Male sterility associated with overexpression of the noncoding *hsrw* gene in cyst cells of testis of *Drosophila melanogaster*

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Abstract

Of the several noncoding transcripts produced by the *hsrw* gene of *Drosophila melanogaster*, the nucleus-limited > 10-kb hsrω-n transcript colocalizes with heterogeneous nuclear RNA binding proteins (hnRNPs) to form fine nucleoplasmic omega speckles. Our earlier studies suggested that the noncoding hsrω-n transcripts dynamically regulate the distribution of hnRNPs in active (chromatin bound) and inactive (in omega speckles) compartments. Here we show that a P transposon insertion in this gene's promoter (at – 130 bp) in the $hsrw^{05241}$ enhancer-trap line had no effect on viability or phenotype of males or females, but the insertion-homozygous males were sterile. Testes of $hsrw^{05241}$ homozygous flies contained nonmotile sperms while their seminal vesicles were empty. RNA : RNA *in situ* hybridization showed that the somatic cyst cells in testes of the mutant male flies contained significantly higher amounts of hsrω-n transcripts, and unlike the characteristic fine omega speckles in other cell types they displayed large clusters of omega speckles as typically seen after heat shock. Two of the hnRNPs, viz. HRB87F and Hrp57A, which are expressed in cyst cells, also formed large clusters in these cells in parallel with the hsrω-n transcripts. A complete excision of the P transposon insertion restored male fertility as well as the fine-speckled pattern of omega speckles in the cyst cells. The *in situ* distribution patterns of these two hnRNPs and several other RNA-binding proteins (Hrp40, Hrb57A, S5, Sxl, SRp55 and Rb97D) were not affected by *hsrw* mutation in any of the meiotic stages in adult testes. The present studies, however, revealed an unexpected presence (in wild-type as well as mutant) of the functional form of Sxl in primary spermatocytes and an unusual distribution of HRB87F along the retracting spindle during anaphase–telophase of the first meiotic division. It appears that the P transposon insertion in the promoter region causes a misregulated overexpression of *hsrw* in cyst cells, which in turn results in excessive sequestration of hnRNPs and formation of large clusters of omega speckles in these cell nuclei. The consequent limiting availability of hnRNPs is likely to *trans*-dominantly affect processing of other pre-mRNAs in cyst cells. We suggest that a compromise in the activity of cyst cells due to the aberrant hnRNP distribution is responsible for the failure of individualization of sperms in $hsrw^{05241}$ mutant testes. These results further support a significant role of the noncoding hsrω-n transcripts in basic cellular activities, namely regulation of the availability of hnRNPs in active (chromatin bound) and inactive (in omega speckles) compartments.

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Introduction

Ever since its discovery as a unique benzamide-induced puff (Lakhotia and Mukherjee 1970), the 93D locus of *Drosophila melanogaster* has remained intriguing. The 93D or the *hsrw* gene is transcribed in most cells under normal conditions to produce several noncoding transcripts, whose levels are further enhanced following heat shock or amide treatment (see recent review by Lakhotia and Sharma 1996). Functions of this gene remained elusive owing to the noncoding nature of its transcripts and absence of mutant alleles with a visible phenotype. Recent studies in our laboratory revealed that the more than 10 kb long hsrω-n transcript, which remains confined

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to nucleus (Garbe *et al*. 1986), is essential for organizing a novel nucleoplasmic structure, the omega speckles (Lakhotia *et al*. 1999; Prasanth *et al*. 2000), which are distinct from the well-known inter-chromatin granule clusters or IGCs (Misteli and Spector 1997). We suggested that the omega speckles regulate nuclear trafficking and storage of a variety of heterogeneous nuclear RNA-binding proteins (hnRNPs) and some other related RNA-binding proteins (Lakhotia *et al*. 1999; Prasanth *et al*. 2000).

The processing of nascent transcripts in a eukaryotic nucleus is a very precise and important activity involving many types of proteins, of which the hnRNPs form a large multimembered family. The hnRNPs are generally associated with RNA polymerase II transcribed nascent transcripts and are involved in an array of functions extending from packaging of pre-mRNAs to post-transcriptional processing including alternative splicing and transport (Krecic and Swanson 1999). The proposed involvement of hsrω-n transcripts in organizing the omega speckles for regulation of the intranuclear trafficking and availability of hnRNPs (Lakhotia *et al*. 1999; Prasanth *et al*. 2000) suggests that perturbations in *hsrw* gene function is likely to affect hnRNP metabolism in the nucleus. This in turn can have cascading pleiotropic effects.

