Heat Shock Response - Regulation and Functions of Coding and Non-Coding Heat Shock Genes in Drosophila

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Heat shock response is a homeostatic adaptive response exhibited by nearly all cells in response to thermal and a variety of other stresses. In this article, the nature, functions and regulation of the various families of heat shock proteins are presented first and this is followed by an account of studies in the author's laboratory relating to the expression and regulation of some of the heat shock protein-coding genes and the non-coding hsr\(\omega\) gene in Drosophila. Our studies showed that unlike the common belief, different cell types of Drosophila exhibit differential activation of the various heat shock genes in a developmental stage and cell type specific manner. Transcripts of the different members of the HSP70 gene family are differentially induced and turned over in various cell types. A unique set of proteins, which includes the HSP64, is induced immediately after heat shock in larval Malpighian tubules while none of the typical heat shock proteins are induced in this tissue till at least one hour after heat shock. Interestingly, in most other tissues, HSP64 is not induced by heat shock. The heat inducible 93D or the hsr\(\omega\) gene of Drosophila melanogaster does not code for any protein. This gene is developmentally active and is also singularly induced by a variety of amides. Recent studies in our laboratory showed that one of its multiple non-coding transcripts is instrumental in the organization of nuclear omega speckles, which seem to be very important for regulation of availability of the heterogeneous nuclear RNA binding proteins (hnRNPs) for RNA processing activities in unstressed and stressed conditions as per contemporary requirements of the cell. Accordingly, over- as well as non-expression of this gene under normal conditions has severe consequences. It is likely that an equivalent of the non-coding hsr\(\omega\) gene is present in all other organisms as well. Future studies are expected to reveal the physiological bases of such pronounced as well as subtle differences in the stress response mounted by different cells of an organism and their evolutionary significance.

Key Words: Stress response; HSP, Omega speckles, hsr\(\omega\); 93D

Introduction

The heat shock response was discovered by Ritossa (1962) as the induction of a specific set of puffs in polytene chromosomes of salivary glands of Drosophila larvae in response to a brief exposure to elevated temperature or to chemicals that disrupt oxidative metabolism of the cell. A little more than a decade later, Tissieres et al. (1974) and Lewis et al. (1975) reported the synthesis of a novel set of polypeptides in Drosophila tissues exposed to elevated temperature. The discovery of heat shock (HS) induced synthesis of a novel set of polypeptides, the HS proteins or the HSPs, stimulated a remarkable flurry of research activity so that within a short time, the HS or the stress response was established as a major field of study to not only understand the survival strategies of living organisms in their everyday life but also to understand the mechanistic details of gene expression and regulation (Schlesinger et al. 1982). It was obvious that the HS response evolved very early in the history of living organisms as a homeostatic mechanism to protect the cellular machinery from damages inflicted by a variety of adverse environmental factors like temperature, oxidative stress, salinity, osmolarity, heavy metals, genotoxic compounds etc. The well orchestrated changes in the transcriptional and translational activities of individual cells in response to such environmental stresses are collectively termed as "heat shock" or "stress" response (Schlesinger et al. 1982).

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As remarked by Ashburner (1982), "The heat-shock response had been shown not only to be universal but also to occur under a wide variety of different stress conditions".

My own interest in the HS genes in Drosophila was aroused because of a serendipitous finding that one of the major HS genes of Drosophila melanogaster, the 93D locus, was singularly inducible with benzamide (Lakhotia & Mukherjee 1970, 1980). Later studies directed at understanding this gene's organization and functions revealed many unusual and interesting aspects, not only concerning this particular gene but also about other HS genes. In the following, the HS paradigm is described first and this is followed by an account of the studies carried out in my laboratory during the past 2-3 decades on these aspects.

Heat Shock Response
Sudden increase in the cellular temperature, beyond the normal physiological range of the species, is recognized by the cell as "heat shock", which results in transcriptional activation of a set of genes, the HS genes. The ongoing transcription of most other genes is generally inhibited under such conditions. In higher organisms, the pre-existing mRNAs are typically sequestered and not translated under the conditions of stress. The ribosomal machinery of a heat shocked cell preferentially translates the newly synthesized HS mRNAs resulting in synthesis and rapid accumulation of HSPs (Tissieres et al. 1974, Lewis et al. 1975, Lindquist 1986). Lewis et al. (1975) made an important observation that all the examined cell types of Drosophila displayed a remarkably similar induction of a new set of HSPs. Interestingly, the temperature at which the cells optimally respond to HS varies considerably in different organisms, e.g., 24-28°C for salmon embryos, 33-35°C for yeast, 35-38°C for Drosophila, 35-40°C for plants and 40-44°C for birds and mammals (Lindquist 1986). Even within a group, there are significant variations in relation to specific environmental adaptations. Thus compared to the temperate species of Chironomus, the tropical species show maximal response to HS at a higher temperature and also survive better at such temperatures (Nath & Lakhotia 1989). Likewise, blow flies and houseflies can withstand much higher temperature than Drosophila (Tiwari et al. 1995, 1997).

Apart from heat stress, other environmental stresses like amino acid analogues, transition heavy metals, uncouplers of oxidative phosphorylation, recovery from anoxia, chemotherapeutic agents, pathophysiological states like viral infection, fever, inflammation, ischemia, hypertrophy, malignancy etc. are also known to induce some or all of the HSPs (Morimoto et al. 1992, 1994, Csermely 1998). The cellular levels of HS proteins are elevated in certain chronic diseases like Hashimoto's thyroiditis, Graves' disease, arthritis and artherosclerosis (Morimoto et al. 1990). Localized tissue injury can also induce HSPs (Gower et al. 1989, Morimoto et al. 1990).

Heat Shock Protein Families
The HS genes and the mechanisms of their induction under conditions of cellular stress are conserved across the different phylogenetic groups (Schlesinger et al. 1982, Nover 1984). The HSPs are broadly classified according to the apparent molecular weight, amino acid sequence homologies and their functional aspects (Nover 1984) into 5 major families: HSP100 family (100-104 kDa), HSP90 family (82-90 kDa), HSP70 family (68-75 kDa), HSP60 family (58-65 kDa) and the small HSP family (15-30 kDa). HSP70 family proteins are the most conserved proteins among the HSP families followed by the HSP90, HSP100, HSP60 and small HSPs. Other than these, several other minor HSPs are also synthesized during HS (see later). Homologs of the HSPs are also often present under normal physiological or unstressed conditions and are termed as HS cognates (HSC). The chromosomal locations of the major HS genes in Drosophila melanogaster are shown in figure 1.

