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The non-coding transcripts of *hsr-omega* gene in *Drosophila*: Do they regulate trafficking and availability of nuclear RNA-processing factors?

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The *93D* or *hsr-omega* (hsr?) is an unusual non-protein-coding gene with multiple transcription products which are dynamically expressed in most cell types of *Drosophila melanogaster* and this gene, besides being a member of the heat shock gene family, is uniquely induced in polytene cells by a variety of amides. The various aspects of this gene's organization, regulation and inducible properties are briefly reviewed. Recent data in our laboratory show that absence of the *hsr-omega* transcripts because of nullosomy or over-expression of the these transcripts in specific cell types due to mutation in the promoter region of this gene results in specific phenotypes. It is known from several earlier and our recent studies that in unstressed cell nuclei a variety of hnRNA binding proteins (hnRNPs) associate with many chromosomal sites, including the 93D, and with extra-chromosomal speckles where the *hsr-omega* transcripts also co-localize. Following heat shock and other stresses, the bulk of these proteins and the hsr-omega nuclear (hsr? -n) transcripts get localized to the 93D site. We propose that one of the important functions of the hsr? -n transcripts is to act as a 'sink' for at least some members of the hnRNPs and related proteins so that any increase or decrease in the abundance of these nuclear transcripts correspondingly modifies the 'sink' size, which in turn affects the availability of such proteins in active nuclear compartments and regulates the nuclear RNA processing activity. It appears that non-availability or over-abundant availability of these transcripts disrupts the regulated and fine-tuned balance of the various RNA-processing factors resulting in *trans*-dominant mutant phenotypes. We believe that binding with specific proteins and consequent regulation of their activity may be a common feature of the functions of non-protein coding genes.

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THE transcripts of heat shock or other stress-induced genes are rapidly translated into specific heat shock proteins or Hsps[1]. However, unlike most of the widely studied genes for Hsps or other stress proteins, the stress-inducible *93D* or hsr? gene of *Drosophila* produces several transcripts but does not code for any protein [2–7]. This intriguing gene is situated at the 93D6-7 band position of right arm of chromosome 3 of *D. melanogaster* [8,9] and because of its unique heat-inducible transcription products it is also designated as *heat shock RNA omega* or *hsr?* gene[10]. Among the heat shock loci in *D. melanogaster*, the *93D* (hsr?) locus forms one of the most transcriptionally active puffs after a temperature shock [11,12]. However, the 93D puff is also uniquely induced by benzamide, colchicine and other amides [13–15]. The *hsr?* gene is conserved in the genus *Drosophila* since one of the major heat shock induced genes in all species of *Drosophila* has similar inducible properties [16].

Besides being highly induced by heat shock and amides, the hsr? transcripts are also normally present in cultured cells and in most cell types during development [17,18]. Interestingly, however, the relative abundance of the different hsr? transcripts varies in relation to developmental stage and in an inducer-specific manner[10,19].

Structure rather than sequence of the non-coding hsr? transcripts is conserved

The genomic organization of this locus in different species of *Drosophila* is remarkably conserved since in all cases analysed so far, the locus spans 10 –20 kb with a 5 to 10 kb or longer stretch of short tandem repeats at its 3¢ end and two exons separated by an intron at the 5¢ end; sizes of the two exons and the intron are comparable in different species (see Figure 1). Surprisingly, the DNA sequence of the *hsr?* homologues in different species is highly

diverged[20–22]. It is interesting that one of the major heat shock induced puffs in polytene nuclei of some *Chironomus* species also produces non-coding transcripts whose structure, sequence divergence and some other properties are reminiscent of the *hsr*? locus

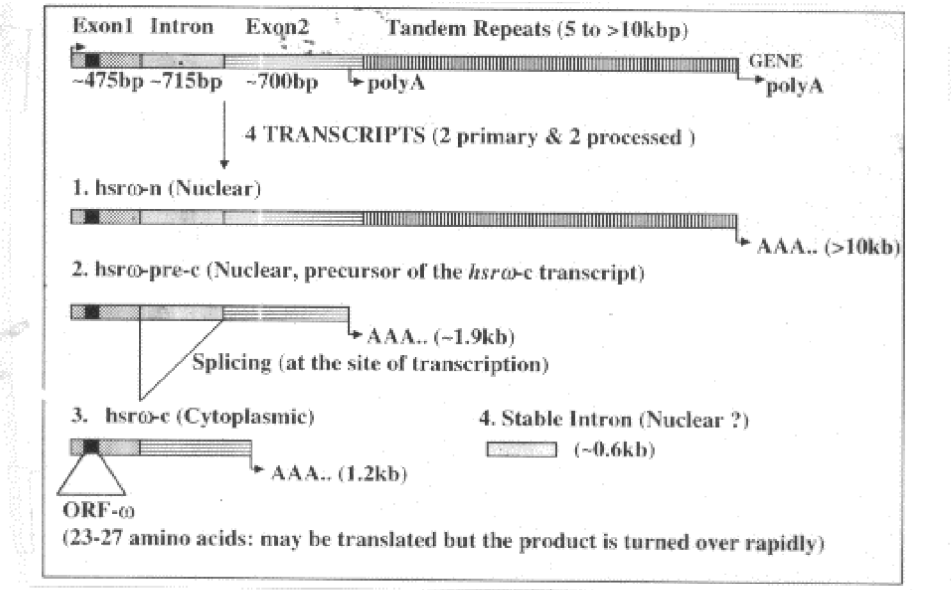

Figure 1. Schematic representation of the architecture of the *hsrω* gene of *Drosophila* and its different transcripts (redrawn after Ray and Lakhotia[7]).

of *Drosophila*; the possible presence of an *hsr*? homologue in *Chironomus* indicates its wider evolutionary conservation[23–25].