With a view to examine such possibilities, we looked for mutations in the *hsrw* gene by P transposon insertion. We identified one such P transposon insertion line, *l(3)05241*, generated during lethal P insertion screen by Spradling *et al*. (1995), as a mutant allele of the *hsrw* locus (Lakhotia *et al*. 2001) and renamed this as $hsrw^{05241}$. The $hsrw^{05241}$ chromosome has a *P-LacZ-rosy*$^{+}$ transposon inserted at – 130 bp position in the promoter region of the *hsrw* gene. Flies homozygous for this P transposon insertion show normal morphology and viability, and as we reported earlier (Lakhotia *et al*. 2001) the expression of *hsrw* in all somatic cells, from embryo to adult, in the $hsrw^{05241}$ homozygotes is essentially similar to that in wild type. Interestingly, however, while the $hsrw^{05241}$ homozygous females are fertile, the males are completely sterile. Our present results showed that the testes of $hsrw^{05241}$ homozygous male flies were full of bundles of nonmotile sperms while the seminal vesicles remained empty. To examine if the abnormal sperm development was associated with altered expression patterns of *hsrw* transcripts and/or various nuclear proteins that may interact with these noncoding transcripts, we examined the *in situ* localization of hsrω-n transcripts and several nuclear RNA-binding proteins in various cell types of testes in wild-type and mutant flies. Our results show that the P transposon insertion in the promoter region in the $hsrw^{05241}$ allele results in a significantly enhanced level of hsrω-n transcripts in the somatic cyst cells of adult testes. This is associated with an altered organization of omega speckles in the cyst cell nuclei. The $hsrw^{05241}$ mutation had no effect on expression of *hsrw* and synthesis of several RNA-binding proteins in any of the meiotic cell types. Since a clustering of omega speckles, as happens after heat shock in wild-type cells, is associated with reduced RNA-processing activities in the nucleus (Lakhotia *et al*. 1999; Prasanth *et al*. 2000), the clustering of omega speckles in $hsrw^{05241}$ mutant cyst cells may be expected to disrupt the normal hnRNP metabolism and RNA processing in the nucleus. Cyst cells have essential functions in growth and differentiation of sperms during their long period of association with a cyst of developing germ cells starting from the founder spermatogonial cell till the individualization of 64 sperms (Fuller 1993). We suggest that the aggregation of nuclear hnRNPs due to the overabundant hsrω-n RNA adversely affects cyst cell functions and this ultimately results in the production of nonindividualized and nonmotile sperms.

Materials and methods

***Fly strains*:** Larvae and flies of wild-type (Oregon R^{+}) and mutant stocks ($hsrw^{05241}$*/TM6B, Df(3R)GC14/TM6B,* $Df(3R)e^{GP4}$*/TM6B,* $Df(3R)e^{P}$*/TM6B,* ry^{506} and *D2-3 Sb/TM6B, Tb*) were maintained on standard *Drosophila* food medium at 22 ± 1°C (for details of the mutant names etc., other than those described below, see Lindsley and Zimm 1992). The $hsrw^{05241}$ chromosome carries a *P-LacZ-rosy*$^{+}$ transposon inserted at – 130 bp position of the *hsrw* gene (Lakhotia *et al*. 2001).

To eliminate background mutations, if any, the $hsrw^{05241}$*/TM6B* chromosome was freely floated for 10 generations through the ry^{506} female germline. The $hsrw^{05241}$*/TM6B* flies were mated with ry^{506} flies and the resulting ry^{+} ($hsrw^{05241}/ry^{506}$) progeny females were pair-mated with $hsrw^{05241}$*/TM6B* males. This process was repeated for 10 generations. In each generation, about 50 male flies that carried two copies of the P insertion were checked for fertility by crossing them with wild-type females.

Complementation mapping of the male-sterile phenotype associated with $hsrw^{05241}$ mutation was carried out using three deficiency chromosomes, viz. *Df(3R)GC14* (93D6-7 to 93D10 region deleted), $Df(3R)e^{GP4}$ (93B12-13 to 93D6-7 region deleted; Mohler and Pardue 1984) and $Df(3R)e^{P}$ (93B6-7 to 93D3-5 region deleted; Lakhotia and Tapadia 1998).