The HSP100 Family
The clp or the HSP100 gene family codes for constitutive and stress inducible proteins in the range of 100-110 kDa (Parsell et al. 1991, Squires & Squires 1992), which function as regulators of energy dependent proteolysis and as molecular chaperones (Clarke 1996). They stabilize certain polypeptides during severe thermal stress and either enable resolubilization of non-functional protein aggregates or target the irreversibly damaged polypeptides for degradation. The eukaryotic HSP100 members play an important role in thermotolerance (Sanchez & Lindquist 1990, Parsell et al. 1991). An HSP100 homolog has not yet been identified in Drosophila.
Besides being heat inducible, the HSP83 is constitutively expressed at high levels during normal growth (Chomyn & Mitchel 1982, Zimmerman et al. 1983, Xiao & Lis 1989, Ding et al. 1993, Yue et al. 1999). HSP83 gene is the only HS protein-coding gene in D. melanogaster with an intron (Hackett & Lis 1983). Like the mammalian HSP90, HSP83 in Drosophila is also mostly cytoplasmic in unstressed as well as heat shocked cells but Morcillo et al. (1993) showed that after heat shock, HSP83 is also present at the heat induced 93D puff. Lange et al. (2000) reported HSP83 in centrosomes in Drosophila and vertebrates (HSP90). Rutherford and Lindquist (1998) made an interesting observation that heterozygosity for HSP83 recessive mutation in Drosophila results in revelation of a wide variety of otherwise cryptic morphological abnormalities, the nature and extents of which depend upon the specific genetic background and environmental conditions in which the heterozygosity for the HSP83 mutation is introduced. Thus this family of proteins has been suggested to provide a simple molecular mechanism for evolvability (Rutherford 2000).

The HSP70 Family
The HSP70 protein family is highly conserved with ~50% amino acid identity among all characterized species from bacteria to man (Feige & Polla 1994, Morimoto et al. 1994, Macario et al. 1999) and is the most abundantly induced protein in stressed cells. An important feature of this family is its multi-member composition, e.g., 14 in yeast and 11 in Drosophila, (Rassow et al. 1997). Some members of the HSP70 gene family, the HS cognate or HSC70 genes, are constitutively expressed while others, the HSP70 genes, are induced by heat and other stresses.

HSP70 plays critical role in thermotolerance in bacteria (the DnaK protein), yeast, Drosophila and mammalian cells (Parsell & Lindquist 1994). A member of this family, the HSP73, is present in the centrosome and seems to help in its reorganization after damage by the heat stress (Perret et al. 1995, Brown et al. 1996). HSP70 also protects cells from oxidative damage with mitochondria as the targets for protection (Polla et al. 1996, Mallouk et al. 1999). The interactions between HSP70 and ATP may decide the cell survival, necrosis or apoptosis (Mallouk et al. 1999). HSP70 has varied roles in modulating the

The HSP90 Family
Members of the HSP90 molecular chaperone family are mainly cytoplasmic (Lindquist & Craig 1988). In mammals, this family includes the two major molecular chaperones of the cytosol, the HSP90 (90 kD) and the 94 kD glucose regulated protein, grp94. In most cells the HSP90 family proteins account for 1-2% of all cellular proteins (Csermely et al. 1998). HSP90 family members are apparently involved in diverse cellular functions in view of their association with a wide variety of cellular proteins like steroid receptors, histone H1, cytoskeleton, HSP56, HSP70 etc. (Jakob & Buchner 1994, Csermely et al. 1998, Caplan 1999).

The HSP90 family member in Drosophila is the HSP83 gene located at the 63BC locus (figure 1).
inflammatory and immune responses (Polla et al. 1998, Basu & Srivastava 2001). There are inter-
individual variations in the basal levels of HSP70/ HSC70, which correlate with the level of stress-
induced synthesis of the major HSPs (Boshoff et al. 2000). Such inter-individual variations may be one of
the factors that make different individuals differentially susceptible/resistant to various disease
conditions (Boshoff et al. 2000). Seasonal and geographical variations have also been reported in
the constitutive and induced synthesis of HSPs (Nath & Lakhotia 1989, Ultimasov et al. 1992). While
protective under stress conditions, over-expression of HSP70 at physiological conditions is deleterious
for cells (Feder et al. 1992). It may also prevent damaged and, therefore, potentially threatening
cells, from apoptosis (Samali & Cotter 1996).

HSP70 family in D. melanogaster includes both
heat inducible (HSP70 family) and constitutively
expressed HSC70 genes. Five different HSC70 genes
are expressed, with spatial and temporal variations,
under normal growth conditions in Drosophila
(Elefant & Palter 1999). Two different loci, 87A7 and
87C1, respectively, on the right arm of chromosome 3
(figure 1) carry clusters of genes coding for the heat
inducible HSP70 in D. melanogaster. The 87A7 site
carries two copies and the 87C1 site carries 3 copies of
the heat inducible HSP70 genes. The two clusters are
separated from each other by ~500 kb of DNA. At the
87C1, the proximal two HSP70 genes are separated
from the third gene by ~38 kb of DNA, which contains
many αβ repeats (see Nover 1984). The αβ repeats
produce heat-inducible but non-coding RNAs of as
yet unknown function. The protein coding sequences
in these five HSP70 genes show 97% identity amongst
themselves; ~400bp upstream region also shows very
high homology between the 5 genes (Ingolia et al. 1980;
Karch et al. 1981). In contrast, the 3' untranslated
regions (3' UTR), comprising ~250bp from the
termination codon till the poly-A+ site, of the two
HSP70 genes at 87A7 locus are similar, but different
from those of the HSP70 gene copies at the 87C1 locus.
Interestingly, the 3'UTRs of the proximal and the
middle HSP70 genes at the 87C1 locus are similar with
each other but are moderately diverged from that of
distal most HSP70 gene at this site (Torok et al. 1982).
All the five HSP70 genes have been shown to
transcribe in response to HS (Ish-Horowicz & Pinchin
1980). Besides these 5 copies of HSP70 genes, another
heat inducible gene belonging to the HSP70 family, the
HSP68 gene, is present at the 95D locus on the right
arm of chromosome 3 and this produces a 68kD
polypeptide (Holmgren et al. 1979).

