# Chapter 14

# The Noncoding Developmentally Active and Stress Inducible *hsrω* Gene of *Drosophila melanogaster* Integrates Post-Transcriptional Processing of Other Nuclear Transcripts

**Subhash C. Lakhotia**

## Summary

The *93D* or the *hsr-omega* (*hsrω*) gene of *Drosophila melanogaster* became an interesting gene more than 3 decades ago in view of its unique inducibility with a brief benzamide treatment. Subsequent studies revealed many unusual features of this gene, a homologue of which is present in all *Drosophila* species examined. This gene is developmentally active in nearly all cells types of *Drosophila*, is induced by heat shock along with the other heat shock genes but is singularly induced by a variety of amides, all of which also inhibit general chromosomal transcription. The *hsrω* gene in all species of *Drosophila* has a characteristic architecture with two exons and an intron and a long stretch (>5 to ~15 kb) of tandem repeats on the 3' end of the gene. Like many other noncoding genes, the base sequence of the unique as well as the tandem repeat region of the *hsrω* gene is not conserved in different species. However, in all species examined, two primary nucleus-limited transcripts, ~2 kb and >10 kb, respectively, are produced but none of them carry any significant open-reading frame. The ~2 kb transcript is spliced to generate a 1.2 kb cytoplasmic transcript, which has a translatable ORF of 23-27 aa. Translation of this short ORF perhaps helps monitor the "health" of cellular translational machinery.

The large nucleus-limited >10kb *hsrω*-n transcript is so far the only known eukaryotic large RNA, which shows a speckled distribution in the nucleoplasm. These transcripts are present, besides at the site of transcription, as many nucleoplasmic speckles close to the chromatin domains. The various nuclear hnRNPs and some other proteins such as Sxl remain bound with the various transcriptionally active chromatin sites, and with the nucleoplasmic speckles formed by the *hsrω*-n transcripts. These speckles, designated as "omega speckles", are distinct from the well-known inter-chromatin granule clusters. The *hsrω*-n transcripts have an essential role in organizing the omega speckles, which serve to dynamically regulate the availability of hnRNPs and related proteins for RNA processing activities at any given time. Mutants that mis-express the *hsrω* gene and thus affect the omega speckles have diverse phenotypic consequences, presumably because of aberrant processing of various nuclear premRNAs due to altered availability of hnRNPs etc.

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The involvement of the noncoding transcripts of *hsrω* gene in metabolism of nuclear hnRNPs and in monitoring the "health" of ribosomal machinery provides new paradigms for roles of noncoding transcripts in integration of cellular regulation. Such activities are vital to all eukaryotic cells and therefore, genomes of all of them should include noncoding genes performing comparable functions.

## Introduction

Much of the very exciting progress in Biology during the past four decades has been propelled by the belief that a "gene", to be functional, must produce RNA, which must be translated into a protein.[1] Studies using this paradigm have enabled us to move from genetic engineering to genomics and now to the post-genomic era. Parallel and equally exciting developments in cell and developmental biology and other areas enabled remarkable correlations between gene expression, protein synthesis and the mechanistic details of cell function. Notwithstanding this most remarkable progress, one of the persisting riddles is the role/s of the bulk of the genomic DNA in eukaryotes. Almost all eukaryotes have much more DNA than accounted for by the protein coding "genes" and a significant proportion of the "noncoding" genomic DNA is nevertheless transcribed. Such "noncoding" sequences have often been brushed aside as "selfish" or "junk" because of the continuing emphasis on the "coding sequences". However, in recent years, the noncoding "genes" are also beginning to gain appreciation as meaningful components of genomes.[2-21]

The *93D* or *hsrω* gene of *Drosophila* was among the few "noncoding genes" known in the early 1980s. The unique organization of polytene chromosomes in dipteran insects and the phenomenon of puffing in polytene chromosomes provide a uniquely convenient approach to identify specific genes, their expression patterns and the proteins they produce. This indeed was responsible for the seminal identification of heat shock genes in *Drosophila*.[22] Subsequent identification of the heat shock proteins[23] provided the much needed demonstration of the causal relationship between gene activity (puffing), transcription and protein synthesis. One of the major heat shock induced puffs, the 93D puff, in polytene cells of *Drosophila melanogaster*[24] was found by Lakhotia and Mukherjee[25] to be uniquely inducible by brief treatment with benzamide and this made it an interesting system for further analysis. Studies during the past few decades revealed this locus to be a rather unusual noncoding gene in *Drosophila*. Unlike some other later discovered noncoding genes like *Xist*[26,27] or r*ox*,[28-30] the unusual properties of this gene's expression and regulation and its functions remained enigmatic for a long time (for reviews see refs. 31-34) and it is only in the past few years that the functions of this gene are beginning to be understood.[35,36] These suggest novel roles for RNA molecules in regulating cellular activities.

## The *93D* or the *hsrω* Gene in *Drosophila* Displays Unique and Conserved Inducibility but Apparently Does Not Produce a Protein

Initially this gene was named as *93D* because of its location in the 93D cytogenetic region of polytene chromosomes of *Drosophila melanogaster* but it was renamed later as the *hsrω* after its transcription products, heat shock RNA omega.[37] As a member of the heat shock gene family, the *93D* or the *hsrω* gene is one of the most active genes[24,38] following heat shock as well as after treatment with carbon dioxide, 2-4 dinitrophenol, arsenic compounds etc or after recovery from anoxia.[39] However, the most interesting feature of this gene, which attracted our attention was its singular inducibility with benzamide (Fig. 1).[25,40] It is now known that many other amides like, colchicine, colcemide, formamide, nicotinamide etc, also singularly induce the 93D puff in salivary glands of *D. melanogaster* larvae.[41,42] All species of *Drosophila* examined to date carry a homologue of the *93D* gene of *D. melanogaster*, since in all of them one of

