a tank with several bluegills showed no sign of changes in Hsp70 mRNA expression. These results suggest that psychological stress may enhance Hsp70 mRNA expression in the brain just via visual perception and such presumably defensive response may somehow modulate the consequences of the stress (Kagawa and Mugiya 2000).

4.6.4 Hsps as Biomarkers of Environmental Pollution

Large scale field studies of stress proteins in various non model organisms indicated that heat shock proteins may be induced in response to abiotic contamination of the environment, in particular to different chemical pollutants. Basing on these data it was suggested to use Hsps as sensitive natural biomarkers providing information about current state of the environment, which may help to estimate the biological impact of toxic chemicals to live organisms and predict adverse consequences of such exposure. Such biological approach based on quantitative analysis of Hsps is often more sensitive and reproducible than direct monitoring of environmental contamination. There are three aspects of stress proteins that are essential for their use as biomarkers of pollution:

1. Hsps are the important components of the cellular protective and adaptive response;
2. synthesis of Hsps is likely to be induced by a large number of xenobiotics;
3. different Hsps are highly conserved in all organisms from bacteria and plants to man and in this regard the same sets of antibodies (kits) can be used for their detection and measurement in various organisms (Bierkens 2000). The estimation of Hsps concentrations as biomarkers for understandable reasons is applied predominantly in marine and soil toxicology (Feder and Hofmann 1999).

It is of note, that measurements of Hsps level as a biomarker of pollution were already applied in a variety of organisms including: plants (algae) (*Enteromorpha intestinalis*); reef coral *Montastraea franksi*; clamps (*Mytilus edulis*, *Mytilus galloprovincialis*, *Mytilus trossulus*); planarian *Dugesia (Girardia) schubarti*; copepods (*Tigriopus japonicas*); amphipods (*Gammarus roeseli*); insect larvae (*Chironomus tentans*); fishes *Cyprinodon nevadensis amargosa*, *Limanda limanda*, *Carassius carassius*, zebrafish; ascidian *Pseudodistoma crucigaster* and many others (Bierkens 2000; Hallare et al. 2005; Lee et al. 2006; Lewis et al. 2001; Mićović et al. 2009; Rios-Sicairos et al. 2010; Venn et al. 2009; Wu et al. 1996).

In several cases, such field studies clearly demonstrated correlation between levels of different pollutants (especially heavy metals) and Hsps levels.

Thus, in *Enteromorpha intestinalis* Hsp70 expression was increased with copper exposure but was not unaltered following exposure to the herbicide Irgarol 1051 (Lewis et al. 2001). Strong induction of Hsp70 occurred in the blue mussels *Mytilus edulis* from Southern Baltic at high concentration of heavy metals. Combination of metals (Cd + Cu) was found to increase the Hsp70 level more intensely than related concentrations of singularly applied metals (Radłowska and Pempkowiak 2002). In *Mytilus trossulus* Hsp70 induction roughly correlated with arsenic and cadmium levels in water (La Porte 2005; Radłowska and Pempkowiak 1998). In the *Mytilus galloprovincialis* Hsp70 and metallothioneins were induced by heavy metals, Pb, Hg and Cd, implying that these stress proteins might be power biomarkers of marine pollution (Mićović et al. 2009).

To identify a sensitive biomarker of freshwater monitoring, pollutant-induced expression of heat shock proteins (Hsps) in the larvae of the aquatic midge *Chironomus tentans* (Diptera, Chironomidae) was evaluated. Different substances, such as nonylphenol, bisphenol-A, 17-alpha-ethynyl estradiol, bis(2-ethylhexyl) phthalate, endosulfan, paraquat dichloride, chloropyriphos, fenitrothion, cadmium chloride, lead nitrate, potassium dichromate, benzo[*a*]pyrene and carbon tetrachloride were used. The response of the Hsps gene expression to chemical exposure was rapid and sensitive to even very low chemical concentrations but it was not stressor specific. Interestingly, an increase in the expression of *Hsps* genes was observed not only for a stress inducible form (Hsp70), but also for a constitutively (HSC70) expressed form (Lee et al. 2006).

Furthermore, zebrafish embryos can be used for water quality assessing with detecting of Hsp70 levels as a biomarker. To evaluate the suitability of these tests for environmental screening, fertilized zebrafish eggs were exposed to water samples collected from five sites of varying levels of pollution from Laguna Lake, Philippines. Reconstituted water was used as laboratory control while water samples from a highly polluted freshwater source were used as a positive control. The levels of Hsp70 in embryos, exposed in water from two sites located closest to Manila, the Philippine capital (Northern West Bay and Central West Bay), showed a pronounced Hsp70 elevation relative to the control probes (Hallare et al. 2005).

Similarly, it was shown that kidney Hsp30 and liver Hsp70 expression can serve as sensitive biomarkers for the presence of field environmental stressors in wild crucian carp populations (An et al. 2014). In field populations of the dab

(*Limanda limanda*) Hsp70 levels correlated with DNA damage level caused by stress conditions (Schröder et al. 2000).

At the present time highly sensitive and cheap RT-PCR approach is commonly used to estimate the levels of *Hsp* genes expression under various environmental conditions. Thus, hepatic expression of Hsp70 and cytochrome P450 1A mRNAs in white mullet (*Mugil curema*) monitored by RT-PCR from July, 2005 until July, 2006 in three coastal lagoons located in the Southern Gulf of California, Mexico, was higher for both genes in the Urias Estuary, which was considered the most polluted among the three systems, especially during the rainy season (summer to fall). These results indicate that fishes *Mugil curema* is a good candidate species to implement biomonitoring programs in tropical coastal environments (Rios-Sicairos et al. 2010).

It is well known that the impacts of marine pollutants on reef corals and their symbiotic algae are an important element of global coral reef decline. It was shown that Hsp70, and to a lesser extent Hsp90 expression increases significantly following exposure to two major marine toxic pollutants (copper and dispertant Corexit 9527) and, hence, their levels may serve as a good indicator of specific coral reef state (Venn et al. 2009).

Hsps as biomarkers in field bioassays were sometimes used in terrestrial invertebrates. Specifically, the Hsp70 response was investigated in isopods *Oniscus asellus* exposed to a heavy metal gradient around smelters near Avonmouth, UK. In the sites closest to the smelter, *O. asellus* showed highest Hsp70 levels. The authors conclude that Hsp70 level in isopod species can be a suitable biomarker of heavy metal pollution in metal-contaminated field sites (Arts et al. 2004).

Likewise, small heat shock protein 20 of the intertidal copepod *Tigriopus japonicus* can be used as a possible biomarker for exposure to endocrine disruptors (Seo et al. 2006).

Robert Tanguay and his group were the first to use the levels of autoantibodies against Hsp27, Hsp60 and Hsp70 for evaluation of deleterious effects of various chemicals and heat on the health state of workers of harmful manufactures, such as chemical industry or steelmaking enterprise (Wu et al. 1996, 1998; Yang et al. 2007).

The goal of these studies was to evaluate lymphocyte and/or plasma Hsp70 levels and levels of autoantibodies against Hsp70 as biomarkers for assessing exposure response of steel workers to complex coke oven emissions (COEs). It was shown that lymphocyte Hsp70 levels increased in the group with intermediate exposure to emissions but decreased in the high-exposure group (Yang et al. 2007). Presence of autoantibodies to heat stress proteins was detected in workers exposed to high temperature and carbon monoxide. Antibodies to Hsp27 and Hsp71 were found more frequently in the high temperature and carbon monoxide-exposed groups than in controls. Anti-Hsp60 antibodies were only detected in workers exposed to high temperature or carbon monoxide (Wu et al. 1996). Exposure to benzene induces autoantibodies to Hsp72 (Wu et al. 1998).

Despite the obvious benefits the wide use of Hsps levels measurements as biomarkers of environmental pollution is by hindered for several reasons. First of all since the induction of Hsps is as a rule is unspecific for different stresses, sometimes

it is difficult or impossible to explain the observed increase to a definite stressor. Second, usually organisms in nature undergo multistress challenges. For example, stress from herbicides may be influenced by the temperature or humidity elevation. Furthermore, sometimes Hsps expression may be modified by certain uncontrolled factors such as parasites contamination. For example, in amphipod *Gammarus roeseli* Hsp70 level increases significantly at response to heavy metals (Pd), but in the case of infection with cystacanths of the acanthocephalan (*Polymorphus minutus*) infected individuals did not exhibit Hsp70 induction (Sures and Radszuweit 2007). Therefore, the monitoring of Hsps levels in field populations should be always carried out in parallel with measurements of many other environmental parameters that may strongly influence the results of such measurements. Specifically, in articles cited above the changes in Hsps concentrations in response to pollution were shown to correlate with: rate of growth (Lewis et al. 2001); cytochrome P450 level (Rios-Sicairos et al. 2010); P-glycoprotein level (Venn et al. 2009); metallothioneins level (Mićović et al. 2009); hemoglobin gene expression (Lee et al. 2006); embryonic lethality (Hallare et al. 2005); alanine aminotransferase (ALT), acid phosphatase (ACP) and alkaline phosphatase (ALP) activity (Wu et al. 1996).

4.7 Peculiar Structure and Functions of Cellular Proteins in Animals from Thermally Contrasting Environments

Apart from exploring a very ancient and universal system of heat shock genes found in all organisms studied so far, in the species dwelling under extremely cold or xeric conditions many cellular house-keeping proteins underwent adaptive evolution and acquired specific traits combining molecular stability on the one hand and structural flexibility on the other. The investigation of many enzymes from the organisms inhabiting thermally contrasting ecological niches established the correlation between the average temperature of environment and proteins stability. Thus, blue mussel (*Mytilus galloprovincialis*), a native of the Mediterranean Sea, is displacing the native blue mussel (*Mytilus trossulus*) from its habitat in central and Southern California apparently due to physiological adaptations. These adaptations enabling the former species to perform better at high temperatures include the structure and function of the enzyme malate dehydrogenase (cMDH) (Fields et al. 2001). The demonstrated relative warm adaptation of *M. galloprovincialis* cMDH may be one of physiological features that increase the competitive ability of the invasive species in warm habitats (Fields et al. 2001). Interestingly, these differences consistent with warm adaptation result from minor changes in sequence: the *M. trossulus* ortholog differs from *M. galloprovincialis* ortholog by only two substitutions.

Likewise, studies of orthologs of lactate dehydrogenase (LDH) and other vital enzymes performed in different laboratories have revealed the importance of conserving kinetic properties and structural stability during adaptation to temperature, and recently identified the types of amino acid substitutions causing such adaptive variations of proteins in ecological aspect (see below).

There are also a number of examples of qualitative adaptive strategies, when expression of distinctly different isoenzymes contributes to seasonal thermal adaptation by adjusting a particular metabolic node to new environmental conditions. Such isoforms were described for acetylcholine esterase, ferritin, choline esterase, LDH and many other enzymes (see Zakhartsev et al. 2007).

It was also shown that stabilization of proteins tertiary structure preventing their heat denaturation in thermoresistant species was usually achieved by means of specific amino acid substitutions resulting in the formation of additional intramolecular links in the vicinity of active center which stabilize the enzyme structure (Fields 2001).

As a result, in organisms resistant to heat proteins denaturation and misfolding begins at significantly higher temperatures and, hence, the activation of HSF after the disruption of HSF-Hsp90 complexes and beginning of abundant Hsps synthesis occur at much higher temperatures in comparison with related thermosensitive species.

The investigation of various (mostly fungi) thermophiles helps to understand major trend in proteins evolution to cope with constant high temperature conditions. Although proteomes of highly thermophilic prokaryotes provided a lot of valuable information in this respect and were successfully exploited in biotechnology, many proteins required for eukaryotic cell functioning under high temperature conditions are absent from bacteria or archaea. Recently, the comparative analysis of the first genome of the thermophilic fungi (*Chaetomium thermophilum*) with the genomes of two mesophilic species (*Thielavia terrestris* and *Thielavia heterothallica*) revealed consistent amino acid substitutions apparently associated to thermophily. Importantly, the same substitutions were also found in an independent lineage of thermophilic fungi (Van Noort 2013). The most consistent pattern observed in almost all fungal lineages of the thermophilic genomes is the substitutions of lysine by arginine and tryptophane (Van Noort 2013).

Furthermore, whole-genome analysis allowed to conclude that in the case of hyperthermophilic archaea the encoded proteins are characterized by an increase in frequency of charged amino acid residues and a decrease in that of polar uncharged residues as compared to the mesophilic counterparts. However, a discrepancy is noticeable in the overrepresentation of positively and negatively charged residues in hyperthermophilic proteins. The percentage of positively charged amino acids residues is significantly higher in hyperthermophiles compared to mesophiles. Furthermore, there are significant increases in aromaticity and average hydrophobicity and a decrease in the usage of polar uncharged amino acid residues (Ser, Thr, Gln and Asn) in thermophilic proteins. Examination of surface charge distribution reveals a marked increase of positive charge in the surfaces of thermophilic proteins as compared to their mesophilic counterparts (Das et al. 2006; Haney et al. 1999; Nakashima et al. 2003). These features apparently determine the extremely high thermal stability of thermophilic arhaea proteins and their ability to function at high temperatures up to near 100 °C. In general, thermophilic archaea demonstrate many adaptive differences from other organisms living in ordinary conditions, non-connected with protein structure and chaperone functions, in particular

unusual structure of membrane phospholipids, DNA supercoiling and many others (Mirambeau et al. 1984; van de Vossenberg et al. 1998).

Although Hsps and in particular Hsp70 are highly conserved in close species there are data implying that temperature selection may modulate the structure and function of the chaperones depending on specific thermal habitats. It was shown in a few studies that orthologous variants of molecular chaperone from differential thermally adapted species significantly vary in their thermal responses. Thus, comparison of Hsc70 orthologs from polar and temperate notothenoid fishes differ in their ability to prevent thermal aggregation of lactate dehydrogenase (Place and Hofmann 2005). Hsc70 from the Antarctic species lost the ability to completely refold denatured LDH at a lower temperature in comparison with Hsc70 from the temperate species. Likewise, Hsc70 from highly eurythermal marine goby (*Gillichthys mirabilis*) exhibits temperature insensitivity across the range of temperatures that are ecologically relevant for this species during luciferase refolding assays (Place and Hofmann 2001).

These data suggest that structure and functions of chaperones maps onto the thermal history of host species and that temperature selection acted on their molecular structure.

4.8 Conclusions

The broad scale analysis performed herein includes the data obtained in various organisms belonging to different taxa from mollusks and insects to mammalian species that dwell under highly variable and sometimes aggressive habitats. The accumulated evidence enables to conclude that certain chaperone-dependent molecular adaptations may be common for highly phylogenetically diverse organisms while others may be restricted to a specific group or environment. The major established regularities are summarized as follows:

1. Most thermoresistant organisms are characterized by comparatively high constitutive level of Hsp70 and other Hsps in relation to close forms from temperate regions. Such preparative defense strategy provides certain degree of preadaptation at the cellular and organism levels and usually does not require massive Hsps induction under conditions of moderate heat stress in comparison with thermosensitive forms (Fig. 4.17).
2. High constitutive levels of Hsp70 and other chaperones often observed in highly eurythermic and/or thermophilic forms may be regulated at different levels that include transcription efficacy; *Hsp* genes copy number, and stability of correspondent mRNAs or chaperones themselves. Specific mechanisms underlying constitutive and inducible levels of Hsps may vary within the same order (e.g. Diptera).
3. Different organisms explore members of specific Hsps families or their combinations (Hsp70, small Hsps or Hsp40) to respond to stressful conditions and relative role of particular chaperones or their isoforms strongly depends on the severity of the challenge

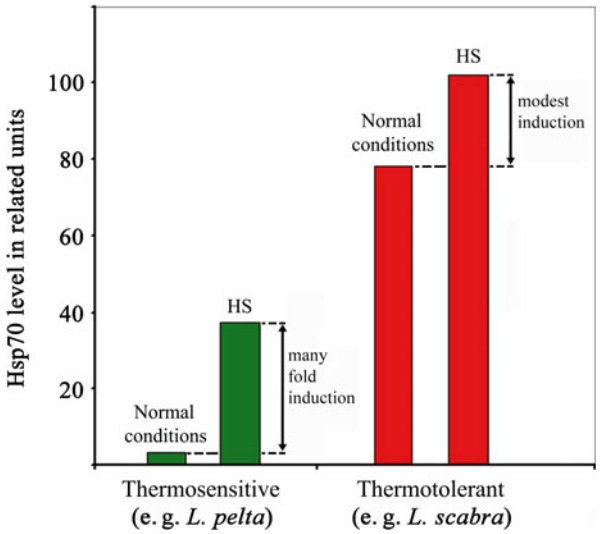

Fig. 4.17 Typical patterns of Hsp70 induction in thermosensitive and thermoresistant organisms. HSR of two thermally contrasting mollusk species are given as example. Two species of limpets (*L. pelta* and *L. scabra*) dwelling in thermally contrasting biotopes were chosen for comparison. Thermoresistant species (i.e. *L. scabra*) usually exhibit high constitutive Hsp70 level and modest induction after HS. In contrast, thermosensitive species (i.e. *L. pelta*) are characterized by low Hsp70 levels and strong induction after temperature elevation (Adapted from Dong et al. 2008 with permission)

4. Activation of HSF and induction of Hsps synthesis (threshold) in heat tolerant organisms occurs at higher temperatures in comparison with related thermosensitive forms, which implies that cellular proteins from thermophilic organisms as well as transcriptional and translational machinery in general are more stable than their mesophilic counterparts.
5. Insect life stages (e.g. larvae versus adults) often differ significantly in the heat shock response and thermoresistance. Thus, larvae of certain stenothermal cold-adapted insect species which constitutively expressing Hsp70, partially or completely lost the ability to activate Hsps synthesis after HS, while their adults preserved this ability.
6. Organisms entering certain life-stages to survive extreme conditions (desiccation, anabiosis, diapause etc.) usually accumulate large amounts of certain Hsps necessary for survival and subsequent recovery.
7. Hsps may be involved in pathogenicity of certain parasites species.
8. Different Hsps may be used as biomarkers of environmental pollution.

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Chapter 5
Different Trends in the Evolution of Heat Shock Genes System

In Chap. 4 we described characteristic differences in the thermoresistance and Hsp synthesis patterns in various organisms including vertebrates, insects, Crustacea etc. Striking differences observed in Hsp synthesis in response to HS in close and phylogenetically distant forms could be due to different molecular mechanisms. It is possible to suggest that thermoresistant species may contain higher copy numbers of *Hsp70* genes as compared to related species from cold and temperate climates. However, this is an exception rather than a general rule. Thus, Southern blot analysis of genomic DNA has shown that lizard species sharply different in the constitutive level and kinetics of Hsp70 synthesis in response to temperature elevation (i.e. *L. vivipara* and *P. interscapularis*) not only preserved the same number of *Hsp70* genes but also retained their practically identical structure and arrangement in the genome. It is noteworthy, that the species compared belong to different subfamilies that diverged millions years ago. On the other hand, the comparison of *hsf1* genes in these two lizard species demonstrated striking differences in their structure (Zatsepina et al. 2000). These data suggest high conservatism for *Hsp70* genes in the investigated lizard species. Therefore, it is plausible to conclude that the observed differences in the HSR resulted not from different copy number but may be due to peculiarities of the regulatory machinery of heat shock genes in various organisms.

The structure of *Hsp70* and other stress-genes are highly conserved in most studied organisms, similar to conservatism observed for other house-keeping genes that often have tandem organization, e.g. histone loci or ribosomal genes (Marzluff et al. 2002). Thus, mammalian *HSP70* genes exhibit approximately 50 % similarity with orthologous genes in *E. coli* while *Drosophila Hsp70* genes are 73 % homologous to the correspondent mammalian genes at the nucleotide level (Hunt and Morimoto 1985). Interestingly, similar homology in phylogenetically distant forms is also observed for the components of HSR at the level of individual Hsp proteins, suggesting the importance of the primary genes structure for high stability and efficient translation of *Hsp70* mRNA (Hunt and Morimoto 1985).

The presence of multiple copies belonging to certain families represents another characteristic feature of the *Hsp70*, *Hsp40* and small *Hsps* genes typically observed

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in various eukaryotes. The investigation of homology within *Hsps* genes families and comparison with related species revealed in a few cases higher similarity in interspecific comparison, which implies early amplification and divergence of the pertinent genes families (Lindquist and Craig 1988). It was shown by many authors that high homology (practical identity) of *Hsp70* genes observed in different Diptera species is achieved by means of gene conversion (Bettencourt and Feder 2002; Garbuz et al. 2011b; Leigh Brown and Ish-Horowicz 1981).

A lack of introns, typical for most eukaryotic *Hsp* genes, represents another feature common for these genes demonstrated soon after the discovery of *Hsps* system (Southgate et al. 1985). The absence of introns enables the transcribed *Hsps* mRNAs to rapidly move from nuclei to cytoplasm without splicing, significantly accelerating the whole HSR. Notably, the splicing machinery is usually strongly disturbed by HS and other stresses, which may favor the selection of intronless copies of *Hsp* genes in the course of evolution.

However, there are several prominent exceptions from this rule, such as *Hsp90* genes which are usually transcribed under non-stress conditions in many organisms and contain one intron (Southgate et al. 1985). Introns were found in cognate (HSC70) genes of *Drosophila* and other organisms. Besides, *Hsp70* genes of the nematode *Caenorhabditis elegans* and mud crab (*Scylla paramamosain*) as well as *Hsp60* and *Hsp10* genes also contain introns (Hansen et al. 2003; Heschl and Baillie 1989, 1990; Yang et al. 2013a, b).

A typical structure of the genomic loci comprising *Hsps* genes and trends in their evolution may be illustrated exploring a few well studied model organisms belonging to different taxa. In higher eukaryotic organisms, initially *Hsp70* genes were cloned and actively studied in *D. melanogaster* (Livak et al. 1978). Following the discovery of *Hsp70* loci in this model species the orthologous genes were cloned and investigated from other multiple *Drosophila* and Diptera species, including geographically diverse strains. At the present time, *Hsp70* genes have been cloned and sequenced from multiple organisms including bacteria, insects, various parasites (e.g. *Leishmania* species); plants, nematodes, mammalian species including mice, rats and humans. Practically in all studied eukaryotes, Hsp70 family proteins are encoded by several alleles and the copy number of *Hsp70* genes may vary from 3–4 to 15 copies and even more (Kampinga et al. 2009). Note, that in bacteria (e.g. *E. coli*) there is only one gene (*DnaK*) orthologous to eukaryotic *Hsp70* (Segal and Ron 1996).