This locus produces two primary nuclear transcripts (Figure 1) of which the larger, the nucleus limited hsr? -n transcript, is about 10 –20 kb long and spans the entire transcription unit composed of the proximal unique region and the distal long stretch of short tandem repeats which are not present at any other site. The second smaller transcript, the hsr? -pre-c, is ~1.9 kb long, and includes the two exons and the intron corresponding to most of the proximal unique region of the gene. The ~700 bp intron in the hsrw -pre-c is spliced out to generate the 1.2 kb cytoplasmic transcript, the hsrw -c, which is transported to cytoplasm and carries a small (23 –27 amino acid residues in different species) translatable open reading frame, but does not produce any detectable product[3–5]. All the three transcripts have the same start point, but while the omega pre-c is precursor for omega-c, the omega-n is not precursor to any of them [3,10,22]. Splicing of the hsrw -pre-c takes place at the site of synthesis [19] and the spliced out intron persists as a 600 bp fragment[10].

Regulation of the*hsr*w gene expression is complex

The production of more than one transcript by the *hsr*w locus, their dynamic developmental expression and their inducibility by heat shock and by amides, indicate presence of multiple regulatory elements in its promoter region. Further complexity in its regulatory organization is suggested by the tissue-specific variations in its expression pattern and also by the fact that its induced expression is modulated by specific conditions. Thus, a variety of experimental conditions and certain genotypes are known to specifically prevent puffing of the 93D locus in heat shocked but not in amide treated salivary gland polytene cells of *D. melanogaster*[6,26]. Promoter analyses studies by Mutsuddi and Lakhotia[18] revealed that the basic regulatory elements for its developmental and heat shock induced activity are mostly within the –844 bp upstream region. The putative amide response elements (AREs), on the other hand, seem to be located far upstream

(>22 kb) from the transcription start point [27]. Other unpublished results in our laboratory further suggest that the induced expression of this gene is differently regulated in polytene and non-polytene cells.

Heat shock and amides increase the levels of all the hsrw transcripts but their relative levels are regulated in an inducer specific manner. Chemicals like benzamide and colchicine that specifically induce 93D, but not the other heat shock loci, result in higher levels of the omega-n transcripts, whereas heat shock leads to a relatively greater increase in the level of omega-c [10,19]. The omega-n transcripts rapidly turnover in the nucleus but conditions that result in inhibition of general nuclear transcription lead to a rapid accumulation of this transcript through its increased stability [28]; likewise drugs that inhibit translation through chain initiation or elongation, stabilize omega-c transcripts which also normally turnover within minutes in control cells [10]. It is notable that heat shock as well as the amide treatments cause a general inhibition of chromosomal transcription [6,13].

It is interesting that under certain conditions of heat shock the 93D puff is not induced in polytene cells and shows little ^{3}H-uridine incorporation and yet, the amount of hsrw transcripts present at the 93D region of polytene chromosomes remains nearly as high as when this is typically induced [19]. This suggests a reduced turnover of the hsrw transcripts in the nucleus and/or withdrawal of these transcripts back to the 93D site from their otherwise more wide distribution in the nucleus (see later).

The *hsr*w gene has important functions

Since the *hsr*w gene does not produce any protein and also since its base sequence in different species of *Drosophila* has changed rapidly, the functional significance of this gene can be questioned and one could argue that a gene like this is a typical example of 'selfish' or 'junk' DNA. However, the above noted complexities of its regulation, its role in thermo-tolerance and the phenotypic effects resulting from this gene 's absence or due to its over-expression, as discussed below, lead us to believe that even though without a typical protein product, the *hsr*w gene has vital functions during normal development and under conditions of stress.

Role in thermotolerance

*hsr*w-nullosomics are relatively poor in acquiring thermo-tolerance [29] and unlike wild type flies do not survive when grown at 31° C (ref. 30). These suggest that the transcripts of this gene play crucial role in the stress response. A more direct evidence for this was obtained by studies of A. A. Hoffmann's group[31,32] who carried out selection experiments for thermo-resistance. Their results showed significant differences with respect to allelic variations and the levels of the nuclear and cytoplasmic transcripts of the *hsrw* gene between the lines selected for thermo-resistance and those not
selected.

*Phenotypes due tonullosomy of the hsr*w *locus*

Intensive mutagenesis at the 93D locus by Mohler and Pardue[8] failed to recover any point mutation but provided two small overlapping deletions, viz. *Df(3R)e^{Gp4}* and *Df(3R)GC14*, whose overlap specifically defines the 93D heat shock and amide-inducible locus [8,9]. Mohler and Pardue[8] found these *hsrw*-nullosomic trans-heterozygotes (*Df(3R)e^{Gp4}/Df(3R)GC14*) to mostly die at embryonic stage while the few (~20%) survivors to adult stages were very weak, unable to properly walk or fly, sterile and died within a few days. A more detailed study of effects of the nullosomy for the *hsr*w locus in *Df(3R)e^{Gp4}/Df(3R)GC14* trans-heterozygotes in our laboratory[33] revealed additional phenotypes as follows:

1. About 10% of the *hsr*w-nullosomic embryos that died after development of cuticular structures

showed defects in anterior terminal structures ranging from near absence of some or all components of the anterior structures to complete fusion of the dorsal arm, dorsal bridge, H-piece, labrum, and the hypopharyngeal organ. Such defects in anterior terminal structures were absent in the nullosomic embryos that hatched.

2. Many of the stage 12 to stage 15 *hsr*w-nullosomic embryos showed (i) fusion of the anterior and posterior commissures on one side of the ladder like CNS and/or (ii) break in the longitudinal axons in the central nervous system in the abdominal segments. Embryos with at least one copy of the *hsr*w locus did not show such abnormalities.
3. The few surviving *hsr*w-nullosomic flies were very weak, unable to walk or fly properly and were short-lived. The *hsr*w-nullosomic males showed apparently normal reproductive organs with bundles of motile sperms in their testes but such males never produced any progeny when crossed with wild type females. On the other hand, out of the 104 *hsr*w-nullosomic females crossed with wild type males, 47% did not lay eggs and 34% laid eggs which did not hatch but the remaining 19% laid a few eggs, at least some of which hatched into normal viable larvae. Examination of ovaries of these different categories of *hsr*w-nullosomic females after Phalloidin and DAPI staining showed that the ovaries of *hsr*w-nullosomic females that did not lay eggs after mating, showed more severe defects than the other two categories of *hsr*w-nullosomic females. These ovaries showed high percentage of degenerating follicles and most interestingly, in some of the egg chambers the nurse cells were not properly demarcated. Associated with the reduced number of nurse cells, the distribution of ring canals was also aberrant. Another interesting defect was the reduced frequency of stage 9, stage 10a, and stage 10b egg chambers in most of the ovarioles. In some follicles, the correct positioning of the oocyte was affected (for details, see ref. 33).