The 2.4-2.5 kb intronless HSP70 transcripts are
undetectable in unstressed cells but are induced more
than 1000 fold upon HS (Velazquez et al. 1983). The
half-life of the HSP70 mRNAs also increases at least
10 fold under these conditions (Petersen & Lindquist
1988). During recovery from heat shock, the HSP70
genes are rapidly repressed and the HSP70 messages
are selectively degraded (Dellavalle et al. 1994).

Although the presence of the inducible forms of
HSP70 family members is regarded as a distinct
indication of a cell under stress and the concerned
genes are believed to be under a state of "readiness"
for rapid activation (see later), our studies have
revealed that regulation of HSP70 genes in different
cell types of Drosophila is much more complex. One
of the first examples of a differential activation of the
HSP70 genes by the thermal stress was the differential
inducibility of their two sets at the 87A and 87C loci,
respectively (see Lakhotia & Sharma, 1996 for review).
Our recent studies (Lakhotia 2001b, Lakhotia & Prasanth 2001) have revealed more
remarkable differences in the HS induction and stability of the transcripts of the HSP70 genes at the
two loci in different embryonic, larval and adult cell
types. For example, during later stages of embryonic
development, transcripts of the HSP70 genes at only
the 87A, but not at the 87C, were detectable upon HS
in the neuronal cells in embryonic central and
peripheral nervous system from stage 13 onwards.
Likewise, cells in the posterior part of the heat
shocked larval mid gut expressed only the 87A genes
and these cells continued to express 87A genes even
2 hrs after recovery from HS. These and other results
(Lakhotia & Prasanth 2001) reveal that the HS
inducibility of the HSP70 genes from the two loci is
differentially regulated during development and the
two sets of transcripts are metabolized in a cell-
and development-stage specific manner. Apparently, the
multiple copies of HSP70 genes are not just to make
more Hsp70 protein during HS. We also found a
constitutive expression of the HS inducible form of
HSP70 in spermatogonial cells of D. melanogaster
The HSP60/Tcp1 Family

HSP60 family proteins are stress inducible as well as constitutively expressed, are essential for growth under all conditions and are found in the cytosol of bacteria (the GroEL protein), in the matrix of eukaryotic mitochondria and in the stroma of chloroplasts (Hartl et al. 1992, Houry et al. 1999). HSP60 family proteins, commonly also called "chaperonins" (Hemmingsen 1992), exert functions similar to those of HSP70 family proteins in assisting protein folding (Houry et al. 1999). The chaperonins require the presence of another co-chaperonin, the GroES or HSP10, for effective binding with and folding of proteins (Hartl et al. 1992, Georgopoulos et al. 1994, Bukau & Horwich 1998). Interestingly while the HSP70/DnaJ complex associates preferentially with short peptides or polypeptides in extended conformations, the HSP60/GroEL proteins show higher affinity towards partially folded structures. These two families of proteins function in sequential reactions during folding of a newly synthesized polypeptide. The cytosolic homologs of HSP60 in eukaryotic cells are classified as TCP-1 or TriC (TCP-1 ring complex, Gupta 1990), which are hetero-oligomeric chaperones involved in the folding of actin and tubulin.

The HSP60 family members in Drosophila have been identified only very recently. My laboratory (Lakhota & Singh 1989) was the first to report the HS induced synthesis of HSP64 as a member of a novel set of polypeptides in Malpighian tubules of Drosophila larvae (figure 2A, Lakhota & Singh 1989). This protein was later shown to be a member of the HSP60 family (figure 2B, Singh & Lakhota 1995, Lakhota & Singh 1996). The HSP64 is not induced by HS in any other cell type of Drosophila. It is interesting that 1 hr after heat shock, synthesis of the typical set of the common Hsp's is induced in the larval Malpighian tubules (Krebs & Feder 1997, Priya Srivastava, Prasanth K. V. & Lakhota, unpublished) but at this time the Hsp64 and other Malpighian tubule-specific HS induced proteins are no longer synthesized. Most intriguing, however, is our observation that the HSP70 transcripts from both the 87A and 87C gene copies are induced immediately in the larval Malpighian tubule cells after HS but their transport to cytoplasm and translation are delayed for about an hour. The significance of the HS induced

Figure 2 Heat shock response in Malpighian tubules of Drosophila larvae is very different from that in other tissues. A. Fluorograph of 35S-methionine labelled and SDS-PAGE separated polypeptides from control (C) and heat shocked (HS) salivary glands (SG) and Malpighian tubules (MT) of D. melanogaster larvae. Note that unlike in salivary glands, none of the typical HSPs (83, 70, 68, 27, 26, 23 and 22 kDa) are induced by heat shock in Malpighian tubules; instead, a different set with a major polypeptide of 64 kDa is induced in this tissue. B. The 64 kDa heat shock induced polypeptide in Malpighian tubules is a member of the HSP60 family as revealed by immunoprecipitation of 35S-methionine labelled polypeptides using an HSP60 antibody and fluorography of the SDS-PAGE fractionated immuno-precipitates from control (C) and heat shocked (HS) Malpighian tubules. Note the specific and stronger labeling of the 64 kDa polypeptide in the heat shock lane (lane 2). The lanes 3 and 4 show fluorograph of total proteins from 35S-methionine labeled control and heat shocked Malpighian tubules. Figures adapted from Lakhota and Singh (1989, 1996).
transcriptional activation of one of the HSP64 genes in Malpighian tubules and its negative correlation with the synthesis of HSP70 and other typical HSPs remains to be understood. It is interesting to note in this context that in several insect species, the HS response in different tissues, especially in Malpighian tubules (in some cases in testes also), shows specific deviations in terms of induction of the “HSP60”, “HSP70” or a “mixed” type response (see Singh & Lakhotia 2000). It appears that tissues like Malpighian tubules have a different stress physiology and, therefore, have evolved special regulatory mechanisms. Further studies on this aspect are expected to provide exciting insights.