Drosophila species belonging to *melanogaster* subgroup were subject of various genetic and molecular studies including whole genome sequencing (Campo et al. 2013). It was demonstrated that *D. melanogaster* genome contains five or six copies of *Hsp70* genes depending on the strain (Maside et al. 2002). *Hsp70* genes are localized at 87A2–A3 and 87B12–B15 loci of chromosome 3R (chromosome loci are given accordingly to the modern nomenclature provided in FlyBase). In some papers localization of *D. melanogaster Hsp70* genes described as 87A and 87C1. The *Hsp70Aa* and *Hsp70Ab* genes, clustered in opposite orientation at chromosomal locus 87A2–A3, are only 2 of the 5–6 *Hsp70* genes in *D. melanogaster*; the others are clustered at 87B where two of them are arranged in opposite orientation

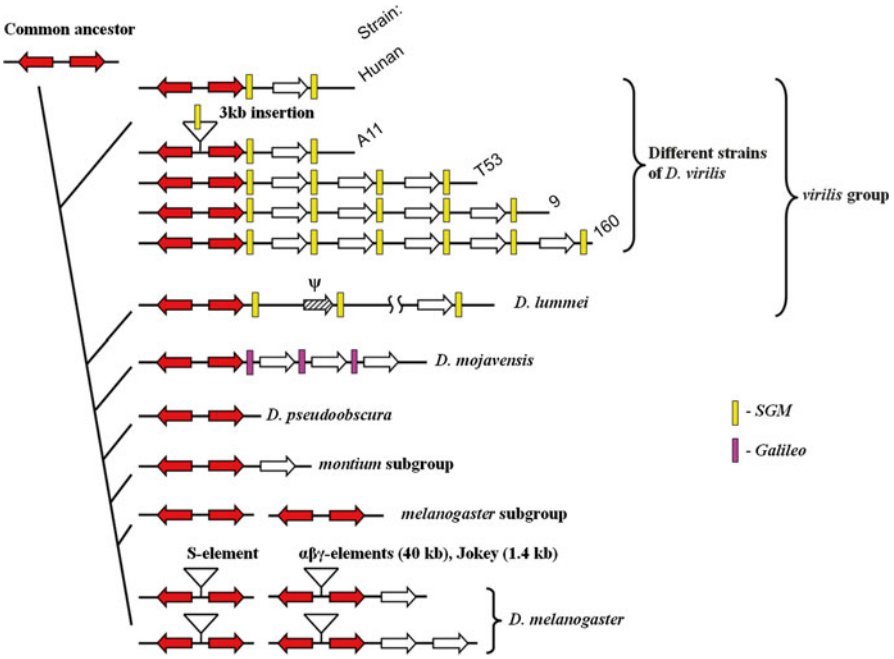

Fig. 5.1 Schematic illustration of the evolution of *Hsp70* locus in selected *Drosophila* groups. Intaspecific differences in *Hsp70* copy number observed in various geographical *D. virilis* strains are indicated (A11 and Hunan China, T53 Tashkent, 9 Caucasus, 160 Japan). Ψ pseudogene in *D. lummei*. *SGM* and *Galileo* mobile elements, integrated into *Hsp70* cluster (see details in the text)

exactly as in 87A while other copies represent tandem organization (Fig. 5.1). All *Hsp70* genes do not contain introns and encode a protein of practically identical sequence with difference in only nine amino acids residues between 87A and 87B groups. The similarity among the *Hsp70* genes in coding sequence, however, is not as extensive for regulatory sequence, and the *Hsp70* genes at the two clusters differ in the kinetics and tissue specificity of their expression (Lakhotia and Prasanth 2002). The inverted copy at 87B is disrupted by 38 kb intergenic region which contains short repeats of very peculiar structure, the so called "αβγ-elements" (Fig. 5.1). The transcription of these repeats is also induced by HS but their RNA is not translated (Hackett and Lis 1981). All *Hsp70* copies found at 87A and 87B loci are 96 % homologous and such high interlocus and intralocus conservatism is apparently maintained by gene conversion mechanism (Bettencourt and Feder 2002; Leigh Brown and Ish-Horowicz 1981).

Molecular analysis revealed the arrangement and evolution of *Hsp70* clusters in all species comprising the *melanogaster* group (Bettencourt and Feder 2001, 2002; Konstantopoulou et al. 1998). Evolution by tandem duplications of *Hsp70* copies occurred in the *Drosophila ananasae* and *montium* subgroups, while in certain other *Drosophila* species e.g. *melanogaster* subgroup, the duplication of the whole ancestral structure took place (Fig. 5.1).

Figure 5.1 illustrates the peculiar arrangement of *Hsp70* genes in *D. simulans*, *D. mauritiana* and few other species. Southern blot analysis coupled with hybridization and sequencing demonstrated the presence of four *Hsp70* genes in two separate clusters located at a single chromosome locus in orienatation "head to head" in different species of *melanogaster* group including: *D. elegans*, *D. ficusphila*, *D. eugracilis*, *D. lucipennis*, *D. takahashii*, *D. orena* and *D. erecta*. Therefore, the accumulated data suggest that duplication of *Hsp70* locus predated the divergence of the *melanogaster* subgroup from some other subgroups within *melanogaster* group (Bettencourt and Feder 2001).

The presence of conversion tracts indicates that *Hsp70* sequence similarity in *D. melanogaster*, *D. orena*, and other species in the *melanogaster* subgroup is maintained by gene conversion, which for some reason is relaxed in *D. mauritiana*. Thus, five out of eight *Hsp70* alleles cloned from the latter species represent pseudogenes because they contained deletions disrupting the ORF and resulted in the generation of a premature termination codons (nonsense codons) (Bettencourt and Feder 2001). In general, the investigation of species of *melanogaster* subgroup demonstrated amazingly conserved arrangement and copy number of *Hsp70* gene within the group (Fig. 5.1). The same is true for *D. melanogaster*, a cosmopolitan species originated in Africa in which multiple laboratory strains and various geographical populations studied exhibit amazingly uniform number and structure of *Hsp70* gene copies (Bettencourt and Feder 2001; Maside et al. 2002).

It is of note that all Diptera species that have been studied so far share an inverted pair of *Hsp70*-encoding genes at a single chromosome locus which likely represents a basic unit which underwent further evolution in the course of species divergence. Characteristically, in mosquitoes (Benedict et al. 1993) and the basal *Drosophila* species *Drosophila pseudoobscura* these two inverted copies are the only known *Hsp70* genes (Fig. 5.1).

In contrast to cosmopolitan *D. melanogaster*, which is associated with humans and found throughout the world, *Drosophila* species of the *virilis* group occupy distinct geographical areas that sometimes overlap (Patterson and Stone 1952; Throckmorton 1982). *D. virilis* distribution throughout the Northern Hemisphere is primarily below 40°N latitude. Cold-adapted *D. lummei*, considered the closest relative of *D. virilis*, occurs from just above 40°N to just above 65°N latitude and from Sweden to West to the Pacific coast of Asia. *D. virilis* and *D. lummei* replace one another along a latitudinal cline and demonstrate striking differences in thermotolerance and HSR (Chap. 4).

D. lummei according to different lines of evidence descended from *D. virilis* or a common ancestor approximately 5 MYA (Spicer and Bell 2002) and invaded higher latitudes than its ancestor. *D. virilis* and *D. lummei* can be crossed under laboratory conditions and produce partially fertile progeny.

Since we observed characteristic differences in thermoresistance and general HSR in these species, e.g. *D. lummei* apparently does not have induced thermotolerance (Chap. 4), it was of significant interest to compare the structure of clusters comprising *Hsp70* genes in *D. virilis* and *D. lummei*.

Based on the above-mentioned correlation between latitude and thermoresistance in *D. virilis* and *D. lummei*, it was possible to speculate that the observed differences in HSR may represent evolutionary adaptations resulted from selection to contrasting habitats (not just by-product of the species divergence).

On the other hand, the intraspecific comparison of thermoresistance and Hsps synthesis in response to HS in geographically diverse strains of *D. virilis* and *D. lummei* did not reveal significant dependence on the temperature of their habitats. All strains within the same species were characterized by similar responses to HS as concerns thermoresistance, irrespective of whether they originated from the Southern or Northern part of the species range (Garbuz et al. 2003). It is possible that such a pattern is not universal for *Drosophila* species, because a lowland population of *D. buzzatii* regularly exposed to high temperature in nature exhibits a lower expression level of Hsp70 at a temperature that generates a strong response in the highland population of the same species that rarely encounters extreme temperatures (Sørensen et al. 2005). See also below pertinent information in the discussion of *D. melanogaster* sub-populations inhabiting different slopes of Evolution Canyon.

In the species of the *virilis* group *Hsp70* genes are localized at a single chromosomal locus. Previously one of us localized *Hsp70* genes in *D. virilis* and *D. lummei* at the 29C site of chromosome 2 (Evgen'ev et al. 1978, 2004).

Contrary to the *melanogaster* pattern, detailed Southern blot analysis revealed striking differences in the *Hsp70* copy number not only between cosmopolitan low-latitude *D. virilis* and closely related Northern species *D. lummei*, but also between geographical strains belonging to both species. We included 12 strains of *D. virilis* and 5 strains of *D. lummei* in such analysis. As expected in both species we revealed the basal structure consisting of two *Hsp70* copies in inverted orientation (head-to-head) with intergenic interval equal 1.5 kb. Surprisingly, *D. virilis* geographical strains contain from three to seven tandemly arranged copies (Fig. 5.1). Interestingly, strains of the Northern thermosensitive species (*D. lummei*) contain significantly less *Hsp70* copies than most strains of the thermophilic *D. virilis*. It is of note, that in all studied *D. lummei* geographical strains without exception, one of the *Hsp70* copies contains large deletions in its ORF and, hence, likely represents a pseudogene originated early after *D. lummei* divergence from an ancestor. Subsequently, when *Hsp70* clusters were cloned from representative strains of both compared species it was demonstrated that in *D. virilis* the distance between tandemly arranged copies is always 4.8 kb while in *D. lummei* this interval is much larger and, hence, *Hsp70* genes in the latter species are more loosely packed in the cluster (Evgen'ev et al. 2004).

The sequencing of complete *Hsp70* clusters in *D. virilis* and *D. lummei* demonstrated that all *D. virilis Hsp70* copies comprising the cluster in the given strain are highly conserved and contain only a few substitutions throughout the coding region. Similarly, promoters (250 bps) of *Hsp70* genes in *D. virilis* and *D. lummei* are also highly homologous and differ at only 17 positions. In contrast, 3′-flanking regions of *Hsp70* in both species are diverged and contain multiple substitutions and

deletions revealed in both intraspecific and interspecies comparisons. Such characteristic differences suggest that gene conversion is involved in maintenance of high similarity of coding sequences of *Hsp70* genes in the *virilis* species, essentially as was observed in the *D. melanogaster Hsp70* cluster. The polyadenylation site apparently represents a 3′-boundary of the *Hsp70* sequences on which conversion machinery operates, because 3′-flanking regions of many genes including *Hsp70* copies usually include multiple substitutions and deletions. In contrast, 5′-flanking sequences of *D. virilis*, *D. lummei*, and *D. melanogaster Hsp70* genes also exhibit high similarity that is comparable to that of the coding regions. This high conservatism of the regulatory regions is probably a result of strong selection pressure on efficient transcription. *Hsp70* promoter regions of *D. virilis* and related species, just like in the case of *D. melanogaster*, include a TATA-box, four HSE canonical sequences, and several GAGA-motifs located approximately 200 bps upstream the transcription start site (Evgen'ev et al. 2004).

Notably, at the 3′-flanking region of all *Hsp70* genes of *D. virilis*, *D. lummei* and another *virilis* group species *D. montana* we detected a fragment of a very ancient *SGM* mobile element (Evgen'ev et al. 2004). This very ancient transposon may execute various structural and regulatory roles in different *Drosophila* species, and constitutes a significant part (about 10 %) of satellite DNA in *D. guanche* (Miller et al. 2000). The occurrence of *SGM* in specific regions of *Hsp70* genes of the *virilis* group species implicates this transposon in the evolution of *Hsp70* cluster in the group. In xeric *D. mojavensis* another mobile element, *Galileo*, has similar distribution in the *Hsp70* claster (Garbuz, personal communication). The role of transposable elements in the evolution of heat shock genes will be discussed in detail in Chap. 6.

The reorganization of the *Hsp70* cluster in the course of the *virilis* group species evolution may have occurred by unequal crossing-over, which leads to the fluctuations in the copy number that are evident at the present time in various geographical strains of the species. Such variations in *Hsp70* copy number provide polymorphism on which natural selection may operate depending on the environmental (predominantly thermal) conditions. Interestingly, we have demonstrated clear-cut positive correlation between the number of *hsp70* copies in the cluster and the level of Hsp70 synthesis after HS when different *D. virilis* strains were compared. Besides, the strains with higher copy number exhibit more Hsp70 isoforms as revealed by 2D electrophoresis (Garbuz et al. 2003; Evgen'ev et al. 2004).

In general, low-latitude thermophilic species belonging to different groups i.e. *D. virilis* и *D. mojavensis* are characterized by more compact arrangement of their *Hsp70* clusters, which likely provides higher levels of *Hsp70* genes expression due to cooperative interaction between regulatory regions and resulting rapid chromatin decondensation. In contrast, the distance between individual *Hsp70* copies is significantly larger in cold-adapted *D. lummei*, which may explain the occurrence of significantly smaller puffs formed at the correspondent loci after HS. This difference is particularly evident when the polytene chromosomes of interspecific hybrids are analyzed (Fig. 5.2) (Evgen'ev et al. 2004).

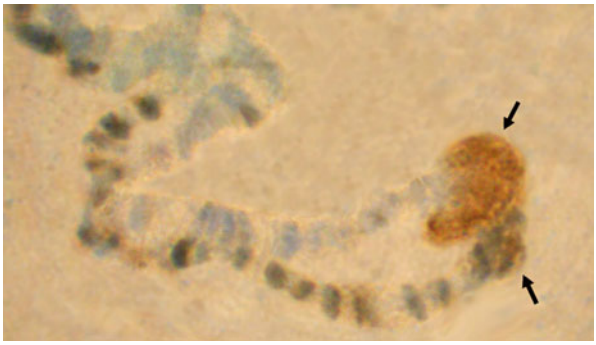

Fig. 5.2 *Hsp70* puffs (marked by *arrows*) on polythene chromosomes of *D. virilis* and *D. lummei* F1 hybrids after heat shock. Much smaller puff at the lower homeologue belonging to *D. lummei* comprising less *Hsp70* copies is evident (From Evgen'ev et al. (2004) with permission)

It is necessary to emphasize that *D. melanogaster* laboratory strains (e.g. Oregon R) synthesize significantly (two fold) more Hsp70 after mild and moderate HS in comparison with thermophilic *D. virilis* and *D. mojavensis*, despite the fact that these species have similar *Hsp70* copy numbers (Bettencourt and Feder 2001; Krebs 1999). The reason of this difference is not quite clear at the present time and can not be explained by different structure of correspondent regulatory regions of the studied species which have highly conserved *Hsp70* promoters containing similar number and the same distance of HSEs and other important motifs (e.g. GAGA-sites) from the TATA box (see Chap. 7).

Figure 5.1 illustrates the presumptive evolution of *Drosophila Hsp70* cluster (Evgen'ev et al. 2004). It is likely that ancestral species contained a basal structure consisting of two *Hsp70* copies in inverse orientation. Such original ancestral structure is preserved in *D. pseudoobscura* and in mosquitos (Benedict et al. 1993). Additional tandemly arranged copies found in *D. virilis* and other species probably resulted from several duplications due to unequal recombination between tandemly arranged copiers.

In cold-adapted *D. lummei* part of the tandemly arranged copies were subsequently lost by unequal crossing-over and pseudogenization, leading to the increase of intergenic intervals between individual functional *Hsp70* copies in the cluster. The size increase of intergenic regions may also occur due to the insertion and amplification of simple repeats, as evidently happened in the case of *Hsp70* genes in *D. melanogaster* where long tracks of αβγ-elements were found between *Hsp70* genes.

We speculate that in the course of divergent evolution the loss of a few *Hsp70* copies probably took place in *D. lummei* due to deletions and/or pseudogenization, a process which may not be deleterious for this cold-adapted species rarely subjected to drastic temperature fluctuations (Evgen'ev et al. 2004). The established characteristic differences in the structure of *Hsp70* clusters between high and low-latitude species of the *virilis* group differing by thermotolerance raised the question concerning the generic nature of the observed patterns.

In order to answer this question several Diptera species belonging to *Stratiomyidae* family were chosen for the investigation of *Hsp70*-containing loci. As was described

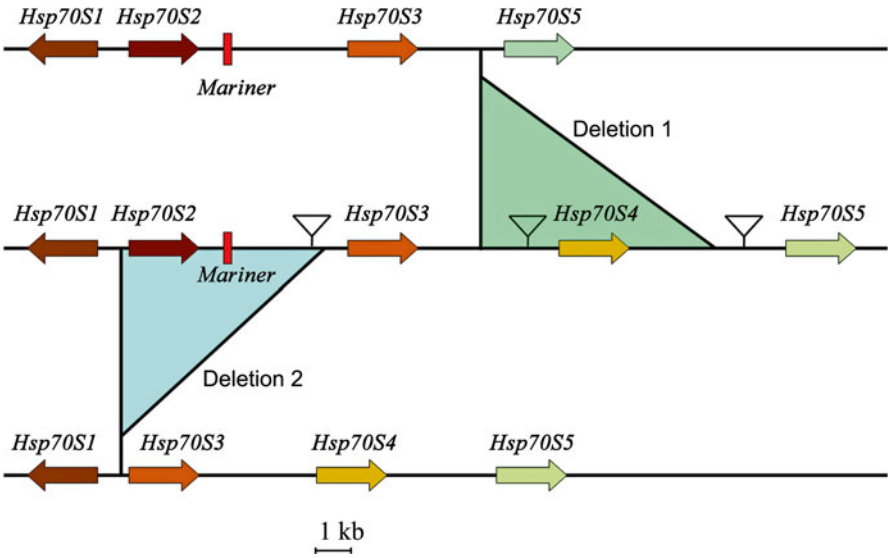

Fig. 5.3 General arrangement of *S. singularior Hsp70* genes cluster. *Large triangles* indicate the locations of presumptive deletions that include certain *Hsp70* copies in the sub-populations of the species. *Deletion 1* includes *Hsp70S4* copy; *Deletion 2* includes *Hsp70S2* copy. *Mariner* an insertion of ancient transposon *Mariner* fragment

in Chap. 4, certain species of this family are typical extremophiles while others may be found in various environments with strikingly different thermal regimes (Garbuz et al. 2008). For comparative analysis of *Hsp70* clusters *S. singularior* was chosen. The larvae of this eurythermal species were collected in the Crimean inland Lake Kirkoyashskoe where water and mud are saturated with H_2S, and mineralization reaches approximately 80 g/l. The measured temperature in the habitats of this Crimean stratiomyid used in our studies varied from 15 to 35 °C, but apparently it can be higher in summer and much lower in winter. For comparison with the above highly eurythermal species, the cold-adapted species *O. pardalina* belonging to the same family was collected from the spring habitat near St.-Petersburg which is characterized by a year-round stable low water temperature (4–8 °C) and total mineralization within 0.38–0.42 g/l. These species exhibit different HSR in terms of Hsps synthesis and thermoresistance (See Chap. 4).

It is of note, that in contrast to the majority of similar studies, this investigation was performed on fresh field material collected from the natural habitats, which helps to describe the patterns existing in natural populations of the compared species. The analysis of cloned sequences as expected revealed the presence of basal universal structure consisting of two inverted *Hsp70* copies in the genomes of both species (Garbuz et al. 2011b). Similar to *Drosophila* species, the organization of *S. singularior Hsp70* includes several tandemly arranged copies (Fig. 5.3). However, in contrast to *Drosophila* case, *S. singularior Hsp70* cluster exhibits amazingly high polymorphism in terms of tandem copy number, mutual arrangement and the

structure of intergenic regions even within a single population investigated. Furthermore, in contrast to the most *Drosophila* species, all *Hsp70* copies in the genome of *S. singularior* are highly conserved only within the ORF and 5′-flanking region, not exceeding 50 bps upstream from the transcription start which comprises TATA-box and the first HSE-sequence.

Surprisingly, in *S. singularior* the regulatory regions upstream of the first HSE and downstream of the stop codon do not show any significant similarity between different *Hsp70* copies. It is of note that conversion tracts were detected in *S. singularior Hsp70* genes (within ORF) and, hence, gene conversion is apparently operating within the indicated highly homologous regions of *Hsp70* genes in this species. The investigation of *S. singularior* specimens from the same area in the Crimea revealed the presence of at least three distinct subpopulations that differ by the number of *Hsp70* copies and the length of the intergenic intervals (Fig. 5.3). Apparently there is a constant exchange and recombination of genetic material between these sub-populations which may play a role in rapid adaptation to fluctuating thermal conditions of the habitat. Notably, high variability of *Hsp70* regulatory regions has been previously demonstrated in a xeric *Drosophila* species (*D. mojavensis*) which inhabits Mexican deserts and, similar to *S. singularior*, often encounters drastic temperature fluctuations (Krebs 1999). The significance of variability in *Hsp70* promoter regions in correspondent genes expression is discussed in detail in Chap. 7.

The analysis of *Hsp70* genes in the cold-adapted *O. pardalina* as expected revealed the presence of a basic inverted pair of copies. However, the distance between these inversely oriented copies was significantly larger in comparison with correspondent spacer of *S. singularior* (4.6 kbs vs. 1.5 kbs respectively). In addition to these inverted repeats, the *O. pardalina Hsp70* cluster contains two apparently functional tandemly arranged copies exhibiting high homology to *Hsp68* and two pseudogenes also belonging to the *Hsp70* family. Characteristically, the spacing between tandemly oriented members of the *Hsp70* family is much larger in *O. pardalina* in comparison with *S. singularior*. Furthermore, the comparison of different individual functional copies of *Hsp70* in *O. pardalina* revealed significantly more substitutions and deletions than in *S. singularior Hsp70* genes. This observation suggests that gene conversion is either altogether absent or does not function efficiently in *O. pardalina Hsp70* genes separated by very long intergenic regions (≥8 kbs). On the other hand when the same *Hsp70* copies (alleles) are compared, they exhibit amazing similarity in terms of both ORF and intergenic regions. High homology (virtually identity) of individual *Hsp70* alleles and intergenic regions observed in *O. pardalina* may be explained by a small size of the studied isolated population and comparatively stable thermal conditions of this particular habitat (4–8 °C) (Garbuz et al. 2011b).

The investigation of *Drosophila* species which replace each other along latitudinal gradient (e.g. *D. virilis* and *D. lummei*) or *Stratiomyidae* family species inhabiting strikingly different thermal niches revealed similar pattern of *Hsp70* organization in the compared organisms. Thus, thermophilic species (*D. virils* and *S. singularior*) seem to have more compact and conversion-prone structure of *Hsp70* clusters, while in related cold-adapted species dwelling under comparatively stable environmental

conditions, the intergenic regions are significantly longer and individual *Hsp70* copies comprising the cluster are subject to pseudogenization and loss by unequal crossing-over.

Compact arrangement of *Hsp70* copies in the cluster observed in thermoresistant species probably ensures rapid and efficient decompactization of the correspondent chromatin regions and highly efficient transcription due to cooperative interactions of *Hsps* regulatory regions. This speculation is corroborated by the kinetics of Hsp70 induction in the compared species after HS as well as by striking differences in the correspondent puff size evident in *D. virilis* x *D. lummei* interspecific hybrids (Evgen'ev et al. 2004).

Compact organization of *Hsp70* genes in the clusters may be also relevant for efficient suppression of Hsps system under normal non-stress conditions observed in *Drososphila* probably because the constitutive transcription of *Hsp70* genes in *Drosophila* species is deleterious and may cause developmental abnormalities e.g. by binding to inappropriate proteins (Feder et al. 1996; Feder 1997). It is known that efficient repression of *Hsp70* cluster localized at 87A locus is executed with the help of scs and scs′ insulators (scs – specialized chromatin structures), that restrict this locus from long-distance interactions and, also represent the boundaries of *Hsp70* decompactization occurring after temperature elevation or other stress (Petesch and Lis 2008). These insulators (scs and scs′) include binding sites for the protein BEAF32 and are able to block the action of enhancers on a reporter gene (e.g. *white*) (Hart et al. 1997).

It is of note, that a fragment of an ancient *Mariner* mobile element detected in *Hsp70* genes of *S. singularior* (Fig. 5.3) is reminiscent of the *SGM* element presence characteristic for *D. virilis Hsp70* cluster (see above) (Garbuz et al. 2011b). A possible involvement of mobile elements in the evolution and function of *Hsp* genes in various organisms will be discussed in Chap. 6.