*Mutations at Ras or hsp83 genes act as dominant enhancers of embryonic lethality due to hsr*w*-nullosomy*

Interestingly all the above phenotypic effects of *hsrw*-nullosmy are shared, to varying degrees, by mutations in the *Ras1* or several other genes that are involved in the Ras-signaling pathways [34–38]. It is, therefore, not surprising that mutations at *RasI* or *RasIII* genes in *Drosophila*, which by themselves have no effect on viability in heterozygous condition, dominantly enhance the embryonic lethality due to *hsrw*-nullosomy since even the 20% of such embryos that develop to adulthood, fail to hatch as larvae when also carrying a mutant *RasI* or *RasIII* allele in heterozygous condition [7]. Likewise, recessive mutations at the *hsp83* locus also act as dominant enhancer of embryonic lethality due to *hsrw*-nullosomy[39]. Hsp83 is known to specifically localize to the 93D puff following heat shock to larval salivary glands [24] and *hsp83* mutations are known to affect some of the Ras-signaling pathways [38,40].

*Male sterility due to over-abundance of hsr*w *transcripts in cyst cells in testes of D. melanogaster*

Recent studies in our laboratory [41] on a P-insertion mutant in which the *P-lacZ-rosy*$^{+}$ transposon is inserted at –133 bp position in the promoter region of the *hsrw* locus revealed that due to this promoter mutation, the hsrw transcript level in the somatic cyst cells of testes was several folds higher. This was associated with the spatial order of the various spermatogenic stages in testes being disorganized and filled with immotile sperms which did not leave testes so that the seminal vesicles were completely empty. A pair of cyst cells encircles the growing cysts of meiotic cells till sperm maturation and provides 'nourishment' to the differentiating male germ cells [42]. It appears that over-abundance of the hsrw transcripts in cyst cells of the mutant testes results in some critical function/s being affected which in turn, disrupts the spatial order of growing germ cells and their maturation within the testes and ultimately results in male sterility (see later).

Hsrw transcripts colocalize with a variety of hnRNPs and some other proteins in the nucleus

A remarkable insight into some of the possible functions of this gene has been gained by analysing the nature of proteins that are known to associate with this gene 's nuclear transcript. The *93D* site of *D. melanogaster* and its homologues in other species are associated, specially after induction, with unique large-sized RNP particles[43,44]. Clusters of such RNP-particles are seen in a variety of cell types and are mostly associated with the site of this gene 's location but are also present in the nucleoplasm[43,45]. The proteinaceous core of such particles is surrounded by RNA[45]. As summarized below, antibodies against an intriguing array of RNA-binding proteins shows specific binding with the hsrw transcripts, and these complexes are localized mostly at the 93D site, specially after its induction.

P11, Q14, Q16 and Q18

The P11, Q14, Q16 and Q18 antibodies, generated against nuclear non-histone chromosomal proteins of *D. melanogaster*, recognize the P11 group of RNA binding proteins[46]. The protein recognized by the P11 antibody is also known as Hrp36 in view of its 36 kDa size and its hnRNA binding properties and as HRB87F in view of its gene being located at the 87F cytogenetic region[47], Q14 and Q16 antibodies recognize different epitopes of Hrp36 or HRB87F. Q18 antibody recognizes a similar-sized protein whose gene is located at the 57A cytogenetic region and is, therefore, also known as HRB57A (ref. 48).

The earlier[44,46,49,50] and our own unpublished immunostaining studies show that all these proteins normally remain bound to a variety of transcriptionally active sites on polytene chromosomes (Figure 2 *a*) but after heat shock are almost eliminated from these sites and mostly get specifically localized to the heat shock induced 93D puff site (Figure 2 *b*) in association with the characteristic large RNP-particles that also accumulate at this site[44]. We have seen that after benzamide and colchicine treatments also, bulk of the HRB87F binding is restricted to the induced puff at 93D in *D. melanogaster* as well as *D. simulans* (Figure 2 *c–e*). Our studies further show that in a variety of other cell types of *Drosophila*, the P11 antibody binding is similar to that in salivary gland polytene nuclei: in all the tissues examined (larval gut polytene and the imaginal cells, Malpighian tubules, brain ganglia of larvae and the cyst cells in testes of adult flies), the unstressed cells show many small speckles of P11 all through the nuclear area along with a larger cluster which corresponds to the 93D chromosomal region (see Figure 3 *c*, *f*) but after heat shock, bulk of the HRB87F protein gets clustered at the 93D site although a few small speckles are still seen in rest of the nucleus (Figure 3 *d*). Hogan *et al.*[28] using radioactive *in situ* hybridization studies, claimed that the hsrw -n transcripts were uniformly distributed in the nucleus. However, our results, using the non-radioactive *in situ* hybridization (colour and fluorescence) technique, which permits better spatial resolution of the hybridization signal, showed that the hsrw -n transcripts in nuclei of all these cell types are mostly present as distinct speckles (Figure 3 *a*, *b* and *e*) very similar to the distribution of HRB87F protein. Indeed, simultaneous *in situ* hybridization and immunostaining (Figure 3 *e–g*) reveals that bulk of the nuclear hsrw RNA and the HRB87F protein co-localize. Such studies revealed that while all the speckled HRB87F protein co-localized with speckles of the hsrw -n RNA, the more diffusely distributed HRB87F protein in the nucleus was apparently not associated with the hsrw transcript. On the other hand, a few speckles of the nuclear hsrw transcript were free of HRB87F (Figure 3 *e–g*). Unlike our present results that the hsrw -n transcripts are located at multiple sites in nuclei of different cell types, Buchenau *et al.*[48], using a short oligonucleotide probe, reported that hsrw-n transcripts in *D. melanogaster* cells were localized exclusively at the 93D site. We think that this discrepancy is related to a reduced sensitivity of the oligonucleotide probe used by Buchenau *et al.*[48] compared to the full length repeat unit (280 bp) or the cDNA (1.2 kb) riboprobes used by us. The distinct speckled distribution of hsrw -n RNA is unique since no other nuclear RNA, other than the snRNAs, etc. is so far known to show a comparable pattern.