The first HSP60 gene in D. melanogaster, localized at the 10A cytogenetic region of the X chromosome (figure 1), was cloned by Kozlova et al. (1997). The Berkeley Drosophila Genome Project has unraveled two more HSP60 genes in D. melanogaster; one at 21D locus and the other at 25E locus (figure 1). The putative protein sequences of the two HSP60 genes located at 10A and 25E are ~80% similar with each other but the 21D gene shows only ~60% homology with the other two. Recent studies in my laboratory (Srivastava & Lakhotia 2000 and other unpublished results) have revealed very interesting functional differentiation of the three HSP60 genes in D. melanogaster. The HSP64 from the 10A gene is widely expressed in nearly all cell types in a developmentally regulated manner while the HSP64 encoded by the 21D gene seems to have male germ cell specific functions (also see Timakov & Zhang 2001). On the other hand, the HSP64 gene at 25E appears to be HS inducible in larval Malpighian tubules, gut and fat bodies (Srivastava & Lakhotia 2000 and other unpublished results).

The Small HSP Family
The small HSPs, a heterogeneous group with sizes ranging from 20-30kDa, are the least conserved among the HSPs (Lindquist 1986). This group of genes also belongs to a multigene family in most organisms (3 in nematodes, at least 4 major proteins in Drosophila, 3 in mammals including crystallins and >20 in plants but only 1 in yeast). Cytoplasmic particles containing the small HSPs (sHSPs) complexed with small RNAs have been isolated from Drosophila and yeast during recovery from HS (Arrigo et al.1985). sHSPs suppress aggregation of denatured proteins and promote their activation but unlike HSP70 and HSP60, the chaperon activity of sHSPs is independent of ATP (Jakob et al. 1993, Arrigo & Landry 1994).

There are four well characterized sHSPs (HSP28, HSP26, HSP23 and HSP22) in D. melanogaster and genes for all four of them are located at the 67B region on the left arm of chromosome 3 (figure 1), within a short stretch of DNA in the order HSP28, HSP23, HSP26, HSP22. Three other HS inducible genes are also localized at this site but these have not been well characterized yet. Each of the sHSP genes has its own promoter, with HSP26 being transcribed in a direction opposite to the other three (Petersen et al. 1979, Craig & McCarthy 1980, Lindquist 1986). The four Drosophila sHSPs have an overall homology of ~50% (Southgate et al. 1983). The sHSPs shuttle between nucleus and cytoplasm during HS and recovery (Arrigo & Landry 1994) and are found associated with cytoskeletal components also (Leicht et al. 1986). All these genes are expressed at normal temperatures with each being independently regulated in a tissuespecific manner (Southgate et al. 1983, Arrigo & Tanguay 1991, Marin et al. 1993, Michaud et al.1997), suggesting their different roles under normal (and stressed) conditions. Their HS inducibility can also be independently modulated. Thus we found (Lakhotia & Singh 1988) that when D. melanogaster larvae were reared at 10°C, their salivary glands showed reduced synthesis of HSP23 not only developmentally but also after heat shock, while the other sHSPs did not appear to be much affected.

Other Members of the Heat Shock Gene Family
Besides the above major HS gene families, several other genes/proteins that are induced by the heat stress have been identified in different organisms.

Several peptidyl prolyl isomerases have been identified as HSPs (Lilie et al. 1993). The DnaJ of E.coli, stimulates the ATPase activity and release of protein substrate from DnaK, the HSP70 homolog in prokaryotes. The eukaryotic counterpart of DnaJ, the HSP40, is also heat inducible and is believed to participate in protein folding in association with HSP70 (Wild et al. 1992). The ubiquitin too is heat inducible. Ubiquitin binds with the heat denatured proteins and targets them for degradation (Hochstrasser 1992, Craig et al. 1994). In addition,
several other eukaryotic proteins like γ-interferon in mammalian cells, albumin in rat liver (Srinivas et al. 1987), histone H2b in *Drosophila*, enolase and glyceraldehyde-3-phosphate dehydrogenase in yeast are also induced by heat (see Lindquist 1986). The ATP-dependent ion protease, lysU, one of the *E. coli* lysyl-tRNA synthetases and rpoD, coding for the σ^30^ subunit of RNA polymerase in prokaryotes, are also heat inducible (see Lindquist 1986).

### Regulation of Heat Shock Response

HS affects gene activity at transcriptional, post-transcriptional as well as translational levels (Table 1). The most obvious effect is the selective and rapid transcriptional induction of HS genes with a concomitant repression of most of the ongoing chromosomal transcription. RNA polymerase II is withdrawn from most chromosomal sites after HS and is redistributed at the HS loci (Bonner & Kerby 1982). To facilitate such rapid activation, the 5' ends of the HS genes (especially HSP70 genes) are maintained in an open configuration (Costlow & Lis 1984, Lu et al. 1992). The GAGA factor, TATA-binding protein (TBP) and the RNA polymerase II remain associated with the uninduced promoter of the HSP genes like *HSP70, HSP26, HSP27* etc with the polymerase remaining paused after transcribing about 25-30 nucleotides of these genes (Rougive & Lis 1988, Giardina et al. 1992, Li et al. 1996, Weber et al. 1997).

HS activates the HS factor (HSF), which binds to the highly conserved HS elements (HSE) in the promoter of all eukaryotic HS genes (Morimoto et al. 1994, Wu et al. 1994). HSE is typically composed of 3-6 alternatively oriented repeats of a 5bp sequence motif, 5mGAAAn-3mAmin (Amin et al. 1988). The number of HSEs at individual genes varies (Amin et al. 1988, Fernandes et al. 1994). The monomers of HSF present in unstressed nucleus trimerize in response to HS, which binds to HSEs to transcriptionally activate the HS genes (Westwood et al. 1991, Westwood & Wu 1993). A single HSF coding gene exists in *Drosophila* as well as yeast, but multiple HSFs are present in other organisms like tomato, mouse and man (Nover & Scharf 1997, Morimoto 1998). Transcription of HS genes under stress is autoregulated (DiDomenico et al. 1982) so that inhibition of the synthesis of functional HSPs during HS causes transcription of HS gene to continue for much longer period; under such conditions the HS mRNAs are very stable.