In addition to inducible *Hsp70* genes, this family in *Diptera* includes *Hsp68* genes and several copies of cognate *Hsp70*. In *D. melanogaster*, the stress-inducible *Hsp68* gene is encoded by a single copy which is 77 % homologous (at the nucleotide level) to *Hsp70* genes (Holmgren et al. 1979). The evolution of *Hsp68* genes has been investigated in five species of *melanogaster* subgroup and five species belonging to *montium* subgroup (Kellett and McKechnie 2005). Species of the *montium* subgroup harbor two distinct types of *Hsp68* genes which differ significantly by the length of a C-terminal domain, which may influence the efficiency of the chaperones' interaction with protein substrates. The more evolutionary ancient species *D. pseudoobscura* contains two copies of *Hsp68* genes, one of which apparently is not functional and represents a pseudogene. Screening of *D. lummei* and *D. virilis* genomic libraries revealed in both species the presence of two functional copies of *Hsp68* genes arranged as an inverted repeat (Velikodvorskaia et al. 2005). It was hypothesized that *Hsp68* genes in *Drosophila* originated by duplication and subsequent divergence of the inverted pair of *Hsp70* genes (basal structure). Possibly, one of the *Hsp68* genes was subsequently lost in the course of evolution of the *melanogaster* and *montium* subgroups, while species of the *virilis* group apparently retained the original structure consisting of two inverted copies. *Drosophila* cognate *Hsp70*

genes (*Hsc70s*) are scattered in different regions of the genome including the X-chromosome. Generally, *Hsc70* genes often exhibit higher homology with correspondent cognate genes in other species than with inducible *Hsp70* genes or other representatives of *Hsc70* copies within the species genome. This feature possibly indicates early amplification of *Hsc70* genes which may predate species divergence. In contrast to inducible *Hsp70* genes, cognate copies belonging to this family often have typical exon-intron structure and may have various functions in the cell under non-stress conditions (http://flybase.bio.indiana.edu/).

Clustered organization of *Hsp70* genes is not restricted to Diptera. Thus, a *Hsp70* cluster was also described in African trypanosomes (*Trypanosoma brucei*) that are found in the bloodstream of their mammalian hosts or the disease vector tsetse flies. However, the general arrangement of *Hsp70* genes forming the cluster is quite different from that of *Drosophila* species. *Trypanosoma* cluster harbours six tandemly arranged *Hsp70* copies. One of these *Hsp70* genes is constitutively expressed and does not contain HSEs in the regulatory regions. This particular copy is 6 kb apart from the rest HS-inducible members of the cluster. The five inducible members of *Hsp70* family are separated by short 200–250 bps intergenic regions that contain regularly spaced TATA-boxes and HSEs. The demonstrated clustered and very compact organization of heat-inducible *Hsp70* copies in parasitic *Trypanosoma* is probably of great importance for the species' adaptation to specific environmental conditions, just like in *Diptera* thermophilic species (see Chap. 4). Thus, such organization of *Hsp70* copies makes possible highly efficient and rapid accumulation of *Hsp70* mRNA and correspondent proteins in response to temperature increase. Different *Trypanosoma* species being transmissive parasites often encounter acute HS after switching host when temperature fluctuations may range from 20–25 °C (insect body temperature) to 36–37 °C (mammalian body temperature) associated with *Hsp70* induction (Glass et al. 1986; Muhich and Boothroyd 1989).

Although *Hsp70* genes are found in many copies in various vertebrate organisms only three members (i.e. *HSPA1A*, *HSPA1B* and *HSPA1L*) belonging to this ubiquitous family of stress genes are always found in a linked cluster (400 kb distance) with the highly conserved major histocompatibility complex (MHC) locus. Besides humans, three *HSP70* copies were linked with MHC locus in rats, mice, bulls, frogs and many other vertebrates (Hunt et al. 1993; Salter-Cid et al. 1994; Walter et al. 1994). The constitutively expressed *HSP70A1L* gene in mammals is localized in inverse orientation in relation to the two major inducible members of the human *HSP70* family namely, *HSPA1A* and *HSPA1B* genes (Milner and Campbell 1990, 1992). All ten other members of human and other mammalian species *HSP70* genes are scattered in the genome and may be found in different chromosomes as single copies. As expected, inducible and highly similar *HSPA1A* and *HSPA1B* genes as well as the other highly inducible gene *HSPA6* lack introns and contain canonical HS promoters which comprise two complete HSE sequences upstream of TATA-box. In contrast to these inducible copies, *HSP70A1L* contains one intron of different length depending on the species and exhibits only 85 % similarity with the inducible members of the family. This gene does not contain HSEs and TATA box at the promoter region and is not induced by heat or other stresses.

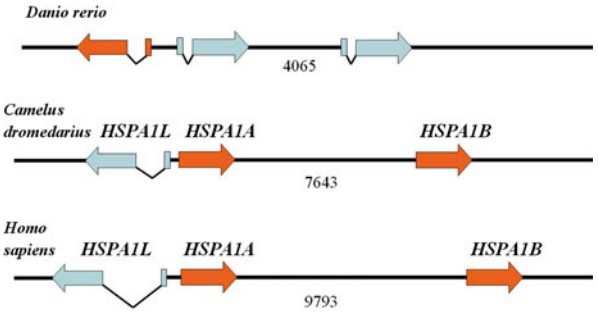

Fig. 5.4 General arrangement of MHC-associated *Hsp70* genes cluster in various vertebrates (fish, camel and human). Heat-inducible *Hsp70* copies are marked by orange color. The sizes of intergenic spacers between tandemly arranged copies in nucleotides are given below the lines. Introns found in *Hsp70* genes are shown by the angles. The cluster of *D. rerio* was constructed basing on sequences from the GenBank (BC056709 and XM_003198110). *4065*, *7643* and *9793* are distances between *HSPA1A* and *HSPA1B* genes in kbs (Camel and human clusters are from Garbuz et al. 2011a)

Interestingly, the zebrafish (*Danio rerio*) genome sequence revealed the presence of a cluster comprised of three *Hsp70* family genes arranged in a fashion similar to that of humans and other mammals (Fig. 5.4). One of these genes judging by the promoter structure is inducible. Surprisingly, all three genes found in the cluster in fish contain a short intron at the same site and exhibit higher similarity with each other in comparison with mammalian clustered *HSP70* genes. Therefore, the accumulated information enables to conclude that a basic unit consisting of three *Hsp70* genes linked with the MHC locus is more conserved than *Hsp70* clusters found in many other groups such as insects. Characteristically, in different phylogenetically distant groups of mammals, a general arrangement of *HSP70* cluster is practically identical. The mammalian species may only slightly differ in the length of intergenic spacer between *HSPA1A* and *HSPA1B* and in the size of the intron uniformly found in constitutively expressed *HSPA1L* gene. This similarity may resemble high conservatism of the whole genomic region which includes a MHC locus demonstrated for various mammalian species (Cameron et al. 1990).

On the other hand, quite a different situation is observed in various Diptera species, described above, where striking differences in the *Hsp70* cluster structure were revealed not only between close species (e.g. *D. virilis* vs. *D. lummei*) and geographically diverse populations of the same species (e.g. *D. virilis*) but even between specimens from the same natural populations as was observed in *S. singularior* (see above).

It is necessary to underline that clustered organization of stress-genes is not restricted to the *Hsp70* family. Certain organisms may contain other classes of *Hsps* genes organized as clusters. Thus, the parasitic organism *Trypanosoma cruzi* harbours several *Hsp90* genes (from 6 to 9 copies) arranged in tandem orientation (head to tail) (Dragon et al. 1978). These genes encode a protein with molecular mass about 85 kD and homologous to *Hsp83* of *D. melanogaster* and *Hsp90* of *S.*

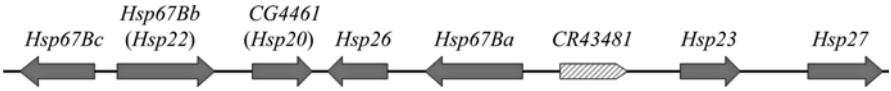

Fig. 5.5 General organization of small heat shock genes cluster in *D. melanogaster. CR43481* is non-protein coding gene whose RNA is not translated

cerevisiae. Similarly, *Hsp90* (*Hsp83*) genes of the parasite *Leishmania mexicana*, represented by four copies, also form a cluster containing arranged in tandem orientation essentially as in *T. cruzi* (Shapira and Pinelli 1989). On the other hand, in *Anopheles* mosquitoes (*A. albimanus*) that transmit human malaria, two highly homologous *Hsp83* genes (99.6 % similarity) are arranged as an inverted repeat and contain a conserved intron (Benedict et al. 1996). Among the species examined, *D. melanogaster* and many other *Drosophila* species contain a single *Hsp83* gene (Holmgren et al. 1979) while Diptera species belonging to *Stratiomyidae* family (*S. singularior*) comprise two *Hsp83* genes arranged in tandem orientation and separated by 12.5 kbs. Characteristically, all studied Diptera *Hsp83* genes have a similar general structure which includes one intron just before the ORF, and a single large HSE unit which consists of three to eight GAA/TTC motifs depending on the species (Astakhova et al. 2013 ;Tian et al., 2010). Besides cytosolic *Hsp90* genes, described above, eukaryotic genomes usually contain endoplasmic and mitochondrial members of the *Hsp90* family, which exhibit low levels of similarity with orthologous genes expressed in cytosol and may be located in other chromosomes (Felts et al. 2000; Maynard et al. 2010). In general, while Diptera *Hsp83* are characterized by higher variability of ORFs, their regulatory regions are more conserved in comparison with those of *Hsp70* family members (Astakhova et al. 2013).

In structure, Hsp90 is rather similar to bacterial gyrases which may be explained by their common origin. These proteins together with certain ribosomal proteins belong to the so called "GHKL-class" designated basing on abbreviations of the founding members of this group: DNA gyrase, Hsp90, bacterial histidine kinases, and MutL (Chen et al. 2006).

In Dipterans, small heat shock proteins genes homologous to crystallins are also arranged in clusters. Thus, in *D. melanogaster* seven highly homologous *sHsps* genes form a cluster at one chromosomal locus. Four of these genes are arranged as inverted repeats and do not contain introns (Ayme and Tissieres 1985). Besides these seven genes, there is another member of the family which is transcribed after HS but does not produce protein. The general scheme of the *sHsps* cluster is illustrated in Fig. 5.5.

On the other hand, although *Hsp40* and *Hsp110* genes found in mammals and other organisms are represented by multiple copies, they usually do not form clusters but instead are scattered in different chromosomes (Kampinga et al. 2009).

Chaperonins belonging to the first (GroEL) and second (CCT) groups (Chap. 2) share high homology in the ATP-domain region but strikingly differ by the structure of the peptide-binding domain. The observed similarity suggests common origin of these groups of genes, while subsequent divergence of eight genes encoding different CCT

subunits apparently took place at the early stages of eukaryotes evolution (Archibald et al. 2000; Kim et al. 1994). It is of note, that in human genome *Hsp60* and *Hsp10* (or *HSPD1* and *HSPE1*) genes are linked head to head and separated by 280 bps fragment containing a bidirectional promoter (Hansen et al. 2003; Ryan et al. 1997).

New rapidly evolving methods enabled to investigate in detail the tertiary structure of various proteins, and shed light on the origin of multiple genes that may play quite different roles in the cell (despite having originated from a common ancestral structure). Thus, X-ray diffraction structure analysis clearly demonstrated that peptide-binding domain of MHC is reminiscent of mammalian HSP70 substrate-binding domain (Flajnik et al. 1991; Rippmann et al. 1991). Basing on this similarity it was speculated that MHC genes originated by means of recombination between *Hsp70* genes and immunoglobulin genes at the early stages of vertebrate evolution (Hughes and Nei 1993).

The tertiary structure of proteins can be more conserved than respective amino acid sequence, because natural selection tends to preserve tertiary structure necessary for normal function of the protein despite mutational input. For example, while the structure of ATP-binding domain of Hsp70 is rather similar to correspondent domains of actin and sugar kinases, the homology between these proteins at the amino acid level is less than 20 %. ATP hydrolysis and phosphate group transfer in the case of sugar kinases are executed by the same basic mechanism common for all three groups of these diverse proteins, which basing on this functional similarity are grouped into actin/heat shock protein 70/sugar kinase superfamily (Bork et al. 1992; Hurley 1996; Smith and Kirley 1999). Therefore, it is possible to speculate that genes encoding actin, sugar kinases and *Hsp70* may have originated from the same ancestral gene encoding ATP-binding domain at the early stages of life on the Earth.

The modern *Hsp70* family is believed to originate from a correspondent single copy gene found in the Archaea (Gupta and Singh 1992). The hypothetical phylogenetic tree which illustrates the evolution of *Hsp70* genes family is depicted in Fig. 5.6. The evolutionary process involving *Hsp70* genes included various mechanisms of amplification and divergence of the resulted copies. Specifically, the role of mobile elements in the evolutions of *Hsp70* loci will be discussed in Chap. 6. Gross rearrangements of the genome included duplications and translocations of *Hsp70* copies, which either formed clusters or become scattered in the genome as single copies. Once dispersed, the genes could acquire different functions and under selection pressure differentiate into heat-inducible, constitutive or glucose-regulated genes which can be expressed either in cytosol, mitochondria or endoplasmic reticulum. It is possible to assume that small heat shock genes underwent similar evolution leading to the formation of present clusters.

Hsp110 family genes exhibit 30 % homology with *Hsp70* genes at ATP-binding domain and, hence, probably diverged from them in the course of evolution (Lee-Yoon et al. 1995), as well as genes giving rise to modern histocompatibility complex (Flajnik et al. 1991). It is also evident that at all levels of organization natural selection favored intronless structure of *Hsps* genes while gene conversion efficiently maintained amazingly high similarity of *Hsps* genes often organized as compact clusters.

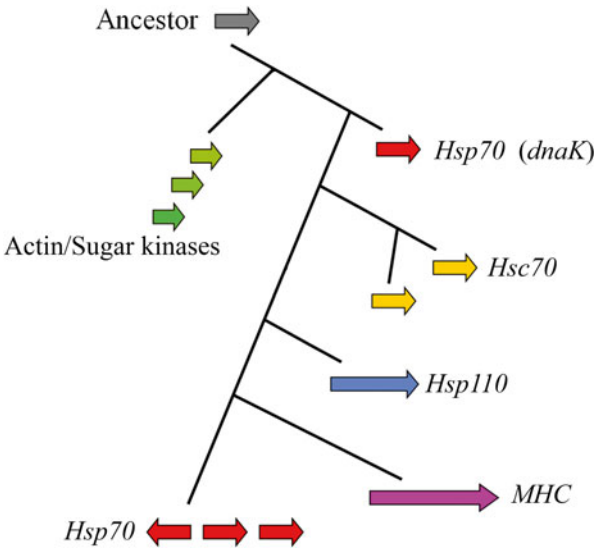

Fig. 5.6 Presumptive origin and general evolution of *Hsp70* genes. At the first stage Hsp70 possibly branched from a family which comprises actins and sugar kinases. Subsequently, in eukaryotes divergence multiple duplications and functional differentiation of ancestral *Hsp70* genes occurred. At the later stages of evolution, the *HSP110* family was separated, and the formation of MHC genes took place possibly by recombination between genes encoding *Hsp70* and immunoglobulins. *MHC* major histocompatibility complex

5.1 Conclusions

1. Most classes of inducible *Hsps* genes include clusters of highly conserved intronless copies arranged either as inverted repeats or/and tandemly arranged sequences.
2. Gene conversion is responsible for high similarity (virtually identity) of copies comprising a cluster of *Hsp70* genes and thermotolerant species often have more compact clusters than related cold-adapted forms.
3. While many phylogenetically distant vertebrate species exhibit amazing similarity in terms of the gross structure of major *Hsp70* cluster, in Diptera interspecific and even intraspecific differences in the number and relative positions of inducible *Hsp70* copies have been detected.
4. Our studies demonstrated that there are different trends in the evolution of Hsps system in different organisms to provide optimal response to fluctuating environmental conditions.

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Chapter 6
The Role of Mobile Elements in the Evolution and Function of HSPS Systems

6.1 Mobile Genetic Elements: The Distribution and Significance

The "selfish DNA" theory postulates that transposable elements (TEs) are intragenomic parasites, and that natural selection against deleterious effects associated with their presence is the main force preventing their genomic spread in natural populations (Orgel and Crick 1980).

On the other hand Barbara McClintok's pioneering studies of transposable elements in maize enabled her to propose that transposons provide the host with important genetic diversity. Her observation in the early 1980s that transposon mobility may be induced by a genomic shock led her to formulate the model that transposons may have an adaptive value and reorganize the host genome as a means of responding to stress (McClintock 1984).

Retrotransposons are the most widespread and abundant class of eukaryotic transposable elements (TEs). A major repeated component of all eukaryotic genomes is comprised of various classes of retrotransposons. For example, more than four million retrotransposons constitute at least 40 % of the human genome while more than 50 % of the maize genome is made up of retrotransposons and this class of elements is estimated to comprise up to 90 % of the genomes of wheat and lilies (Flavell 1986; Kidwell and Lish 1997, 2002).

With the completion of numerous DNA sequencing projects over the last few years, an abundance of data has emerged that is relevant to the issue of adaptive value of TEs. The analysis of sequenced genomes demonstrated that in vertebrates alone there are now hundreds of examples of transposons or fragments of transposons being associated with functional genes and executing regulatory roles. Characteristically, the majority of gene-TE associations involve TEs or transposon fragments found within gene regulatory regions (Brosius 1999; Evgen'ev 2007).

Various studies have shown that environmental variations can promote genome plasticity through transcriptional activation and mobilization of different classes of

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TEs, especially retroelements, often in response to specific stimuli such as biotic stress and abiotic environmental changes such as temperature fluctuations (Capy et al. 2000; Garcia Guerreiro 2012; Junakovic et al. 1988; Liu et al. 1995; Ratner et al. 1992; Walbot 1999). Therefore, it became clear that TEs apparently have a large impact on genome structure and stability. They may contribute to variations in genome size, and are considered as one of the major sources of genetic variability in all classes of eukaryotes studied so far (Arkhipova et al. 2003; Ewing and Kazazian 2011; Kazazian 2004; Kidwell and Lish 2002). It is evident at the present time that although TEs may be capable of "selfishly" maintaining themselves in populations and species on a day-to-day basis without providing selective advantage to their hosts, over longer spans of evolutionary time TE-mediated mutations may arise that are of adaptive evolutionary significance and may facilitate species distribution over diverse environments including aggressive ones.

In this respect, large scale investigation of various hydrothermal crustacean organisms showed a particularly great diversity of *DIRS*-like elements with five families of shrimps and three families of crabs. *DIRS1*-like retrotransposons elements are a particular group of retrotransposons according to their mode of transposition that implies a tyrosine recombinase (Piednoël and Bonnivard 2009). It is a challenge to speculate that the observed extremely high diversity of this class of TEs may have adaptive value providing survival of crustacean species in such particularly unstable microhabitat as hydrothermal vent.

6.2 Natural Occurrence of TEs Within *Hsps* Genes

In *D. melanogaster* populations TEs are usually found at various frequencies in most genomic locations. A few cases of fixation of TE insertions have been reported, usually in regions of low recombination such as telomeres and pericentromeric heterochromatic regions, where selection is expected to be less effective (Evgen'ev 2007; Kaminker et al. 2002; Pardue and DeBaryshe 2003). However, some time ago the apparent fixation of an *S* element in a highly recombining region in two natural populations of *D. melanogaster* has been described. Thus, three similar fragments of an *S* element are inserted into the 5′-regions of three members of *Hsp70* family in this species. A PCR-based analysis suggested that the insertions were fixed or present at high frequencies in the entire species. A population survey of the levels of nucleotide sequence variation at the insertion site in 87B in two natural populations of *D. melanogaster* provided evidence for reduced levels of variation in the region, normal levels of recombination, and selection, reflected in a significant departure from neutrality of the variant frequency spectrum. This was particularly strong for the *S* element inverted repeats (IRs) and suggests that these are very ancient components of *D. melanogaster* genome and may have functional significance for the host (Maside et al. 2002, 2003).

Considering their mutational abilities, TEs are potent agents of genomic change during evolution. However, TEs require access to chromatin for insertion and not all

genes apparently provide equivalent access. It was interesting to test whether the regulatory regions of heat-shock genes that represent "open" chromatin lacking most histones under normal non-heat-shock conditions (Farkas et al. 2000; Karpov et al. 1984; Lis and Wu 1993) render their proximal promoters especially susceptible to the insertion of transposable elements in nature. At this end, studies of Martin Feder's group from The University of Chicago indicated that the proximal promoter regions of heat-shock genes harbor a remarkable number of *P* transposable element (TE) insertions relative to both positive and negative control proximal promoter regions in various geographical populations of *D. melanogaster*. An unbiased screen of the proximal promoters of 18 heat-shock genes in 48 natural populations of this species demonstrated that more than 200 distinctive transposable elements had inserted into these promoters; while greater than 96 % of all detected TEs for unknown reason are *P* elements. By contrast, few or no *P* element insertions segregate in natural populations in a "negative control" set of proximal promoters lacking the distinctive regulatory features of heat-shock genes. Furthermore, the natural *P* element insertions cluster in specific sites ("hot spots") in the promoters, with several separate geographical populations exhibiting *P* element insertions at exactly the same position. By contrast, a "positive control" set of promoters resembling heat-shock promoters in regulatory features surprisingly harbors few *P* element insertions in nature. It was concluded that the distinctive regulatory features specific for heat-shock genes (in *Drosophila*) are especially prone to mutagenesis via *P* elements in nature. Thus, in nature *P* elements create significant and distinctive variation in heat-shock genes expression, upon which evolutionary processes may act providing fine tuning of the battery of heat shock genes under fluctuating environmental conditions (Walser et al. 2006). It is not clear at the present time why *Hsp* genes promoters are so "attractive" specifically for insertion of *P* element but not other multiple TEs found in *Drosophila*.

At the next step the sequenced genomes of 12 species of *Drosophila* have been screened to monitor the distribution of various TEs in their genomes (Stark et al. 2007). These species lack *P*-element in their genome. Surprisingly, in the 12 species genomes, transposable element insertions are no more abundant in promoter regions of single-copy heat-shock genes than in other promoters with similar or dissimilar architecture. Also, insertions appear randomly distributed across the promoter region. In contrast, insertions clustered near the transcription start site in promoters of single-copy heat-shock genes in *D. melanogaster* natural populations. On the other hand, *Hsp70* promoters exhibit more TE insertions per promoter than all other gene sets in the 12 species, similarly to the pattern observed in natural populations of *D. melanogaster*. Insertions in the *Hsp70* promoter region, however, cluster away from the transcription start site in the studied 12 species, but near it in natural populations of *D. melanogaster*. These results suggest that *D. melanogaster* heat-shock promoters are unique in terms of their interaction with specifically *P* elements. The analysis performed confirms that *Hsp70* promoters are distinctive in terms of TE insertions across *Drosophila* species studied (Haney and Feder 2009). Furthermore, there is convincing evidence that in *D. melanogaster* besides described above case of *Hsp70* genes other groups of *Hsp*

genes also represent "hot spots" for *P* elements insertions. Thus, it was demonstrated that *Drosophila* small heat-shock genes located in the same genome locus are distinctively evolvable because of frequent insertions of *P* elements (Chen et al. 2007). Thus, detailed analysis of two natural populations of *D. melanogaster* revealed 16 distinctive *P* transposable elements collectively segregating in proximal promoters of the two small heat-shock genes (*Hsp26* and *Hsp27*). These elements vary in size, orientation and insertion site. Frequencies of *P* element-containing alleles varied from 5 to 100 % in these populations. Two *P* elements inserted into *Hsp26* reduced or abolished *Hsp26* expression. The element which reduced *Hsp26* expression increase or did not affect inducible tolerance to high temperature, increased fecundity, but decreased developmental rate. The element which abolished *Hsp26* expression decreased thermotolerance and fecundity. In lines founded in the 1980 year and subjected to different experimental evolution, the allelic frequency of the inserted *P* elements varied considerably, and was at lower frequencies in lines selected for increased longevity and for accelerated development than in controls. It was concluded that transposable element insertions into small *Hsp* genes as well as in the members of *Hsp70* family in *Drosophila* populations can have immediate dramatic fitness consequences, and therefore create variation on which selection can act (Chen et al. 2007). It is intriguing to speculate that peculiar pattern of *P* elements insertions observed in *D. melanogaster Hsp* genes may somehow result from cosmopolitan distribution of the latter species around the world.