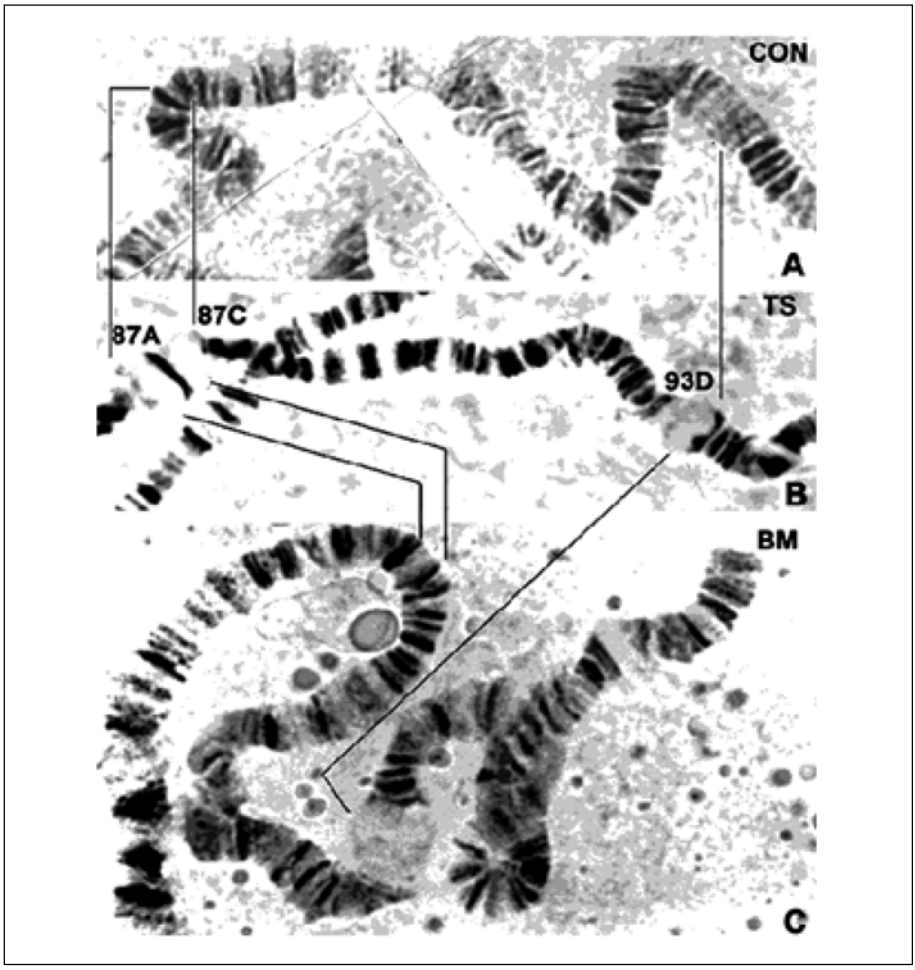


Figure 1. The 93D puff in salivary gland polytene chromosomes of *Drosophila melanogaster* is induced along with other members of the heat shock family following temperature shock (TS, B) or alone by benzamide (BM, C). The chromosome in A is from untreated control (CON) salivary gland. The 87A and 87C puffs, carrying multiple copies of the *hsp70* genes and the 9D puff are marked. Reprinted with permission from ref. 82.

the major heat shock genes, located at evolutionarily comparable region of the genome, is uniquely inducible by amides (Fig. 2).[43,44] Although an amide inducible heat shock puff has not been found in *Chironomus* and *Anopheles*,[45] heat shock inducible telomeric Balbiani rings in some species of *Chironomus* show properties that are in some ways comparable to the *hsrω* locus of *Drosophila*.[33,46-48]

Studies on activation of this locus in *Drosophila* documented a number of interesting, but as yet little understood, aspects of this gene's activation in larval salivary glands (for details, see reviews in refs. 31,32). Observations on RNA metabolism at the 93D locus by Spradling et al[49] and Lengyel et al[50] suggested that this gene harbors repetitive sequences and that bulk of its transcripts do not leave the nucleus. A more definitive suggestion that this gene may be a

