Interaction of the non-protein-coding developmental and stress-inducible *hsrω* gene with *Ras* genes of *Drosophila melanogaster*

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The *93D* or *hsrω* (*hsr-omega*) is an unusual non-protein-coding gene which shows a dynamic developmental expression in most cell types of *Drosophila melanogaster* and which, besides being a member of the heat shock gene family, is uniquely induced in polytene cells by a variety of amides. We briefly review the various aspects of this gene's organization, regulation and inducible properties and present our recent data to show that, similar to our earlier report of interaction between the *hsrω* and *hsp83* genes, mutations at the *Ras1* or the *Ras3* gene of *D. melanogaster* also dominantly enhance the pre-pupal and pupal lethality of embryos that are nullosomic for this gene. In the absence of any protein product, the *hsrω* transcripts are suggested to interact with the Ras1 signaling cascade. The interaction of a non-translatable, developmentally produced as well as heat and other stress inducible RNA with the Ras signaling proteins and with the Hsp83 chaperone protein opens new possibilities for understanding of functions of this intriguing gene.

1. Introduction

Unlike the well known heat shock and other stress inducible genes that exert their actions through protein products, the developmentally regulated as well as stress inducible *93D* or *hsrω* gene of *Drosophila* produces several transcripts but apparently does not code for any protein (Lakhotia and Mukherjee 1982; Garbe and Pardue 1986; Hovemann *et al* 1986; Fini *et al* 1989). This intriguing gene is situated at the 93D6-7 band position of right arm of chromosome 3 of *D. melanogaster* (Mohler and Pardue 1984; Burma and Lakhotia 1986) and because of its unique heat-inducible transcription products it is designated as *heat shock RNA omega* or *hsrω* gene (Bendena *et al* 1989). Unique features of the *93D* or *hsrω* locus will be briefly reviewed in the following before presentation of our data on genetic interaction of this gene with the Ras pathway genes.

1.1 *Conservation of inducible properties and molecular design, but not of the base sequence of the hsrω locus in the genus Drosophila*

Like other heat shock loci in *D. melanogaster*, the *93D (hsrω)* locus is highly active after heat shock (Ashburner 1967; Mukherjee and Lakhotia 1979). However, it differs from rest of the heat shock genes in being uniquely induced by benzamide, colchicine and other amides (Lakhotia and Mukherjee 1980, 1984; Tapadia and Lakhotia 1997). All species of *Drosophila* harbour a homologue of the *hsrω* locus with similar inducible properties (Lakhotia and Singh 1982). The genomic organization of this locus is remarkably similar in all species of *Drosophila*: 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 (see figure 1). In spite of the unique molecular design shared by the *hsrω* homologues in different species,

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its DNA sequence is highly diverged (Garbe *et al* 1986, 1989; Ryseck *et al* 1987). The possible presence of an *hsrω* homologue in *Chironomus* further indicates its evolutionary conservation in other dipterans as well (Nath and Lakhotia 1991; Morcillo *et al* 1993; Morcillo and Diez 1996).

1.2 *hsrω produces multiple, non-protein-coding transcripts*

This locus produces two primary nuclear transcripts (figure 1). The hsrω-n is about 10–20 kb long, spanning the entire transcription unit composed of the proximal unique region followed by the long stretch of short tandem repeats. The second nuclear transcript, the hsrω-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 hsrω-pre-c is spliced out to generate a 1·2 kb third transcript, the hsrω-c, which resides in cytoplasm and carries a small (23–27 amino acid residues in different species) translatable open reading frame, but does not produce any detectable product (Garbe and Pardue 1986; Hovemann *et al* 1986; Fini *et al* 1989). 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 (Garbe and Pardue 1986; Ryseck *et al* 1987; Bendena *et al* 1989). Splicing of the hsrω-pre-c takes place at the site of synthesis (Lakhotia and Sharma 1995) and the spliced out intron persists as a 600 bp fragment (Bendena *et al* 1989).

Like many other heat shock genes, *hsrω* gene is also expressed in cultured cells and during normal development (Bendena *et al* 1991). The presence of *hsrω* transcripts in all cells, except in pre-blastoderm stage embryos and in primary spermatocytes, reflects the essentiality of this non-protein-coding gene during normal development of *Drosophila* (Bendena *et al* 1991; Mutsuddi and Lakhotia 1995). The developmental expression pattern of *hsrω* transcripts is dynamically regulated. In early embryos, low level of ω-c and very little of ω-n and ω-pre-c are present but levels of ω-n and ω-c increase significantly during later stage. During larval development, *hsrω* transcripts are low in second instar but high in third instar and pupal stages. *hsrω* loci in all species of *Drosophila* form developmentally regulated puff during post ecdysone late third instar larval stage and the pattern of changes in RNA levels follow the changes in ecdysone titer