6.3 Role of Transposable Elements in the Regulation of *Hsp70* Genes Expression

It is clear that insertions of TEs into *Hsps* genes in most cases should lead to decreased transcription and may be deleterious for a population which encounters frequent temperature fluctuations and should rapidly respond to such challenges.

Multiple cases were reported in which disruption of *Hsp70* regulatory regions by transposable element (TE) insertions underlies natural variation in expression of the stress-inducible molecular chaperone Hsp70 in *D. melanogaster*. Thus, three *D. melanogaster* populations from different continents were found to be polymorphic for *Jockey* or *P* element insertions in the promoter of the *Hsp70Ba* gene. All three detected TE insertions are within the same 87 bps region of *Hsp70Ba* promoter, and it was demonstrated (see above) that the distinctive promoter architecture of *Hsp* genes may make them vulnerable to TE insertions. As expected each of the TE insertions reduces *Hsp70* levels, and RNase protection assays demonstrated that such insertions actually reduced transcription of the *Hsp70Ba* gene. In addition, the TEs insertions alter two components of organismal fitness, such as inducible thermotolerance and female reproductive success. Thus, TE transposition can create quantitative genetic variation in gene expression within populations, on which natural selection can act (Lerman et al. 2003; Michalak et al. 2001).

Furthermore, in order to determine the role of insertion site position on *Hsp* genes expression a series of *D. melanogaster* strains with *P* element insertions from −28 to −144 nucleotides upstream to the transcription start site of the *Hsp70A* genes were compared. These sites corresponded to the range of naturally occurring *P* element insertion sites were explored to elucidate the consequences of insertion site for *Hsp70A* gene expression. Although all insertions reduced *Hsp70A* expression below that of a control strain, the magnitude of the reduction was inversely related to the number of nucleotides between the transcription start site and the insertion location.

A pre-existing hypothesis was that naturally occurring transposable element insertions in *Hsp* promoters may be beneficial in some circumstances, which may account for their retention in certain natural populations. Along these lines Chen et al. (2008) has demonstrated that in a control line heat shock reduced fecundity, whereas in lines with *P* element insertions heat shock typically increased fecundity. Finally, according to cluster-specific quantitative RT-PCR, expression of the *Hsp70A* cluster genes was typically greater than that of the *Hsp70B* cluster genes, although the latter are more numerous and, in this case, free of *P* element insertions (Chen et al. 2008).

At the next step the quantitatively measurement of the input of TEs insertions into *Hsp70* promoters was performed exploring *in vitro* luciferase assay. Notably, naturally occurring TEs insertions that disrupt *Drosophila* promoters were often correlated with modified promoter function and implicated in regulatory evolution, but their phenotypes have not been measured directly. To establish the functional consequences of the TE insertions, the constructs were created with either TE-bearing or TE-lacking *Hsp70* promoters fused to a luciferase reporter gene. Subsequently, luciferase luminescence has been assayed in transiently transfected *Drosophila* cells. It was shown that each of the four TEs investigated reduces luciferase signal after heat shock and heat inducibility of the *Hsp70* promoter. To test if the differences in *Hsp70* promoter "strength" are TE-sequence dependent, each of the TEs was replaced with multiple intergenic sequences of equal length. These replacement insertions similarly reduced luciferase signal, suggesting that the TEs affect *Hsp70* promoter strength function by altering promoter architecture. These results are consistent with differences in Hsp70 expression levels, inducible thermotolerance, and fecundity previously associated with the TEs. That two different varieties of TEs in two different *Hsp70* genes have common effects suggests that TE insertion represents a general mechanism modulating promoters strength through which selection may manipulates *Hsp70* gene expression (Lerman and Feder 2005).

On the other hand, it is evident that switching off a part of the *Hsp70* genes may in some cases be adaptively advantageous, and one of the mechanisms providing for such "switching off" has been described in several *D. melanogaster* strains grown under conditions of elevated temperature (Michalak et al. 2001; Zatsepina et al. 2001). Thus, when studying *D. melanogaster* laboratory line T collected in subequatorial Africa in the 1970s, several unexpected facts were reported. This line was capable of reproducing at 31 °C, under laboratory conditions while other lines of this species become sterile at this temperature. Interestingly, line T was kept at the St.-Petersburg University for many years at 31 °C without a noticeable decrease in

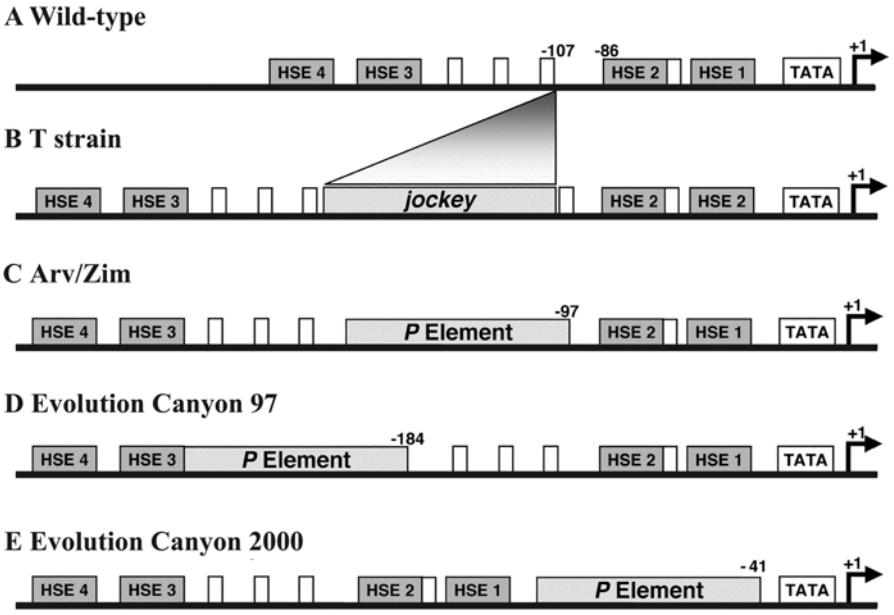

Fig. 6.1 Transposable elements disrupt the *Hsp70Ba* promoter in different strains of *D. melanogaster* (T strain from Central Africa, Arv/Zim – cross of California and Zimbabwe parents, and two strains from Evolution Canyon, Israel). GAGA elements are indicated as *white bars* (From Lerman et al. (2003); Lerman and Feder (2005) with permission)

fertility in contrast to other *D. melanogaster* strains. Thus, flies of this line are remarkably tolerant of sustained laboratory culture above 30 °C and of acute exposure to much higher temperatures. Importantly, inducible thermotolerance of high temperatures, which in *Drosophila melanogaster* is due in part to the inducible molecular chaperone *Hsp70*, is only modest in this strain. Expression of Hsp70 protein and *Hsp70* mRNA is likewise reduced and has slower kinetics in this strain (T) than in a standard wild-type strain (Oregon R). These strains also differed in constitutive and heat-inducible levels of other molecular chaperones. The lower Hsp70 expression in the T strain after mild HS apparently has no basis in the activation of the heat-shock transcription factor HSF, which is similar in T and Oregon R flies (see Chap. 4). Rather, the reduced expression may stem from insertion of two transposable elements, *H.M.S. Beagle* in the intergenic region of the 87A *Hsp70* gene cluster and *Jockey* in the *Hsp70Ba* gene promoter. The arrangement of *Hsp70* gene locus in Oregon R and strains containing different TEs is shown in Fig. 6.1. Characteristically flies from T strain are more thermoresistant and synthesize more Hsp70 after acute HS at 39 °C (see Chap. 4 for details).

It is noteworthy, that in other similar cases as well, long-term cultivation of various *D. melanogaster* lines at an elevated temperature in the laboratory led to switching off some copies of *Hsp70* genes due to incorporation of a certain mobile element (Bettencourt et al. 2002).

It became clear that constitutive or chronic expression of Hsps is not uniformly beneficial. For example, experiments on transgenic *D. melanogaster* strains with extra copies of *Hsp70* genes have demonstrated that overexpression of Hsp70 could be harmful and increase lethality during the development (Krebs and Feder 1997; Bettencourt et al. 1999; Roberts and Feder 1999; Roberts and Feder 2000). Apparently, when organisms are under constant conditions of elevated temperature like in the case of strain T or desert snails described by Arad and his co-workers (Arad et al. 2010) or other stress factor, decreasing expression of *Hsp70* gene battery or switching off a part of *Hsp70* genes may be adaptively advantageous by minimizing the deleterious effects of Hsps. Thus, probably the observed by several authors reduced *Hsp70* expression in *D. melanogaster* strains living chronically at intermediate temperatures represents an evolved suppression of the deleterious phenotypes of Hsp70.

Evidently the expression level of Hsps in each species and geographical population is a balance between benefits (high thermotolerance) and costs (e.g. negative impact of Hsps overexpression on growth, fertility and other vital characteristics).

Notably, the insertions of TEs in the promoters of *Hsp* genes not always lead to the transcription inhibition as was described above. Thus, it has been demonstrated in rice that the insertion of a miniature inverted-repeat transposable element (MITE), considerably enhanced *Hsp70* expression (Zhang et al. 2012).

To quantify the influence of the TE MITE insertion a series of *Hsp70* promoter deletion constructs was established. Analysis of beta-glucuronidase activities from the promoter deletion constructs in transient expression assays identified a cis-element, located from −493 to −308 bp upstream of the ATG start site. This element with several characteristics of MITE transposons designated as "HS185" turned out to have a crucial role in *Hsp70* promoter activity in rice. Three hundred and sixty two copies of homologous sequences of this TE were detected in the rice genome, which are preferentially located in the non-coding regions. Transient expression assays showed that HS185 inhibited the enhancer activity of the cauliflower mosaic virus 35S promoter. These results demonstrate that not only is HS185 necessary for *Hsp70* promoter activity, but it also has a functional role as an insulator (Zhang et al. 2012).

Another example of enhancing effect of TEs upon insertion into stress genes has been recently described in yeast. It was shown that, *Tf1*, a long-terminal repeat retrotransposon in *Schizosaccharomyces pombe*, integrates into various promoters with a preference for the promoters of stress response genes. To determine the biological significance of *Tf1* integration, the authors took advantage of saturated maps of insertion activity and studied how integration at hot spots affected the expression of the adjacent genes. These studies demonstrated that *Tf1* integration did not always reduce gene expression. On the contrary, *Tf1* integration increased the expression of 6 of 32 genes studied. It is known that the long terminal repeats (LTRs) of *Tf2* are transcribed, and in rare cases, the RNAs can continue into adjacent sequence and read-through neighboring genes (Feng et al. 2013). However, in the examples studied, the increases in mRNA of genes caused by *Tf1* insertion were not the result of read-through transcripts. Instead, the results of RNA blots and 5′-RACE assays revealed that *Tf1* carried enhancer that increased the promoter activity of genes

adjacent to *Tf1* insertions. It was necessary to explain why *Tf1* insertion enhanced the expression of some genes and not others.

Subsequently it was found that genes induced by heat were the only genes with expression that was increased by *Tf1* insertion. This together with finding that the transcription of *Tf1* itself was induced by heat suggested that the *Tf1* enhancer and the stress response genes were recognized by the same or similar activators of transcription. Indeed, the identification of a motif common to the promoters of *Tf1* and the genes that had expression increased by *Tf1* insertions supported this model. Similar factors bound to adjacent sequences often multimerize and cause synergistic increases in transcription. According to this model, the insertion of *Tf1* next to a stress response gene would be effectively increasing the number of binding sites for the same transcription activators and thus stimulate transcription (Feng et al. 2013). Furthermore, it was demonstrated that Atf1p activator of transcription plays specific and direct role in targeting integration sertions of *Tf1* into promoters of heat-induced genes (Majumdar et al. 2011).

Interestingly, in the pioneer studies of the interaction between heat shock response and TEs mobilization it has been demonstrated that "heavy" heat shock in *Drosophila* induced amplification of several families of transposable elements (*copia* etc), that contain heat shock elements (HSEs) in their LTRs and were apparently induced by temperature elevation (Junakovic et al. 1988; Ratner et al. 1992).

Thus in such experiments, performed by Ratner's group, males of a *D. melanogaster* isogenic line with a wing mutation (*radius incompletes*) were treated by standard light heat shock (37 °C for 90 min) and by heavy heat shock (transfer of males from 37 °C for 2 h to 40 °C for 1 h and back). In the F1 generation of treated males mated with non-treated females of the same isogenic line, mass transpositions of *copia*-like mobile genetic element *Dm-412* were found. The altered positions of the element seem nonrandom and five "hot spots" of transposition were found. Three-quarters of all transpositions were localized in the hot spots positions. It was shown that, as a result of heat shock treatment, the probabilities of transpositions were two orders of magnitude greater than those of the control sample in the next generation after HS-induction. Comparison of the results with those after stepwise temperature treatment shows that the induction depends on the intensity of the stress action (temperature of treatment) rather than on the type of the stress action (Ratner et al. 1992).

Heat shock genes in all organisms represent rapidly inducible loci and, hence, with a few prominent exceptions (see above) usually do not contain introns often resulted from TEs insertions not to spend time on splicing. However, there are multiple examples when *Hsp* genes harbored transposable elements which probably play important roles in their fine tuning and evolution. Thus, pseudogenes and cognate genes derived from heat shock genes by different mechanisms often contain TEs in their sequences and in introns in particular.

Thus, recently three new repetitive sequences from the bivalve mollusk *Mytilus galloprovincialis*, designated Mg1, Mg2, and Mg3, with monomer lengths of 169, 260, and 70 bps, respectively, were characterized. Surprisingly, these three repeats constitute approximately 7.8 % of the *M. galloprovincialis* genome and were found

inside the introns of two genes of the *Hsp70* family, *Hsc70* and *Hsc71*. Both the monomer length and the genomic content of the repeats indicate satellite sequences. The Mg1 repetitive region and its flanking sequences exhibit significant homology to CvE, a member of the *Pearl* family of mobile elements found in the eastern oyster (*Crassostrea virginica*). Thus, the whole homologous region was designated MgE, and represents the first putative transposable element characterized in this mollusk. The ApaI, Mg2, and Mg3 repeats are continuously arranged inside the introns of both the *Hsc70* and *Hsc71* genes. The presence of perfect inverted repeats flanking the ApaI-Mg2-Mg3 repetitive region, as well as a sequence analysis of the repeats, indicates a transposition-like insertion of this region (Kourtidis et al. 2006).

In general, the genes of the *Hsp70* family are highly conserved, and the presence of repetitive DNA or of mobile elements inside their introns is of significant interest and may have important functional significance. It is of note, that transcription of certain satellite sequences is induced by HS and correspondent cDNA copies may be probably inserted into various genomic sites exploring retrotransposition mechanism (Enukashvily and Ponomartsev 2013).

6.4 Possible Involvement of TEs in the *Hsp70* Genes Copy Number Variation Within and Between Species

In principle various TEs may be involved in *Hsps* copy number modulation in different ways. First, some TEs which induce gross genomic rearrangements sometimes may change *Hsp* genes locations and copy number by means of ectopic pairing and/or unequal crossing-over. Second, retroelements may provide reverse transcriptase activity to produce multiple pseudogenes from the cDNAs of actively transcribed heat shock genes. Such pseudogenes may subsequently either degenerate or serve as cognate heat shock genes expressed under normal non-stress conditions. Below we shall discuss a few typical example of possible involvement of TEs in heat shock genes evolution accompanied by significant copy number changes.

An amplification of *Hsp70* genes comprising the clusters in the *melanogaster* species subgroup represents a classical example of *Hsps* genes evolution accompanied by significant increase in copy number of the pertinent genes (see above). It has been demonstrated that *Hsp70* genes in the subgroup proliferated rapidly by means of duplication of an ancestral two-*Hsp70* gene unit and subsequent tandem duplication of a single gene in *D. melanogaster* species alone (Bettencourt and Feder 2001).

The authors speculated that the two-to-four duplication event observed in a few species of the subgroup was likely a retrotransposition. Importantly: no other duplicated sequences flank the 87A7 and 87C1 (87B in FlyBase) gene clusters of *D. melanogaster* where these two-*Hsp70* cassettes are located in *D. melanogaster* genome. Besides, it is known that the *Hsp70* genes have no introns and are often surrounded by simple repetitive DNA. These genes bear polyA tails at least in the case of the ancestral two-copy *Hsp70* cluster of *D. auraria* (Bettencourt and Feder 2001). The origin of the fifth *Hsp70* gene detected in *D. melanogaster* was more

complex. It was speculated that tandem duplication and remodeling via gene conversion likely formed the mosaic *Hsp70Bb*/*Hsp70Bc* region in the latter species (Bettencourt and Feder 2002).

It is well known that in general, duplicated genes often either diverge toward new functions or degenerate toward non- or subfunctionality and often form pseudogenes (Kim et al. 1998; Kidwell and Lish 2002; Shapiro and von Sternberg 2005; Evgen'ev 2007). Instead, the *Hsp70* genes of *D. melanogaster* and *D. virilis* persist as functional duplicate copies, consistent with important adaptive role of *Hsp70* expression to achieve very rapid, efficient, and extremely high Hsp70 protein expression after HS and provide inducible thermotolerance (Garbuz et al. 2003; Evgen'ev et al. 2004; Feder and Hofmann 1999; Feder and Krebs 1998). Interestingly, the described arrangement of *D. melanogaster* 5–6 *Hsp70* genes in the genome (87A and 87B regions) is practically identical in all laboratory strains and geographical populations of this well studied cosmopolitan species (Leigh Brown and Ish-Horowicz 1981; Bettencourt and Feder 2002).

On the other hand, molecular investigation and sequencing of the *Hsp70* gene cluster in *D. virilis*, *D. lummei* and other species belonging to the *virilis* group (Evgen'ev et al. 2004) showed that most of strains of the Southern thermophilic species (*D. virilis*) carry significantly more *Hsp70* copies than the strains of Northern thermosensitive one (*D. lummei*). Moreover, in contrast to *D. melanogaster* different strains of these two species differ characteristically by copy number of *Hsp70* genes.

In the course of sequencing of *D. virilis* and *D. lummei Hsp70* clusters sequences of very ancient *SGM* mobile element (Evgen'ev et al. 2004) have been detected at the 3′-flanking regions of all copies of the *Hsp70* genes comprising the clusters in these species. Figure 5.1 depicts a scheme illustrating the localization of *SGM* fragments at the *Hsp70* cluster in the two species mentioned above as well as presumptive ancestral arrangement of the cluster.

Presumptive "fresh" insertion of *SGM* between inverted copies comprising the *Hsp70* cluster in only one particular strain of *D. virilis* was detected (Fig. 5.1). This observation favors the conclusion that the process of *SGM* amplification and transposition is still operating in *D. virilis* at the present time (Evgen'ev et al. 2004). Notably, this ancient *SGM* element may play various functions in *Drosophila* species, thus in *D. guanche* 10 % of satellite DNA consists of *SGM* sequences (Miller et al. 2000). Ubiquitous occurrence of *SGM* at the same position in all *Hsp70* copies of the cluster in all *virilis*-group species studied enables to suggest the important role of this specific mobile element in the evolution of the whole cluster in this group. Probably pairing and unequal crossing over occurring in functionally insignificant *SGM* sequences located close to the 3′-end of *Hsp70* copies represent the molecular mechanism underlying the differences in the *Hsp70* copy numbers observed between different geographical strains of the *virilis*-group species and between the sibling species belonging to this group (e.g. *D. virilis* and *D. lummei*) as well. Apparently natural selection subsequently regulates the differences in the *Hsp70* copy number depending on the environmental conditions requirements.

In *D. mojavensis*, a species from the *repleta* group, another ancient mobile element, *Galileo*, integrated into 3′-flanking regions of tandemely arranged *Hsp70*

copies. Thus, the participation of mobile elements in the *Hsp70* cluster formation is regularly for the *virilis* and *repleta* groups of *Drosophila* (Garbuz, personal communication).

Similar pattern of *Hsp70* genes evolution with participation of TEs probably took place in the other distant Diptera species belonging to *Stratiomyidae* family. The heat shock response in several species of belonging to this family that dwell in thermally and chemically contrasting habitats including highly aggressive ones was described in Chap. 4 (Garbuz et al. 2011).

Although all studied *Stratiomydae* species exhibit high constitutive levels of Hsp70 accompanied by exceptionally high thermotolerance, characteristic interspecies differences in Hsp expression and survival after severe heat shock were detected. The analysis of genomic libraries of two *Stratiomyidae* species inhabiting thermally and chemically contrasting ecological niches indicated that though the genomes of both species contain similar numbers of *Hsp70* genes, the spatial distribution of *Hsp70* copies differs characteristically. In a population of the highly eurythermal species *S. singularior*, which lives in thermally variable and chemically aggressive (hypersaline) conditions, the *Hsp70* copies form a tight cluster. In contrast, in a population of the stenotopic *Oxycera pardalina* that dwells in a stable cold spring, the distance between individual *Hsp70* copies in the genome is very large, if they are linked at all (Garbuz et al. 2008). Although the *Hsp70* coding sequences of *S. singularior* are highly homogenized *via* conversion, the structure and general arrangement of the *Hsp70* clusters are highly polymorphic, including gross and complex aberrations, and various deletions in intergenic regions. The insertions of incomplete *Mariner* transposon in close vicinity to the 3′-UTRs were detected in one of *Hsp70* copies in *S. singularior* reminiscent to *SGM* insertions found in *D. virilis* and *D. lummei Hsp70* copies and possible role of this TE in the evolution of *Hsp70* gene cluster in the species of the *Stratiomydae* family has been suggested (Garbuz et al. 2008).

Besides the above described case of *Hsp70* genes proliferation in *D. melanogaster* where retrotransposition has been suggested as a primary mechanism providing duplication of an ancestral two-*Hsp70* gene cassette (Bettencourt and Feder 2001), there are other examples that implicated reverse transcription in the amplification of heat shock genes in various organisms. Generally speaking, pseudogenes are often found in different *Hsps* genes families (Kampinga et al. 2009). The frequent occurrence of pseudogenes is apparently due to extremely high transcription level of *Hsps* genes after various stressful stimuli. The source of the reverse transcriptase necessary for the presumptive heat shock genes retrotransposition may be provided by various endogenous retroelements but it was never demonstrated.

Thus it has been speculated that abundant human L1 elements may provide necessary reverse transcriptase activity resulted in very high *Hsp70* pseudogenes quantity described in the human genome.

For a long time there were discrepancies in estimation of the number of members of the human *HSP70* genes family (11 counted over 10 years ago). Some have been described but the information is incomplete and inconsistent. A coherent body of knowledge encompassing all family components that would facilitate their study individually and as a group was lacking. Nowadays, the study of chaperone genes

benefits from the availability of genome sequences and a new protocol, chaperonomics, which was applied to elucidate the human *HSP70* family.