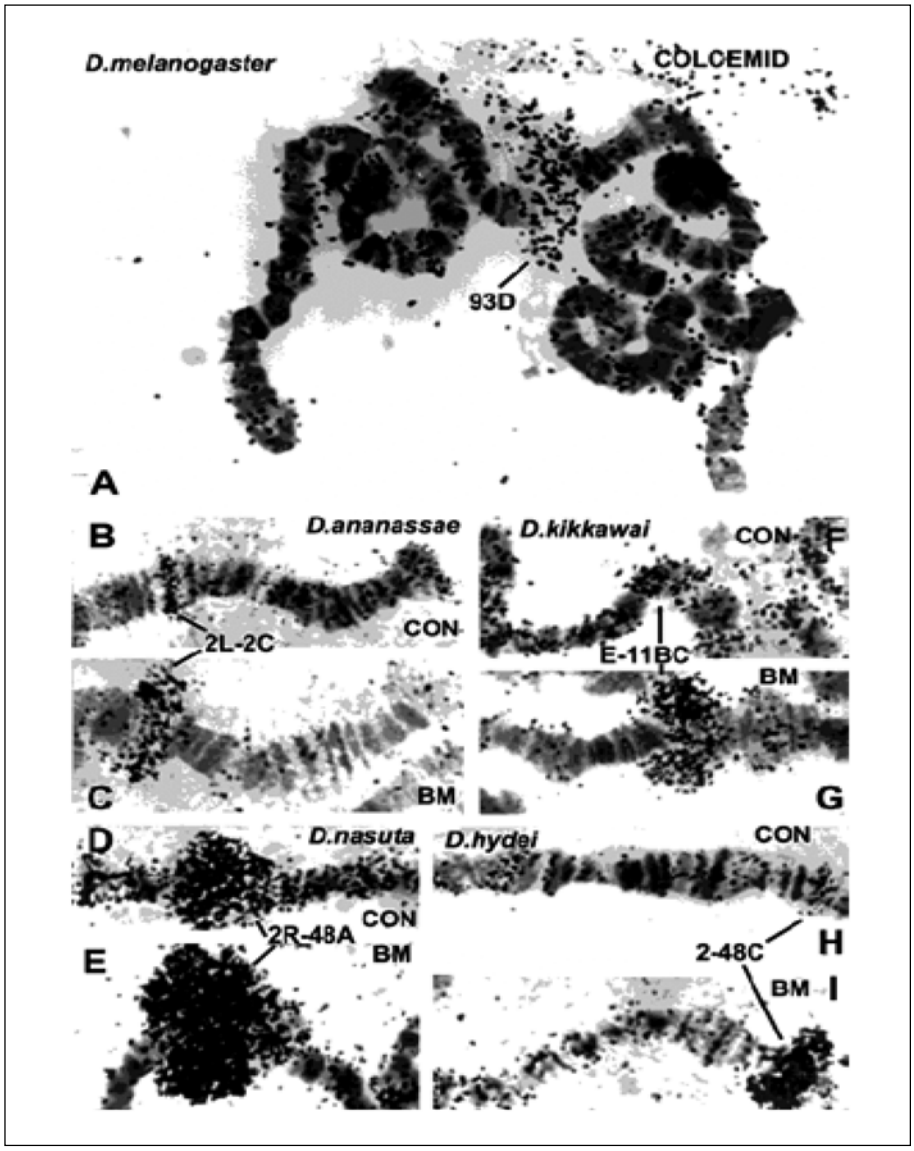


Figure 2. Amides selectively induce one member of the heat shock puffs in different species as revealed by $^{3}$H-uridine incorporation following colcemid (A) or benzamide (BM, C, E, G. I) treatment of salivary glands of different species, as mentioned in each case. The amide-inducible puff is also active in untreated (CON) glands (B, D, F, H). Reprinted with permission from ref. 82.

noncoding one came from the studies by Lakhotia and Mukherjee,[51] who examined protein synthesis following the selective activation of the 93D puff in benzamide treated larval salivary gland cells and concluded that in spite of this gene's evolutionary conservation, it does not code for a protein.

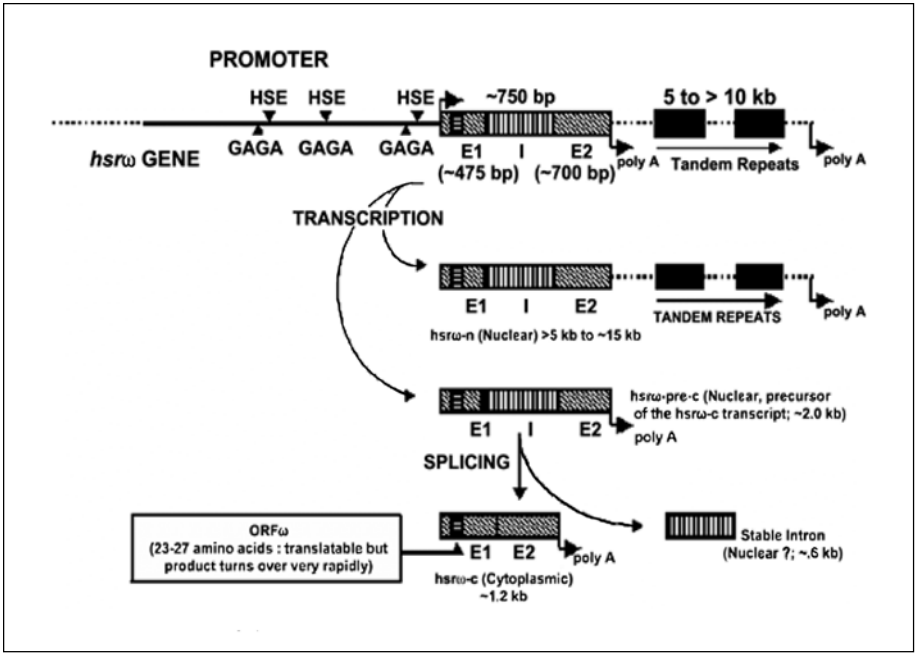


Figure 3. Architecture of the *hsr*w gene and its transcripts in different species of *Drosophila* (for details see text and the references mentioned therein)

## The *hsrω* Gene Shows a Unique Conserved Architecture but with Little Conservation of Base Sequence in *Drosophila* Species

An unusually strong conservation of the organization of the gene (see Fig. 3) but little conservation of the base sequence is evident from a comparison of the *hsrω* homologues from *D.hydei*,[52] *D. melanogaster*[53,54] and *D. pseudoobscura*.[55] In all the three species, the *hsrω* transcription unit comprises a characteristic unique region (~2.5 kb) at the 5' end, which includes 2 exons (~500 bp and ~700 bp long, respectively) and one intron (~700 bp), with a poly-A site at ~1.9 kb from the transcription start site. This poly-A site is followed by another unique region (~600 bp) and then by a long stretch (varying from 5 kb to ~15 kb) of short tandem repeats unique to this locus.[55-58] In all species, the *hsrω* gene has two transcription termination sites, the first after the 2nd exon (~2 kb from the transcription start point) and the second after the last tandem repeat unit[56] and accordingly, two primary transcripts are produced (see later). Neither of these transcripts or their processed product has any significant open reading frame. The rapid divergence of the base sequence in its transcribed region in different species of *Drosophila*, in spite of its conserved architecture, further supports the noncoding nature of this gene.[52,54,56,59,60] A strong conservation of the "architecture" but not so much of the primary base sequence at the *hsrω* locus is comparable with the noncoding *Xist* gene in mammals.[27,61]

Short stretches of high conservation interspersed between regions of high degree of sequence divergence are notable in the nonrepeated part (till the first transcription termination site) in *D. melanogaster*, *D. hydei* and *D. pseudoobscura*.[55] The most striking regions of sequence conservation include the acceptor and donor splice sites (the conservation extends beyond the splice junction),[55] the polyadenylation site and transcription start site. Significance of these conserved regions is not known. Finally, an open reading frame (ORFω) coding for 23 to 27