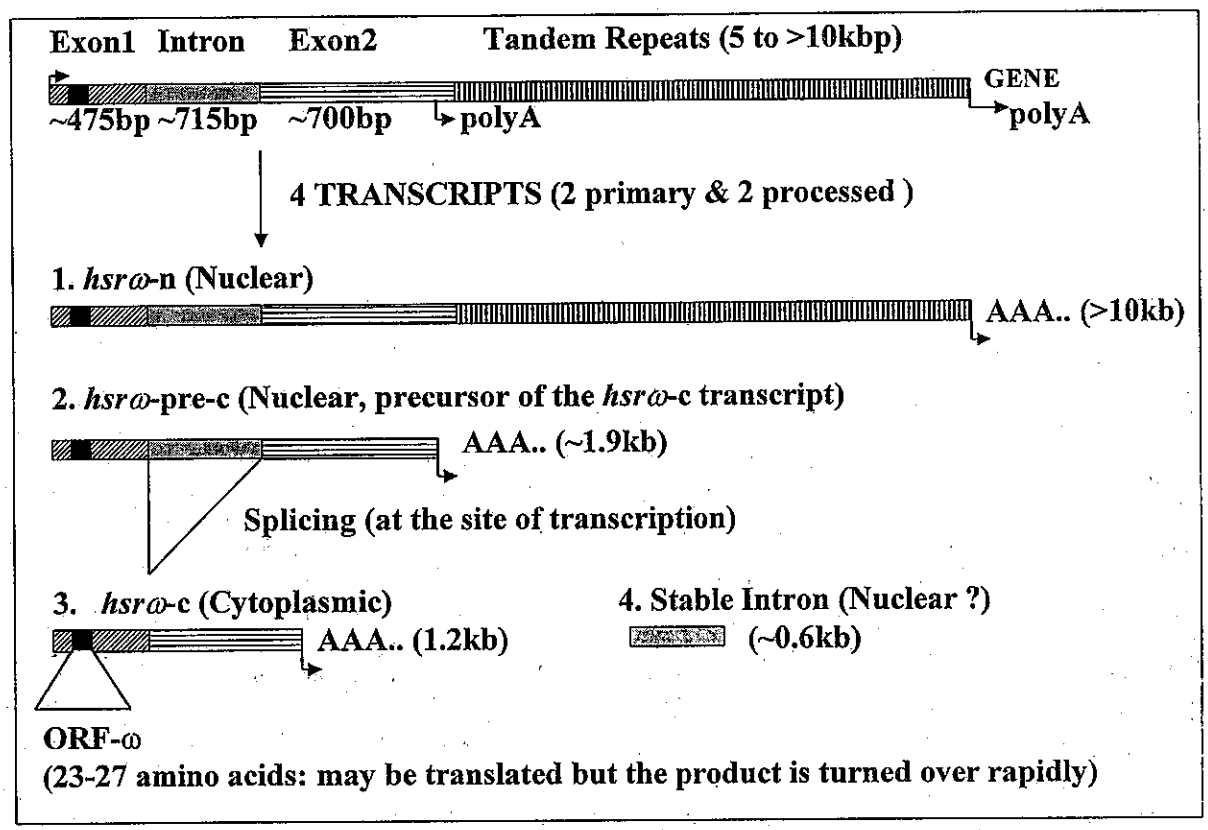

Figure 1. Architecture of the *hsrω* gene and its multiple transcripts in different species of *Drosophila* (not to scale). See text for details.

(Lakhotia 1989; Bendena *et al* 1991). It is also noteworthy that 93D transcripts are highly expressed in prothoracic gland which is the site of ecdysone synthesis (Mutsuddi and Lakhotia 1995).

1.3 *Regulation of the hsrω gene is complex*

The *hsrω* gene displays a complex regulation. A variety of experimental conditions specifically prevent puffing of the 93D locus in heat shocked but not in amide treated salivary gland polytene cells of *D. melanogaster* (reviewed in Lakhotia 1989; Lakhotia and Sharma 1996). Promoter analysis studies by Mutsuddi and Lakhotia (1995) revealed that the regulatory elements for its developmental and heat shock induced activity are mostly located within the −844 bp upstream region. On the other hand, the putative amide response elements (AREs) map far upstream (>22 kb) from the transcription start point (Lakhotia and Tapadia 1998).

Although this locus remains highly active even 30 min after heat shock, the association of heat shock transcription factor (HSTF) with the promoter of *hsrω* gene is very transient, being only for ~5 min (Westwood *et al* 1991). Lakhotia and Mutsuddi (1996) showed that the −844 bp upstream sequence from the transcription start point of *hsrω* gene is sufficient to confer heat inducibility to a *lacZ* reporter gene and that this −844 bp region has three typical heat shock element consensus motifs at −496 bp, −240 bp, and −56 bp, respectively. Reasons for a transient binding of HSTF with the locus in spite of the presence of typical HSEs are not known.

The relative levels of ω-c and ω-n transcripts can be regulated independent of each other in an inducer specific manner. Chemicals like benzamide and colchicine that specifically induce 93D, but not the other heat shock loci, lead to higher induction of the ω-n transcripts, whereas heat shock leads to a relatively greater increase in the level of ω-c (Bendena *et al* 1989; Lakhotia and Sharma 1995). All drugs that inhibit translation, either through initiation or through elongation, stabilize ω-c transcripts which normally turn over within minutes in control cells (Bendena *et al* 1989).