Using this approach recently 47 *HSP70* sequences, which include 17 genes (with *HSPH* group), and 30 pseudogenes were identified. The genes belong to several evolutionarily distinct groups with distinguishable subgroups according to phylogenetic and other data, such as exon-intron and protein features. The N-terminal ATP-binding domain (ABD) was conserved at least partially in the majority of the proteins but the C-terminal substrate-binding domain (SBD) was not. Nine proteins were typical Hsp70s (65–80 kD) with ABD and SBD, two were lighter lacking partly or totally the SBD, and six were heavier (>80 kD) with divergent C-terminal domains. The exon-intron features, and transcriptional variants of all these genes have been recently described. Besides protein structure, isoforms, and patterns of expression in various tissues and developmental stages have been also analyzed (Brocchieri et al. 2008). Evolutionary analyses, including human *HSP70* genes and pseudogenes, and other eukaryotic *Hsp70* genes, showed that six human genes encoding cytosolic *HSP70* and 27 pseudogenes originated from retro-transposition of *HSPA8* sequence, a gene highly expressed in most tissues and developmental stages. Therefore, the human *HSP70* gene family is characterized by a remarkable evolutionary diversity that mainly resulted from multiple duplications and retrotranspositions of a highly expressed gene, *HSPA8*. Human HSP70 proteins are clustered into seven evolutionary groups, with divergent C-terminal domains likely defining their distinctive functions. These functions may also be further defined by the observed differences in the N-terminal domain.

Within Group VI, only the coding region of *HSPA8* is encoded by multiple exons (eight or seven in two isoforms), whereas the coding regions of all other genes are encoded within a single exon. Similarly, most pseudogenes related to *HSPA8* did not show signs of exon-intron structures. This suggests that the sequences of group VI and related pseudogenes were all derived from *HSPA8* by retrotransposition. The impressive retrotransposition activity (perhaps L1-associated) involving *HSPA8* is also consistent with the very high level of expression of this gene in comparison with the other members of the *HSP70* family. The multi-exon structure of all other genes suggests instead that sets of similar sequences (e.g. the *HSPA4* and *HSPA12* subgroups) were generated by duplication events (Brocchieri et al. 2008).

The possibility of direct involvement of retroelements in the amplification and regulation of various stress genes including Hsps system raised a question regarding a role of RNAi machinery in such presumptive crosstalk.

6.5 Interaction Between RNAi and Heat Shock Genes Systems

RNA interference (RNAi) pathways have evolved as efficient modulators of gene expression that operate in the cytoplasm by degrading RNA target molecules through the activity of short RNAs (21–30 nucleotides). RNAi components play an

important role in the nucleus, as they are involved in epigenetic regulation and heterochromatin formation and remodeling (Brennecke et al. 2007; Kawasaki and Taira 2004; Malone and Hannon 2009).

Recent studies exploring a genome-wide RNAi screen demonstrated that there are multiple genomic factors involved in small RNA biogenesis and specifically factors of the germline piRNA pathway (Czech et al. 2013).

The discovery of different classes of small RNAs and RNA interference phenomenon responsible for various transposons silencing gave a new switch to our understanding of complex interaction between molecular mechanisms underlying heat shock response and expression of mobile elements comprising a significant fraction of all eukaryotic genomes. Specifically, several lines of evidence demonstrated that different classes of small RNAs are involved in epigenetic modifications of histones and remodeling of heterochromatin in response to different forms of stress including heat shock (Malone et al. 2009; Place and Noonan 2014).

Most of the data regarding the functional interaction between Hsps system especially Hsp90 and various components of RNAi machinery have been accumulated in *Drosophila* and mice (Iwasaki et al. 2010; Olivieri et al. 2012; Xiol et al. 2012).

It was shown that major proteins involved in RNAi functioning such as: Dicer2 (DCR2) and Argonaut2 (AGO2) are non-randomly associated with multiple sites of *D. melanogaster* chromosomes preferentially occupying transcriptionally active loci and interacting with basic transcription machinery under normal temperature conditions (Cernilogar et al. 2011). Specifically, these proteins are abundant at 87A and 87B loci of polytene chromosomes containing *Hsp70* genes. Furthermore, after heat shock *Dcr2* and *Ago2* null mutations that compromise RNAi system function, strongly impaired global dynamics of RNA PolII. Moreover, in the strains with mutated *Dcr2* and *Ago2*, clear-cut decondensation of chromatin is observed at these sites which apparently resulted in the increased transcription of *Hsp70* genes without heat shock (Cernilogar et al. 2011). It was also demonstrated by the deep sequencing that AGO2 is strongly enriched in small RNAs that encompass the promoter regions and other regions of heat-shock and certain other genetic loci with a strong bias for the antisense strand, under normal conditions and particularly after HS. It was shown that certain miRNAs play important role in RNAPII positioning at the promoter region of *Hsp70* genes. Furthermore, immunoprecipitation experiments exploring anti-AGO2 antibodies revealed drastic increase of siRNA homologous to *Hsp* genes after temperature elevation which suggests the involvement of RNAi system in HS response regulation (Cernilogar et al. 2011).

Taken together, the accumulated results show that DCR2 and AGO2 are globally associated with transcriptionally active loci including all *Hsp* genes and have a pivotal role in shaping the transcriptome by controlling the processivity of RNA polymerase II both under non-stress conditions and after heat shock.

On the other hand, chaperones themselves are apparently involved in many ways in the function of RNAi system. It is known, that protein Argonaute2 (AGO2) and associated small interfering RNAs (siRNAs) form the RNA-induced silencing complex (RISC) for target messenger RNA cleavage and post-transcriptional gene silencing (Martinez et al. 2013). Although it was demonstrated that AGO2 is

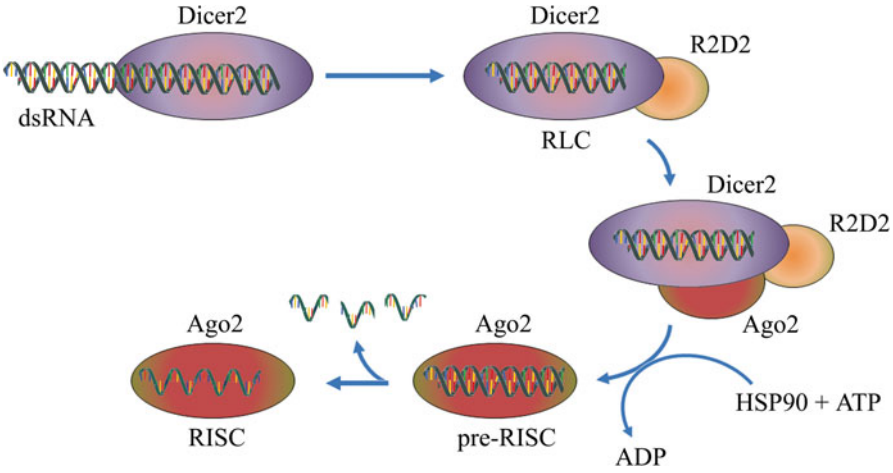

Fig. 6.2 A model for Hsp90 function in the RNAi pathway. Hsp90 promotes a conformational change in Ago2, enabling it to receive siRNA duplex from RISC-loading complex (*RLC*). Dicer2 and R2D2 are major protein components of RLC (From Miyoshi et al. 2010)

essential for RISC activity, the mechanism of RISC assembly was not well understood, and not all factors controlling AGO2 protein function are described so far.

It was demonstrated by several authors that Hsc70/Hsp90 chaperones machinery participate in loading small RNA duplexes (siRNAs and miRNAs) into the RISC and assembly of the RISC complex (Fig. 6.2). The constitutively expressed Hsp90 regulates conformational changes in human, *Drosophila* and yeast Argonautes required to accommodate the loading of double stranded siRNAs and miRNAs into Argonaute proteins the core components of RISC (Iwasaki et al. 2010; Miyoshi et al. 2010; Pare et al. 2013). It was also shown that such loading requires ATP, whereas separating the two small RNA strands within Argonaute does not. Thus, the Hsc70/Hsp90 chaperone machinery is required to load small RNA duplexes into Argonaute proteins, but not for subsequent strand separation of small dsRNAs or target cleavage. It was suggested that the chaperone machinery uses ATP and mediates a conformational opening of AGO proteins so that they can receive bulky small RNA duplexes (Iwasaki et al. 2010).

The important role of chaperones in RISC assembly was repeatedly demonstrated in various organisms including plants, where posttranscriptional gene silencing is also mediated by RNA-induced silencing complexes (RISCs) that contain AGO proteins and single-stranded small RNAs. The assembly of plant AGO1-containing RISCs like in animals depends on the molecular chaperone Hsp90 synthesized under physiological conditions and after HS (Iki et al. 2012).

Epigenetic silencing of transposons by Piwi-interacting RNAs (piRNAs) constitutes a universal and ancient RNA-based genome defense mechanism controlling expression and amplification of various TEs and viruses which may be harmful for the host (Malone and Hannon 2009).

Piwi endonuclease action amplifies the piRNA pool by generating new piRNAs from target transcripts by not completely understood mechanism.

Furthermore, it has been recently demonstrated that various chaperones and co-chaperones are apparently involved in piRNA biology. Thus, in *Drosophila* "Shutdown" protein, an evolutionarily conserved co-chaperone collaborates with Hsp90 during piRNA biogenesis, probably at the loading step of RNAs into PIWI proteins. It was clearly demonstrated that this co-chaperone is essential for both primary and secondary piRNA populations in *Drosophila* (Olivieri et al. 2012).

Independent lines of evidence demonstrated that several other co-chaperones associated with the molecular chaperone Hsp90 are also involved in piRNA biogenesis pathway delivering piRNAs to the Piwi group proteins in various organisms, including mice and *Drosophila* (Xiol et al. 2012). Thus, mice lacking co-chaperone Fkbp6 derepress LINE1 (L1) retrotransposon and display reduced DNA methylation. Inhibition of the ATP-dependent Hsp90 activity in an insect cell culture model results in the accumulation of short antisense RNAs in Piwi complexes (Xiol et al. 2012).

In summary, one may assume that the two very ancient and universal genome defense mechanisms such as heat shock genes system and RNA interference machinery are apparently interact in many complex and very sophisticated ways and we only begin to understand the details of their cross-talk.

6.6 Conclusions

Mobile genetic elements apparently play various sometimes opposite functions in the regulation and evolution of heat shock genes in all eukaryotic organisms. The insertions of TEs into regulatory regions or ORFs of *Hsp* genes may significantly modulate their expression and provide material for fine tuning of the whole Hsps system in response to rapidly changing environmental conditions. The presence of TEs in certain regions of *Hsp* genes makes them prone to recombination and fast propagation in a species or loss by unequal recombination. It is likely, that certain highly expressed *Hsp* genes may explore retrotransposition machinery of certain retroelements for their amplification and spread in the genome. Recent data demonstrate co-evolution and close interaction between RNAi system responsible for biogenesis and silencing of TEs and chaperones functioning.

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Chapter 7
Fine Tuning of the HSR in Various Organisms

It is widely assumed that heat shock response system in eukaryotes is amazingly conserved. Thus, all described organisms possess one or several genes encoding transcription factors belonging to HSF family that recognize the same sequences (HSEs) in the promoters of various *Hsps* genes (Åkerfelt et al. 2010; Morimoto 1998; Wu 1995). Furthermore, HSF family members in different organisms contain two highly similar domains responsible for DNA-binding and heat-induced trimerization as described in detail in Chap. 3. HSF recognizes practically the same simple sequences within promoters of *Hsp* genes (GAANNTTCNNGAA) that usually present in several copies at a regular distance from the transcription start. Various lines of evidence demonstrated that *Hsp70* gene promoter is able to efficiently function in the cells of phylogenetically distant organisms even belonging to different phyla. Thus, reporter constructs under the control of *Drosophila melanogaster Hsp70* gene promoter were readily expressed in the cells of mosquito *Aedes aegypti*, silkworm *Bombix mori* transgenic strains and in sea urchin embryos (Berger et al. 1985; McMahon et al. 1984; Uhlirova et al. 2002). Furthermore, constructs with *D. melanogaster Hsp70* regulatory region were efficiently transcribed in *Xenopus* oocytes, rat fibroblasts and monkey COS cells (Bienz and Pelham 1982; Burke and Ish-Horowicz 1982; Mirault et al. 1982; Voellmy and Rungger 1982). Importantly, all such constructs exhibited clear-cut heat-inducible pattern of expression in the cells of the foreign hosts, thus corroborating the presumed high conservatism of HS response in unrelated organisms.

However, subsequently several other groups described species-specific differences in the HS promoter efficiency (Kalosaka et al. 2006). These differences were attributed to the presence of specific regulatory elements (e.g. GAGA sites in *Drosophila* or ATRS in leaf-miner fly *Liriomyza*) within *Hsp* promoter regions of various species (see below).

Notably, early studies of the expression of such constructs in heterologous cells in 80th usually dealt with qualitative analysis of expression because at that time there was no technique such as Q-RT-PCR enabling to accurately measure the

M.B. Evgen'ev et al., *Heat Shock Proteins and Whole Body Adaptation to Extreme Environments*, DOI 10.1007/978-94-017-9235-6_7

transcription levels. Therefore, it was not possible to accurately compare the efficacy of *Hsp* promoters in the homologous cells with that in the cells of a "foreign" host species.

Later, a few investigations appeared demonstrating that sometimes even in Diptera species *D. melanogaster Hsp70* promoter functions less effectively in comparison with endogenous host promoter. Thus, the heat-inducible activity of the promoter region of the *D. melanogaster Hsp70* gene, which contains motifs for HSF and GAF binding, assayed in transgenic medfly *Ceratitis capitata* germ-line carrying the *lacZ* reporter, was found to be several fold lower than the activity of the orthologous region of the medfly *Hsp70* gene with the same *lacZ* reporter (Kalosaka et al. 2006). Similarly, in transgenic Australian sheep blowfly *Lucilia cuprina* carrying reporter chloramphenicol acetyl transferase gene under *D. melanogaster Hsp70* promoter the reporter gene was expressed with 10–100-fold lower efficiency than in *Drosophila* cells and, characteristically, did not exhibit the inducible pattern of transcription (Atkinson and O'Brochta 1992).

These data indicate that certain organisms developed specific individual mechanisms underlying heat shock response in the course of divergent evolution. In this regard mechanisms that may provide the adaptation of a population or a species to highly fluctuating environments are of special interest. The recent studies demonstrated that, indeed, heat shock response system may undergo many changes providing "fine tuning" necessary for optimal expression of individual *Hsp* genes and, hence, adaptation of a given species or a population to specific environmental conditions including extreme ones.

It was shown that activity ("strength") of HS promoter is determined by the number and context of HSEs. Interestingly, it may also depend on the presence of other regulatory elements that may be specific for a give taxon or even species (Chen et al. 2011). As was shown by different studies the optimal sequence of a classical HSE (GAANNTTCNNGAA), contains three nucleotide sense units GAA/TTC. It was also demonstrated that a nucleotide before G in the unit may be also of importance, thus, it is assumed that AGAA represents an optimal combination. On the other hand within the unit itself only the first nucleotide G (or C in the case of complementary block) is strictly conserved while the second and especially the third one may be substituted by other nucleotides and these changes does not prevent HSF recognition of the modified HSEs (reviewed by Tian et al. 2010). Promoters of individual *Hsp* genes may vary in different species by the number of GAA/TTC units. Thus, in the majority of Diptera species investigated in this respect *Hsp83* promoters at least contain one HSE, consisting of six to eight elementary three nucleotides units (Astakhova et al. 2013; Tian et al. 2010). Furthermore, certain promotors may contain the so called "gap-type" and "step-type" HSEs (see Chap. 3) and such elements are also effectively recognized by HSF (Hashikawa et al. 2006; Yamamoto et al. 2005). Gap-type HSEs were detected within *Hsp* genes promoters in yeast and *Drosophila* (Tian et al. 2010; Yamamoto et al. 2005). It is of note, that surrounding nucleotides may also significantly influence individual HSE activity.

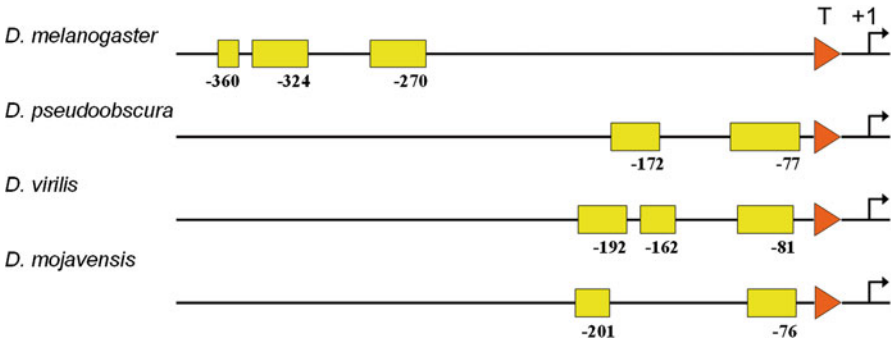

Fig. 7.1 The structure of regulatory regions of low molecular weight *Hsp* genes in *Drosophila* species. *Yellow rectangles* – HSEs, *T* TATA-box. Size of rectangles represents the amount of GAA units within HSEs (From Tian et al. 2010)

The strength of promoter is determined not only by the number of HSEs but may also depend on their distance from the transcription start site. Usually related species belonging to the same taxon comprise similar numbers of HSE copies within their *Hsp* genes promoters and HSEs have similar spacing regarding each other and transcription start. In 13 *Drosophila* species *Hsp70* promoters comprise four functional HSEs and, importantly, the position of the first two proximal HSEs regarding the transcription start is more conserved than the localization of the more distal ones (Tian et al. 2010). Furthermore, *Hsp83* promoters of *melanogaster* species group contain a single HSE, while the number of GAA/TTC units within this promoter may be species-specific. Other *Drosophila* species such as *D. mojavensis*, *D. virilis* and *D. grimshawi* have additional more distal HSE in *Hsp83* promoter. Within *Drosophila* species the *Hsp27* promoter exhibits the highest level of structural variability both in terms of HSEs number and relative position of HSEs when species of *melanogaster*, *repleta* and *virilis* groups are compared (Fig. 7.1). One may only speculate whether the observed structural polymorphism of small heat shock genes promoters resembles adaptation to specific environments or represent the result of random mutation and rearrangements in the course of species evolution. Mobile elements may be involved in the "fine tuning" of *Hsps* genes promoters as discussed in Chap. 6.

When studying heat shock genes clusters in two eurithermal Diptera species, *Stratiomys singularior* and *Oxycera pardalina*, members of the *Stratiomyidae* family (common name "soldier flies"), we described very unusual structure of *Hsp70* promoters (Garbuz et al. 2011). In contrast to all *Drosophila* species studied so far, *S. singularior* exhibits high variability of promoters of *Hsp70* genes comprising a cluster in the latter species. In *Drosophila* species high level of homology is observed not only in the promoters of neighboring *Hsp70* comprising a cluster but, is evident when promoters of different species are compared. In contrary the promoters of *S. singularior Hsp70* regulatory regions display significant similarity only within the first 50 bps upstream of transcription start site while the preceding

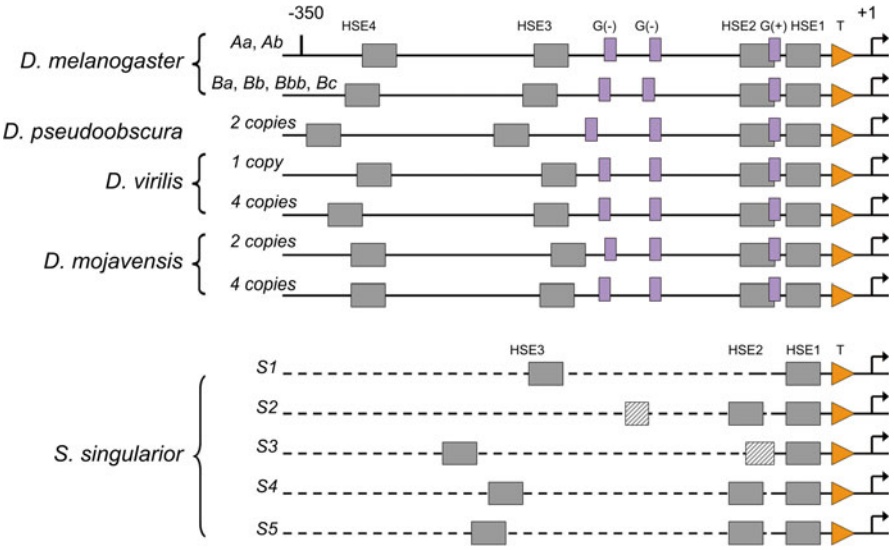

Fig. 7.2 The variability of *Hsp70* regulatory regions in *Drosophila* species and in *S. singularior*. *G* GAGA motifs in «+» or «−» orientation, *T* TATA-box (Modified from Tian et al. 2010; Garbuz et al. 2011). Lighter rectangles represent the position of putative gap-type HSEs

regions do not exhibit any significant homology (Fig. 7.2). Moreover, the number and localizations of HSEs within *S. singularior Hsp70* promoters also vary with the exception of the first HSE localized in the short (50 bps) proximal highly conserved region. Surprisingly, in *O. pardalina*, the another species of the same family (Stratiomyidae) promoters of all four sequenced *Hsp70* genes are highly homologous and differ by only a few substitutions that do not affect HSEs. The differences at 3'-regions characteristic for all five *Hsp70* genes of *S. singularior* enable to perform 3'-RACE analysis, which demonstrated that all the genes comprising *Hsp70* cluster are actively transcribed (Garbuz et al. 2011). The observed high variability of the regulatory regions of *Hsp70* genes in this species probably indicates the absence of conversion process operating in these areas and reveals a peculiar mechanism of the *Hsp70* cluster origin in this species. The observed variability in *S. singularior Hsp70* promoters may be of adaptive value providing differentiated expression of individual *Hsp70* genes after various forms of stress or at different developmental stages.

In contrast to *Hsp70* promoters, *Hsp83* promoters of *S. singularior*, *O. pardalina*, different *Drosophila* species and several other Diptera such as *Anopheles albimanus* are highly conserved not only regarding the number and positions of HSEs but share high sequence similarity as well (Fig. 7.3). There are Diptera species such as *Aedes aegypti* and *Culex pipiens* that contain additional HSEs in *Hsp83* regulatory regions localized at a greater distance from the transcription start (Astakhova et al. 2013; Tian et al. 2010).

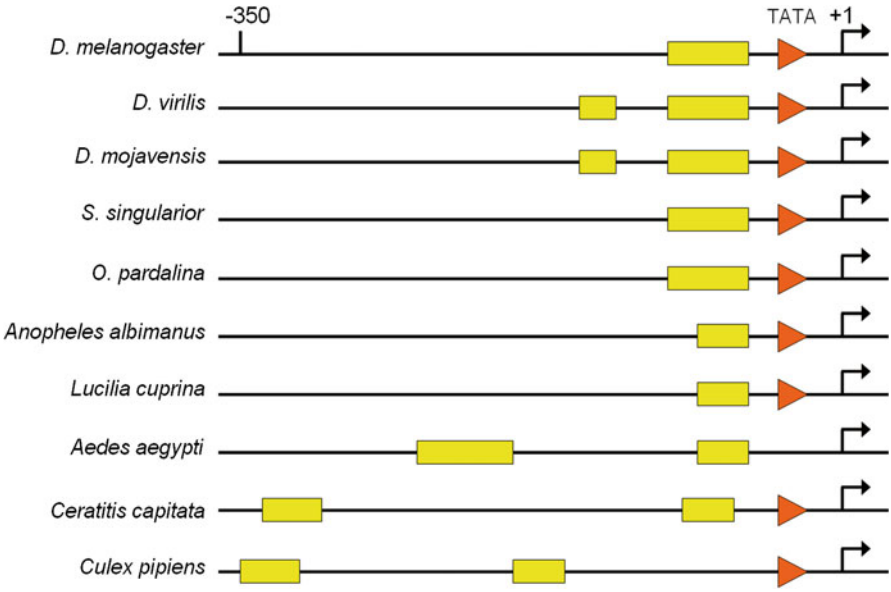

Fig. 7.3 The structure of *Hsp83* genes regulatory regions in different Diptera species. *Yellow rectangles* – HSE, *T* TATA-box. The length of rectangles resembles the number of GAA/TTC units within HSEs (Modified from Astakhova et al. 2013; Tian et al. 2010)

Previously we demonstrated that all species belonging to Stratiomyidae family used in our experiments were characterized by high constitutive levels of Hsp70 and exhibited extraordinary high thermotolerance independently on the temperature of their habitats (Garbuz et al. 2008). It was of significant interest to investigate the strength of Stratiomyidae species promoters in the genome of the foreign species. To this end, we developed constructs containing sequences of *S. singularior Hsp70S3* gene for transformation of *D. melanogaster* strain where all five *Hsp70* endogenous copies were deleted (Gong and Golic 2004). In these experiments we obtained several transgenic strains carrying one or more inserts of such constructs in *D. melanogaster* genome. Surprisingly, we failed to observe puffs in polytene chromosomes at the sites of construct insertions after HS. As expected when *S. singularior Hsp70* promoter has been substituted by that of *D. melanogaster* such constructs produced puffs after HS and were strongly induced judging by Q-RT-PCR and Northern hybridization experiments. More illustrative results of Q-RT-PCR for comparison of *S. singularior* and *D. melanogaster Hsp70* promoters strength are depicts in Fig. 7.4.