The P transposon in $hsrw^{05241}$ was mobilized by crossing $hsrw^{05241}$ female flies with *D2-3 Sb/TM6B, Tb* males following standard procedures (Bier *et al*. 1989) and lines that had lost the P transposon were selected. Four lines that showed full excision of the *P-LacZ-rosy*$^{+}$ transposon were named $hsrw^{05241\text{–R1}}$ to $hsrw^{05241\text{–R4}}$.

***PCR mapping of P transposon insertion in* $hsrw^{05241}$ *and the excision-revertant lines*:** Genomic DNAs from wild-type, $hsrw^{05241}$ and the four excision-revertant lines ($hsrw^{05241\text{–R1}}$

to *hsrw*$^{05241-R4}$) were subjected to PCR with combinations of primers corresponding to different regions of the *hsrw* gene and the P-element end. The following primers were used: (i) RIP, a 27-bp primer corresponding to + 145 bp to + 171 bp region (5′-ACTCGTACTGCTGCTGCTCGTCTGCTG-3′) of the first exon of *hsrw* gene (for sequence, see Ryseck *et al*. 1987); (ii) L226P, a 27-bp primer for – 222 bp to – 196 bp region (5′-CCATATGTATGTGCTTAACCGGCTTA-3′) of the *hsrw* promoter (for sequence, see Mutsuddi and Lakhotia 1995); (iii) PP, a 31-bp primer for the 31-bp P-element terminal inverted repeat (5′-CGACGGGACCACCTTATGTTATTTCATCATG-3′).

The PCR amplification was carried out in a final volume of 50 µl containing 20 pM each of two primers (RIP and PP or L226P and PP or L226P and RIP combinations), 200 µM of each dNTP (New England Biolabs, USA) and 3 U of *Taq* polymerase (Bangalore Genei, India). The cycling parameters included an initial denaturation for 3 min at 94°C followed by 30 cycles of denaturation at 94°C for 1 min, 1 min annealing at 62°C, and extension at 72°C for 1 min, except the last cycle where the extension time was 5 min. Ten µl of the PCR product was loaded on a 1.5% agarose gel to check for amplification and size of the amplicons.

***Phenotype of male reproductive organs*:** Internal reproductive organs of 3-day-old male flies of different genotypes (see Results) were dissected and directly mounted in Poels' salt solution (PSS, Lakhotia and Tapadia 1998) under a coverslip or were teased open and then mounted under a coverslip.

***Giemsa staining of adult testes*:** Testes from 3–4-day-old flies of different genotypes were dissected in PSS, fixed in 4% paraformaldehyde for 10 min and stained with Giemsa (pH 10) for 15 min following Bonaccorsi *et al*. (1988).

***Whole testis immunostaining*:** Intact testes from 3-day-old wild-type and *hsrw*^{05241} males were immunostained using antibody against the Rb97D protein (Heatwole and Haynes 1996) or the 7Fb antibody against Hsp70 (Velazquez and Lindquist 1984) as described earlier (Prasanth *et al*. 2000).

***RNA : RNA in situ hybridization (RISH)*:** To compare expression of *hsrw* in testes in wild-type and *hsrw*^{05241} mutant and *hsrw*$^{05241-R}$ flies, a mix of digoxigenin (dig)-labeled riboprobes derived from pJG10 and pDRM30 clones of the *hsrw* gene (Lakhotia and Sharma 1995) were hybridized *in situ* to RNA in intact testes as described earlier (Prasanth *et al*. 2000). Hybridization was detected colorimetrically using alkaline phosphatase (AP)-conjugated anti-dig antibody (Roche, Germany).

***In situ hybridization to hsrw transcripts and immunolocalization of RNPs in testis cells*:** Testes from 3–4-day-old adult males (wild type, *hsrw*^{05241}, *hsrw*$^{05241-R1}$ or *Df(3R)e*^{Gp4}*/Df(3R)GC14)* were dissected in PSS and either incubated at room temperature (control) or heat-shocked (HS) at 37°C for 40 min. Lightly squashed preparations of control and heat-shocked testes were processed for RISH using dig-labeled pDRM30 riboprobe. Rhodamine-tagged anti-dig antibody was used to detect the hybridization as described earlier (Prasanth *et al*. 2000). After the RISH, the squash preparations were processed for immunostaining with antibodies against various RNA-binding proteins like Sxl (Samuels *et al*. 1994), HRB87F (hnRNP A1 homologue, Hovemann *et al*. 1991), Hrb57A (hnRNP K homologue, Hovemann *et al*. 2000), S5 (hnRNP M homologue, H. Saumweber and K. H. Glaetzer, personal communication; Saumweber *et al*. 1980), Hrp40 (or Squid, hnRNP A1/2 homologue, Matunis *et al*. 1992), SRp55 (or B52, a 52-kDa RNA-binding protein of the SR family, Champlin *et al*. 1991) and Rb97D (Heatwole and Haynes 1996), and antibody binding was detected through FITC-conjugated secondary antibody as described earlier (Prasanth *et al*. 2000).