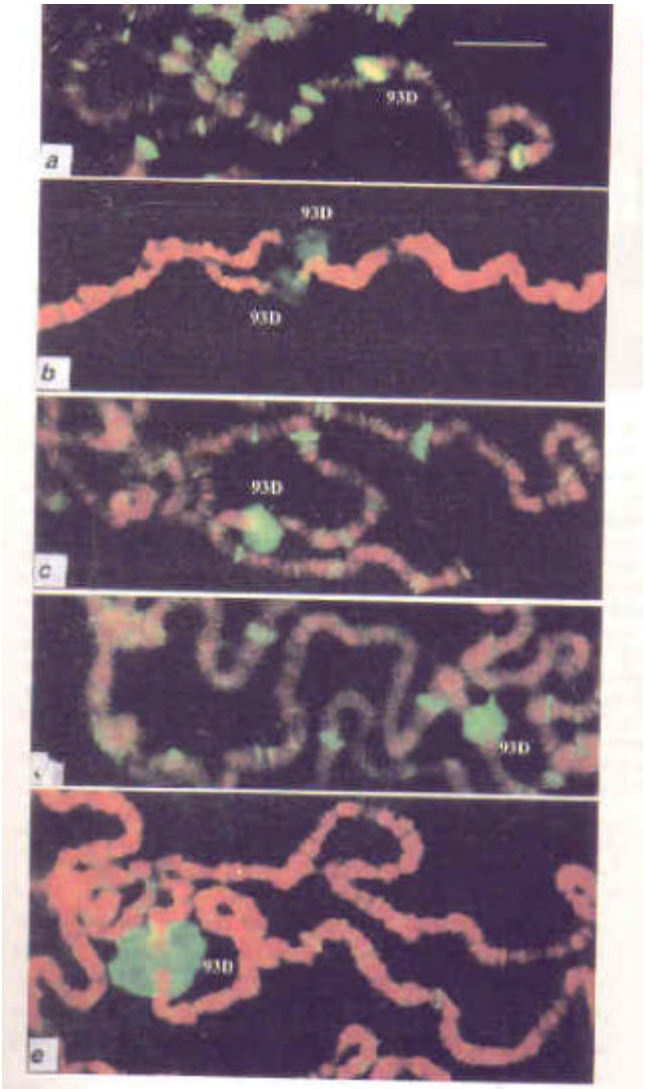

Figure 2. Immunofluorescent localization of the HRB87F or HRP36 protein (PII antibody) on polytene chromosomes of *D. melanogaster* (*a–d*) and *D. simulans* (*e*). The HRB87F protein is widely distributed (yellowish green fluorescence) on many transcriptionally active polytene chromosome sites, including the 93D, in unstressed cells (*a*) but a 40′ heat shock at 37°C (*b*) causes this protein to be withdrawn from all sites and to get exclusively localized at the 93D puff (the two homologs are partly asynapsed in the example in *b* and thus two 93D puffs are visible). Benzamide (1 mM for 10′ at 24°C) and colchicine (100 µg/ml for 40′ at 24°C) also cause bulk of the HRB87F protein to accumulate at the 93D puff although it continues to be present at several other sites also (*c* and *d*, respectively). The example in *e* shows the massive localization of HRB87F protein at the 93D puff in polytene chromosomes of *D. simulans* following the benzamide treatment. The immunostaining was carried out following Saumweber *et al.*[46] and Dangli and Bautz[16] with some minor modifications. The polytene chromosomes were counter-stained with propidium iodide (red fluorescence) to show chromosome bands. All photomicrographs were recorded with a Nikon E800 fluorescence microscope on Fujicolour 200ASA film. The photographic prints were scanned and reproduced using the Adobe Photoshop software. The scale bar represents 10 µm.

The binding properties of HRB87F or Hrp36 suggest that its association with the hsrw transcripts is due to indirect interaction [51,52]. HRB87F is a homologue of hnRNP-A1 of vertebrates [47,50,53]. HnRNP-A1 is known to act as RNA helicase, to catalyse RNA-RNA annealing activity and may play a role in pre-mRNA splicing, alternative splice site selection and other aspects of nuclear mRNA metabolism [53–55]. HnRNP-A1 may also function as RNA chaperone to prevent misfolding of RNAs and resolve RNAs that are misfolded [56]. In agreement with these roles in hnRNA processing,

over-expression of *Hrb87F* in a transgenic line was associated with aberrant splicing of the *ddc* (*dopa-decarboxylase*) transcripts in larval brain ganglia [57]. However, its null mutants do not have any phenotype, which suggests existence of redundant pathways.

The HRB87F or Hrp36 has been seen to bind mostly to those chromosomal sites whose transcripts are generally not immediately transported to cytoplasm and on this basis it has been suggested to have a role in nuclear retention of such transcripts [44,58]. However, studies in our laboratory (Prasanth, K. V. and Lakhotia, S. C., unpublished) show this protein 's transient binding with the *hsp70* loci during initial stages of their heat shock induction. Since the hsp70 transcripts are immediately transported to cytoplasm, it seems that the Hrp36 has other roles as well.

HRB57A protein recognized by the Q18 antibody is homologous to the hnRNP K family of proteins[48,59] which have roles in nuclear metabolism of hnRNAs. HRB57A, like the HRB87F, is withdrawn from other sites and recruited specifically to the 93D puff upon heat shock and gets rapidly redistributed during recovery from stress [48].