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<th>Level of regulation</th>
<th>Heat shock genes</th>
<th>Other genes</th>
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<tr>
<td>Transcriptional</td>
<td>H5 genes are rapidly transcribed due to binding of activated HSF with HSEs in HS gene promoters</td>
<td>Transcription of most other genes is inhibited and RNA polymerase II is redistributed on HS genes</td>
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<tr>
<td>Post-transcriptional</td>
<td>Most HS mRNAs are without introns and thus are rapidly transported to cytoplasm without the need for much processing</td>
<td>Splicing and polyadenylation of non-HS mRNAs are generally reduced/inhibited and the RNA-processing factors are reorganized in the nucleus</td>
</tr>
<tr>
<td>Translational</td>
<td>HS mRNAs are selectively translated by ribosomes in heat shocked cells</td>
<td>In higher eukaryotes translation of other normal transcripts is inhibited and the mRNAs are sequestered till the cell recovers from HS</td>
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RNA splicing and polyadenylation of the pre-existing transcripts are also affected by HS (Yost et al. 1990, Shen et al. 1993). Since most of the HS genes do not contain introns, their transcripts are rapidly transported to the cytoplasm without the need of the usual post-transcriptional processing (Yost & Lindquist 1986, 1988, 1991, Bond 1988). About 40% of the *HSP70* transcripts in *D. melanogaster* lack a poly-A tail because of a rapid and selective removal of poly-A from previously adenyated transcripts (Dellavalle et al. 1994). High temperatures block the turnover of the normally short-lived *HSP70* mRNA, allowing its rapid accumulation (Petersen & Lindquist 1988). The 3′ UTRs on *HSP70* transcripts destabilize them during unstressed conditions and during recovery (Petersen & Lindquist 1988). Since new transcription and processing of pre-existing transcripts of most of the non-heat shock genes is inhibited, the nuclear RNA processing proteins also get redistributed in heat shocked cells (see later).

Translational regulation during HS in *Drosophila* involves a rapid decay of pre-existing polysomes (Kruger & Benecke 1981, Duncan 1996). Ribosomes in heat shocked cells selectively translate the HS mRNAs which bear unusually long and A-rich 5′ untranslated leader sequences (Klemenz et al. 1985, McGarry & Lindquist 1985, Lindquist 1986, Kozak...
1988, Duncan 1996). Though synthesis of most of the proteins is rapidly inhibited following heat shock, translation of some mRNAs like those of core histones continues unaffected (Farrel-Towt & Sanders 1984).

It is obvious that the above elaborate regulatory strategies ensure a rapid production of the different HSPs to salvage the damages inflicted by the stress and to prevent further damages. Also since HSPs can have a negative effect under non-stress conditions (Feder & Hoffmann 1999), the regulatory pathways ensure their quick disposal after the stress is withdrawn. As already noted above, studies in our (Lakhotia 2001b, Lakhotia & Prasanth 2001) and other laboratories (see Feder & Hoffmann 1999) also show that specific cell types can over-ride these apparently universal regulatory circuits so that some cells do not synthesize any of the HSPs under stress while in other cell types, some or all HSP genes may be subject to different transcriptional and/or post-transcriptional regulations.

The 93D or the hsro is an Unusual Heat Shock Gene in Drosophila

The 93D (named after its location in the 93D cytogenetic region of polytene chromosomes of Drosophila melanogaster) or the hsro (named after its transcription products, HS RNA omega) locus is an unusual member of the HS gene family in Drosophila. This gene does not code for any protein but seems to have very important cellular functions under normal as well as stressed conditions.

The Inducibility and Architecture of the hsro Gene are Conserved in the Genus Drosophila but the Base Sequence is not

The hsro gene is developmentally expressed in most cell types (Lakhotia et al. 2001) and is strongly induced by HS along with the other HS genes (Mukherjee & Lakhotia 1979, figures 1B, 3B). However, the most interesting feature of this gene which attracted our attention was its singular inducibility with benzamide (Lakhotia & Mukherjee, 1970, 1980, figure 3C). As a member of the HS gene family, the 93D gene is induced by carbon dioxide, recovery from anoxia, 2,4-dinitrophenol, arsenic compounds etc (Ashburner & Bonner 1979, Mukherjee & Lakhotia 1979, Lakhotia & Sharma 1996, Lakhotia et al. 1999). But it is uniquely induced by chemicals like benzamide (Lakhotia & Mukherjee 1970, Lakhotia & Mukherjee 1980), colchicine or colcemid (Lakhotia & Mukherjee 1984, figure 4A) and several other amides (Tapadia & Lakhotia 1997). These chemicals also inhibit most of the chromosomal R DNA synthesis (Lakhotia & Mukherjee 1970, 1980, Tapadia & Lakhotia 1997).

An equivalent of the hsro gene is present in all species of Drosophila (figure 4B, Lakhotia & Singh 1982). In all species of Drosophila examined so far, the hsro transcription unit includes a characteristic 5' end (with 2 exons and an intron spanning ~1.9kb length) followed by a long stretch (more than 5kb to ~15kb) of short tandem repeats, which are unique to this locus (Garbe et al. 1986, Hovemann et al. 1986, Lakhotia et al. 1999). An intriguing feature of the hsro gene is the rapid divergence of base sequence in its transcribed region in different species of Drosophila. In spite of the high sequence diverg-ence, it shows comparable inducible properties and produces several transcripts of similar sizes and properties (Garbe et al. 1986, Hovemann et al. 1986, Fini et al. 1989).

The hsro gene does not code for any protein (Lakhotia & Mukherjee, 1982, Garbe & Pardue 1986,