The 93D puff site is associated with unique large-sized RNP particles (Dangli *et al* 1983) and shows specific binding of an intriguing array of antibodies, specially after its induction by heat shock. These include anti-cyclic-guanosine-mono-phosphate (Spruill *et al* 1978) and antibodies against the P11 and Q18 class nuclear proteins (Dangli *et al* 1983; Hovemann *et al* 1991). The P11 and Q18 proteins share homologies with sn-RNP and hnRNP binding proteins (Hovemann *et al* 1991; Buchenau *et al* 1997). Recent observations in our laboratory (Prasanth Kumar K V and Lakhotia S C, unpublished) reveal interesting dynamics of the binding of anti P-11 antibody with the 93D and the Hsp70 coding twin puffs at 87A and 87C sites following heat shock. In control wild type polytene cells, the P11 antibody binds, as reported earlier (Dangli *et al* 1983; Hovemann *et al* 1991), with a large variety of active sites but soon after heat shock, the P11 binding becomes more and more concentrated at the 93D puff site, with a corresponding withdrawal from other sites so that by 40 min of heat shock, the P11 antibody is localized exclusively at the 93D puff only. Interestingly, however, between 5 and 10 min of heat shock, the P11 antibody also binds with the 87A and 87C puff sites. In benzamide and colchicine treated wild type cells, the P11 antibody binding to the 93D puff is greatly increased but many other sites still show the presence of the P11 protein. Wild type cells given heat shock following benzamide treatment or in the presence of colchicine, do not show the 93D puff and correspondingly, the P11 antibody binding to this site is less but not absent. Intriguingly, however, the 87A puff now shows a significant presence of P11. In *hsrω*-nullosomic [*Df(3R)e*^{Gp4}/*Df(3R)GC14*] polytene nuclei, the P11 antibody always shows a strong presence at the 87A and 87C puffs following heat shock (Prasanth Kumar K V and Lakhotia S C, unpublished). It appears that the *hsrω* transcripts function as "sink" for the P11 family of hnRNP and snRNP-binding proteins (Hovemann *et al* 1991; Buchenau *et al* 1997): it remains to be known if the binding of these proteins with the *hsrω* transcripts activates or inactivates these proteins.

Another very interesting observation reported by Morcillo *et al* (1993) was that within 5 min of heat shock, the Hsp83 gets localized on the 93D puff in polytene chromosome of 3rd instar larvae of *D. melanogaster*. Intriguingly, Hsp83 was not found to be associated with benzamide induced puff indicating that this association is inducer specific (Morcillo *et al* 1993).

1.4 *Possible functions of hsrω locus*

The *hsrω* locus has been suggested to influence transcription and/or turnover of the transcripts from the twin sites at 87A and 87C which carry multiple copies of the heat inducible *hsp70* genes (Lakhotia 1989; Sharma and Lakhotia 1995, 1996). In addition, the presence of a short open reading frame in the smaller hsrω-c transcript has been suggested to be related to its role in monitoring the efficiency of cytoplasmic translation process (Lakhotia 1989; Lakhotia and Sharma 1996). Dynamics of the binding of the P11 class of nuclear proteins with the *hsrω* locus, as noted above, is perhaps related to a role of these transcripts in processing and transport of various other transcripts. The *hsrω* gene is also important for acquisition of thermo-tolerance (Lakhotia 1987). McKechnie *et al* (1998) have recently shown an interesting correlation between allelic variation at the *hsrω* locus and thermoresistance in flies. The specific manner

in which the different hsr-ω transcripts mediate these varied activities is not understood.

Absence of point mutations at the locus has been a major handicap in analysing its functions. In an extensive screen to obtain mutations at this site, Mohler and Pardue (1982) failed to obtain any point mutations at this locus but recovered small deficiencies in the 93D cytogenetic region some of which, like *Df(3R)e*^{Gp4} and *Df(3R)GC14*, have been very useful in analysis of the possible functions of this locus. A detailed cytogenetic analysis by Mohler and Pardue (1984, also see Burma and Lakhotia 1986) showed that in *Df(3R)e*^{Gp4}*/Df(3R)GC14* deficiency trans-heterozygotes, only the 93D6-7 band is homozygously absent and this band harbours the heat and amide inducible site. These deficiency trans-heterozygous individuals are characterized by high mortality and the few escapee flies that survive to adult stage are very weak and die within a few days (Mohler and Pardue 1984).

1.5 *Phenotypic effects of hsrω nullosomy resemble some of the phenotypes due to Ras pathway gene mutations*

Examination of the *hsrω*-nullosomic embryos and the few surviving adult flies revealed (Lakhotia and Ray 1996; P Ray and S C Lakhotia, in preparation) interesting phenotypes: majority of such nullosomics die as early embryos but among some of the embryos that continue to develop, defects are seen in the terminal cuticular head structures and in the organization of ganglia and axonal commissures in abdominal central nervous system. The hatching of the ~20% surviving *hsrω*-nullosomic embryos is delayed; the hatched larvae develop more or less normally to pupal stage but emerge as very weak adult flies which can neither walk properly nor fly (Lakhotia and Ray 1996). All the *hsrω*-nullosomic males are sterile although they seem to have normal-looking testes and motile sperms; on the other hand the ovaries in *hsrω*-nullosomic females show variable defects in differentiation of nurse cells and formation of the ring canals between the nurse cells and the growing oocyte (P Ray and S C Lakhotia, in preparation). It is remarkable that none of the above phenotypes are displayed by all of the *hsrω*-nullosomics which has lead to a suggestion (Lakhotia and Sharma 1996) that this locus is a member of redundant genetic pathway.