HRB57A and HRB87F are present in cytoplasm during very early embryonic development but are transported to nuclei from stage 12 onwards of embryonic development [48,60]. It is interesting to note that this stage corresponds to initiation of zygotic gene expression, including that of the *hsr*w (refs. 17, 18).

S5 and T29

S5 and T29 antibodies, also generated against non-histone chromosomal proteins of *Drosophila*, together form the S5-group [46]. These antibodies bind to a variety of transcriptionally active sites on unstressed polytene chromosomes but after heat shock, they lo- calize mostly at the 93D puff [49]. However, unlike

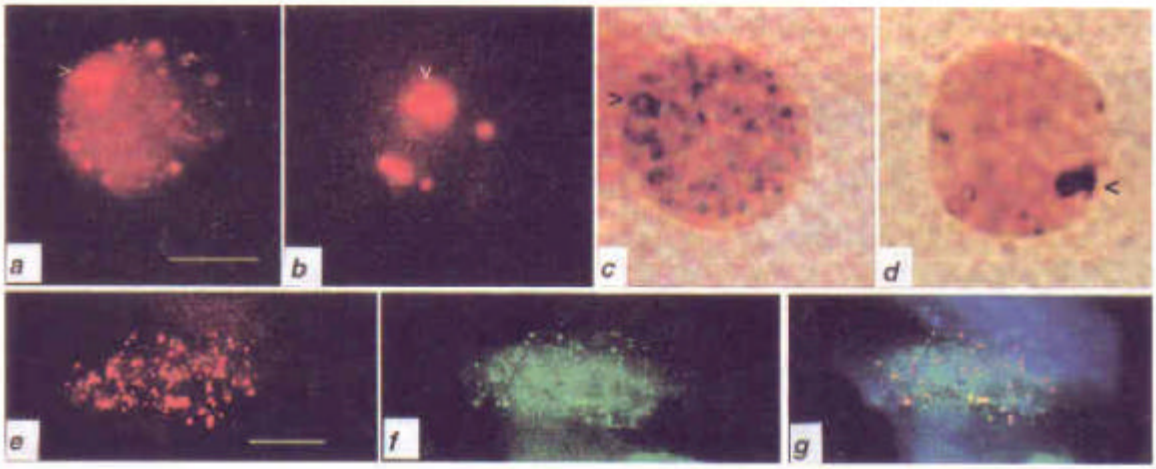

Figure 3. The hsrω-n RNA (*a*, *b*, *e* and *g*) and the HRB87F protein (*c*, *d*, *f* and *g*) are distributed in overlapping speckles in unstressed (*a*, *c*) and 40' heat shocked (*b*, *d*) larval Malpighian tubule (*a–d*) and adult testes cyst cell (*e–g*) nuclei. The hsrω-n RNA was localized in intact Malpighian tubule (*a*, *b*) or testis cyst cells (*e*, *g*) by *in situ* hybridization under non-denaturing conditions with a dig-UTP labelled anti-sense riboprobe transcribed *in vitro* from a clone[19] of the repeat unit of the 93D locus (*pDRM30*) while the HRB87F protein was localized through immunostaining using the P11 monoclonal antibody[46]. In *a*, *b* and *e*, the RNA hybridization was detected using the TRITC conjugated anti-dig-antibody (Boehringer Mannheim, Germany). The HRB87F immunostaining in *c* and *d* was detected by anti-mouse-HRP secondary antibody (Amersham, UK) and in *f* with anti-mouse-FITC secondary antibody (Sigma, USA). The hsrω-n RNA and the HRB87F protein in cyst cell in *e–g* were simultaneously detected by *in situ* hybridization with the dig-labelled riboprobe and immunostaining with the P11 antibody: *e* shows only the localization of hsrω-n RNA (TRITC fluorescence detected by the G2A filter block), *f* shows the distribution of HRB87F protein in the same cell (FITC fluorescence detected by the B2A filter block) while in *g*, the localizations of both are simultaneously seen using the DFT fluorescence filter block in the Nikon E800 Fluorescence microscope. Note that both the hsrω-n RNA (*a*) and the HRB87F protein (*c*) are present in the unstressed Malpighian tubule cells as many speckles, one of which is larger (arrow head in *a*) and represents the *hsrω* locus. Heat shock (*b* and *d*) causes most of the small speckles to disappear while the large cluster at the *hsrω* locus (arrowheads) becomes more enlarged. Co-localization of the hsrω-n RNA and the HRB87F protein in unstressed cyst cells of adult testis (*e–g*) shows that almost all the speckles of the HRB87F protein are superimposed on speckles of the hsrω-n RNA (orangish colour in *g*) while the more diffuse green fluorescence in nuclear area (*f*) is not matched by similarly distributed hsrω-n RNA (*e*). Photomicrographs were taken on Nikon E800 microscope and processed as in Figure 2. The scale bars (common for *a–d* and for *e*, *f*) represent 10 μm.

the above noted P11-group, they continue to decorate several non-heat shock loci as well. S5 is also known to bind to the Y chromosomal loops A and C in primary spermatocytes [61,62].

V4, P75 and T7

These antibodies were also generated against non-histone nuclear proteins by Saumweber *et al.*[46] and bind with many chromosomal sites in unstressed polytene cells. After heat shock the V4 antibody binds to 93D and the other heat shock puffs. T7 decorates all major heat shock puffs but its presence over other inactivated loci is reduced. After 5 min of heat shock, T7 binding is considerably higher at the 93D puff than the other heat shock puffs [49]. The proteins recognized by V4, P75 and T7 antibodies are yet to be characterized.