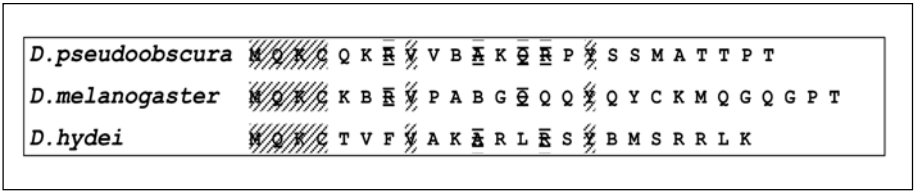


Figure 4. Amino acids coded by the ORFω in three species of *Drosophila*. Note that only the first four amino acid residues and two others (diagonal shading) are conserved in all the three species. A few others (horizontal shading) show identity only in any two species. Data from ref. 55.

amino acids is present in all the three species at about +120 position from the transcription start site. Intriguingly however, except for the conservation of the first 4 amino acids and two others interspersed in between, the rest of the amino acids are not conserved, although a few of them are identical between two species (see Fig. 4). This ORF is translatable but the translated product is not detectable.[62]

Base sequence of the repeat units is also remarkably diverged between species, although within a species a very high conservation exists.[52,56] Thus the repeats in the *hsrω* genes of *D. hydei* and *D. melanogaster* are highly diverged. The repeat unit in *D. hydei* is 115 bp while in *D. melanogaster* it is 280 bp. Interestingly a nonamer (ATAGGTAGG) is the only sequence motif that seems to be conserved between the *hsrω* repeats of the two species, occurring once per ~115 nucleotides in both cases.[34] The length of the stretch of tandem repeats at the *hsrω* gene of *D. melanogaster* varies between 5 and 15 kb in different populations but for a given population the length seems to remain fairly constant.[58]

## The *hsrω* Gene Produces Multiple Noncoding Transcripts

The *hsrω* gene does not code for any protein[51,54,57] although the small ORFω is translatable.[62] *hsrω* gene produces two primary transcripts (Fig. 3), viz., the *hsrω*-n transcript of ~10 to 15 kb length, which spans the entire transcription unit and remains localized in the nucleus, and the *hsrω*-prec transcript of ~2 kb length, spanning only the 5' region comprising of the two exons and the intron. The ~2 kb *hsrω*-prec transcript is spliced to give rise to the cytoplasmic 1.2 kb *hsrω*-c transcript.[37,56,57,62] Splicing occurs at the site of transcription[63] and the spliced out intron seems to be relatively stable.[37] The rapid sequence divergence at this locus in *Drosophila* species compares with most other noncoding genes. The production of transcripts of comparable properties suggests that the structure of the transcripts is more important than the base sequence itself.[33,35]

The different *hsrω* transcripts show inducer specific profiles. Heat shock causes marked increase in levels of all the three transcripts while the amide treatment leads to a significant increase of only the >10 kb long nuclear *hsrω*-n.[37,62] This difference seems to be related to the different effects of heat shock and amides on transcriptional and translational activities. Heat shock affects transcriptional as well as translational activities while the amides affect only chromosomal transcription without any effect on translational activity.[40,51] The *hsrω*-n as well as the *hsrω*-c transcripts display rapid turnover in normal cells but inhibition of transcription and translation has different effects on their stability.[37,64] The *hsrω*-n is stabilized when new transcription is inhibited with Actinomycin D,[64] while the *hsrω*-c is stabilized by inhibitors of protein synthesis.[37,65] The differential sensitivity of the two *hsrω*-n and *hsrω*-c transcripts to inhibitors of transcription and translation correlates with their nuclear and cytoplasmic roles, respectively (see later).

## The *hsrω* Gene Shows Widespread Developmental Expression

Several in situ RNA localization studies[65-67] established that this gene is expressed under normal developmental conditions in almost all cells except the early embryonic pole cells and the spermatocytes in testis. The level of its transcripts in different cell types varies and heat shock induced increase in the amount of these transcripts in different cell types is generally proportional to its normal level of expression. It is interesting that the pole cells and the spermatocytes do not express this gene even after heat shock.[67]

The *hsrω* gene responds to ecdysone.[65,66] The level of this gene's developmental expression is tightly regulated from very low to very high levels in a cell type specific manner.[67] In embryos, *hsrω* transcripts are particularly abundant in the developing central nervous system; likewise the larval and adult brain ganglia also show high levels of *hsrω* gene expression.[67,68] In this context it is interesting that an allele of *hsrω* has been reported to enhance ataxin-1-induced neurodegeneration in *Drosophila* model.[69]

## Promoter Region of the *hsrω* Gene Is Complex

The *hsrω* gene produces independently regulated multiple transcripts, and, besides being developmentally expressed, is inducible, either as a member of the heat shock gene family or individually, by a variety of experimental conditions. Therefore as expected, experimental studies revealed *hsrω* gene's promoter to be complex. Flies carrying transgenes with defined lengths of the *hsrω* promoter and the *lacZ* reporter gene suggested that while the heat shock inducibility and developmental expression are regulated by elements located within the proximal 845 bp of the promoter region, the amide inducibility was not included in this interval.[66,70] These and other[67] studies also indicated existence of regulators/enhancers that regulate this gene's expression in individual cell types/tissues. In a unique approach using small chromosomal deficiencies, Lakhotia and Tapadia[71] mapped the amide-response element/s to be more than 21 kb upstream of the transcription start point. Another aspect of the complexity of the *hsrω* gene's promoter region has been revealed through studies on an enhancer-trap line ($hsr\omega^{05241}$) in which a P-element with the *lacZ* reporter gene is inserted at –130bp position of the *hsrω* gene.[67,72] In spite of the insertion of ~13 kb of the P-transposon construct in the promoter region at –130 bp position, expression of the *hsrω* gene remains essentially unaffected in embryonic, larval and adult tissues of the $hsr\omega^{05241}$ homozygotes.[67] Only the cyst cells of adult testis show a distinctly perturbed expression of the *hsrω* transcripts in the $hsr\omega^{05241}$ homozygotes.[72] Interestingly, the *lacZ* reporter gene expression pattern in this enhancer-trap line is also comparable to the expression of the *hsrω* gene itself in most of the nonpolytene cells in embryonic, larval and adult stages. However, several larval polytene cell types do not show any expression of the lacZ, neither under normal developmental conditions nor after heat shock.[67] Yet, the *hsrω* transcripts are strongly induced by heat shock in nonpolytene cells as well as in all the polytene cell types, in spite of the fact that only one heat shock element (HSE), located at –57 to -43,[70] is close to the *hsrω* transcription unit while the other two (at -466 to –452 and at -250 to –235 nucleotide positions, respectively[70]) are far removed due to the insertion of P-transposon in this chromosome. It remains to be seen if only the proximal HSE is enough for the *hsrω* transcription unit or the more distal ones are still able to exert their role. Notwithstanding the long P-transposon insertion, the $hsr\omega^{05241}$ allele is also strongly induced by amides.[67] Apparently, the amide response element/s, already located more than 21 kb upstream,[71] can still act even after further displacement due to the insertion.