It is interesting that the four major phenotypic effects of *hsrω*-nullosomy, viz., early embryonic lethality, abnormal embryonic anterior terminal structures, defects in embryonic CNS and in adult ovarian follicle differentiation in survivors, are shared to varying extents by mutations in several different genes which are involved in the Ras signaling pathways. Thus mutations in the *torso* or *Ras1* genes have a variable effect on the terminal structures in embryos (Sprenger and Nusslein-Volhard 1993; Schnorr and Berg 1996). Mutations in genes like *fra* and *DER* also produce defects in embryonic CNS of *Drosophila* of the kind seen in some of the *hsrω* nullosomics (Kolodzlej *et al* 1996; Schejter and Shilo 1989). *DER*, the *Drosophila* homologue of epidermal growth factor receptor, acts exclusively through Ras pathway and Ras is known to be concentrated in the embryonic CNS (Ezer *et al* 1994). Ovaries of females homozygously mutant for *Ras1*^{5703} or of females carrying the *Ras1*^{5703}*/Ras1*^{E62K} heteroallelic combination show abnormalities like degenerating egg chambers and chambers with fewer nurse cells or misplaced oocyte (Schnorr and Berg 1996). Furthermore, mutations in genes like *Dsrc-kinase*, *hu-li-tai* (*hts*), *kelch*, *cherio* etc., are known to affect growth of the ring canals (see review by Robinson and Cooley 1996) and interestingly, *Dsrc-kinase* mediates its signal through Ras proteins in different organisms.

We have earlier shown that recessive point mutation at the *hsp83* locus has a dominant enhancing effect on the high lethality associated with nullosomy of the *hsrω* in *Df(3R)e*^{Gp4}*/Df(3R)GC14* deficiency trans-heterozygotes (Lakhotia and Ray 1996). Since Hsp83 is also known to be involved in Ras signaling (Doyle and Bishop 1993; Cutforth and Rubin 1994) and since, as noted above, the phenotypic effects of *hsrω* nullosomy are similar to those due to mutations in some of the Ras signaling pathway genes, we have examined if mutations at the *Ras* genes in *D. melanogaster* interact with *hsrω* gene. Three *Ras* genes, mapped at 85D (*Dras1*), 64B (*Dras2*) and 62B (*Dras3*) band positions on the third chromosome, are known in *Drosophila* (Neuman-Silberberg *et al* 1984).

Our present results show that mutations at *Dras1* and *Dras3* loci dominantly enhance pre-pupal as well as pupal lethality of the *hsrω* nullosomics and thus for the first time suggest an interesting role of *hsrω* transcripts in the Ras1 mediated signaling cascade during normal development of *D. melanogaster*.

2. Materials and methods

2.1 *Fly strains and culture*

A wild type (Oregon R$^+$), three deficiency mutations in the 93D cytogenetic region viz., *Df(3R)e*^{Gp4}, *Df(3R)GC14* and *Df(3R)e*^{P} (Mohler and Pardue 1984; Lakhotia and Tapadia 1998), two loss of function alleles of the *Ras1* gene (*Ras1*^{E62K} and *Ras1*^{D38N}) and one gain of function mutant allele of the *Ras3* (*Roughened* or *R*) gene (Simon *et al* 1991; Hariharan *et al* 1991) were used in the present study. It is known (Mohler and Pardue 1984; Burma and Lakhotia 1986) that the *Df(3R)e*^{Gp4} (deletion from 93B11-13 to 93D6-7) and *Df(3R)GC14* (deletion from 93D6-7 to 93D10) deletions do not complement

each other and if both these deletions are brought together in trans, only the 93D6-7 band (the site of the *hsrω* gene) on the right arm of chromosome 3 is homozygously deleted. The *Df(3R)e*^{P} chromosome bears a small deletion extending from 93B10-11 to 93D3-5 and in this deletion the far upstream amide response elements of *hsrω* gene are missing but the coding region and the heat shock and developmental response sequences are intact (Lakhotia and Tapadia 1998). The *Df(3R)e*^{Gp4} was maintained against the *TM6B* balancer while the *Df(3R)e*^{P} and *Df(3R)GC14* deficiencies were maintained against *TM6B* as well as the *In(3R)C, Sb e l(3)e* balancers (for details of these balancers and other markers, see Lindsley and Zimm 1992).

Roughened (*R*), identified by Wallace in 1935 (Lindsley and Zimm 1992) and later characterized by Hariharan *et al* (1991) is a gain of function mutation of *Ras3*; it has been renamed by Hariharan *et al* (1991) as *Rap1* due to its high degree of homology with human *rap1A* and *rap1B* genes. In the present study this gene will be designated as *Ras3* and its mutant allele as *R* (*Roughened*) (Hariharan *et al* 1991). *Ras1*^{E62K} and *Ras1*^{D38N} alleles bear point mutations in the coding region of the *Ras1* gene which inactivate the Ras1 protein (Simon *et al* 1991). As all these mutations are recessive lethal, they were maintained against the *TM6B* balancer. Through a series of appropriate crossings, each of the three mutations were separately recombined with *Df(3R)e*^{Gp4} and *Df(3R)e*^{P} chromosomes for genetic interaction studies. In addition, the *R* mutation was also recombined with *Df(3R)GC14* deletion chromosome. The following stocks were thus generated: (i) *Ras1*^{E62K} *Df(3R)e*^{Gp4}*/TM6B*; (ii) *Ras1*^{D38N} *Df(3R)e*^{Gp4}*/TM6B*; (iii) *R Df(3R)e*^{Gp4}*/TM6B*; (iv) *Ras1*^{E62K} *Df(3R)e*^{P}*/TM6B*; (v) *Ras1*^{D38N} *Df(3R)e*^{P}*/TM6B*; (vi) *R Df(3R)e*^{P}*/TM6B*; (vii) *R Df(3R)GC14/TM6B*.