The P75 antibody of Fleischmann *et al.*[63] seems to be different from the P75 of Saumweber *et al.*[46]. It recognizes a non-histone chromosomal protein of 75 kDa, which though preferentially associated with transcriptionally active regions on larval salivary gland polytene chromosomes, in many cases shows an inverse correlation with the RNA polymerase II content of the given site. While its binding with the 93D site is strong in unstressed as well as heat shocked cells, the other heat shock loci show little binding except during recovery from heat shock [63]. Unlike the P11 group of antibodies that bind only on a subset of RNA Pol II transcribed regions of polytene chromosomes, the P75 also decorates at least some Pol III transcribed sites. Since the P75 was generally more abundant at sites whose transcripts are retained for a longer period in the nucleus, its role in nuclear retention of transcripts has been suggested [63].

Hrp40 or squid

Hrp40 or squid is another member of hnRNPA1/B class of hnRNA binding protein family and was

first identified as part of the complex that contains at least ten major hnRNPs of which the Hrp36 (HRB87F) also is a member [64,65]. *hrp40* (*squid*) encodes transcripts of different lengths and produces at least three different protein isoforms presumably due to alternative splicing. *hrp40* gene is also localized at 87F band position of polytene chromosomes, very close to the gene coding for the Hrp36 or HRB87F protein [64]. Hrp40 is a nuclear protein which directly binds to poly-A containing nuclear RNAs in most somatic cells and which is mostly associated with actively transcribing loci of polytene chromosomes [64]. Recent studies in our laboratory (Prasanth, K. V. and Lakhotia, S. C., unpublished) revealed that after 40¢ at 37 ºC, Hrp40 gets highly localized to 93D puff, much like the earlier noted Hrp36 (P11). It is, however, interesting to note that the 87A7 puff site, one of the *hsp70* loci, also showed a weak staining with Hrp40 antibody even after 40 min of heat shock, while the 87C1 puff, the other *hsp70* locus, did not show any binding. Female flies homozygous for null mutation of *hrp40* or *squid* have been reported to be sterile and to show variable degree of degeneration of oocytes and some other abnormalities [66,67] which are somewhat reminiscent of the defects seen in ovaries of *Df(3R)e*^{Gp4}/*Df*(*3R*)*GC14* that are nullosomic for the *hsrw* gene (see above and ref. 33).

Sxl (Sex-lethal) andSnf (Splicing necessary factor)

The *Sxl* gene has a key role in sex determination and dosage compensation in *Drosophila* [68] since the Sxl is a RNA-binding protein that regulates the sex-specific alternative splicing of a number of pre-mRNAs of sex-determining and dosage compensating genes as well as of its own pre-mRNA [69–74]. Sxl complexes with Snf (**s**plicing **n**ecessary **f**actor) to prevent spliceosome assembly at the male-specific exons of pre- mRNAs of downstream genes in females [75]. Snf is a 28 kD nuclear protein and shows extensive sequence similarity to vertebrate RNA-binding proteins U1A and U2B² with greater similarity to U2B² with respect to its size and structure [76]. Immunostaining of female larval salivary gland polytene chromosomes indicates that Sxl decorates more than 50 cytological sites (Sxl-antibody does not bind to any site in salivary gland chromosomes of normal male larvae). Results of Samuels *et al.* [77] and our own observations show that after heat shock, Sxl gets localized exclusively to the 93D locus. Though Snf cooperates with Sxl in sex-specific splicing, the Snf stains many sites on polytene chromosome without sex bias. Furthermore, unlike Sxl, the Snf is present after heat shock on all the major heat shock puffs, including 93D (ref. 77). Other than Snf, some hnRNA-binding proteins are also known to interact with Sxl. For example, hnRNPL helps Sxl bind to transcripts to which Sxl alone does not bind and Sxl helps Hrp36 or P11 (HRB87F) bind to RNA to which the latter alone binds only weakly [78]. The Sxl protein strongly binds with RNA at AUUUUUUU or AUUUUUUUU sequence motifs and relatively weakly with motifs that have fewer Us following the 5¢ A (ref. 77). Our analysis of the base sequences available in the EMBL database revealed that there are a number of potential Sxl-binding motifs in the hsrw -n transcripts of *D. melanogaster* as well as *D. hydei.*

Sera of patients suffering fromankylosing spondylitis (AS), mixed connective tissue disease (MCTD) and from systemic lupus erythematosus (SLE)

Patients suffering from AS, MCTD and SLE are known to possess antibodies against hnRNPs, snRNPs and other packaging proteins [79]. Sera from certain AS patients bind strongly to the heat shock induced 93D puff, with a weak staining at other heat shock loci; MCTD sera bind with 93D and other heat shock loci after heat shock while SLE sera binds with the 93D, 87A and 87C puffs as well as with some other non-heat shock loci after heat shock [80].

Hsp83

Morcillo *et al.* [24] reported that after heat shock, Hsp83 (the Hsp90 homologue of *Drosophila*) was present in a fibrillar network in the entire cytoplasm of polytene cells but within the nucleus, it was

specifically localized to the 93D puff and disappeared from this site as the puff regressed during recovery. It is interesting to note that the *hsr*w homologues in other species of *Drosophila* and *Chironomus* also show specific binding of Hsp90 family protein and the other HRPs noted above after heat shock. Our results [7,39] on interactions of *hsp83* and *Ras* genes with the *hsr*w locus are significant in the context of the binding of Hsp83 with the *hsr*w locus after heat shock. Hsp90 family proteins are known to possess DNA and RNA binding properties [81,82] and the LKVIRK-region of the Hsp90 is homologous to the RNA-binding domain of cauliflower mosaic virus RNA-movement protein[83]. Although similar properties of *Drosophila* Hsp83 are yet to be discovered, the highly conserved structure and function of the Hsp90 family protein in different organisms suggest that *Drosophila* Hsp83 may also possess RNA binding activity. Furthermore, many RNA binding proteins are also involved in Ras/Src signaling [84,85]. It may also be mentioned that an earlier report [86] indicated that antibody to cyclic guanosine monophosphate (cGMP) also showed specific binding with the 93D puff after heat shock. It is not known if the cGMP localization at the 93D puff is in some way related to the interaction of *hsr*w gene with Ras pathway genes.