In another set, squash preparations of testes from wild-type or *hsrw*^{05241} or *Df(3R)e*^{Gp4}*/Df(3R)GC14* flies were directly processed for immunostaining with the antibodies against RNA-binding proteins. Antibody binding was detected through FITC-conjugated or rhodamine-conjugated secondary antibody as described earlier (Prasanth *et al*. 2000). In some cases (see Results), the slides were counterstained with DAPI to reveal chromatin. The slides were stored at – 20°C till examination in a Nikon E800 fluorescence microscope.

***Polyacrylamide gel electrophoresis and Western blotting*:** Protein samples were prepared from adult testes, adult ovaries, or all internal tissues, excluding the testes, of wild-type male flies. The desired tissues were dissected in PSS and dissolved in the sample buffer for SDS–polyacrylamide gel electrophoresis and subsequent Western blotting (Lakhotia and Singh 1993). The blot was treated with a mouse anti-Sxl primary antibody at 1 : 100 dilution (Samuels *et al*. 1994). A horseradish-peroxidase-conjugated anti-mouse secondary antibody was used in conjunction with the ECL detection reagents (Amersham Pharmacia Biotech, UK) according to the manufacturer's instructions to detect presence of Sxl protein.

Results

The *hsrw*^{05241} mutation is due to *P-LacZ-rosy*$^{+}$ transposon insertion at – 130 bp position in the promoter of the *hsrw* gene (Lakhotia *et al*. 2001). Expression of the *hsrw* gene in this insertion chromosome is remarkably similar to that of the wild-type allele in all embryonic and somatic cells of larvae and adults, correlating with a normal phenotype and viability of the *hsrw*^{05241} homozygous flies (Lakhotia *et al*. 2001). However, the *hsrw*^{05241} homozygous males are completely sterile.

Male-sterile phenotype of hsrw^{05241}

To ascertain if the sterility of *hsrw*^{05241} homozygous males was due to defects in reproductive organs, the testes and associated internal reproductive organs of wild-type and *hsrw*^{05241} males were compared. As shown in figure 1,a&b, the most remarkable difference between wild-type and *hsrw*^{05241} male reproductive organs was in the seminal vesicles, which in the mutant males were very small. Testes of mutant males did not show any apparent abnormality except that these appeared to be a little more 'full'. The accessory glands, vas deferens, etc. appeared similar in wild-type and mutant males. When unfixed testes from wild-type and *hsrw*^{05241} males were teased open in PSS, the mutant testes revealed presence of thick bundles of completely nonmotile sperms (figure 1d) rather than the freely moving individualized sperms seen in the wild-type (figure 1c). The seminal vesicles in wild-type flies were full of sperms (figure 1e) but in the *hsrw*^{05241} the vesicles were devoid of any sperm (figure 1f).

Giemsa staining of intact testes following the method of Bonaccorsi *et al*. (1988) revealed an abnormal spatial organization of developing germ cells in the *hsrw*^{05241} testes. As seen in figure 1g, the cysts of spermatocytes in