To investigate the role of the observed characteristic differences in the structure of the *D. melanogaster* and *S. singularior Hsp70* regulatory regions, we investigated the ability of these highly diverged *Hsp70* promoters to drive transcription of the luciferase reporter gene in a Schneider-2 (S2) *D. melanogaster* cell culture, both at a steady state and after temperature elevation.

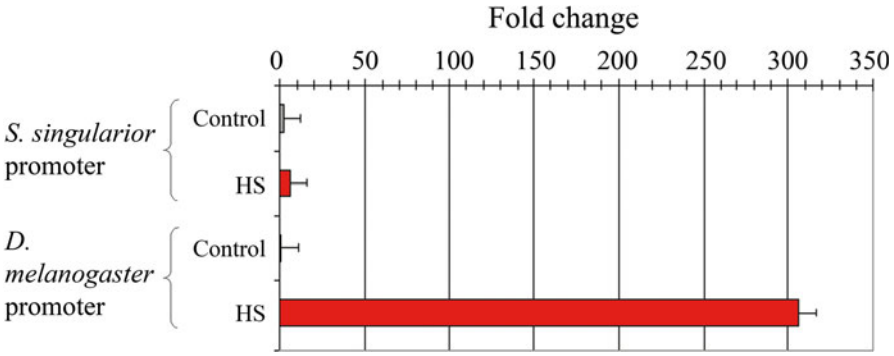

Fig. 7.4 Transcription of constructs with various *Hsp70* promoters in transformed *D. melanogaster* strains. Q-RT-PCR results exploring RNA isolated from transgenic strains transformed with constructs containing *Hsp70* genes under control of *S. singularior* or *D. melanogaster* promoters. Fold change was determined relative to the control points. *HS* heat shock. It is clearly seen that *D. melanogaster Hsp70* promoter is 100-fold more efficient, than *S. singularior* promoter

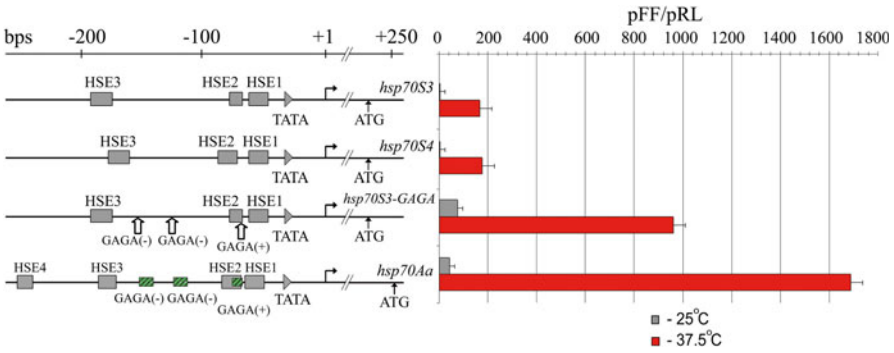

Fig. 7.5 *Left*: detailed structure of constructs used in luciferase assays. All regulatory elements within *D. melanogaster Hsp70* and *S. singularior Hsp70S3* and *Hsp70S4* promoters are shown. GAGA sites in *D. melanogaster* promoter are marked by small green boxes. Transcription start site is marked by a *bended arrow*. *ATG* – start codon of the luciferase ORF. Experimentally inserted GAGA elements in the *S. singularior Hsp70S3* promoter are indicated by open vertical arrows below the sequence of the construct. *Right*: luminescence levels as the ratio between *Firefly* (pFF) and *Renilla* (pRL) luciferase luminescence

We developed constructs with the ORF of the luciferase gene under the control of various *Hsp70* promoters (Fig. 7.5). In the construct designated *Hsp70S3*, the luciferase ORF was placed under the control of the *Hsp70S3* gene promoter used in the above experiments to obtain transgenic strains. We used a promoter of another *Stratiomys Hsp70* gene (*Hsp70S4*) to check whether the low efficiency observed in *D. melanogaster* cells was due to a specific structure of the *Hsp70S3* gene or if it represents a characteristic feature of all *Stratiomys Hsp70* genes. Our analysis indicates that promoters of *Hsp70S3* and *Hsp70S4* genes exhibited similar "strength" when tested in cell-culture based luciferase reporter system. The construct with a

promoter from the *D. melanogaster Hsp70Aa* gene served as a positive control. Except for the upstream regulatory 5'-region, all constructs contained the 5'-UTRs necessary for normal translation under HS conditions (Fig. 7.5).

The experiments demonstrated that the *D. melanogaster Hsp70A*a promoter exhibits at least a ten-fold higher efficiency, both in steady-state conditions and after HS (37.5 °C) in comparison with *Hsp70S3* and *Hsp70S4* promoters in the luciferase reporter system (Fig. 7.5).

Although HSE sequences differ slightly in *D. melanogaster* and *S. singularior Hsp70* promoters *D. melanogaster* HSF efficiently recognizes them in both species as was demonstrated by *in vitro* experiments. Therefore, the observed drastic differences in the strength of *Drosophila* and *Stratiomys Hsp70* promoters in *D. melanogaster* cells are independent of the differences in the number and/or structure of HSEs, but rather associated with the presence of other recognition sites. It is well known that the presence of HSEs is necessary but not sufficient for high efficacy of *Hsp* promoters after HS and other factors should be involved. Thus, in the case of *Drosophila Hsp70* and small *Hsps* genes the presence of GAGA elements necessary for binding of GAGA-binding factor (GAF) is prerequisite for rapid and efficient induction of protein synthesis. The position and number of GAGA elements is also very conserved in *Drosophila* species. It was shown that experimental deletion of GAGA elements leads to dramatic decrease of *D. melanogaster Hsp70* expression after HS (Georgel 2005). Functional GAGA element in *Drosophila* usually looks like GAGAG or GAGAGAG or consists of several trinucleotides units (GAG), separated by spacers comprised by uneven number of nucleotides (one, three or five). In some cases the changes in the GAG units are admitted (Georgel 2005; Omelina et al. 2011). In contrast to *Drosophila* species promoters of *Hsp70* in *S. singularior* and *O. pardalina* contain only single trinucleotides GAG (or CTC). Although there are experimental evidences that such units may also bind GAF with low efficiency (Wilkins and Lis 1998), EMSA analysis performed in our laboratory failed to reveal significant binding of *Drosophila* recombinant GAF with *Stratiomys* promoters sequences containing GAG *in vitro*. We cannot exclude, however, that endogenous Stratiomyidae GAF is able to bind with such short units more efficiently.

In order to investigate the role of GAGA elements in the efficiency of *Stratiomys* promoters in *D. melanogaster* S2 cells we developed constructs containing one or several consensus GAGA elements in different orientation in *S. singularior* promoters (Fig. 7.5). These experiments exploring luciferase reporter system demonstrated that insertion of three GAGA elements into *Hsp70S3* promoters resulted in the pronounced increase (five to sixfold) in the level of reporter gene expression which is close to the efficiency of endogenous *D. melanogaster Hsp70* promoters (Fig. 7.5). Therefore, it is evident that the absence of functional GAGA sites in *Stratiomys Hsp70* regulatory regions represents the key factor responsible for dramatic differences in *Hsp70* promoters strength of the two species in *D. melanogaster* cells. It is noteworthy, that *Hsp70* genes in both species (*S. singularior* and *D. melanogaster*) in spite of the drastic differences in their constitutive expression are strongly induced by temperature elevation (Garbuz et al. 2008; Garbuz et al. 2011). Therefore, it is not clear what additional transcriptional factors similarly to GAF in *Drosophila* are

involved in the regulation of *Hsp70* genes in thermotolerant Stratiomyidae species. Importantly, in other Diptera species e.g. *Ceratitis capitata*, just like in the case of *S. singularior Hsp70* genes promoters do not contain GAGA elements (Kalosaka et al. 2006). Furthermore, in other Diptera species such *Liriomyza sativae* and *L. huidobrensis*, there is only one complete GAGA element located at −135 position in relation to transcription start site (Chen et al. 2011). However, it was shown that efficient function of *Hsp70* promoter in *Drosophila* cells requires the presence of at least three full-size GAGA elements within the first 150 bps of regulatory region, with the first proximal GAGA element in direct orientation and two more distal elements in inverse orientation (Georgel 2005). Interestingly, even in *D. melanogaster* functional GAGA elements were detected in the promoters of small *Hsp* genes and *Hsp70* copies while the induction of *Hsp83* and *Hsp68* genes apparently does not require the presence of these motifs and these loci are induced by HS and produce large puffs. The search of various databases using MEME and MatInspector gene analisis programs failed to identify any motifs for binding of known transcriptional factors besides HSEs and TATA-boxes in *S. singularior Hsp70* promoters. The demonstrated involvement of GAF in heat-induced induction of *sHsps* and *Hsp70* genes is probably unique for *Drosophila* and other Diptera species evolved other mechanisms for *Hsp70* genes induction. It is also possible that the induction of *Hsp70* genes in other Diptera species may require long distance interactions with not yet identified sequences similar to scs/scs' elements described in *D. melanogaster* (Hart et al. 1997; Petesch and Lis 2008).

Species-specific features in heat shock response mechanisms seem to be characteristic for *Hsp70* family. Thus, when we compared the expression of constructs containing luciferase reporter gene under *S. singularior* or *D. melanogaster Hsp83* promoters in S2 cells we observed even higher expression of constructs with *Stratiomys Hsp83* promoter both in normal conditions and after HS (Fig. 7.6). *Hsp83* gene in the genome of most Diptera species is represented by only one or two copies that are involved in the regulation of multiple vital signaling pathways both

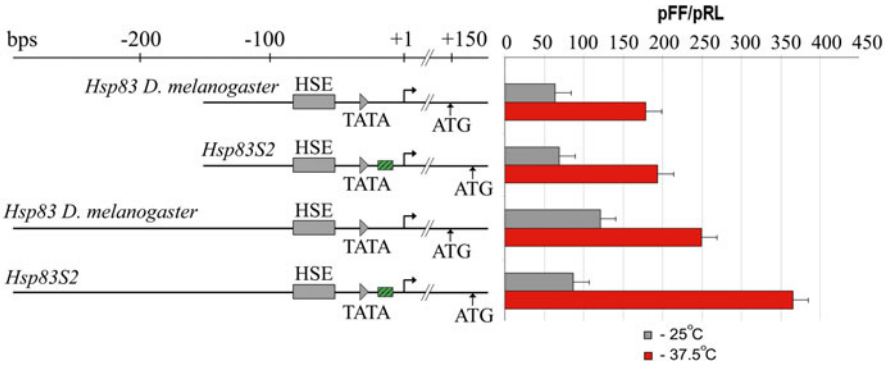

Fig. 7.6 Different efficiency of *S. singularior* and *D. melanogaster Hsp83* promoters in S2 cells. Transcription start site is marked by a *bended arrow*. Putative GAGA site in *Hsp83S2* promoter is marked as *green box*. *ATG* – start codon of the luciferase ORF

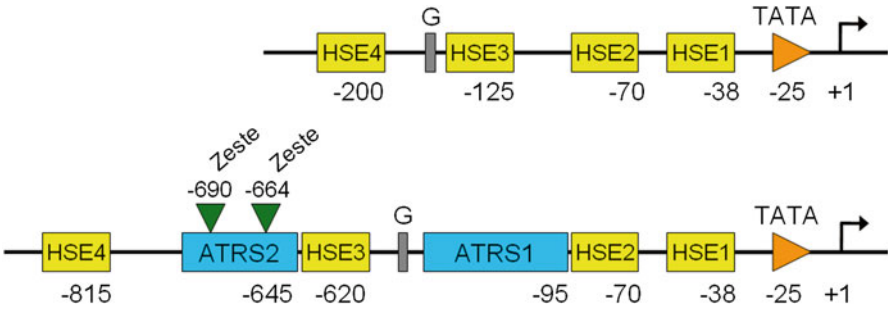

Fig. 7.7 The structure of *Hsp70* promoters in two close related species of flies – *Liriomyza huidobrensis* (*top* panel) и *Liriomyza sativae* (*bottom panel*). *G* – putative GAGA box (From Chen et al. 2011)

under normal conditions and after stress (see Chap. 3) which probably requires high conservatism of *Hsp90* genes including regulatory regions.

Besides GAGA elements described in a few *Drosophila Hsp* genes other regulatory elements were detected in the promoters of *Hsps* genes of other Diptera species that may be involved in modulation of environmental stress response.

Multiple studies have shown that GAGA elements are not the only sequences that may play an important role in *Hsp70* gene induction by HS. In fact, *Hsp70* promoters of the leaf-miner fly *Liriomysa sativa* harbour AT-rich sequence elements (ATRS) that are absent in the congeneric species.

Thus, when comparing the *Hsp70* regulatory regions of two related leaf-miner fly species *Liriomyza huidobrensis* and *Liriomyza sativae*, it was shown that the latter species is characterized by the presence of two AT-rich blocks designated "ATRS1" and "ATRS2". Apart from this difference the species exhibited highly conserved localization of HSEs in their promoters (Fig. 7.7). Intriguingly, the authors detected sites for the binding of *Zeste* transcription factor within these ATRSs, which may play a role of transcription activator in this particular Diptera species, somehow enhancing the strength of the *Hsp70* promoter (Chen et al. 2011). It was shown that this protein plays an important role in the transcription activation in *Drosophila* (Kostyuchenko et al. 2009). Furthermore, the deletion of the whole ATRS2 or *Zeste* recognition sites significantly decreased the *Liriomyza sativae Hsp70* promoter strength in luciferase reporter system while cotransfection of the construct with Zeste overexpression vector enhanced the promoter efficiency.

It can be concluded, that in the case of *Hsp70* promoters HSEs represent highly conserved basic elements, described in all Diptera species studied so far. However, mechanisms underlying fine regulation of *Hsp70* in response to stress are highly variable and usually in addition to HSF, require the participation of several other factors, providing chromatin modifications and other changes in transcription machinery after stress. Genome-wide studies have shown that evolution of regulatory regions of *Hsp* genes in relevance to HSE sequences mostly included the duplications of the GAGA motifs and single nucleotide substitutions within HSEs most of which as we showed above for *D. melanogaster* and *S. singularior* promoters did

not significantly affect their binding activity. On the other hand, taxon divergent evolution may involve dramatic changes within *Hsp* genes promoters that do not necessary involve HSF but may depend on other transcriptional factors.

In Chap. 5 we consider the major trends in the evolution of *Hsp70* genes in flies (Diptera) and mammalian species. Generally speaking, high conservatism demonstrated for genes comprising *HSPA1* cluster is also evident when regulatory regions of these three genes are aligned. We carried out comparative analysis of regulatory regions of *HSPA1A*, *HSPA1B* and *HSPA1L* genes in eight unrelated mammalian species belonging to different families and demonstrated practically identical pattern and localization of major regulatory elements in all species studied (Garbuz et al. 2011).

The demonstrated interspecific differences were represented by nucleotide substitutions and a few deletions. Previously we demonstrated that HS-induced expression of *HSP70* genes in Arabian camel *Camelus dromedarius* characteristically differ from that in human cells (Ulmasov et al. 1993) and, hence, it was of significant interest to compare the regulatory regions of camel's *HSP70* with orthologous sequences of other mammalian species. It is well known that camel is highly adapted to desert conditions and one may expect to find some specific features in the structure of its heat shock genes and in particular in their regulatory regions. The results of sequence comparison of camel's *HSPA1* cluster with the orthologous genes from other mammals are depicted in Fig. 7.8.

At the next step we developed several constructs where a reporter gene (firefly luciferase) was placed under the control of promoters of different origin. Thus we used promoters of all three genes comprising *HSPA1* cluster in humans and in camel and investigated the expression of such constructs in human culture cells

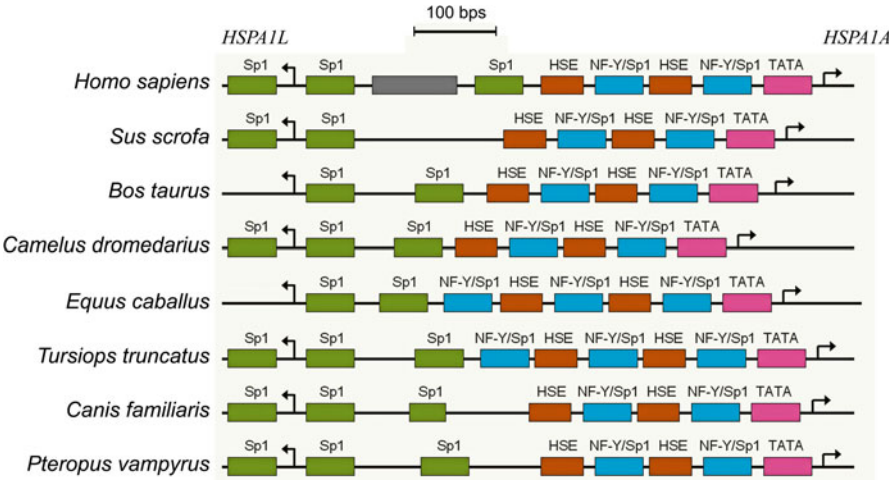

Fig. 7.8 The arrangement of *HSPA1L* и *HSPA1A* regulatory regions in different mammalian species. *Grey rectangles* represent a sequence with unknown functions which is present only in primates

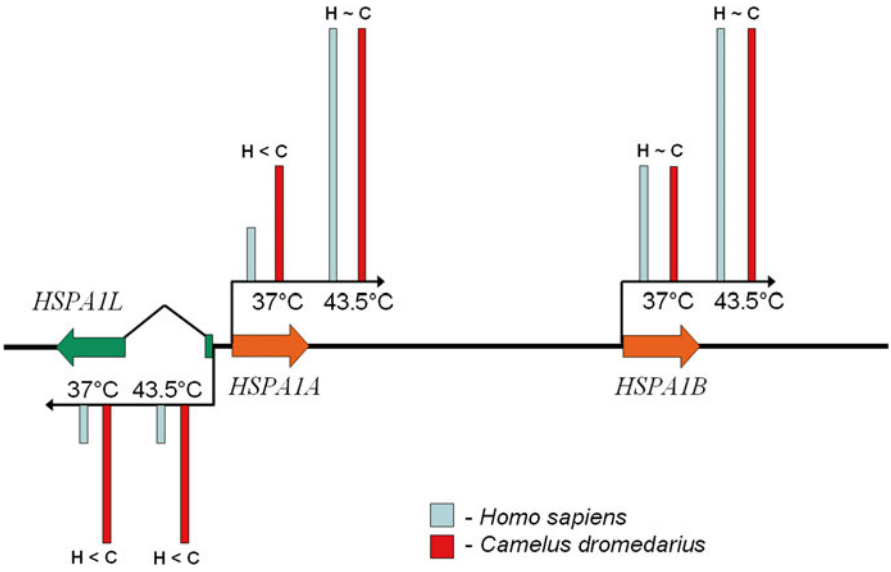

Fig. 7.9 The comparison of expression levels of camel and human genes *HSPA1A*, *HSPA1B* and *HSPA1L* at different temperature regimes. *H Homo sapiens*, *C Camelus dromedarius*

HEK293. In spite of the fact that in general the architecture of human and camel *HSP70* promoters was rather similar we demonstrated significant differences in the activity of *HSPA1A* and *HSPA1L* regulatory regions studied. Characteristically, under normal physiological conditions the activity of camel's *HSPA1A* promoter significantly exceeded (two to three times) the activity of orthologous human promoter (Fig. 7.9). However, after temperature elevation both promoters were equally efficient. Furthermore, in the case of *HSPA1L* gene the camel's promoter exhibited seven to eightfold higher efficiency that the orthologous human sequence, both under normal conditions and after heat shock (Fig. 7.9). At the present time we cannot determine with confidence the exact sequence responsible for the observed differences. In principle, the presence of ~100 bps sequence characteristic for only primates *HSP70* genes promoters (Fig. 7.8) may explain the observed differences if this sequence serves as transcription attenuator.

Actually, several lines of evidence indicate that evolution of mechanisms underlying the regulation of stress response may also act at the level of translation. Thus, soon after the discovery of heat shock response it was demonstrated that in *Xenopus* oocytes translation of *D. melanogaster Hsp70* mRNA is strongly inhibited by HS while endogenous mRNAs are efficiently translated (Bienz and Pelham 1982). On the other hand, it was shown that in *D. melanogaster* cells translation of *Hsp70* mRNAs is strongly activated by HS (Hernández et al. 2004). Therefore, it is thought that sophisticated regulatory mechanisms exist in different organisms operating at the translation level.

Along these lines, when camel *HSPA1* cluster was investigated we detected the presence of upstream silenced start (AUG) codon in the 5′-UTR of correspondent mRNA with stop codone (UAG) following right after it. Moreover, this additional start codon was found in the context not optimal for effective translation initiation (Garbuz et al. 2011). As a rule such codons are missed in the process of scanning 5′-UTR by 40S ribosomal subunit, but occasionally may be involved in translation initiation exploring non-canonical mechanisms (Kozak 1987; Sheikh and Fornace 1999). In the bull *Bos taurus* we also detected upstream AUG in a few *HSPA1A* alleles which is included into a short upstream ORF before *HSP70* coding ORF (Garbuz et al. 2011). At the present time the functional significance of such additional start codons is not clear, because as we demonstrated its substitution in the camel's promoter did not affect the translation of correspondent construct mRNA in the HEK293 cells either under normal conditions or after HS. Possibly, the revealed upstream start codons are involved in tissue-specific translation regulation or just represent the result of random mutations that predated the divergence of these species (*Camelus dromedarius* and *Bos taurus*) and do not affect the *HSPA1A* mRNA translation efficiency.

7.1 Conclusions

In contrast to high conservatism of heat shock genes coding sequences and heat shock response system as a whole, promoters of many *Hsp* genes may exhibit a high degree of variability in different organisms, even including members of phylogenetically close forms. Besides highly conservative heat shock elements (HSEs) present in heat shock promoters of all organisms, in certain species the *Hsp* promoters may contain specific regulatory elements (motifs) which are recognized by special regulatory factors restricted to the particular species or species group. This variability in terms of *Hsp* promoters structure and function may play an important role in the fine tuning of Hsps expression in response to rapidly changing environmental conditions.

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Chapter 8
Experimental Modulation of Heat Shock Response

8.1 Induced Thermotolerance, Hardening, Acclimation

Soon after the discovery of molecular mechanisms underlying the heat shock response and, specifically, the synthesis of a restricted set of proteins with different molecular weights (heat shock proteins) multiple experimental approaches were applied with the goal to somehow modify the HSR and monitor the biological consequences of such manipulations (see reviews of Feder and Hofmann 1999).

It was found in different organisms that preliminary exposure to moderate temperatures (heat hardening) strongly increased the viability observed after subsequent severe heat shock and many other stressful factors (Lindquist 1986). This universal phenomenon termed induced thermotolerance usually correlates with the accumulation of Hsps after mild heat shock which presumably protects the cells from the subsequent severe otherwise lethal heat shock treatment. Thus, Jedlicka obtained mutation of *D. melanogaster* where all Hsps are not synthesized after HS. Correspondingly, the mutant flies do not exhibit induced thermotolerance (Jedlicka et al. 1997).