Hsp70

Laran *et al.* [87] reported that following heat shock, the Hsp70 also binds with the *hsr*w locus in heat shocked salivary glands of *D. hydei* (2-48C puff). However, the heat shock induced 93D puff of *D. melanogaster* did not show detectable binding of Hsp70, although Shopland and Lis[88] have reported that Hsp70 generally 'paints' all polytene chromosome regions after heat shock.

hsrw -n transcripts function as 'sink' for nuclear RNA-processing factors: A model

It is clear from the above that many of the proteins that bind with nuclear transcripts of the *93D* or the *hsr*w locus in polytene and a variety of other cells belong to the hnRNP family of nuclear proteins[53,64,89] which are involved in regulated splicing and mRNA transport. Sxl and Snf are also RNA-binding proteins and are involved in regulated splicing while the Hsp83 or Hsp70 belong to the molecular chaperone family. Recent studies in our laboratory (Figures 2 and 3 and other unpublished results) have extended the earlier observations made mostly on salivary gland polytene chromosomes to many other cell types of *Drosophila* and in all cases hnRNPs like Hrp36, Hrp40 and presumably other similar proteins co-localized with the nuclear hsrw transcripts. In unstressed cells the hsrw transcript and the hnRNPs are distributed as small speckles while heat shock causes them to aggregate mostly at the *hsr*w gene locus (Figures 2 and 3). It may be noted that members of the SR family of proteins, so named due to their serine/ arginine rich domains and which are involved in regulated alternative splicing reactions and modulating chromatin structure during transcription [90–92], do not appear to bind to the 93D site or its transcripts [93,94] (also our unpublished observations) in a manner as the hnRNP family members do. In mammalian cells, the SR family proteins associate with the interchromatin speckles or interchromatin granules which contain little hnRNPs [95,96]. *Apparently the speckles or clusters of the hsrw-n transcripts and the hnRNPs in Drosophila cells also are distinct from speckles or the inter-chromatin granules that contain the SR-family proteins.* Earlier ultrastructural studies[44,45] also showed that the particles containing the P11-family of hnRNPs and the hsrw -transcripts had a unique organization

We believe that the dynamic co-localization of the hnRNP and some other RNA-binding and processing proteins with the hsrw -n transcripts is very significant and suggests a possible role for the non-coding hsrw nuclear transcripts. *We propose that one of the important functions of the nuclear hsr*w*-n transcript is to act as a 'sink' for at least some members of the hnRNA-processing proteins so that any increase or decrease in the abundance of the hsr*w*-n transcripts correspondingly modifies the 'sink' size, which in turn affects the availability of such proteins in active nuclear compartments and thereby regulates the nuclear RNA processing activity.* It is likely that the speckles or clusters of hsrw -n transcripts and the associated hnRNPs in the nucleus represent the sequestered form of these proteins while the more diffusely distributed hnRNPs are

perhaps associated with active chromatin sites. In view of the known antagonistic roles of the SR proteins and hnRNPs[53,90–92,97], RNA processing activities are expected to be affected by the specific sequestering of hnRNPs by hsrw -n transcripts and consequent change in the balance between the SR proteins and hnRNPs.

Cellular stresses like heat shock significantly inhibit chromosomal transcription and RNA-processing activity and since most of the heat shock transcripts do not need splicing or other processing prior to their rapid transport to the cytoplasm for translation [98–100], it is logical to presume that much of the hnRNPs, whose half-life is generally long [101], remain unengaged under these conditions. Heat shock may also thermally denature or cause other damage to these proteins due to which they, if allowed to remain available in active compartment, may carry out aberrant RNA processing functions. To avoid such possibilities, these proteins must be prevented from being active and therefore, need to be sequestered away from active compartment. The amount of hsrw -n transcripts also rises rapidly during stress due to new synthesis and reduced turnover [10,19]. The increased pool of hsrw -n transcripts can thus 'soak' the unengaged hnRNPs. We believe that the rapid and correlated redistribution of hsrw -n transcripts and some of the RNA-processing factors in heat shocked nuclei serves to sequester a subset of RNA-processing proteins away from the active compartment. The hnRNPs are not found free of RNA in the nucleus [53] and therefore, it is likely that the pool of hnRNPs that is not productively engaged with hnRNA processing remains anchored to some other RNA. The hsrw -n RNA would be one such RNA. Like the interchromatin granules which probably are the storage sites for the SR family of proteins [95,102], the unengaged hnRNPs presumably also aggregate with the hsrw -n RNA as speckles or granules for storage till required. It is significant in this context that RNA is not present in core region of the large RNP particles that characterize the *hsrw* locus[45]. Therefore, it appears to us that the hsrw -n transcripts mask various hnRNPs that aggregate in these large particles and thereby keep them sequestered. Dangli *et al.*[44], while characterizing the large RNP-particles associated with the 93D puff, suggested the large RNP-particles to have a 'storage' function so that the 93D transcripts could be retained in the nucleus. However, we believe that it is the 93D or the hsrw transcripts that help 'store' a variety of hnRNPs, etc. during periods of their inactivity. As the cells recover from stress and resume their normal transcriptional and RNA-processing activities, the hsrw transcripts are rapidly turned over [10] and redistributed in the nucleus and in parallel, the RNA-processing factors are released and also redistributed to active nuclear compartments.