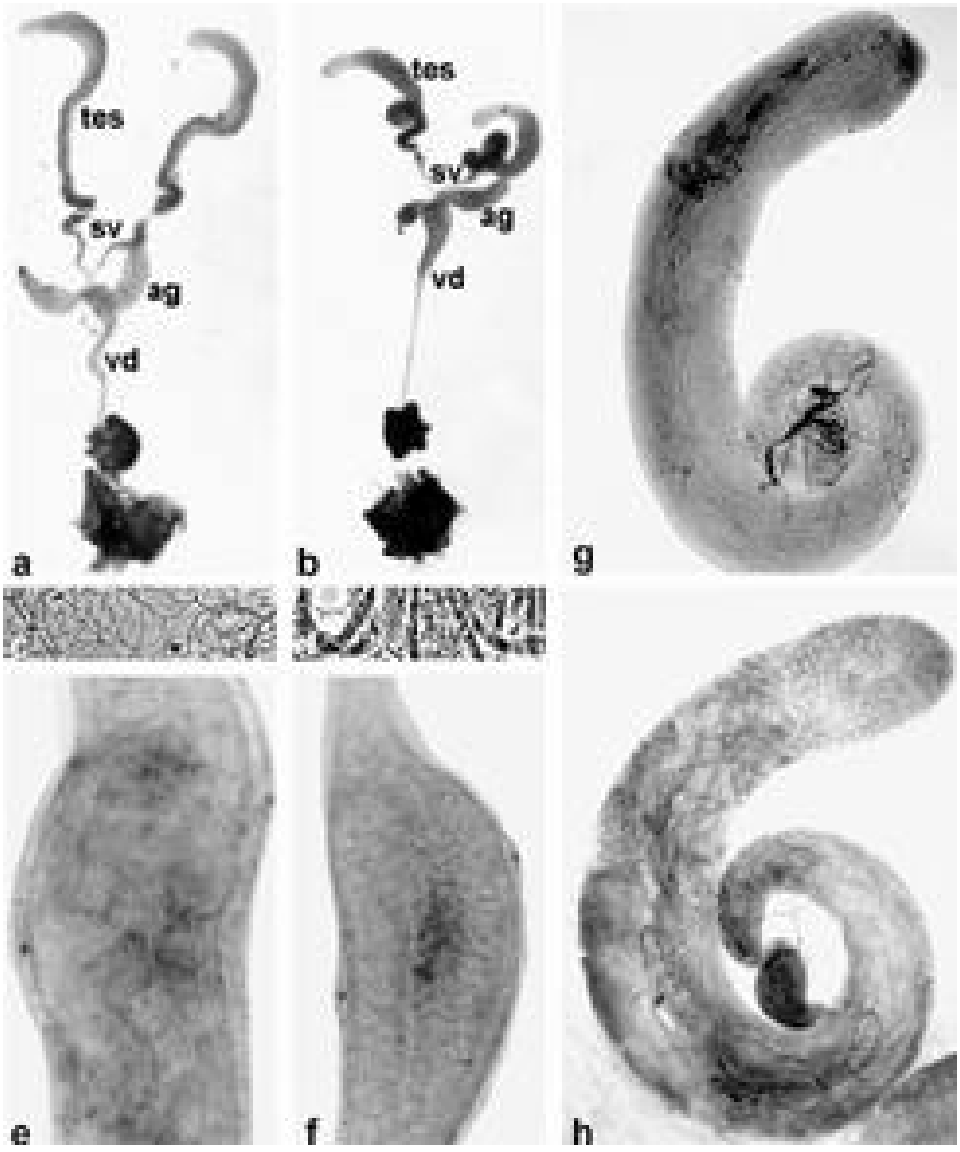

Figure 1. a and b: Internal reproductive organs of wild-type (a) and *hsrw*^{05241} (b) mutant male flies; note the very small seminal vesicles (sv) in the *hsrw*^{05241} mutant flies (ag, accessory glands; tes, testis; vd, vas deferens). c and d: Individualized and freely motile sperms from unfixed testis of wild-type male (c) and thick bundles (non-individualized) of nonmotile sperms of *hsrw*^{05241} mutant male (d). e and f: Seminal vesicles from wild-type (e) and *hsrw*^{05241} mutant (f) male flies. g and h: Whole testes of wild-type (g) and *hsrw*^{05241} mutant (h) flies stained with Giemsa to reveal the distribution of spermatocytes and sperms; the spermatocytes are visible as light, rounded structures.

wild-type testes were distributed in the anterior part and along the concave face of the coiled testes while bundles of individualized sperms filled its convex face. In the *hsrw*^{05241} mutant testes, however, thick bundles of sperms appeared to fill much of the internal space, which displaced the cysts of spermatocytes and resulted in their disorganized distribution in the anterior half as well as on the convex face of the testes (figure 1h). Immunostaining of intact testes with antibody against Rb97D protein, which is specifically synthesized in primary spermatocytes (Heatwole and Haynes 1996), also revealed a similar disorganization in the mutant testes (not shown).