To check the effect of mutations in *Ras1* and *Ras3* genes (*Ras1*^{E62K} or *Ras1*^{D38N} and *Roughened* or *R* alleles, respectively) on the viability of *hsrω*-nullosomic larvae and pupae, flies of the stocks i–vi above were crossed with *Df(3R)GC14/TM6B* to obtain trans-heterozygotes carrying either of the above recombinant chromosomes as one homologue and the *Df(3R)GC14* chromosome as the other homologue of the chromosome 3. Such larvae, pupae and adult flies show non-tubby phenotype while the sibs heterozygous for the *TM6B* balancer and one of the deficiency chromosomes show a tubby phenotype due to the *Tb* marker on the balancer chromosome (see Lindsley and Zimm 1992). *R Df(3R)GC14/TM6B* and *Df(3R)e*^{P}*/TM6B* flies were inter crossed to generate *R Df(3R)GC14/R*$^{+}$ *Df(3R)e*^{P} progeny which also could be easily distinguished from *R Df(3R)GC14/TM6B* and *Df(3R)e*^{P}*/TM6B* sibs by being non-tubby.

Since *TM6B* balancer carrying embryos cannot be phenotypically distinguished from those not carrying the balancer and because the *TM6B* homozygous embryos also die early, the viability of *hsrω*-nullosomic embryos carrying mutation at the *Ras1* or *Ras3* genes was examined by crossing flies of stocks i–vi above with *Df(3R)GC14/ In(3R)C, Sb e l(3)e* flies rather than with *Df(3R)GC14/ TM6B* flies. Since the *In(3R)C, Sb e l(3)e/TM6B* embryos have more or less normal viability, only the *hsrω*-nullosomic embryos are expected to die in the progeny of this cross. Eggs from these crosses were collected at fixed time intervals and the eggs that did not hatch till 48 h after laying were counted to determine the embryonic lethality. Frequency of eggs that did not likewise hatch in the *Df(3R)e*^{Gp4}*/TM6B* x *Df(3R)GC14/In(3R)C* cross served as control.

All fly cultures were maintained on standard agar-maize powder-sugar-yeast food at 21°C±1°C.

2.2 *Cuticular preparations of embryos*

Ras1^{E62K} *Df(3R)e*^{Gp4}*/TM6B*, *R Df(3R)e*^{Gp4}*/TM6B*, *Df(3R)e*^{Gp4}*/ TM6B* and *Df(3R)GC14/TM6B* flies were separately crossed with wild type (Oregon R^{+}) flies and the non-tubby progeny flies carrying the *Df(3R)e*^{Gp4} or the *Df(3R)GC14* deficiency chromosomes were inter-crossed as: (i) *Ras1*^{E62K} *Df(3R)e*^{Gp4}*/+* x *Ras1*$^{+}$ *Df(3R)GC14/+*; (ii) *R Df(3R)e*^{Gp4}*/+* x *R*$^{+}$ *Df(3R)GC14/+*; (iii) *Df(3R)e*^{Gp4}*/+* x *Df(3R)GC14/+*.

Eggs were collected from the above mass matings and the embryos that did not hatch even 48 h after egg collection were directly mounted in a mixture of 2 : 1 : 1 Hoyer's medium: lactic acid: water (Wieschaus and Nusslein-Volhard 1986). After overnight incubation at 60°C, they were examined under microscope to see if mutation at the *Ras1* or *Ras3* gene has additional effect on the abnormalities in anterior terminal structures seen in *hsrω*-nullosomic embryos.

3. Results

3.1 *Mutations in Ras1 or Ras3 genes dominantly enhance post-embryonic but pre-pupal lethality of hsrω nullosomics*

It is known from our earlier studies (Lakhotia and Ray 1996) that nearly 80% of the *hsrω*-nullosomic embryos (*Df(3R)e*^{Gp4}*/Df(3R)GC14*) do not hatch and the remaining ones are delayed in hatching but once hatched, nearly all of the *hsrω*-nullosomic larvae develop normally to pupal stage. Viability of *hsrω*-nullosomics in the presence of *Ras1* or *Ras3* mutant allele in heterozygous condition was examined by looking for the relative proportions of tubby (monosomic for *hsrω*) and non-tubby (*hsrω*-nullosomic) pupae in the progeny of appropriate crosses.

As shown in figure 2, presence of *Ras1*^{E62K} or *R* in heterozygous condition drastically reduced the survival of the *hsrω*-nullosomics to pupal stage from 20% to

8%. Similar reduction in survival of *hsrω*-nullosomics to pupal stage was also seen when in combination with the $Ras1^{D38N}$ mutant allele (data not shown). To find out whether this lethal enhancer effect of $Ras1^{E62K}$ or *R* mutant alleles was specifically due to absence of the *hsrω* gene, additional crosses were carried out using the $Df(3R)e^{P}$ deletion which maps just outside the coding region of *hsrω* gene (Lakhotia and Tapadia 1998). For this, the $Ras1^{E62K}$ and *R* mutations were recombined with $Df(3R)e^{P}$ and *Df(3R)GC14* chromosomes, respectively, and appropriate crosses were carried out to bring these recombined chromosomes ($Ras1^{E62K}$ $Df(3R)e^{P}$ or *R Df(3R)GC14*) in trans to *Df(3R)GC14* or $Df(3R)e^{P}$ chromosome, respectively. The survival of such transheterozygotes i.e., $Ras1^{E62K}$ $Df(3R)e^{P}/Ras1^{+}$ *Df(3R)GC14* (110%) and *R Df(3R)GC14*/R^{+} $Df(3R)e^{P}$ (114%), having only one functional copy of *hsrω* gene, was only marginally reduced when compared to the viability of $Df(3R)e^{P}$/*Df(3R)GC14* (131%) individuals not carrying any mutant allele at the *Ras1* or the *Ras3* locus (figure 2).