## The *hsrω* Gene Is Functional in Spite of Its Noncoding Transcripts

Conservation of the *93D* or *hsrω* gene, as a heat and amide-inducible gene in different species of *Drosophila* suggested its importance for flies although its noncoding nature and rapid

sequence divergence caused persistent doubts about its functional capability. The following evidence, however, clearly establish that the noncoding transcripts of the *hsrω* gene have important functions.

## *Mutant Phenotype*

An intensive screen for mutations at the 93D locus by Mohler and Pardue[73,74] did not recover any point mutations and for nonbelievers in the functions of a noncoding gene, this was as expected of a "junk" or "selfish" DNA. In hindsight, however, this appears to be related to the noncoding nature of this gene so that base changes are well tolerated, as have actually taken place at this locus during evolution of the different species. Mohler and Pardue identified two small deletions, viz. *Df(3R)e*$^{Gp4}$ and *Df(3R)GC14*, whose overlap specifically defines the HS and amide-inducible *hsrω* locus.[73-75] It is significant that most of the *Df(3R)e*$^{Gp4}$/*Df(3R)GC14* trans-heterozygotes, which are nullisomic only for the *hsrω* gene, die as embryo; a few (~20%) embryos are delayed in hatching but develop as weak flies unable to properly walk or fly, and which die within a few days.[35,73,74,76,77] Imaginal discs from the *hsrω*-nullisomic larvae fail to differentiate in vitro in response to ecdysone.[31] The *hsr*ω nullisomic flies are essentially sterile: the males produce motile sperms but are physically too weak to mate. On the other hand, oogenesis in the *hsrω* nullisomic females is affected so that only a few (~3%) of their eggs actually develop into viable progeny.[35] This correlates with high expression of the *hsrω* gene in the ovarian nurse cells.[66] Compared to wild type flies, *hsrω*-nullisomics are poorer in acquiring thermo tolerance and do not survive when grown at 31ºC.[31,78] A role of the *hsrω* gene in thermo tolerance is further supported by changes in *hsr*ω allele frequencies in unselected lines and those selected for resistance to knockdown by 39ºC heat stress with or without prior hardening.[79] McKechnie et al[80] also observed significant differences in the constitutive levels of the nuclear and cytoplasmic *hsrω* transcripts between these lines.

The *hsrω*$^{05241}$ enhancer-trap line, with a *P-lacZ* transposon insertion at –130 bp position (see above) displays an interesting phenotype. All stages of development of *hsrω*$^{05241}$ heterozygotes as well as homozygotes appear normal, except that the *hsrω*$^{05241}$ homozygous males are sterile.[72] As noted earlier, the expression of *hsrω* transcripts in different tissues and developmental stages of *hsrω*$^{05241}$ homozygotes is more or less comparable to that in wild type.[67] An exception is the cyst cells in adult testis. A pair of cyst cells remains associated with a bundle of 64 developing male germ cells and these cells have significant functions in proper maturation and individualization of the sperms.[81] The promoter dysfunction due to the P-transposon insertion at –130 bp position causes the *hsrω*-n transcripts to be much more abundant in cyst cells of *hsrω*$^{05241}$ homozygotes. This seems to prevent individualization so that bundles of nonmotile sperms are produced, resulting in male sterility.[72]

## *Interaction of* hsrω *with Other Genes*

Interaction of the *hsrω* gene with a number of other genes has been studied either through studies on puffing in polytene chromosomes or through modification of the mutant phenotype of the target gene.

### *Interaction with* hsp70 *Gene Loci*

*D. melanogaster* has two clusters of nearly identical two and three *hsp70* genes at the 87A and 87C cytogenetic regions, respectively.[82,83] Under the usual heat shock condition (37ºC for 30 min), the 87A and 87C sites form nearly equal sized puffs in salivary gland polytene nuclei with comparable $^{3}$H-uridine incorporation.[38] However, whenever the 93D puff fails to actively transcribe during heat shock, transcription at the twin puffs at 87A and 87C sites is affected (see reviews in refs. 31-33). As discussed later, the *hsrω* gene's transcripts regulate movements of heterogeneous nuclear RNA-binding proteins (hnRNPs) between active and

inactive compartments in the nucleus. Our unpublished studies (Prasanth KV and Lakhotia SC) on the distribution of some hnRNPs show that their binding on the 87A and 87C puff sites also gets affected when the 93D puff is not induced by heat shock. Apparently, in the absence of the *hsrω* gene's continuing transcriptional activity during heat shock the hnRNP movement gets affected and this in turn affects RNA metabolism at the *hsp70* gene loci. A recent study in our laboratory[83] revealed that in a variety of embryonic, larval and adult cells, the *hsp70* genes at 87A and 87C loci respond differently even after a typical heat shock. It is not known if this is in some way related to the *hsrω* transcripts also being induced to different levels by heat shock in these cell types.[67]

### *Other Gene Mutations as Dominant Enhancers of Embryonic Lethality of* hsrω*-Nullisomics*

As noted above, about 20% of *Df(3R)e*$^{Gp4}$/*Df(3R)GC14* (*hsrω*-nullisomic) embryos survive to adulthood. However, heterozygosity for recessive mutations at the *hsp83* gene[77] or at one of the Ras loci[84] causes 100% death of *hsrω*-nullisomic embryos. The *hsp83* or the Ras mutant alleles used in these studies by themselves do not cause any lethality in heterozygous condition. The nature of interaction between the *hsrω*, *hsp83* and Ras genes is not clear but this seems to be related to the fact that Hsp83 specifically binds with the *hsrω* locus in polytene nuclei after heat shock[48] and that *hsp83* mutations enhance phenotypes of genes involved in the Ras signaling pathway.[85]