Figure 3 The 93D puff is induced by heat shock in larval salivary gland polytene chromosomes of D. melanogaster along with the other heat shock puffs (like 87A and 87C) but is singularly induced by a 10min treatment with 10mM benzamide at 24°C. Parts of the right arm of chromosome 3 (3R) are shown from control (CON, A), heat shocked (TS, B) and benzamide (BM, C) treated salivary glands. Locations of the 87A, 87C and 93D sites are indicated.
But the production of transcripts of comparable properties suggests that the architecture of the gene and its transcripts is more important than the base sequence itself (Lakhota et al. 1999). Profiles of the different hsro transcripts show inducer specific variations. Following HS, levels of all the three transcripts increase markedly. However, the benzamide and colchicine treatments lead to a significant increase of only the >10kb long nuclear hsro-n (Bendena et al. 1989, Lakhota & Sharma 1995). This difference seems to be related to the different effects of HS and amides on transcriptional and translational activities. As already noted, HS affects transcriptional as well as translational activities while the amides affect only chromosomal transcription without any effect on translational activity (Lakhota & Mukherjee 1982). Turnover patterns of the hsro-n and the ~1.2kb cytoplasmic hsro-c transcripts are also different since while the hsro-n is stabilized by inhibition of transcription with Actinomycin D, the hsro-c is stabilized by protein synthesis inhibitors (Bendena et al. 1989, Bendena et al. 1991). This differential stability relates to the nucleus-limited functions of the hsro-n transcript (see below) and to a cytoplasmic role of the hsro-c RNA. In view of a very small but translatable ORF in the hsro-c (Fini et al. 1989) and the stabilization of this transcript species by translational inhibitors (Bendena et al. 1989), it has been suggested that translation of the small ORF in the hsro-c RNA serves to monitor the “health” of the cell’s translational machinery (Lakhota 1989, Lakhota & Sharma, 1996).

The hsro Gene is Important for Viability and Thermo-resistance

In addition to its strong induction by HS and amides, the hsro gene is also developmentally expressed in a dynamically regulated manner (Bendena et al. 1991, Mutsuddi & Lakhota 1995, Lakhota et al. 2001). As may be expected from the production of multiple transcripts, their dynamic developmental expression in different tissues and their differential inducibility by HS and amides, our studies (Mutsuddi & Lakhota 1995, Lakhota & Tapadia 1998, Lakhota et al. 2001) revealed the promoter to be complex with multiple regulatory elements in promoter region of the hsro gene.

An intensive screen for mutations at the 93D locus by Mohler & Pardue (1982) did not recover any point

Figure 4A-I A 93D-like puff site is present in all species of Drosophila. 3H-uridine-labeled autoradiograms of polytene chromosomes of D. melanogaster (A, colcemid treated), D. ananassae (B & C), D. nasuta (D & E), D. kikkawai (F & G), and D. hydei (H & I) CON, Control and BM, BM-treated. Note the intense labeling of a single puff site (93D in D. melanogaster, 2L-2C in D. ananassae, 2R-48A in D. nasuta, E-11BC in D. kikkawai and 2-48C in D. hydei) in the colcemid or BM-treated nucleus (A, from Lakhota & Mukherjee, 1984 and B-I, from Lakhota & Singh 1982).

Hovemann et al. 1986). hsro gene in all species of Drosophila, that have been examined, produces two primary transcripts, viz., the hsro-n transcript of ~10 to 15kb length (which spans the entire transcription unit and remains localized in the nucleus) and the hsro-pre-c transcript of ~1.9kb length (spanning only the 5' region comprising of the two exons and the intron). The 1.9kb hsro-pre-c transcript is typically spliced to give rise to the cytoplasmic 1.2kb hsro-c transcript (Hovemann et al. 1986, Garbe et al. 1986, Bendena et al. 1989, Fini et al. 1989). The rapid sequence divergence at this locus in Drosophila species is perhaps related to its non-coding nature.
mutations. In hindsight this appears to be related to
the non-coding nature of this gene so that base
changes are well tolerated, as has actually happened
at this locus during evolution. However, two
small overlapping deletions, viz. \( Df(3R)e^{cpd} \) and
\( Df(3R)GC14 \), whose overlap specifically defines
the HS and amide-inducible \( hsro \) locus (Mohler &
Pardue 1982, Burma & Lakhota, 1986) have been
informative about functions of this enigmatic gene.
Most of the \( Df(3R)e^{cpd}/Df(3R)GC14 \) trans-
heterozygotes (\( hsro \)-nullosomic) die as embryo
while the few (~20% of the nullosomics) who survive
to adult stage are weak flies, unable to walk or fly
properly, and which die within a few days (Mohler
1999). Unlike wild type flies, \( hsro \)-nullosomics are
relatively poor in acquiring thermostolerance and do
not survive when grown at 31°C (Lakhota 1987,
Pardue et al. 1990). This suggests that, like the HSP70,
\( hsro \) also plays important role in thermostolerance.
This is further supported by significant differences
in the levels of the nuclear and cytoplasmic \( hsro \)
transcripts between lines selected and unselected for
thermo-resistance (McColl et al. 1996, McKechnie
et al. 1998). In addition, our recent studies on mutations
due to insertion of P-transposon in the promoter
region of the \( hsro \) locus of \( D. melanogaster \) have also
provided significant insight in the way this non-
coding RNA functions (see below).

The \( hsro \)-n Transcripts Organize “omega” Specckles
in Nucleoplasm to Regulate the Availability of
hnRNPs for RNA Processing Activities
It has been known for some time (Dangli & Bautz
1983, reviewed by Lakhota et al. 1999) that antibodies
against several nuclear non-histone proteins, mostly
belonging to the heterogenous nuclear RNA-binding
family of proteins (hnRNPs, Krecic & Swanson 1999),
specifically bind with the 93D puff in heat shocked
salivary glands of \( D. melanogaster \) larvae. We
exploited this information to understand the
functional significance of the association between the
\( hsro \) transcripts and hnRNPs under different genetic
and environmental conditions (Lakhota et al. 1999,
Unlike any known RNA-polymerase II dependent eukaryotic
transcripts, the \( hsro \)-n transcripts are present in the
nucleus, besides at the site of transcription, as small
granules or speckles distributed in nucleoplasm in
close vicinity of chromosomes and whose abundance
varies in cell type specific manner (figure 5, Prasanth
et al. 2000, Lakhota et al. 2001). The hnRNPs and the
related proteins remain bound either to transcriptionally active chromatin sites or to the \( hsro \)-n RNA speckles (figure 5). Significantly, the SR-family of RNA-binding nuclear proteins, which form the well
known inter-chromatin granule clusters (Spector 1993),
do not localize with the \( hsro \)-n RNA speckles (Prasanth et al. 2000, Rajendra et al. 2001). Therefore, the speckles
containing the \( hsro \)-n RNA and hnRNPs and the related
proteins are novel nuclear structures and have been
designated as “omega speckles” (Prasanth et al. 2000).
These studies suggested that this gene's large nuclear-
limited \( hsro \)-n transcript sequesters the various hnRNPs
and related proteins to regulate their availability for
nuclear RNA processing activities (Lakhota et al. 1999,
Prasanth et al. 2000). As illustrated in figure 5, all the nuclear hnRNPs, which are not associated with the
transcriptionally active chromatin sites, colocalize
exclusively with the \( hsro \)-n transcripts in the form of
omega speckles in all the cell types of Drosophila
(Lakhota et al. 1999, 2001, Prasanth et al. 2000, Rajendra
et al. 2001). The presence of the \( hsro \)-n transcripts is
essential for organizing the omega speckles since in the
\( hsro \)-nullosomic \( (Df(3R)e^{cpd}/Df(3R)GC14) \) cells,
the omega speckles are completely absent so that the
hnRNPs remain distributed in a diffuse manner through
the nucleoplasm (Prasanth et al. 2000).