To check if the enhanced lethality of *hsrω*-nullosomics in the presence of mutant allele at the *Ras1* or *Ras3* locus was embryonic or post-embryonic, the percentages of the dying embryos of $Ras1^{E62K}$ $Df(3R)e^{Gp4}$/*TM6B* X *Ras1*$^{+}$ *Df(3R)GC14/In(3R)C* and *R* $Df(3R)e^{Gp4}$/*TM6B* X R^{+} *Df(3R)GC14/In(3R)C* were compared. It was found that 18·5% and 19·6%, respectively, of total embryos died in these crosses and about 5% hatched later than the sibs carrying one or two copies of the *hsrω* locus. These values are similar to those seen for *hsrω*-nullosomic embryos carrying wild type alleles of the *Ras1* or *Ras3* genes.

Since the embryonic lethality of *hsrω*-nullosomics was not enhanced by *Ras* mutations but fewer of such hatched larvae pupated (see above and figure 2), it was suspected that there was a greater death at larval stage. To examine this, larval stages in the progeny of crosses $Ras1^{E62K}$ $Df(3R)e^{Gp4}$/*TM6B* X *Ras1*$^{+}$ *Df(3R)GC14/TM6B* and *R* $Df(3R)e^{Gp4}$/*TM6B* X R^{+} *Df(3R)GC14/TM6B* were periodically examined for the presence of dead individuals. While the *hsrω*-nullosomic larvae were non-tubby, those carrying one of the deficiency chromosome and the TM6B balancer were tubby in appearance. Only non-tubby 1st as well as 2nd instar larvae were seen to die. Interestingly, some non-tubby larvae of genotype $Ras1^{E62K}$ $Df(3R)e^{Gp4}/Ras1^{+}$ *Df(3R)GC14* (~30% of the delayed hatched larvae) persisted as second instar for a prolonged duration which in some cases was up to ~164 h after hatching.

3.2 *Ras1 or Ras3 gene mutation does not enhance embryonic defects due to hsrω-nullosomy*

The *hsrω*-nullosomic embryos are known to die at varying stages and among those that die at later stages of development, some show abnormal anterior terminal structures (P Ray and S C Lakhotia, in preparation). Examination of dead embryos (those that did not

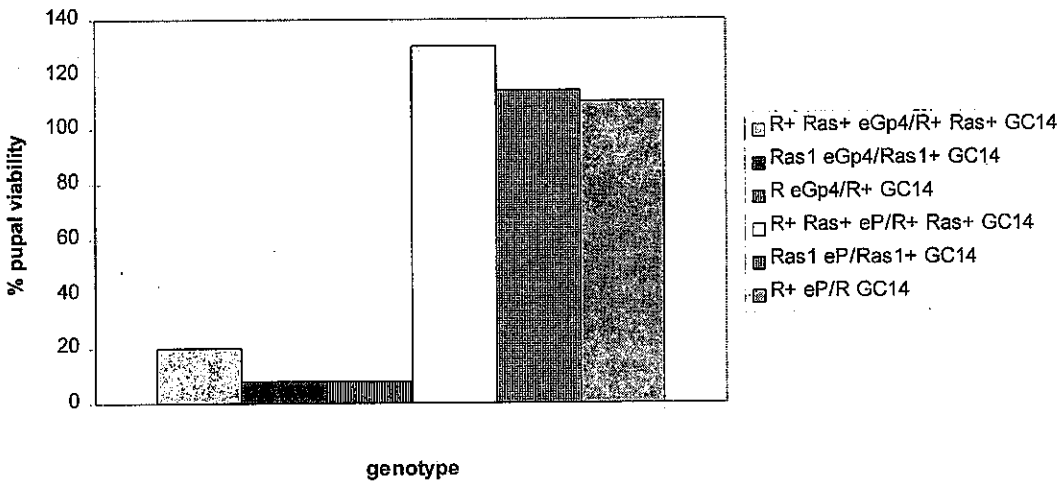

Figure 2. Relative survival of embryos carrying mutations in both the homologues in the *93D* cytogenetic region in the presence of wild type or $Ras1^{E62K}$ or *Roughened* alleles to pupal stage (relative survival was calculated as described by Lakhotia and Ray 1996). Survival of *hsrω*-nullosomics to pupal stage is greatly reduced in the presence of $Ras1^{E62K}$ or *Roughened* alleles (compare column 1 with 2 and 3). $Ras1^{E62K}$ and *Roughened* mutations have only a marginal effect on the survival to pupal stage of embryos carrying one functional copy of *hsrω* gene (compare column 4 with 5 and 6). eGp4, $Df(3R)e^{Gp4}$; GC14, *Df(3R)GC14*; eP, $Df(3R)e^{P}$; Ras1, $Ras1^{E62K}$; R, *Roughened* (~500 pupae were examined for each cross).