### *Enhancement of Poly-Q Induced Neurodegeneration*

Several poly-Q repeat expansion induced neurodegenerative disorders are known in man[86] and for some of them elegant *Drosophila* models have been established.[69,87] Two of the P-insertion mutant alleles of *hsrω* have been found to enhance the neurodegeneration caused by either over-expression of normal or poly-Q expansion carrying mutant allele of human SCA1 (spinocerebellar ataxia type 1) in transgenic *Drosophila* model.[69] The mechanism of enhancing effect of the *hsrω* mutant alleles noted in this study is not clear. Another aspect that needs further analysis is the nature of the two *hsrω* mutant alleles used by Fernandez-Funez et al[69] While little is known about the functional status of the P292 allele of *hsrω*, the other allele, *hsrω*$^{05241}$ appears to be the same as studied in our laboratory.[67,72] Although Fernandez-Funez et al[69] identified this allele as a loss of function allele, our studies showed that this is rather a gain of function allele, at least in cyst cells of testis.[72] It is, therefore, necessary to reexamine the expression of the *hsrω* gene in the specific cell types in which the neurodegeneration is enhanced. It will also be interesting to study if other poly-Q expansion models in *Drosophila* (like for huntingtin) also display comparable enhancement due to *hsrω* mutant expression.

## Functions of *hsrω* Transcripts

The above evidence suggest that the *hsrω* gene does have widespread and vital housekeeping functions in fly's life[31-33] so that this gene's complete absence results in extensive lethality and a variety of other phenotypes in the rare survivors. Recent studies have provided some insights into the novel housekeeping functions of these noncoding transcripts.

### The Small Cytoplasmic *hsrω*–C Transcripts May Monitor the Translational Activities in Cell

Fini et al[62] showed that one or two ribosomes associate with the *hsrω*-c RNA and translate the small ORF, coding for 23-27 amino acids in different species (Figs. 2, 3), although the translated product is not detectable. Bendena et al[37] observed that inhibition of protein but not RNA synthesis results in quick stabilization of the *hsrω*-c transcript, which otherwise shows a

high turnover. In view of these observations and the fact that the amino acid sequence encoded by the omega-ORF is not conserved (see Fig. 4), it has been suggested that the act of translation of the omega ORF is important rather than the translated product.[31-33,62] It appears that the act of translation of the omega-ORF is coupled with degradation of the template so that partial or complete inhibition of translation due to drugs or some other cellular perturbations results in proportionate accumulation of the *hsrω*-c transcripts. This may provide a mechanism for monitoring "health" of the translational machinery through smooth passage of a ribosome through the ORFω and the levels of *hsrω*-c transcripts in the cytoplasm.[32] An additional and/or alternative role for the *hsrω*-c RNA molecules may be to serve as docking sites for unengaged ribosomes. Every cell has a large number of ribosomes, all of which are unlikely be actively engaged with mRNAs all the times. Furthermore, each cell is also likely to experience significantly varying translational activities so that at times many ribosomes get unengaged. The unengaged ribosomes may need storage till later recruitment for active translation. It is possible that RNA molecules like *hsrω*-c provide the storage or docking sites for such unengaged ribosomes.

### The *hsrω*-n Transcripts Organize "Omega" Speckles in Nucleoplasm to Regulate the Availability of hnRNPs for RNA Processing Activities

Antibodies against several nuclear nonhistone proteins, mostly belonging to the heterogeneous nuclear RNA-binding family of proteins (hnRNPs[88]), display more or less exclusive binding with the *hsrω* puff in chromosome spreads from heat shocked salivary glands of *Drosophila* larvae (see Table 1).[89-98] In squash preparations of unstressed polytene cells, these antibodies decorate a large number of transcriptionally active chromosome regions, including the *hsrω* site (Fig. 5). In nonpolytene interphase nuclei the binding of these proteins with active regions is seen as a diffuse staining of chromatin but in these cells also, heat shock causes accumulation of the hnRNPs and related proteins at the 93D site on the chromatin.[36] As the cells recover from heat shock, the hnRNPs move back from the *hsrω* site to the different chromosomal regions within one hour. This rather intriguing phenomenon of the binding of the various nuclear proteins more or less exclusively at the *hsrω* locus under heat shock conditions has now provided clues to possible functions of the large nuclear *hsrω*-n transcripts. Our studies[35,36,67,72] utilizing immuno-fluorescent localization of the various hnRNPs and related proteins in conjunction with in situ hybridization of *hsrω* specific riboprobes to cellular RNA in intact cells of *Drosophila* proved very informative. Among the known RNA-polymerase II dependent eukaryotic transcripts, the *hsrω*-n transcripts are unique in being present in the nucleus, besides at the site of transcription, as small granules or speckles distributed in nucleoplasm in close vicinity of chromosomes (Fig. 5).[35,36,67] These nucleoplasmic speckles of *hsrω*-n RNA also contain a variety of hnRNPs and related proteins (see Table 1). The speckles containing the *hsrω*-n RNA and hnRNPs and the related proteins were designated as "omega speckles".[36] The omega speckles are undetectable in squash preparations in which the nuclear envelope is disrupted, suggesting that they are not closely bound to chromatin. It is significant that the well-known nuclear speckles and inter-chromatin granule clusters with which the SR-family of RNA-binding nuclear proteins are associated,[99,100] are distinct from the omega speckles.[35,36,72] The omega speckles appear to be same as the large RNP-particles observed at the *hsrω* site and free in nucleoplasm in some of the earlier ultra structural studies on *Drosophila* cells.[91,101]

The omega speckles are completely absent in the *hsrω* nullisomic (*Df(3R)e*$^{Gp4}$/*Df(3R)GC14*) cells; in such cells the hnRNPs remain diffused through the nucleoplasm.[36] Furthermore, the immunoprecipitate obtained with antibodies against the hnRNPs contains *hsrω* RNA.[36] It is also noted that in different types of normal cells, there is a close correlation between the amount of *hsrω*-n RNA and the number of omega speckles in nucleus.[36,67] All these observations show that the *hsrω*-n transcript is essential for organizing the omega speckles.