We suggested that the omega speckles are storage
sites for the unengaged hnRNPs and depending upon
the cellular needs, the hnRNPs are dynamically released
from or sequestered in the omega speckles (figure 6,
Lakhota et al. 1999, Prasanth et al. 2000). It is believed
that such a regulated release/withdrawal of the hnRNPs
is important for ensuring the desired splicing and other
processings of the nascent transcripts. As already noted
earlier (table 1), HS causes a drastic reduction in general
transcription and RNA processing activities in the
nucleus. Consequently, most of the nuclear hnRNPs in
heat shocked cells have no substrates (nascent transcripts)
for processing and, therefore, need to be
kept in an inactive state. We have shown (Lakhota
et al. 1999, Prasanth et al. 2000) that under these conditions,
the omega speckles increase in size (due to the greater
amounts of the hnRNPs being sequestered) and
coalesce with each other to form large aggregates and
finally all of them get localized to the 93D chromosomal
site itself (see figure 6). It is likely that the massive
clustering of omega speckles in fully stressed cells
Figure 5. Omega speckles in a nucleus in cyst cell from testes of adult D. melanogaster. The hsrω-n RNA is present at the hsrω gene locus (arrowhead) and as numerous speckles in the nucleoplasmic space close to the chromatin area (red speckles in A, seen after RNA:RNA fluorescence in situ hybridization). The HRB87F (hnRNPA1 homolog in D. melanogaster) localizes diffusely over the chromatin but as speckles in the nucleoplasmic space (green speckles in B, seen after immuno-fluorescent staining with HSB87F antibody of the same cyst cell as in A). As shown in C (overlap of RNA:RNA in situ hybridization in A and immunostaining in B), nucleoplasmic speckles formed by the hsrω-n RNA and HSB87F are the same (for details, see Prasanth et al, 2000).

Figure 6. Dynamic re-distribution of the omega speckles in nucleus following heat shock. The upper panel shows the nuclear distribution of hsrω-n transcripts (red fluorescence following in situ RNA:RNA hybridization; chromosomal DNA shows blue DAPI fluorescence) in partially squashed cells from control (A), 30min heat shocked (B) and 40min heat shocked (C) Malpighian tubules of D. melanogaster larvae. The lower panel shows diagrammatic representation of the distribution of hsrω-n transcripts and the hnRNPs in these three situations. Chromosome bands are shown in black, the hnRNPs bound to transcriptionally active chromosomal sites in green and the omega speckles (containing the hsrω-n RNA and the hnRNPs) in orange. In unstressed nuclei (A), the omega speckles are present in close proximity to, but not on, the chromosomal domains (CD); the omega speckles are also absent from the inter-chromatin domain (ICD). The hsrω-n transcripts are not present anywhere on the chromosomes, except the 93D site itself (arrowheads in upper panel), where they remain complexed with the hnRNPs. Following heat shock (B), in parallel with increasing inhibition of chromosomal transcription and processing of nascent transcripts, the hnRNPs are gradually withdrawn from chromosomal locations and accumulate as clusters of omega speckles in close proximity to chromosomal domain in addition to the 93D site. As the heat shock condition continues and there is near complete inhibition of RNA processing activities, the clusters of omega speckles completely disappear from the nucleoplasmic space and get exclusively localized at the 93D site itself (C). Figure modified from Prasanth et al. (2000).
ensures against illegitimate RNA processing activities under adverse conditions (Lakhotia et al. 1999, Prasanth et al. 2000). When the cells recover from heat stress and resume their normal transcriptional and RNA processing activities, the hnRNPs are released from clusters of omega speckles and within 1 hr, the hnRNPs get restored to active chromosomal sites and typical omega speckles also reappear in the nucleoplasm. Recent studies in our laboratory on over-expressing P-insertion mutant alleles of \textit{hsr0} have provided further evidence for the importance of association between hnRNPs and hsr0 transcripts. An over-expression of these transcripts due to promoter mutation would result in excessive sequestering of the hnRNPs. Since the quantity and quality of the available hnRNPs affects processing and alternative splicing of nascent transcripts (Krecic & Swanson 1999), excessive sequestering of hnRNPs by \textit{hsr0} transcripts in the inactive compartment (omega speckles or their clusters) is expected to have trans-dominant effect on processing of several nuclear transcripts. One such P-insertion mutant allele, \textit{hsr0}^{P241}, shows over-expression of hsr0-n transcripts only in cyst cells in testes, which have important role in sperm maturation and individualization. The \textit{hsr0}^{P241} mutant cyst cells display large clusters of omega speckles even without HS. The excessive sequestering of hnRNPs in the large clusters of omega speckles apparently disrupts the normal RNA processing activity in cyst cells, the consequent compromise in their function seems to be responsible for sterility of the \textit{hsr0}^{P241} homozygous male flies (Rajendra et al. 2001). In another P-insertion promoter mutant allele, the \textit{hsr0} gene is over-expressed in a variety of 3rd instar larval tissues and this is associated with prolongation of the larval period and ultimate lethality (Sengupta & Lakhotia 2000 and other unpublished results). Other recent results (Lakhotia, Surajit Sarkar & Noopur Thakur, unpublished) show that splicing of the Ddc (dopa decarboxylase) transcripts in the central nervous system of \textit{hsr0}-nullosomic larvae is aberrant; this seems to correlate with the absence of omega speckles in these nullosomics (Prasanth et al. 2000) and a consequent abnormal availability of the hnRNPs etc for the RNA processing machinery.