The dynamically regulated expression of *hsr*w gene in almost all cell types from embryo to adult stage of *Drosophila*[17,18] presumably provides for a rapid increase or decrease in the 'sink' size in relation to the varying demands on the nuclear RNA processing machinery in different cell types; one of the consequences of the developmental alterations in the 'sink' size would be a dynamic change in the ratio of the functionally antagonistic SR proteins and hnRNPs in active compartments. Inhibition of nuclear transcription by actinomycin D results in a rapid stabilization and accumulation of the hsrw -n RNA[28]. We think that such stability serves to provide adequate 'sink' for the hnRNPs that remain unengaged in the absence of new transcription. A positive role of the hsrw -n transcripts in regulating the spatial distribution of the various hnRNPs is further suggested by our unpublished observations that in unstressed and more particularly heat shocked cells of the few surviving *hsr*w-nullosomics, these proteins mostly remain distributed in a diffused manner. The disorganized spatial distribution of the RNA-processing proteins due to absence of these transcripts in *hsr*w-nullosomics can be expected to result in malfunctioning of the nuclear RNA-processing machinery. Indeed, as noted above, *hsr*w-nullosomy is associated with a high degree of embryonic lethality and other phenotypic effects. Survival of about 20% of the nullosomic embryos is suggestive of existence of redundant genetic pathways as expected for vital developmental functions [103]. As noted earlier, the *hsrw*^{05241} promoter mutation due to a P-transposon insertion at –133 bp region of the *hsr*w gene is associated with a several-fold higher level of expression of hsrw nuclear transcript in the cyst cells of adult testes and also male sterility. The hsrw nuclear transcript in these mutant cyst cells colocalize with the various hnRNPs in the form of larger clusters, reminiscent of those seen in wild type cells after heat shock [41]. We believe that the over-abundance of hsrw transcripts in cyst cells due to the *hsrw*^{05241} gain of function

mutation traps a larger proportion of the nuclear RNA-processing factors in inactive compartment [41]. This may have a trans-dominant effect on the processing of many nuclear transcripts so that a variety of cyst cell functions are affected. Since cyst cells have important roles in the growth and maturation of spermatogenic stages, their malfunction results in male sterility [42].

It is not clear if the massive accumulation of the hsrw transcripts at the 93D chromosomal site during heat shock is due to increased synthesis alone or is also due to the 'withdrawal' of these transcripts from the speckles distributed throughout the nucleus. Lakhotia and Sharma [19] showed that even when there is no ^{3}H-uridine incorporation at the 93D site under certain conditions of heat shock, the *in situ* level of the hsrw transcripts at this site remained high. Therefore, we believe that the very high level of hsrw transcripts at the 93D site after heat shock and their concomitant disappearance from most of the other nuclear regions is also due to these transcripts being actively 'withdrawn' to the site of synthesis. It is interesting to note here that the 93D locus has been shown to be highly dynamic and mobile in living cells [48]. An attractive possibility is that the dynamicity of this locus in live cells plays a role in its 'soaking' function.

Recent observations of Philips *et al.* [104] and Lu *et al.* [105] on the consequences of the CUG triplet expansion in the 3¢ - untranslated region of the myotonic dystrophy or *DM* gene are very significant in the present context. Transcripts from the expanded gene accumulate in the form of aggregates in the nucleus and work of Philips *et al.* [104] indicates that the CUG-binding protein (CUG-BP), a splicing factor, gets titrated out by the triplet-repeat expanded mutant *DM* transcripts and this disrupts the normal function of the CUG-BP in splicing of transcripts of a certain family of genes [106]. Lu *et al.* [104] have further identified a new member of the CUG-binding proteins, elav-type ribonucleoprotein (ETR-3), which is highly expressed in heart and is able to interact with CUG repeats.

A comparable regulation of certain DNA-binding proteins by sequestering during periods of their inactivity also exists. During interphase stage, the *Drosophila* GAGA factor remains bound to sites of high GA content in the regulatory regions of a variety of genes to maintain a transcriptionally competent state of chromatin [107]. However, during mitosis when transcriptional activity is absent, the GAGA factor molecules get displaced from the euchromatic sites and transiently bind to simple AG-rich repeats in peri-centromeric heterochromatin. Almost a similar situation is observed for the protein encoded by the *proliferation disrupter* (*prod*) gene of *Drosophila* [107]. Thus, in these cases also, the DNA binding regulatory factors are sequestered by heterochromatic repeat blocks when not required.

We believe that during heat shock, the chaperones like Hsp83 (and possibly also Hsp70) accumulate at the *hsr*w locus to protect the RNA-processing proteins, that aggregate there, from irreversible thermal damage. It has recently been reported that heterozygosity for recessive mutation at the *hsp83* gene of *D. melanogaster* has a trans-dominant effect on many other genes resulting in mutant phenotypes that do not show up when both copies of the *hsp83* are functional [108]. Since the *hsp83* mutations have a dominant interaction with the *hsr*w locus, it is tempting to speculate that at least a part of the trans-dominant effect of *hsp83* mutations on other genes may be mediated through the hsrw transcripts: in the absence of threshold levels of functional Hsp83, a variety of hnRNPs that bind with hsrw transcripts, especially during conditions of cellular stress, may not function appropriately and this would affect the functioning of genes whose transcripts need to be processed by these hnRNPs.

Concluding remarks

The 93D or the *hsr*w gene, like the several other 'non-coding' genes, has remained interesting but enigmatic ever since its serendipitous discovery nearly 30 years ago [109]. The puzzling array of its inducible properties, genomic organization and its complex regulation kept the belief alive that transcripts of this and similar other genes have important cellular roles without coding for

conventional protein product/s [6,110]. A combined genetic and molecular cell biological approach has now provided the first insight into one of the several possible ways in which its nuclear transcripts regulate vital functions relating to processing, etc. of pre- mRNAs. The hsrw -c (the 1.2 kb cytoplasmic RNA) binds to ribosomes and its short ORF is presumably translated [3–5]. In parallel with the above noted role of the hsrw -n transcripts, it is tempting to speculate that the hsrw -c has a comparable role in regulating the availability of ribosomes. The stable hsrw -intronic RNA may also have some nuclear functions. All these remain exciting possibilities for further studies. It is obvious that such regulations are essential functions in all eukaryotic cells. Therefore, homologues and analogues for *hsr*w-like gene must exist in all eukaryotes. Since the *hsr*w gene sequences show rapid change, sequence homology may not unravel such genes in other organisms but analysis of the ever-increasing DNA sequence databases for the *hsr*w-like gene architecture and/or protein-binding properties of non-coding RNA species may be expected to permit identification such genes in organisms that do not have the advantage of polytene chromosomes.

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