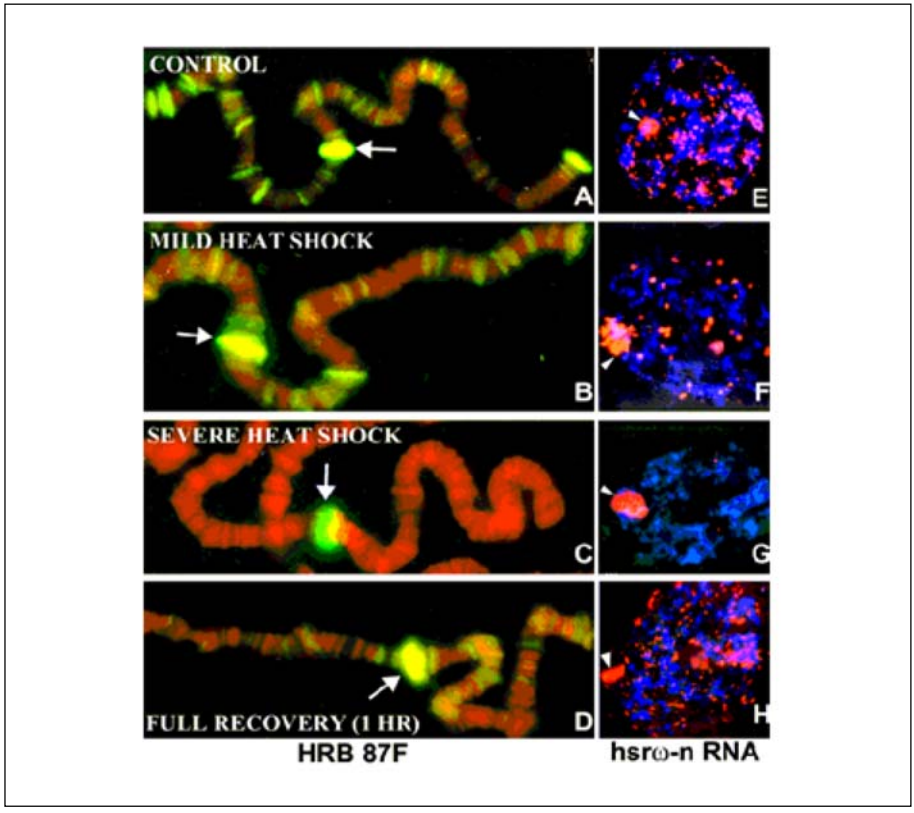


Figure 5. A-D. Heat shock causes all the hnRNPs (green/yellow fluorescence after immunostaining with antibody against HRB87F) to withdraw from the different active regions on polytene chromosomes (dull fluorescence) and finally accumulate at the 93D puff site (arrow) as in C; the hnRNPs return to their normal chromosomal locations within 1 hour of recovery frown heat shock (D). E-H. The *hsrω*-n RNA (localized by fluorescent in situ hybridization, dull fluorescence) in control cells (E) is present in speckles in the nucleoplsm close to chromatin (bright fluorescence) and at the 93D site (solid arrows) but following heat shock, the free speckles tend to coalesce with each other and accumulate at the 93D site (F) so that after about 40min of heat shock, all the *hsr*w-n transcripts are seen only at the 93D site (G). Although not shown here, the hnRNPs follow the same pattern as the *hsrω*-n RNA (see ref. 35,36). Like the hnRNPs (D), the *hsr*w-n transcripts also show the normal distribution within one hour recovery (H). Images adapted from ref 103.

The omega speckles are suggested to function as dynamic storage sites for the unengaged hnRNPs and the release of hnRNPs from them is coupled to degradation of the associated *hsrω*-n transcripts.[35,36] Depending upon the nuclear needs, the hnRNPs are either released from or sequestered in the omega speckles, and correspondingly the levels of *hsrω*-n transcripts go down or up.[35,36] The widely varying levels of *hsrω*-n transcripts in different cell types during normal development[66,67] reflect the varying needs of RNA processing in these cell types since the levels of hnRNPs in these cells also vary correspondingly. Heat shock severely inhibits general transcription and RNA processing activities in the nucleus.[82,102] Under conditions of reduced nuclear RNA processing, the *hsrω*-n level goes up, the omega speckles increase in size, possibly due to the greater sequestration of the hnRNPs, and coalesce with each other to form large aggregates. The aggregates finally get exclusively localized to the 93D chromosomal site itself

***Table 1.*** ***Antibodies to the following proteins specifically bind with the* hsrω *puff in heat shocked polytene nuclei of* D. melanogaster *and other* Drosophila *species***

| Protein | Reference |
|---|---|
| **hnRNPs**: Hrp40 (hnRNP A, Squid), HRB87F (hnRNP A1/A2), Hrb57A (hnRNP K), S5 (hnRNP M) | 89-95 |
| **Sxl** | 96,97 |
| **Nuclear non-histone proteins** recognized by Q14, Q16, T29, P75 antibodies | 89 |
| **Snf** | 97 |
| **Sera from auto-immune disorder patients** | 98 |

(see Fig. 5).[35,36] It is likely that the massive clustering of omega speckles in fully stressed cells ensures against illegitimate RNA processing activities under adverse conditions.[35,36] As the cells recover from heat stress and resume their normal transcriptional and processing activities, the hnRNPs are released from clusters of omega speckles and within one hour, the hnRNPs get restored to active chromosomal sites and typical omega speckles also reappear in the nucleoplasm. The level of *hsrω*-n transcripts also quickly goes down to the normal.

It is significant that in heat shocked *hsrω*-nullisomic cells, the hnRNPs do not completely move out of their chromosomal binding sites, do not form clusters and also do not regain their normal chromosomal distribution till about 2 hours of recovery from heat shock.[103] Thus in the absence of *hsrω*-n transcripts, the hnRNPs are not properly chaperoned and this may be the reason for the earlier noted thermo-sensitivity of *hsrω*-nullisomics.