Long-term, in contrast to rapid heat hardening, heat or cold acclimation described in detail in Chap. 4 represents another widely used experimental approach to modify natural thermoresistance (Somero 2005; Tomanek 2005). Acclimation procedure which may take several days is usually very effective in increasing the thermoresistance (Loeschcke et al. 1997; Tomanek 2005). However, the efficacy of acclimation procedure strongly depends on the microenvironmental conditions of species thermal niche and sometimes leads to paradoxal results. Thus, comparison of various marine invertebrates such as snails and crabs demonstrated that most warm-adapted species apparently live close to their thermal tolerance limits and, hence, usually have lower abilities to enhance heat resistance through acclimation in comparison with more cold-adapted congeneric species (Somero 2005). Crucial physiological system which is a "weak link" in the thermotolerance of many warm-adapted poikilothermal species is heart function. Thus, the comparison of two porcelain crab

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species revealed the maximal habitat temperatures for the two species are approximately 31 and 16 °C, respectively (Stillman and Somero 2000) while thermal limits of heart function are 31.5 and 26.5 °C. Therefore, whereas the subtidal species has 10 °C range between its highest habitat temperature and the upper thermal limit of heart function, in the intertidal warm-adapted species upper habitat temperature and (LT50) and heart disfunction temperature are essentially the same (Somero 2005). Therefore, despite being eurythermal, many marine warm-adapted species are in greater danger from further increase in maximal habitat temperature. It was demonstrated that highly eurythermal snails (*Tegula*) which live close to their thermal limits have a limited acclimation plasticity. On the other hand, snails species occupying moderately variable thermal environments like the subtidal zone are characterized by increased ability to modify their physiological functions (e.g. Hsps synthesis) in response to acclimation and they have a wider temperature range above their body temperature range over which they can synthesize Hsps (Tomanek and Somero 1999). The same pattern of Hsps synthesis have been described in certain Diptera species belonging to the Stratiomyidae family and living in thermally contrasting environments (see Chap. 4). Thus, larvae of cold-adapted *O. pardalina* dwelling under stable conditions (5–8 °C) survive HS more than 35 °C above its habitat normal temperature (Garbuz et al. 2008).

It was concluded that due to such variation in the HSR, species from extreme and highly variable environments are likely to be more affected by climate change than species from moderately variable environments (Somero 2005). Experimental studies of closely related species also demonstrated in many taxa that sessile organisms at least within invertebrates exhibited lower acclimation in the HSR set-points compared to mobile organisms, and there was no difference in acclimation between eurytherms and stenotherms (Barua and Heckathorn 2004). Furthermore, as a rule small Hsps showed limited role in acclimation compared to HSF, Hsp90 and Hsp70. This probably reflects the different roles of these various classes of Hsps in the induction of the HSR, where small Hsps probably do not play an important role (Barua and Heckathorn 2004).

Another attempt to modify thermal adaptation of an organism was to grow many generations of a species under conditions of elevated temperature. To reach this goal *D. melanogaster* flies collected in sub-equatorial semiarid tropical zone of Africa were used. This strain designated "T-strain" described in Chap. 4, was grown in Russia in continuous culture for many years at 31–32 °C where the flies were fertile while all other known *D. melanogaster* lines become 100 % sterile at this temperature. The observed lower Hsps expression in the T strain after moderate HS represents its characteristic feature which apparently has no basis in the compromised activation of the heat-shock transcription factor (HSF), which is similar in T and Oregon R flies. Subsequently, it was demonstrated that the observed reduced expression of Hsp70 likely stems from insertion of two transposable elements, *H.M.S. Beagle* in the intergenic region of the 87A7 *Hsp70* gene cluster and Jockey in the *Hsp70*Ba gene promoter (See Chap. 6).

There exist a growing list of *Drosophila* studies suggesting that, under certain conditions, experimental evolution at elevated temperatures leads to decreased

expression of Hsp70, which is a paradoxical outcome given the presumed role of this chaperone in thermotolerance in *Drosophila* and other organisms. Thus, five *D. melanogaster* strains undergoing natural selection at different temperatures (18, 25 and 28 °C) at 28 °C express less Hsp70 after HS than 25 °C controls during all developmental stages and exhibited induced thermotolerance to less degree than do either the 18 or 25 °C lines (Bettencourt et al. 1999). Later to determine whether and how laboratory and natural selection act on the *Hsp70* expression level of *D. melanogaster*, *Hsp70* allele frequencies in these lines were examined. It was found that insertion/deletion (indel) polymorphism of the 87A7 *Hsp70* cluster ("56H8" and "122" variants) and a single nucleotide polymorphism at the 87B *Hsp70* cluster was differentially fixed in the lines grown for many years under different temperature conditions. 28 °C populations have fixed the 56H8 cluster type containing insertions whereas the 18 and 25 °C populations have fixed the more compact 122 type. Mobile-element insertions in the regulatory regions of *Hsp70* alleles such as 56H8 may reduce *Hsp70* transcription and, hence, Hsp70 expression in 28 °C population (Bettencourt et al. 2002; Lerman et al. 2000; Zatsepina et al. 2001) It is likely, that in warm but non-stressful environments, selection reduces Hsp70 expression and increases the frequencies of insertion-bearing *Hsp70* alleles (Michalak et al. 2001; Zatsepina et al. 2001). This correlates with the results of investigation of allele distribution (56H8 and 122) from flies collected along a latitudinal transect of eastern Australia (Krebs and Feder 1997; Roberts and Feder 1999).

It was suggested that natural selection imposed by temperature and thermal variability may affect *Hsp70* allele frequencies (Bettencourt et al. 2002). The authors hypothesized that the reduced Hsp70 expression in *Drosophila* flies living chronically at intermediate temperatures may represent a common evolved suppression of the deleterious phenotypes of Hsp70.

Likewise, selection for high temperature resistance was performed in cactusophilic species *Drosophila buzzatii* adults or larval stages, in order to test if it leads to the changes in Hsp70 expression pattern.

Lines were selected for increased thermal resistance for up to 64 generations. In adult selection lines, every second generation adults received a 41.9 °C, 90 min heat shock 18–20 h after pre-treatment (heat hardening) at a non-lethal temperature of 38 °C for 75 min.

In larval selection lines, larvae were exposed each generation to mild HS selection (6 h at 35 °C and 18 h at 25 °C). The level of Hsp70 expression induced by a non-lethal high temperature was examined in selected and in corresponding control lines. Lines selected as adults showed a higher Hsp70 expression than controls. All lines which were selected during larval development demonstrated decreased expression of Hsp70. The results suggest that a trade off between heat resistance in the form of Hsp70 expression and fecundity/fertility is responsible for the level of Hsp70 expression in such selection experiments (Sørensen et al. 1999).

Along these lines, changes in heat and desiccation resistance of adult *Drosophila simulans* after short-term exposures to different temperatures (35, 31 and 18 °C) in combination with high and low relative humidity (*ca.* 90 and 20 %, respectively) were investigated. It is well known that hardening under extreme conditions (35 or

31 °C and low relative humidity) commonly resulted in higher resistance to heat and desiccation as compared with other less stressful combinations of temperature and humidity levels. The concentration of the heat-shock protein Hsp70 in the experimental flies increased following almost all applied treatments. Interestingly, life span of the hardened flies under non-stressful conditions was reduced irrespective of the stress dose, indicating a fitness cost for the plastic responses. The results of the study showed that hardening using combined heat and desiccation stress can be very efficient with regard to induction of plastic responses improving tolerance of flies to both types of stress. Therefore, such procedure may facilitate adaptation to hot and dry climatic conditions, though the negative effects on fitness are likely to constrain evolution of such plastic responses (Bubliy et al. 2013).

Probably, natural selection reducing low levels of Hsp70 in flies grown at intermediate temperatures reduces Hsp70 levels at all temperatures as a correlated response to selection (Zatsepina et al. 2001). Interestingly, soil arthropods populating soils contaminated with high levels of heavy metals representing another effective inducer of Hsp70 also evolve decreased Hsp70 expression (Köhler et al. 2000).

8.2 Mutagenesis of Specific *Hsp* Genes to Monitor Their Biological Significance

The first approach to estimate specific role of individual *Hsp* genes and their combinations in thermotolerance and other physiological functions was to get mutations and knockdowns of the pertinent genes. The pioneer studies in this field in unprecedented scale were carried out by the group of Elizabeth Craig in early 1980s exploring great genetic potential of baker's yeast *S. cerevisiae*. In the course of fulfillment of this project numerous strains with mutations of certain stress-related genes and their combinations were developed. *Ssa1* and *Ssa2* were the first genes to be studied in this manner. These genes encode two highly homological proteins belonging to the cytosolic Hsp70 subgroup and both express constitutively (see Chap. 1). Mutants of these two genes were constructed *in vitro* and substituted in the yeast genome in place of the wild-type alleles. No phenotypic effect of single mutations of either gene was detected. However, cells containing both the *Ssa1* and *Ssa2* mutations grew slowly at 30 °C and could not form colonies at 37 °C. The temperature sensitive phenotype can be overcome by inserting either an intact *Ssa1* or *Ssa2* gene into the genome. Pretreatment at 37 °C before shift to a normally lethal temperature of 51 °C protected the double mutant as well as the wild type, indicating that *Ssa1* and *Ssa2* genes products are needed for resistance to constant moderate hyperthermia but not for survival at more high temperatures for a short period (Craig and Jacobsen 1984; Werner-Washburne et al. 1987).

At the next step mutations in other *S. cerevisiae Hsp70* genes were obtained and strains carrying corresponding mutations were analyzed. Knockouts of two inducible *Hsp70* genes, *Ssa3* and *Ssa4*, that are not normally expressed at 23 °C, were added to *Ssa1Ssa2* mutants. Strains carrying mutations in *Ssa3* or *Ssa4* individually,

as well as both in *Ssa3* and *Ssa4*, were indistinguishable from the wild type. However, *Ssa1Ssa2Ssa4* negative strains were unviable. Nevertheless, an intact copy of *Ssa3* regulated by the constitutive *Ssa2* promoter was capable of rescuing a *Ssa1Ssa2Ssa4* strain (Werner-Washburne et al. 1987). In the case of *Ssb1* or *Ssb2* genes, encoding cytosolic Hsp70 members, knockouts strains appear wild type, but growth of a strain containing mutations in both genes is relatively cold-sensitive. Cells containing both *Ssb1* and *Ssb2* mutations showed altered growth properties; double-mutation cells possess an optimal growth temperature of 37 °C rather than 30 °C and grow increasingly poorly as the temperature is lowered. In addition, the *Ssb1 Ssb2* deletion mutant strain had lower barotolerance than the control strain. The strain that expresses the *Ssb1* gene in high copy number had a higher barotolerance than the strain that expresses this gene in low copy number (Craig and Jacobsen 1985). A functional *Ssc1* gene, which encodes Hsp70 protein with mitochondrial localization, is essential for vegetative growth. A spore lacking a wild-type *Ssc1* gene is able to germinate but ceases growth after several divisions (Craig and Jacobsen 1985; Craig et al. 1987).

Mutations in yeast *Hsp90* and *Hsp110* genes have strong effects on viability and growth at different temperatures. *S. cerevisiae* contains two closely related genes belonging to *Hsp90* family (*Hsc82* and *Hsp82*). *Hsc82* expresses constitutively at a very high level and is moderately induced by high temperatures. *Hsp82* expresses at a much lower level at normal temperature and more strongly induced by heat shock. Site-directed disruption mutations were produced in both genes. Cells homozygous for both mutations did not grow at any temperature. Cells carrying other combinations of the *Hsp82* and *Hsc82* mutations grew well at 25 °C, but their ability to grow at higher temperatures was lower than in the case of the wild type strain. Between 36 and 38 °C, differences between mutant and wild-type cells were more pronounced the higher the temperature (Borkovich et al. 1989). In the next work knockouts of two *Hsp110* genes (*Sse1* and *Sse2*) were obtained. Mutation in the *Sse1* gene causes slow growth at all temperatures (20–27 °C) (Mukai et al. 1993), whereas mutations disrupting *Sse2* have no effect on growth. However, overexpression of Sse2 protein from a multicopy plasmid can suppress the slow growth effect of a defective *Sse1* gene (Shirayama et al. 1993). Overexpression of Sse1 can suppress temperature-sensitive defects in different signaling pathways such as the Ras signaling pathway as well as different other temperature-sensitive mutations effects: disruption of the *Ira1* gene, which is involved in activating guanosine 5′-triphosphatase, or deletion in the gene *Bcy1*, which encodes the regulatory subunit of the cyclic adenosine 3′,5′-monophosphate–dependent protein kinase in yeast (Easton et al. 2000).

Strong phenotypic effect was shown in *S. cerevisiae* strains with mutations in *Hsp104* gene which encodes AAA+ disaggregase. Interestingly, mutant cells grew at the same rate as wild-type cells and died at the same rate when exposed directly to high temperatures. For example, growth rates at 25 °C were identical for wild-type cells and cells carrying the mutation. When *log* phase cells were transferred from 25 to 37.5 °C, mutant cells continued growing at approximately the same rate as wild-type cells and reached similar stationary phase densities. However, when

given a mild pre-heat treatment, the mutant cells did not acquire tolerance to heat, as did wild-type cells. For induced thermotolerance, cells were incubated for 30 min at 37 °C before being transferred to 50 °C. The mutant cells died at nearly the same rate as wild-type cells when shifted directly to 50 °C. However, when incubated initially at 37 °C, the mutant cells showed a 100-fold reduction in survival compared to wild-type cells after 20 min at 50 °C in rich media. In minimal media, the difference in killing rates was greater than 1,000-fold. Transformation with the wild-type gene rescued the defect of mutant cells. The effect of Hsp104 in yeast is based on ability of this protein to reactivate the splicing system after heat shock. Authors have shown, that when splicing in *S. cerevisiae* was disrupted by a brief heat shock, it recovered much more rapidly in wild-type strains than in strains containing *Hsp104* mutations (Sanchez and Lindquist 1990; Vogel et al. 1995).

Subsequently, different genetic manipulations with various members of heat shock response system in different model organisms besides yeast were used. The studies on the role of individual components of heat shock system in thermoresistance included whole body analysis as well as investigations using cell cultures.

Thus most thorough investigation exploring knockouts of different *Hsp* and *Hsf* genes in animals was carried out in laboratory mice *Mus musculus* and fruit fly *D. melanogaster. Mus musculus* is a very common model for disruption (knockout) of different genes and, hence, their functions discovery (Austin et al. 2004). In the case of mice the main focus was to study the functions of different HS proteins at the molecular and physiological level under normal non-stress temperature conditions. Thus, active participation of HSP70 in the regulation of various cellular processes in mammals was described (see Chap. 2). Mouse strains defective for different *HSP70* genes, in the first place two highly stress-inducible genes, *HSPA1A* and *HSPA1B* were developed. Inactivation of *HSP70A1A* or *HSP70A1B* resulted in deficient maintenance of thermotolerance and increased sensitivity of different tissues to heat stress-induced apoptosis under long-time whole body hyperthermia (Huang et al. 2001). In *HSP70A1B*-deficient embryonic fibroblasts, osmotic stress markedly reduced cell viability. Furthermore, when osmotic stress was applied *in vivo*, *HSP70A1B*-deficient mice exhibited increased apoptosis in the rental medulla (Shim et al. 2002).

It was shown that expression of C-terminal peptide-binding domain of *HSP70* protects mice cells against heat stress as well as full-size protein expression (Li et al. 1992). Different *HSP70* genes knockouts resulted in increase in sensitivity of different tissues, heart in the first place, to physiological stresses such as ischemia (Hampton et al. 2003). Therefore, targeted *HSP70A1B* disruption increases infarction volume after focal cerebral ischemia in mice (Lee et al. 2001). At the present time different scientists groups obtained mice with knockouts of *HSPA1L*, *HSPA9*, and *HSP110*, in addition to deletions of *HSP70A1A* and *HSP70A1B* (Chen et al. 2011; Eroglu et al. 2010; Mohamed et al. 2012).

At the early stages of heat shock system studies several attempts have been performed to find mutations (temperature sensitive (ts) lethals) that regulate the firing of the whole Hsps system. Temperature sensitive lethals are usually quite viable at normal temperature, but die at restrictive temperatures either at some

specific stage of development or at any stage ("polyphasic lethals"). Since Hsps are induced at practically all stages and in all organs (with a few exceptions) the mutation of any regulatory gene controlling the whole Hsps system should be polyphasic by definition. In the course of such investigations we have described a gene in *Drosophila* responsible for general RNA transport from the nuclei (Evgen'ev et al. 1979; Tret'iakova et al. 2001; Ivankova et al. 2010), while Carl Wu in *Drosophila* and Richard Morimoto in humans discovered specific heat shock transcriptional factors (HSF) that after temperature elevation binds with regulatory elements (HSEs) located in the promoters of all *Hsps* genes and provided extremely fast induction of the whole system. In the course of such analysis various mutations at HSF loci including temperature sensitive ones have been recovered in mice and *D. melanogaster* (Rabindran et al. 1993; Zimarino and Wu 1987).

In mouse strains knockouts of different *Hsf* genes allow to study HS system functions under different physiologically conditions (Christians and Benjamin 2006; Jin et al. 2011). For example, it was shown that *Hsf1*(–/–) mice are characterized by increased sensitivity to heavy metal-induced injury (Wirth et al. 2003). Mitochondria of *Hsf1* negative mice are more sensitive to oxidative damage than in wild type (Yan et al. 2002). Knockouts of *Hsf2* and *Hsf4* have in general normal Hsp expression patterns but different abnormalities in embryonic development and postnatal growth that is the evidence of essential role of HSF family in regulation of different house-keeping genes activity besides HS response regulation (Fujimoto et al. 2004; Wang et al. 2003).

In *D. melanogaster* temperature-sensitive HSF mutant (hsf^4) does not survive heavy heat shock and the mutant line demonstrates delayed HSF activation, later and lower induction of stress genes and strongly decreased heat resistance. Although the importance of Hsps synthesis for resistance to heat stress is well documented there are a few cases when such correlation is not so evident and it seems that other factors besides Hsps may play an important role in induced and basic thermotolerance. Thus, this *hsf* mutation does not affect high temperature-induced knock-down resistance in *D. melanogaster* (Nielsen et al. 2005).

In the course of the *Drosophila* Gene Disruption Project (Bellen et al. 2011) a number of *D. melanogaster* strains with insertions of genetic constructs based on different mobile elements into 5'-part of coding regions of many *Hsp* genes were obtained. This insertion mutagenesis approach allows to obtain full inactivation of any targeted gene in this model species.

Besides, the investigation of various mutated *Hsp* genes at the whole body level the analysis of cell culture transfected or transformed with constructs overexpressing certain stress-related genes represent another widely used approach to investigate specific roles of individual Hsps. Heat shock genes contained in such constructs may be controlled either by their own promoters or by foreign promoters providing their overexpression in the transformed cells. It was demonstrated in numerous papers that cell cultures transformed with plasmids overexpressing different Hsps exhibit higher resistance to certain forms of stress in comparison with wild-type original cells.

For example, transgenic over-expression of αB-crystallin confers simultaneous protection against cardiomyocyte apoptosis and necrosis during myocardial ischemia and reperfusion (Ray et al. 2001). HSP70 overexpression in rat brain reduces oxidative stress, prevents mitochondria damage and protects from focal ischemia (Xu et al. 2009). Overexpression of mitochondrial HSP70 protects astrocytes against ischemic injury *in vitro* (Volobouеva et al. 2008). Rodent cells expressing higher levels of the HSP70 protein generally tolerate thermal stress better, whereas cells expressing mutated nonfunctional *HSP70*-encoding genes, are heat sensitive. These results provide strong evidence that expression of HSP70 in mammalian cells leads directly to thermal tolerance (Li et al. 1992). Such studies demonstrate high efficacy of Hsps and in particular members of Hsp70 family as universal protectors against various kinds of stress.

8.3 Mutagenesis of Regulatory Regions of Individual *Hsp70* Copies to Modulate Their Expression

Finding the spontaneous incorporation of mobile elements predominantly in promoter regions of the *Hsp* genes (Chap. 6) makes them "hot" sites in mutagenesis caused by the transposition of mobile elements. We described the consequences of TE incorporations into various regions of *Hsps* genes in Chap. 6. In order to explore the presumed ability of *P* elements to insert specifically into promoter regions of *Hsp70* genes experimentally, we used the system developed for studying the incorporation of a construct designed on the basis of *P* element. To address this issue, we have exploited a variant of local transposition, which Timakov et al. (2002) have used to mutagenize specific regions of the *Drosophila* genome. It is known that *P* element (the most extensively studied mobile element of *Drosophila*) moves predominantly into the regions of chromosomes located in the neighborhood of preexisting copies. The phenomenon was called "local jumps". In our "bombardment experiments" we used the genetic construct called *EPgy2* which contained *Drosophila white* gene and was flanked by *P* element inverted terminal repeats recognized by *P* element transposase which provides local transpositions of construct similar to those of natural *P* element (Shilova et al. 2006). The expression of this construct is dose-dependent and allows distinct detection of new transpositions basing on the eye color. Two *D. melanogaster* lines (US-2 and US-4) carrying the necessary marked constructs (EPgy2) near locus 87A which contains an inverted repeat of two *Hsp70* genes were obtained (Fig. 8.1). Combination of genetic and molecular-biological methods allowed us to analyze the frequency of *P* element-based construct incorporation in the region of *Hsp70* genes (Shilova et al. 2006). To our surprise, all described transpositions of *P* element-construct in the area of these genes took place exclusively at the nucleosome-free promoter region of *Hsp70* genes (Shilova et al. 2006). Moreover, there were a few "extremely high hot spots" at the level of specific nucleotides in this area (Fig. 8.1). Later extensive analysis of multiple natural populations of *D. melanogaster* has demonstrated that the localization of *P* elements transposed into *Hsp70* gene cluster is also restricted to the

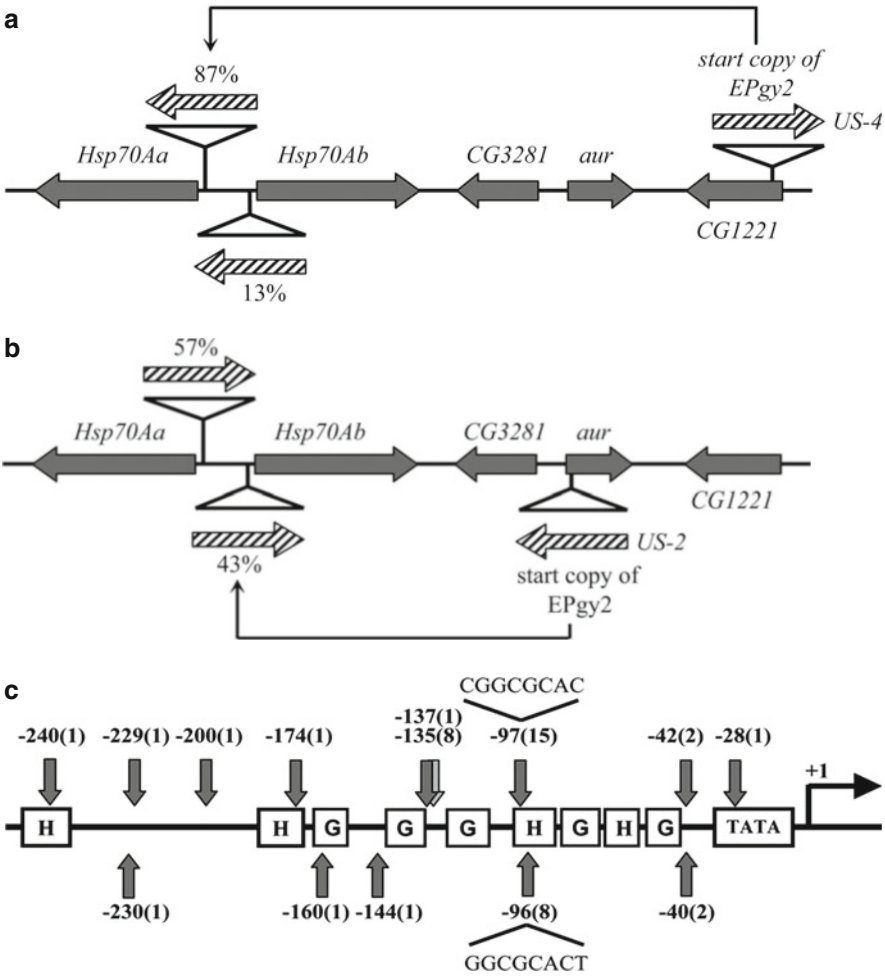

Fig. 8.1 (**a**, **b**) Organization of *Hsp70Aa* and *Hsp70Ab* loci in *D. melanogaster* and bombardment experiments. Localization of the start construction *EPgy2* in strains US-4 (**a**) and US-2 (**b**) is indicated by *hatched arrows. Horizontal arrows* show the transcription direction. The frequency of insertions detected in the progeny of both strains in percents is indicated for each strain. All insertions have been found only in the regulatory regions of *Hsp70* genes. (**c**) Insertion sites for local transpositions of the *EPgy2* construction into promoter region of *Hsp70Aa* and *Hsp70Ab* of *D. melanogaster*. Numbers before parentheses refer to nucleotide position relative to transcription start; numbers of independent transpositions into the indicated nucleotide are in parentheses. Approximate positions of heat shock (H) and GAGA (G) elements and the TATA box are indicated. Target site duplications are provided for "hot spots" located at −135, −97 and −96 nucleotides. Transcription start site marked as +1 (Reproduced with permission from Shilova et al. 2006)

promoter region and is the same to that described in our "bombardment" experiments (Walser et al. 2006, see Chap. 6).