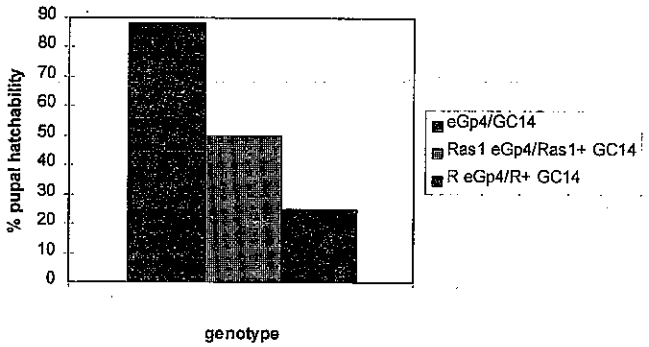

Figure 3. Histograms showing hatchability of *hsrω*-nullosomic pupae in the presence of mutant alleles of *Ras1* or *Ras3* loci. Note the greatly reduced viability of *hsrω*-nullosomic pupae in the presence of mutant alleles of *Ras1* ($Ras1^{E62K}$) or *Ras3* (*R*). eGp4, $Df(3R)e^{Gp4}$; GC14, *Df(3R)GC14*; eP, $Df(3R)e^{P}$; Ras1, $Ras1^{E62K}$; R, *Roughened* (~ 300 pupae were examined for each cross).

hatch even after 48 h) from the crosses $Ras1^{E62K}$ $Df(3R)e^{Gp4}$/+ X $Ras1^{+}$ *Df(3R)GC14*/+, and *R* $Df(3R)e^{Gp4}$/+ X R^{+} *Df(3R)GC14*/+ showed that, as in the case of dead embryos from the cross $Df(3R)e^{Gp4}$/+ X *Df(3R)GC14*/+, these embryos had also been arrested at different stages of embryonic development i.e., either at very early stage (without any cuticular structures) or at later stages of development (with cuticular structures). The dead *hsrω*-nullosomic embryos carrying *Ras1* or *Ras3* gene mutation that had developed cuticular structures were again of two types viz., (i) embryos with normal anterior and posterior terminal structures and ventral denticular bands and (ii) embryos with normal ventral denticular bands and posterior terminal structures but with defective head structure. The frequency of dead embryos with defective anterior terminal structures in progeny of crosses $Ras1^{E62K}$ $Df(3R)e^{Gp4}/Ras1^{+}$ *Df(3R)GC14* (11·3%), and *R* $Df(3R)e^{Gp4}/R^{+}$ *Df(3R)GC14* (15%) was only marginally greater than that in the progeny of cross $Df(3R)e^{Gp4}$/+ and *Df(3R)GC14*/+ (10%).

3.3 *Ras1 or Ras3 mutations dominantly enhance lethality of hsrω-nullosomic pupae*

About 12% of the *hsrω*-nullosomic pupae, carrying all wild type alleles at the *Ras1* or *Ras3* loci, do not eclose as adults. However, in the presence of one mutant allele at either of these loci, the viability of *hsrω*-nullosomic pupae is greatly reduced (see figure 3) with only about 50% and 25%, respectively, of such pupae eclosing.

4. Discussion

As discussed elsewhere (Lakhotia and Ray 1996; Ray and Lakhotia, in preparation), the lethal and other mutant phenotypes seen in $Df(3R)e^{Gp4}/Df(3R)GC14$ genotype are essentially due to nullosomy of the *hsrω* locus only and not due to monosomy or nullosomy of any other flanking genes. We had shown earlier (Lakhotia and Ray 1996) that only about 20% of the $Df(3R)e^{Gp4}/Df(3R)GC14$ (*hsrω*-nullosomic) embryos develop to pupal stage and that this poor viability of *hsrω*-nullosomic embryos was further drastically affected in the presence of a recessive lethal mutation of the *hsp83* gene so that only about 1–2% of the *hsrω*-nullosomic embryos that were heterozygous for the *hsp83* gene mutation survived to pupal stage. Our present study showed that heterozygosity for *Ras1* or *Ras3* mutant alleles also severely affected survival of *hsrω*-nullosomics to pupal and imago stages. It is significant that the survival of $Ras1^{E62K}$ $Df(3R)e^{P}/Ras1^{+}$ *Df(3R)GC14* or *R* *Df(3R)GC14/R*$^{+}$ $Df(3R)e^{P}$ genotypes carrying one, at least partly functional, copy of *hsrω* on the $Df(3R)e^{P}$ homologue (see Lakhotia and Tapadia 1998), was not as much reduced as in the case of *hsrω*-nullosomics. It may further be noted that the lethal enhancing effect of the *Ras* mutations on *hsrω*-nullosomics is unlikely to be due to some other genetic factor that may have been inadvertently introduced while recombining the Ras mutant alleles with the $Df(3R)e^{Gp4}$ chromosome since crossing of *R* *Df(3R)GC14/TM6B* flies with R^{+} $Df(3R)e^{Gp4}$*/TM6B* flies also showed a comparable enhancement in lethality of the *hsrω*-nullosomic progeny (data not presented). Therefore, the dominant lethal enhancing effect of the *Ras1* or the *Ras3* mutant alleles, as of the earlier reported *hsp83* mutation (Lakhotia and Ray 1996), seems to be specifically related to complete absence of both copies of the *hsrω* locus in $Df(3R)e^{Gp4}$/ *Df(3R)GC14* individuals.