The presence of Hsp83 chaperone protein also at the *hsrω* gene locus under heat shock condition[48] and the interaction of *hsrω* with *hsp83* gene mutation (see above and ref. 77) are significant. It is likely that the Hsp83 chaperone activity keeps the hnRNPs protected from thermal damage.[35] It has been reported that heterozygosity for *hsp83* null mutation in *Drosophila* causes epigenetic appearance of a variety of recessive mutant phenotypes, which normally remain masked.[104,105] It is likely that dysfunction of Hsp83/Hsp90 (e.g., reduced quantity in null mutant heterozygotes) affects the chaperoning of hnRNPs, which in turn may affect the desired splicing and/or other aspects of RNA processing. The *hsrω* transcripts may facilitate the chaperoning of hnRNPs by the Hsp83 by providing a common platform.[35]

Post-transcriptional processing of nascent transcripts involves a wide variety of protein and RNA-protein complexes. Among these, the hnRNPs constitute a large family of RNA-binding proteins with important roles in packaging, splicing, transport and degradation etc of RNA.[88,106-108] One of the important functions in which many of the hnRNPs and other proteins like the Sxl, Snf etc have a significant role, is the alternative splicing.[107-109] In recent years, a variety of other well defined nuclear speckles or granules/clusters have been identified and most of them are believed to function as storage sites for specific sub-sets of the various RNA processing factors.[99,100,109-119] It has been well established that the ratio of SR-proteins and hnRNPs in general, and the specific sub-sets of each of these families of proteins that are available for splicing, influence the selection of donor and acceptor splice sites in multi-intronic transcripts.[120,121] Therefore, it appears likely that a regulated release of hnRNPs from the omega speckles and of the SR proteins and other splicing factors by the other storage sites provides an efficient and self-organized machinery for modulation of alternative splicing of various transcripts. Disassembly of interchromatin granules affects the coordination of transcription and post-transcriptional processing[122] apparently due to disruption in the regulated release of RNA-processing factors. Likewise, since the *hsrω*-n transcripts have a pivotal role in organizing

omega speckles as a mechanism for regulated availability of the hnRNPs and related proteins for RNA processing, any change in the levels of these transcripts in a cell may be expected to adversely affect splicing and/or transport of a variety of nuclear transcripts. This in turn would seriously compromise the cell's normal functioning. Therefore, the lethality of *hsrω*-nullisomic embryos[73,74,77] or the sterility of *hsrω*$^{05241}$ mutant males[72] seems to result from widespread disruption of RNA processing due either to complete absence or over-abundance, respectively, of the *hsrω* transcripts. We are currently studying (Sengupta S, Lakhotia SC, unpublished) another *hsrω* mutant allele derived through local hopping of the P-transposon in the *hsrω*$^{05241}$ chromosome; this derived mutant line displays late larval/pupal lethality and it is significant that the omega speckles show extensive clustering in different larval tissues, comparable to that seen[72] in cyst cells of the *hsrω*$^{05241}$ mutant males. It is likely that a new mutation in the *hsrω* promoter in this line mis-regulates *hsrω*-n transcripts in most larval cells, causing over-sequestration of hnRNPs in large clusters of omega speckles. This in turn would cause a widespread mis-regulation of RNA processing and finally in larval/pupal lethality.

## Future Prospects

The *hsrω*-n RNA is the first noncoding RNA to be distinctly shown to be responsible for organizing a well-defined nuclear domain, the omega speckles. Although it has been considered that some RNA species provide a structural role in sequestering the unengaged RNA processing factors,[115] no specific RNA has as yet been identified. The role played by the noncoding *hsrω*-n transcripts in organizing the omega speckles provides new paradigm for understanding the regulation of nuclear RNA processing activities and also for the novel roles that RNA can perform. Since the post-transcriptional processing events in the nucleus are highly conserved[88] and since every cell needs to fine tune the availability of RNA-processing factors in the nucleus to ensure that the RNA-processing events progress smoothly in a well coordinated manner as required by the specific and dynamically changing cellular needs, it is expected that noncoding RNAs comparable to the *hsrω* transcripts are present in all eukaryotic organisms. Furthermore, it is likely that all the different classes of nuclear domains/speckles are organized through one or more species of noncoding RNAs. A pro-active search for their identification will be rewarding. hsrω-like RNA species in humans may have clinical significance as well in view of the fact that this gene's dysfunction enhances poly-Q based neurodegeneration in *Drosophila* models.[69] Furthermore, since many of the fertility factors in man are RNA-binding proteins,[123] and since a dysfunction of the *hsrω* RNA in *Drosophila* testis causes sterility,[72] it will be interesting to examine if any of the infertility cases in human have a comparable etiology.

Functions of the small *hsrω*-c transcript have not been explored much and it will obviously be interesting to examine how the act of translation of the ORFω causes degradation of the template. Likewise, we know little about the spliced out intronic fragment, which is reported to be relatively stable.[37] Does this serve as a sink for some nuclear proteins or can it function as RNA regulator? These possibilities need to be examined. Detailed analysis of the secondary/tertiary structures of *hsrω* gene's various transcripts may provide a basis for search for similar transcripts in other eukaryotes.

Although amides have been instrumental in bringing this gene into focus, little is known about the action of amides on *Drosophila* cells that leads to the selective activation of the *hsrω* locus. The mechanism by which the *hsrω*-n transcripts are brought back to the *hsrω* locus after heat shock also remains intriguing. It will be interesting to examine if some components of nuclear lamins or other nuclear skeletal elements are involved in this movement.

It is interesting that a noncoding gene like the *hsrω* can have such varied functions in nucleus as well as cytoplasm, which help integrate cellular regulation. Such functions are of fundamental importance to cell's housekeeping activities and provide good examples of self-organizing systems, so characteristic of living organisms.[117] It is certain that as we explore

deeper into the mysteries of genome with no preconceived notions, we will uncover many more instances of such noncoding RNA based self-organizing systems.

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