The observed high specificity of *P* element insertions, is possibly determined by formation of DNA loops in the presence of GAGA-factor (GAF), binding with *GAGA*-motifs of *Hsp70* promoter (Shilova et al. 2006). It is known that GAF, in

addition to participating in the chromatin decondensation, can form oligomeres and organize chromatin into nucleosome-like domains in which the chromatin wraps around the GAFs (Georgel 2005). These structures represent a distinctive chromatin conformation to transposase complexes. Nucleotides −96 and −97 are in the middle of a window (from −80 to −111) flanked by GAF-binding sites but unprotected by bound GAF (Georgel 2005; Gilmour et al. 1989; Weber et al. 1997). Finally, the sequence centered at −96 and −97 (GGCCAGAC) may favor transposition because *P* element prefers GC-rich sequences for insertions (O'Hare and Rubin 1983).

To monitor biological consequences of the insertions the obtained strains with the *P* element-based construct inserted at sites ranging from −28 bps (at the TATA box) to −240 bps nucleotides upstream of the transcription start site at the *Hsp70Aa* and *Hsp70Ab* genes (Shilova et al. 2006) were screened for *Hsp70A* mRNA levels after HS. *P* element-based insertion into the *Hsp70A* proximal promoters decreased mRNA levels. The magnitude of the decrease was inversely related to the distance of the insertion from the transcription site, ranging from 56 % of control levels at nucleotide −28 to 3 % at nucleotide −144. Interestingly, in the absence of heat shock the transformed strains differed in fecundity, and, hence, each strain's fecundity after heat shock was compared with its own pre-heat-shock level. Heat shock treatment increased fecundity relative to controls in the lines with *P* element insertions in *Hsp70A*. In contrast, in the control line used for transformation experiments HS decreased fecundity (Shilova et al. 2006). Along these lines, Lerman et al. (2003) showed that lines with naturally occurring *P* elements in the proximal promoter region of *Hsp70Ba* can have greater fecundity than wild-type lines. It is likely, that *P* element insertions into the *Hsp70* promoter region, reducing the Hsp70 expression, should be favored by natural selection in hot climate areas because the reduction of the Hsp70 level may increase fecundity (e.g. by eliminating the negative effect of Hsp70 on the proliferation). These experimental findings corroborate the data accumulated in *Drosophila* strains grown at elevated temperatures for many generations, where Hsp70 level was usually reduced in the course of such selection (see above in Chap. 6).

The data accumulated suggest that the promoter regions of *Hsp70* genes are indeed "hot" sites for *P* element transpositions, and we developed a simple scheme for the *P*-induced mutagenesis specifically of the promoter regions of *Hsp70* genes. It is a challenge to understanding why representatives of other multiple TEs families existing in *D. melanogaster* genome were rarely detected in the promoters of *Hsps* genes.

8.4 Experimental Manipulations with the Goal to Change *Hsp* Genes Copy Number

Soon after the discovery of heat shock systems in various organisms it was directly demonstrated by different independent approaches that Hsps apparently play important role in the adaptation to rapidly fluctuating conditions of the

environment and comprise very robust, ancient and evolutionary fixed SOS system (Lindquist 1986; Feder and Hofmann 1999; Feder 2007) and the first experiments have been performed to increase thermoresistance mechanistically by increasing the number of major heat shock genes. Such experiments exploring transgenic *D. melanogaster* lines with an artificially increased number of copies of *Hsp70* genes were carried out at the Chicago University (Krebs and Feder 1997; Welte et al. 1993).

To increase *Hsp70* copies number a novel method for transgene analysis, based upon the site-specific FLP recombinase was developed. The method employs site-specific sister chromatid exchange to create an allelic series of transgene insertions that share the same integration site, but differ in transgene copy number. Using this method *D. melanogaster* containing additional copies (6 or 12 of extra copies) of *Hsp70* at 87B site were obtained (Welte et al. 1993). These strains in contrast to wild type flies were characterized by elevated concentration of Hsp70 during the whole development from the larvae to adults under normal physiological conditions. As expected the immediate survival after acute HS (i.e. thermoresistance) was significantly higher in the transgenic flies in comparison with the original line. On the other hand, high frequency of developmental defects was observed in the transgenic flies leading to high lethality of the 1st instar larvae (Krebs and Feder 1997). Heat shock of the transgenic third instar larvae resulted in high lethality at the following stages and only 1 % of the treated specimens reached adult stage. Furthermore, even under non-stress conditions the viability of transgenic flies is significantly lower than that of the original strain (80 %). It was concluded that differentiation in the course of ontogenesis is disturbed in the flies with extra copies of *Hsp70* genes. Specifically, it was demonstrated that cell proliferation is slow down in the transgenic flies (Feder et al. 1996). The increased level of Hsp70 observed in the transgenic strains results in the decrease of fertility (Krebs and Feder 1997). The deleterious effects of elevated Hsp70 concentration may be explained by the well known strong antiapoptotic effects of Hsp70 because apoptosis represents a normal process occurring in the embryogenesis necessary for differentiation and clearing process.

Furthermore, the increased gene expression is predicted to increase energy allocation to transcription, translation and protein functions and this cannot be ignored when modeling the strains with significantly enhanced gene expression (e.g. strains with extra *Hsp70* copies). At this end the metabolic rates of larvae with different copy numbers of the *Hsp70* copies were measured to quantify energy expenditure before, during, and after HS (Hoekstra and Montooth 2013). The magnitude of the increase in metabolic rate was positively correlated with *Hsp70* gene copy number. The obtained results enabled to conclude that the increased energetic demand of expressing extra copies of *Hsp70* in *Drosophila* may contribute significantly to known tradeoffs in physiological performance of extra-copy larvae (Hoekstra and Montooth 2013).

Therefore, although *D. melanogaster* flies with extra *Hsp70* copies were as expected more thermoresistant at some stages, as compared to the control, at other developmental stages, superexpression of *Hsp70* could be harmful and increase

lethality. It is evident that the number of *Hsp* genes and their position in *D. melanogaster* genome are apparently important adaptive characters and are under the strict control of natural selection and further increase in heat tolerance may be achieved exploring other adaptive systems. On the other hand, our experiments with other Diptera species (*Stratiomyidae* family and *virilis* group of *Drosophila*) demonstrated that close species, geographical strains and even specimens of the same population may differ by the number of *Hsp70* copies (Chap. 5).

The data accumulated in the process of the investigation of *D. melanogaster* transgenic strains with extra *Hsp70* copies help to understand comparatively low induction of Hsp70 in the strains constantly grown at elevated temperature for many generations (see above) and in the organisms often subjected to high temperature challenge where selection should favor lower levels of Hsp70 induction after HS.

Therefore, experiments with mechanistic increase of *Hsp70* copy number as expected resulted in short-term thermoresistance but also demonstrated obviously deleterious consequences of the presence of additional *Hsp70* copies in *D. melanogaster* genome. It became evident that adaptations to the extreme conditions cannot be achieved by a simple increase in the number of copies of certain *Hsp* genes.

A few years ago Gong and Golic decided to address the same issue from the other side by means of genetic manipulations with the goal to get the flies completely devoid of part or all *Hsp70* copies (Gong and Golic 2004). Using site-specific deletions they developed *D. melanogaster* strain lacking part or all copies belonging to *Hsp70* family (Fig. 8.2). The investigation of such strain enabled to reveal the role of other *Hsp* genes families in resistance to high temperature and phenomenon of induced thermotolerance and genetically to dissect the role of individual Hsps in HSR in *Drosophila*.

In the course of these deletion experiments several strains devoid of two, four or all six copies of *Hsp70* were obtained (Gong and Golic 2004). Fortunately, even flies lacking all copies of *Hsp70* were quite viable but exhibit significantly lower fertility in comparison with the original strain. Interestingly, the thermoresistance of the strains with deleted two or four copies do not differ drastically from the original strain, however, strain completely devoid of *Hsp70* copies designated "*Hsp70-*" was highly sensitive to HS at all developmental stages. The analysis of other heat shock puffs dynamics revealed drastic delay in the puffs regression in the strain lacking *Hsp70* copies which implies the delay in HSF inactivation after HS. It was also shown that *Hsp70-* flies are able to survive moderate HS (36–37 °C for 30 min), but die after severe HS (39 °C). Another characteristic feature of *Hsp70-* strain is the absence of inducible thermotolerance (Gong and Golic 2006). Notably, Ish-Horwitz was the first who obtained deletions of both major heat shock puffs (87A and 87C1 regions) in 1977 (Ish-Horowicz et al. 1977).

Interestingly, thermosensitive HSF1 mutant (hsf^{4-}), described above which does not bind DNA at the temperatures above 33 °C exhibits very similar thermal phenotype (Jedlicka et al. 1997). In this particular strain (hsf^{4-}) the synthesis of all Hsps is not observed immediately after HS but takes place after the recovery period at normal temperature (Nielsen et al. 2005).

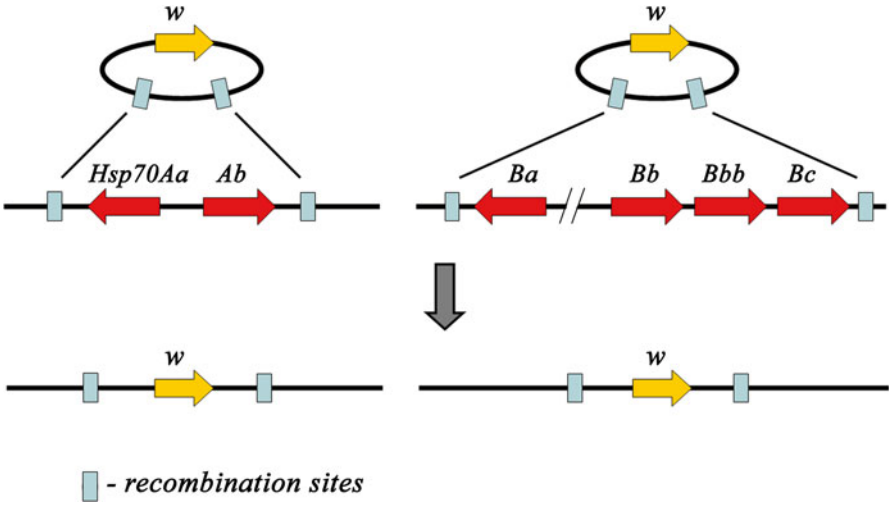

Fig. 8.2 A simplified scheme of site-specific recombination experiments used for deleting all *Hsp70* copies from *D. melanogaster* genome. *w* – *D. melanogaster white* gene (From Gong and Golic (2004) with permission)

Insertions mutants with disruption of different HS genes from *Drosophila* Gene Disruption Project (see above) were also used to study Hsps influence on resistance to oxidative stress after ionizing radiation influence (Moskalev et al. 2009). In wild type strain Canton S chronic irradiation induced adaptive response for subsequent treatment by superoxide radical inductor paraquat in males, but not in females. In *Hsp22Hsp67Bb* mutant homozygotes sex differences were also observed: the radio-adaptive response persisted in males, but not in females. In hsf^4 and *Hsp70Ba* defective homozygotes the radiation-induced adaptive response was not shown in either sex.

All these results suggest that Hsp70 at least in *Drosophila* plays pivotal role in thermoresistance specifically at sub-critical temperatures and corroborated the presumed important role of Hsp70 in the phenomenon of inducible thermotolerance.

8.5 The Development of Transgenic Strains with Experimentally Modified Stress-Resistance

There are multiple data showing correlation between tolerance to various forms of temperature stress, oxidative stress, starvation and longevity. Such correlation has been repeatedly demonstrated in various organisms from bakers yeast and snails to *Drosophila* species and rodents (Johnson et al. 2001; Longo 2003; Perez et al. 2009). The selection of individuals to certain form of stress e.g. high temperature or starvation usually results in correlated increase of tolerance to other forms of stress and sometimes longevity (Harshman et al. 1999; Saunders and Verdin 2009).

Such correlative response may be explained by modulation the levels of specific factors (e.g. FOXO, Hsp70, catalase or sirtuins) involved in response to many forms of stress, occurring in the process of selection for heat or cold resistance or longevity etc.

Along these lines occasionally observed drastic differences in the longevity of related forms are probably due to natural selection for stress tolerance which took place in the course of divergent evolution of certain species. Thus bats (e.g. *Tadarida brasiliensis*) characterized by significantly higher longevity in comparison with ordinary mice also exhibit significantly higher resistance to oxidative damage of proteins (Salmon et al. 2009). Likewise, Naked Mole-Rat with extraordinary high life-span is also more stress-resistance than many other rodents including house mice (Lewis et al. 2012).

The analysis of spontaneous and induced mutations with extended life-span in *D. melanogaster*, nematodes and yeast provided independent corroboration of such correlations and demonstrated that the long-lived mutants usually exhibit high tolerance to various stress factors, including HS, oxidative stress etc. (Lithgow and Walker 2002).

The description of specific and generic roles exercised by various heat shock genes as well as existence of numerous mutations and natural forms differing by expression of Hsps and, hence, tolerance to various forms of stress represent the theoretical grounds for development of transgenic organisms with modified stress resistance. There are several successful attempts to use various stress genes to get transgenic organisms with increased stress tolerance.

Thus, transgenic mice expressing human Hsp70 have improved post-ischemic myocardial recovery and decreased ischemic area in brain infarct. These transgenic mice also showed significant decreases in cell injury and apoptosis in the ischemic hemispheres (Plumier et al. 1995). The authors concluded that high level constitutive expression of the human inducible Hsp70 plays a direct role in the protection of the myocardium from ischemia and reperfusion injury. Similarly, the trehalose-6-phosphate synthase gene (*tps1*), which participates in trehalose synthesis, was cloned from *D. melanogaster* genome and the effect of tps1 overexpression as well as mutation in this gene were examined on the resistance of *Drosophila* to anoxia. Upon induction of *tps1*, trehalose level increased, and this was associated with increased tolerance to anoxia. Furthermore, *in vitro* experiments showed that trehalose reduced protein aggregation caused by anoxia. Homozygous *tps1* mutant (*P* element insertion into the third intron of the gene) leads to lethality at an early larval stage, and excision of the *P* element rescues totally the phenotype. Therefore, it was concluded that trehalose contributes to anoxia tolerance in flies; this protection is likely to be due to a reduction of protein aggregation (Chen et al. 2002).

The existence of close forms strikingly differing by HSR and/or mutations that strongly modify tolerance to various stresses evoked experiments with the goal to use stress genes from certain resistant species and introduce them into a susceptible one. Thus, we decided to use *Hsp70* genes cloned from *S. singularior* genome and introduce them into *D. melanogaster* genome by means of *P*-mediated transformation. *S. singularior* (see above) is very thermoresistant species belonging to

Stratiomyidae family (Diptera) characterized by a high constitutive level and stability of Hsp70.

In our transformation experiments we explored a strain of *D. melanogaster* devoid of all *Hsp70* endogenous copies (Gong and Golic 2004). In these experiments we substituted *Hsp70* genes of *D. melanogaster* by orthologous gene from a highly thermotolerant eurithermal species (*S. singularior*) from the same order (Diptera).

As we reported above, *Drosophila* species differ characteristically from all studied *Stratiomyidae* species in terms of Hsp70 synthesis. Briefly, in *Drosophila* Hsp70 is synthesized only after heat shock or other stress and apparently deleterious under normal physiological conditions. Additionally, *Drosophila* Hsp70 apparently has lower stability compared with that of *S. singularior* (6 h vs. more than 48 h half live, respectively). In contrast, *Stratiomyidae* species larvae exhibit high constitutive levels of Hsp70 and only modest induction after HS (Garbuz et al. 2008). The main goal of the experiments was to monitor the behavior of *Stratiomyidae* Hsp70 in the *Drosophila* cells in terms of induction patterns and half-life duration. Preliminary experiments demonstrated that Hsp70 of *Stratiomyidae* synthesized in *Drosophila* cells provides certain degree of induced thermotolerance in the transformed strains but does differ significantly from correspondent *Drosophila* chaperones in terms of half-life and other features (paper in preparation).

Therefore, it is necessary to keep in mind that mechanistic insertion of additional stress-related gene or combination of such genes from highly resistant organisms cannot be considered as a universal and straightforward approach for the development of organisms with enhanced tolerance to heat and other stressful factors. The investigation of *D. melanogaster* strains with additional copies of *Hsp70* (see above) clearly demonstrated that manipulations with relative positions or/and copy number of individual stress genes usually result in multiple side-effects including deleterious ones. It is evident that genes that respond to heat shock and other environmental challenges described in all organisms studied so far represent highly balanced system which functions in the context of the whole genome and any artificial modification of its components should have multiple and often unpredictable consequences.

8.6 Conclusions

We believe that long continued large-scale studies of the system of *Hsps* genes in the species that differ in the temperature of habitats disclosed the general patterns of functioning and evolution of this system and will allow reinterpretation of the genetic mechanisms underlying selection of organisms with artificially increased stress tolerance. The results accumulated in the course of experimental manipulations of *Hsps* system may be used when developing new approaches to explore mutagenesis with the goal to regulate functioning of this most important SOS-system of the eukaryotic cell.

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Glossary

Now we provide a brief description of the terms used in the text, and indicate basic biochemical methods for analysis of the genes structure and expression explored. We hope that this will make the text more accessible not only for biochemists and molecular biologists, but also for geneticists and field biologists.

DNA sequencing Is the process of determining the order of nucleotides within a DNA molecule. It includes all methods or technology that are used to determine the order of the four bases – adenine, guanine, cytosine, and thymine – in a DNA strand.

Electrophoretic mobility shift assay (EMSA) or mobility shift electrophoresis, gel shift assay, gel mobility shift assay Is a common affinity electrophoresis technique used to study protein-DNA or protein-RNA interactions (transcription factors and promoter sequences for example). This procedure can determine whether a protein or mixture of proteins is capable of binding to a given DNA or RNA sequence. Gel shift assays are often performed *in vitro* when studying regulation of transcription. Method is based on electrophoretic separation of a protein-DNA or protein-RNA complexes in a polyacrylamide or agarose gel. Unbound DNA and DNA-protein complexes differ by electrophoretic mobility and are divided into different fractions by electrophoresis (Garner and Revzin 1981).

Luciferase Is a term for the class of oxidative enzymes exhibiting bioluminescence in the process of catalyzed reactions. The term is derived from Lucifer, the root of which means 'light-bearer' (*lucem ferre*). One example is the firefly luciferase from the *Photinus pyralis*. In biological research, **luciferase reporter assay** is a method commonly used to assess the transcriptional activity in cells that are transfected with a genetic construct containing the luciferase gene under the control of a promoter of interest when the luminescence intensity is proportional to the transcriptional level (Fan and Wood 2007). The other application of the assay is estimation of chaperones activity by measurement of luminescence depending on refolding level of the thermally denatured luciferase when chaperones are subsequently added into reaction mix.

M.B. Evgen'ev et al., *Heat Shock Proteins and Whole Body Adaptation to Extreme Environments*, DOI 10.1007/978-94-017-9235-6

Northern blot (or Northern hybridization) Is a molecular technique used in molecular biology to study gene expression levels by detection of specific RNA species in the sample by hybridization method. Northern blotting takes its name from its similarity to the first blotting technique, the Southern blot (see below). The major difference is that RNA, but not DNA, is separated by electrophoresis and subsequently analyzed using labeled probes (Alwine et al. 1977).

Polymerase chain reaction (PCR) Is a technology routinely used to amplify a specific DNA fragment generating millions of copies of identical DNA molecules. PCR applications include DNA cloning, sequencing, analysis of gene expression and others. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and subsequent replication. Primers (short DNA fragments) containing sequences complementary to the target region along with a thermostable DNA polymerase (after which the method is named) are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. In the first step, the two strands of the DNA double helix are physically separated at a high temperature in a process called DNA melting. In the second step, the temperature is lowered and the two DNA strands become templates for DNA polymerase to selectively amplify the target DNA. The selectivity of PCR results from the use of primers that are complementary to the DNA region targeted for amplification under specific thermal cycling conditions.

Southern blot (or Southern hybridization) Is a method routinely used in molecular biology for detection of a specific sequence in DNA sample. Southern blotting combines electrophoretic separation of DNA fragments in agarose gel, denaturation of double-strand DNA, transfer of electrophoresis-separated and denatured DNA to a filter membrane and subsequent fragment detection by probe hybridization with specific DNA probe labeled with fluorescent dye or radioactive isotope. The labeled probe is also denatured into single strained DNA and recognizes complementary DNA sequences. The method is named after its inventor, the British biologist Edwin Southern (1975). In particular, Southern blotting, which is carried out with genomic DNA, processed by restriction endonucleases, can be used to determine the genes copy number in the genome. Other blotting methods (i.e. Northern blot and Western blot) that employ similar principles, have later been named in reference to Edwin Southern's name.

Quantitative real-time PCR (Q-RT-PCR) Is a modification of the PCR method, allowing to perform a quantitative analysis of nucleic acids, in particular, measurement of the mRNA transcription level.

Transfection Is the process of nucleic acids (mainly DNA in the form of plasmids) introducing into eukaryotic cells by non-viral methods. In the case of transient transfection DNA is introduced into the cell and not integrated usually into the nuclear genome, later it subsequently diluted after mitosis or degraded. Stable transfection leads to the integration of the foreign genetic material into the genome.

Two-dimensional (2D) electrophoresis Is a step by step separation of proteins basing on the values of their isoelectric points and then on molecular weight by electrophoresis in polyacrylamide gel with subsequent detection by immunoblotting or other methods (O'Farrell 1975).

Western blot (or protein immunoblotting) Is a technique used to detect specific proteins in a sample of tissue homogenate or extract. It uses gel electrophoresis to separate proteins by their molecular weights and transfer them to a membrane where they are stained with antibodies specific to the target protein (Renart et al. 1979).

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Index

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