It is interesting that while the enhanced lethality of *hsrω*-nullosomics due to heterozygosity for *hsp83* gene mutation was most pronounced in the first instar larval stage (Lakhotia and Ray 1996), the enhanced lethality due to mutation in *Ras1* or *Ras3* genes occurred in both 1st and 2nd instar larval stages. Like the *hsp83* mutant homozygotes, the $Ras1^{E62K}$, or *R* homozygotes also survive till at least the second instar stage (Hariharan *et al* 1991; Simon *et al* 1991) due to the availability of these gene products from the maternal source till the first or the second instar stages. Therefore, it appears that as the maternal source for these proteins (Ras1 or Ras3) begins to attenuate, the heterozygotes do not make enough Ras1 or Ras3 protein and this begins to affect the *hsrω*-nullosomic survivors. While a limiting supply of zygotically synthesized normal Ras1 or Ras3 due to heterozygosity for a mutant allele at respective locus does not have any deleterious effect in embryos carrying at least one functional copy of the *hsrω* gene, this becomes lethal when no functional copy of the *hsrω* locus is available. The prolonged second instar stage of some *hsrω*-nullosomic larvae heterozygous for $Ras1^{E62K}$ mutant

allele also suggests essential interaction between these gene products for normal development.

While most of the surviving *hsrω*-nullosomic larvae pupate and develop to adulthood (figure 3, Lakhotia and Ray 1996), heterozygosity for mutation at the *Ras1* or *Ras3* loci considerably reduced the viability of the very rare such larvae that pupated (figure 3). This suggests that another critical period for interaction of *Ras* and *hsrω* genes exists during pupal development. Viability of *hsrω*-nullosomic pupae in the presence of a mutant *hsp83* allele is also similarly affected (unpublished).

More or less similar dominant enhancing effects of mutations in three (*hsp83*, *Ras1* and *Ras3*) different genes on the *hsrω*-nullosomy phenotype suggests that all of them may be involved in some common pathway. The Hsp83 of *D. melanogaster* is a member of the Hsp90 family which, besides being heat inducible, constitutes a major component of the normal cellular proteins. The Hsp90 family chaperone proteins are known to be associated with a variety of cellular proteins (see review by Csermely *et al* 1997). Genetic interaction studies have demonstrated a role for the Hsp83 protein of *Drosophila* in the Sevenless and Torso receptor tyrosine kinase (RTK) signaling cascades (Cutforth and Rubin 1994; Doyle and Bishop 1993). Both these RTKs are known to mediate signals through a common Ras1 signaling cascade (Simon *et al* 1991; Hafen *et al* 1993; Lu *et al* 1993). Ras1 is also known to be required in *Drosophila* to relay signal from D-Src, a non-receptor tyrosine kinase, in embryonic or photoreceptor development (Kussick *et al* 1993) and possibly in other unidentified cascades as well (Schnorr and Berg 1996). Involvement of Ras in Src mediated signaling cascades is also known in other organisms, e.g., injection of neutralizing anti-Ras monoclonal antibodies (Smith *et al* 1986) and expression of a dominant interfering allele of *c-ras* (Feig and Cooper 1988) suppress *v-src* transformation in fibroblasts. In mammalian cells, Hsp90 is well known for its association with pp60src, the viral counterpart of *c-src* gene product in mammalian cells (Brugge 1986). Likewise, a reduction in the level of Hsp82 in yeast suppresses the lethality caused by expression of *v-src* (Boschelli 1993). The striking parallels in the involvement of Ras1 and Hsp90 in different protein tyrosine kinase mediated signaling cascades in diverse species suggests that these two gene families interact in important steps in signaling cascades mediated by a wide variety of receptor and non receptor tyrosine kinases.

Ras3, another member of the three *Drosophila Ras* genes and closer to the human *rap1A* and *rap1B* genes than human *ras* genes, is also known to be involved in the Sevenless signaling cascade probably by antagonizing the function of *Ras1* (Hariharan *et al* 1991). A similar antagonistic role of *Ras1* and *Ras3* has also been supported by the observation that the phenotype of *ras*-transformed fibroblasts can be reverted by overexpression of the human *rap1A* gene (Kitayama *et al* 1989). The *Roughened* mutation is a gain of function mutation of *Ras3* gene and, therefore, its phenotypic effect is similar to the effect of a loss of function mutation of *Ras1* (Hariharan *et al* 1991; present study).

Since the *hsrω* transcripts do not give rise to any protein (see § 1.2), it is obvious that effect/s of the *hsrω* gene are mediated through its transcripts. Although an increasing numbers of genes are now known to function without producing translatable RNAs (Lakhotia 1996), the specific ways in which "non-coding" transcripts function remain little understood. The involvement of a non-coding *hsrω* transcripts in a conserved signaling cascade operating through various membrane bound and cellular kinases is rather intriguing. Nevertheless, it has become apparent in recent years that in addition to their classical roles as tRNAs, rRNAs and mRNAs etc., RNAs have a myriad of other functional roles in both cytoplasm and nucleus (Lakhotia 1996; Moore 1996; Tycowski *et al* 1996). Hsp90 is also known to posses DNA and RNA binding properties (Csermely *et al* 1997; Soti and Csermely 1998) and the LKVIRK-region of the Hsp90 is homologous with the RNA-binding domain of cauliflower mosaic virus RNA-movement protein (Koonin *et al* 1991). 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 involved in Ras/Src signaling (Taylor and Shalloway 1994; Neel *et al* 1995), although the nature of such RNAs and the reasons for their binding are not yet known.

Results from present study provide indirect evidence for association of a non translatable, heat and other stress inducible RNA with these signaling proteins and with a chaperone protein. Further studies will unravel the significance and mechanism of this interesting interaction.

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