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

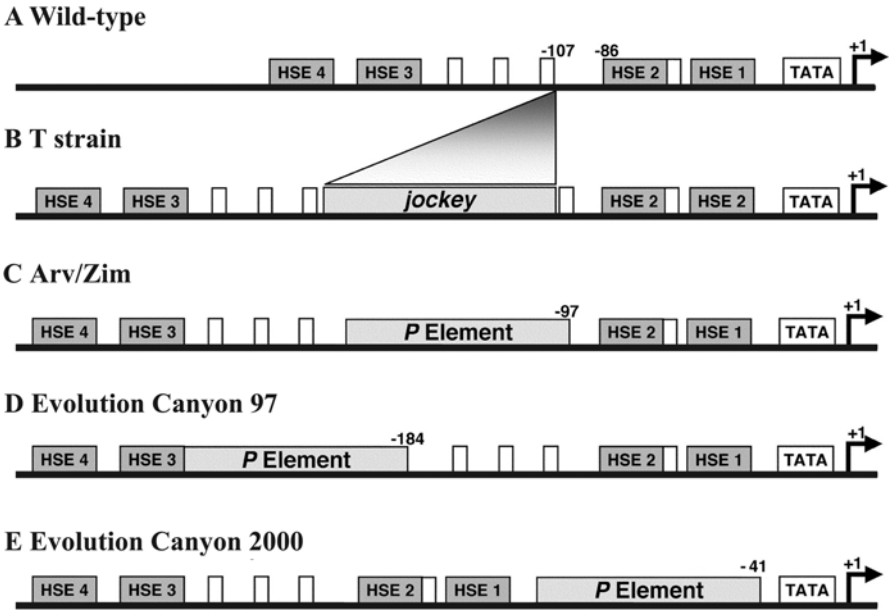

Fig. 6.1 Transposable elements disrupt the *Hsp70Ba* promoter in different strains of *D. melanogaster* (T strain from Central Africa, Arv/Zim – cross of California and Zimbabwe parents, and two strains from Evolution Canyon, Israel). GAGA elements are indicated as *white bars* (From Lerman et al. (2003);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

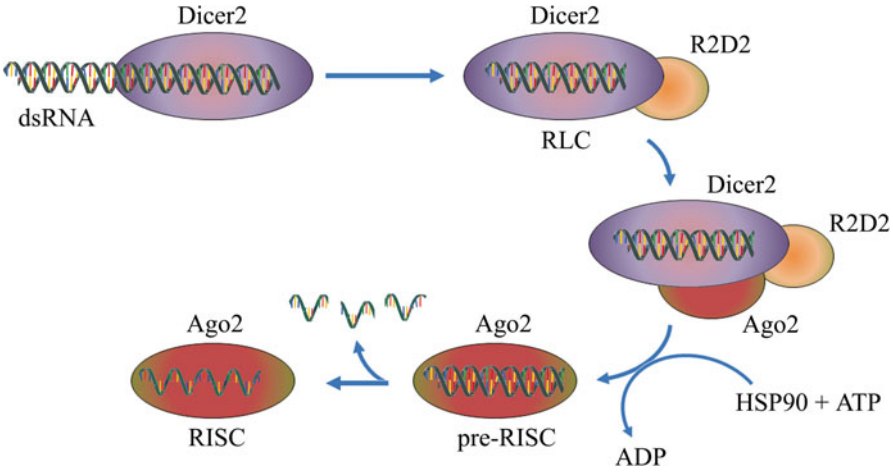

Fig. 6.2 A model for Hsp90 function in the RNAi pathway. Hsp90 promotes a conformational change in Ago2, enabling it to receive siRNA duplex from RISC-loading complex (*RLC*). Dicer2 and R2D2 are major protein components of RLC (From Miyoshi et al. 2010)

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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