Although the possibility of some RNA species providing a structural role in sequestering the RNA processing factors that are not actively engaged in the post-transcriptional processing at a given time has been raised in past (Wieghardt et al. 1999), our studies (Lakhotia et al. 1999, Prasanth et al. 2000) for the first time actually identified the hsr0-n RNA as one such RNA species. The discovery of the omega speckles and the role played by the hsr0-n transcripts in organizing these speckles thus provides a new paradigm to understand the regulation of nuclear RNA processing activities. It is very likely that a regulatory system, comparable to the omega speckles of \textit{Drosophila} cells, exists in other higher organisms, including man, since the post-transcriptional processing events in the nucleus are highly conserved (Krecic & Swanson 1999). In certain pathological conditions in man, it is known that some of the RNA processing proteins are abnormally sequestered and this trans-dominantly affects processing of several other nascent transcripts (see Singer 1998). Obviously, it is essential to fine tune the availability of RNA-processing factors in the nucleus in relation to the specific and dynamically changing cellular needs to ensure that the RNA-processing events progress smoothly in a well coordinated manner. Therefore, a non-coding RNA, similar to the hsr0-n transcript in \textit{Drosophila}, must exist in other higher organisms as well and an active search for this would be rewarding not only from the basic cell biology point of view, but from human health point of view also.

**Interaction of hsr0 with Other Heat Shock Genes**

Our earlier studies (for review see Lakhotia 1989, Lakhotia & Sharma 1996) revealed an intriguing relation between activities of the 93D, 87A (containing two copies of \textit{HSP70} genes) and 87C (containing three copies of \textit{HSP70} genes) loci. A standard HS results in high transcriptional activity at the 93D, 87A and 87C puffs with the 87A and 87C puffs being equal in size and showing similar 3H-uridine incorporation (Mukherjee & Lakhotia 1979). However, when HS and another inducer of 93D (e.g., an amide or recovery from anoxia) were applied together, the 93D puff was seen not to incorporate 3H-uridine and at the same time the 87A and 87C loci puffed unequally. Interestingly, depending upon the specific combination of HS and the other 93D inducer, the 87A or the 87C puff was the larger of the two in a highly reproducible manner (Lakhotia 1987, 1989, Lakhotia et al. 1990, Sharma & Lakhotia 1995, Lakhotia & Sharma 1996). We suggested that the hsr0 transcripts influence RNA metabolism at the 87A and 87C sites (Lakhotia 1989, Sharma & Lakhotia 1995). Our recent observations (Lakhotia & Prasanth 2001) on the binding of hnRNPs provide a novel basis for this effect. Some of the hnRNPs have been reported to bind with the HSP70
RNA in Drosophila (Hamann & Stratling 1998, Reim et al. 1999), which during heat shock are normally completely sequestered by the hsrω transcripts (see above). Our studies show that under conditions when the hsrω locus is not transcriptionally induced by heat shock, the hnRNPs continue to remain associated with several other chromosomal sites, including the 87A and 87C puffs (Lakhotia & Prasanth 2001). It appears that in the absence of a threshold level of hsrω transcripts during HS, the removal of hnRNPs from 87A and 87C (and some other chromosomal) sites is affected. Since the hnRNPs have roles in RNA processing and transport (Krecic & Swanson 1999), their continued presence at the 87A and 87C sites in differential amounts may affect puffing of these sites differently. The functional significance and long term consequences of the unequal puffing of the two HSP70 puffs and the altered hnRNP binding at these sites needs to be examined further.

Morcillo et al. (1993) reported that the HSP83 protein gets quickly localized on the 93D puff in the polytene chromosomes of D. melanogaster after heat shock. Lakhotia and Ray (1996) showed that recessive mutation at HSP83 acts as a dominant enhancer of the lethality associated with the nullosome for the hsrω gene. As noted earlier, wild type HSP83 chaperone protein masks a variety of developmental abnormalities caused by genetic background (Rutherford & Lindqquist 1998). In this context, the interaction of hsrω with HSP83 is interesting. The hsrω transcripts may have a role in this homeostasis by virtue of their involvement in regulation of the metabolism of hnRNPs, and thus in the pre-mRNA processing. The binding of HSP83 with hsrω transcripts may also help keep the associated hnRNPs etc properly chaperoned (Lakhotia et al. 1999). Mutations at RasI or RasIII genes of D. melanogaster, which by themselves have no effect on viability in heterozygous condition, also dominantly enhance the embryonic lethality due to hsrω-nullosomy (Ray & Lakhotia 1998, Lakhotia et al. 1999).

Fernandez-Funez et al. (2000) reported that P-transposon insertion mutant alleles of hsrω enhance the ataxin-1 induced neurodegeneration in Drosophila. In this context it is interesting that the hsrω gene is highly expressed in the nervous system (Lakhotia et al. 2001). Like the earlier noted altered splicing of Ddc transcripts in the hsrω nullosomic condition, it appears that the enhancement of ataxin-1 phenotype is also related to perturbations in nascent RNA processing in hsrω mutant background. Alterations in the dynamics of nuclear hnRNPs resulting from under- or over-expression of hsrω would have wide-ranging trans-dominant effects on other gene activities via its role in modulation of the availability of hnRNPs.

The hsrω gene provides a new paradigm for the roles of non-coding RNAs in cell’s intricate and elaborate mechanisms for maintaining homeostasis.

Concluding Remarks
The HS or the stress response has been an extremely useful paradigm to understand the hierarchies of regulation of gene activity in a cell. A geneticist exploits mutations to understand the “normal” function of a gene. Likewise, the rapid and all pervading, but transient, perturbations in cellular activities by HS allow a deeper understanding of normal cell functions. The stress response paradigm has many applications in biotechnology and human health care (see Lakhotia 2001a). Increasing numbers of studies are revealing subtle or more apparent differences in stress response of different cell types and organisms and their possible ecological and evolutionary consequences (Feder & Hoffmann 1999). Since the cell’s survival under the omnipresent stress conditions is most critical for the species’ survival, the stress response is a vital component of any cell’s defense mechanisms. Therefore, stress biology will continue to remain a fertile field of study (Lakhotia 2001a). Most of the studies on stress response have hitherto remained confined to a few model organisms maintained under laboratory conditions. It will be necessary to extend these studies to a variety of organisms living under naturally varied environmental conditions.

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