The male-sterile phenotype of hsrw^{05241} is due to P transposon insertion and is a recessive gain-of-function mutation

Other studies (also see below) have established that the *hsrw*^{05241} chromosome carries a single P transposon insertion on the right arm of chromosome 3 in the 93D6-7 cytogenetic region at – 130 bp in the *hsrw* promoter (Lakhotia *et al*. 2001). To find out if the recessive male-sterile phenotype was indeed due to the P transposon insertion, the fertility of *hsrw*^{05241}/*Df(3R)e*^{P}, *hsrw*^{05241}/*Df(3R)e*^{Gp4} and *hsrw*^{05241}/*Df(3R)GC14* male flies was examined. Intriguingly, all the three deficiencies complemented the *hsrw*^{05241} P insertion since all the hybrid males showed normal reproductive organs and fertility (not shown). These results suggested that either the male-sterile phenotype was due to a second-site mutation mapping outside the 93B6-7 to 93D10 interval (collectively covered by the three deficiency chromosomes tested) or it was due to a recessive gain-of-function mutation in the *hsrw* gene which required two copies of the mutated allele to manifest male sterility.

That the male-sterile phenotype of *hsrw*^{05241} homozygotes is indeed due to the P transposon insertion at – 130 bp in the promoter region of the *hsrw* gene and not due to any other second-site mutation was established by the following two lines of evidence. Firstly, extensive outcrossing of the *hsrw*^{05241} chromosome with *ry*^{506} chromosome was carried out for 10 generations and more than 500 progeny male flies homozygous for the *P-LacZ-rosy*$^{+}$ transposon were tested for fertility. However, no recombinant fly was obtained which when homozygous for the P transposon insertion was fertile.

A more direct confirmation for the sterility of *hsrw*^{05241} homozygous males being associated with the P insertion was obtained through excision of the transposon by crossing with the 'jump-starter' line (Bier *et al*. 1989) and selection for flies that had lost the transposon. A total of 63 excision lines, identified by rosy eye colour resulting from loss of the *rosy*$^{+}$ marker in the transposon, were obtained. Four of them (named *hsrw*$^{05241–R1}$ to *hsrw*$^{05241–R4}$) displayed normal viability and fertility of females as well as males. The genomic DNAs of these four normally viable and fertile excision-revertant lines did not generate any amplicons following PCR amplification with primers for P element termini and the *hsrw* sequences flanking the site of insertion (PP-RIP or PP-L226P primer combinations) but generated the expected wild-type amplicon with primers (RIP-L226P combination) for genomic sequences flanking the – 130 bp region (see figure 2). This established that the P element is completely excised from the *hsrw* promoter region in all the four revertant lines and the wild-type genomic DNA sequence restored. Genomic DNAs of several other rosy-eyed excision lines that still displayed recessive male sterility were also examined for precision of the excision. All such lines that

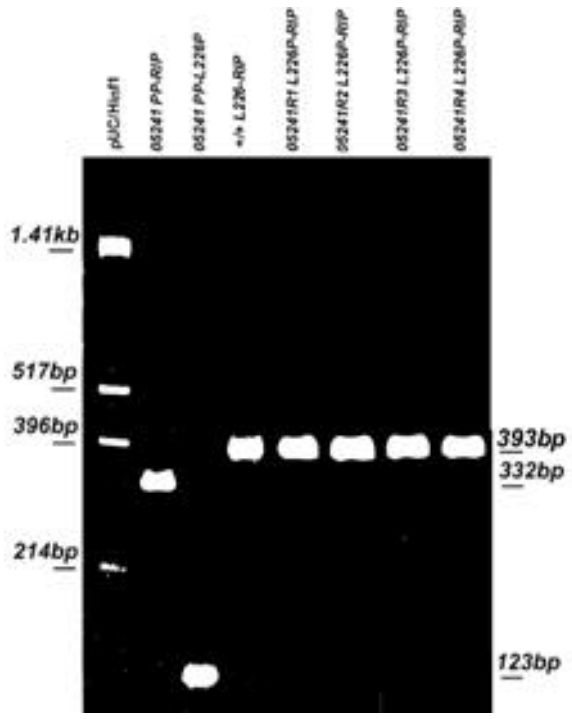

Figure 2. Ethidium bromide stained agarose gel showing PCR amplicons generated with genomic DNAs from *hsrw*^{05241} (lanes 2 and 3), wild-type (+/+, lane 4), *hsrw*$^{05241–R1}$ (lane 5), *hsrw*$^{05241–R2}$ (lane 6), *hsrw*$^{05241–R3}$ (lane 7) and *hsrw*$^{05241–R4}$ (lane 8) homozygous flies using the primer combinations specified on top of each lane. Lane 1 shows marker bands of pUC12 DNA digested with *Hin*fI. Note that the *hsrw*^{05241} genomic DNA generated 123-bp (lane 2) and 332-bp (lane 3) amplicons with PP-RIP and PP-L226P primer combinations (see Materials and methods for details of primers), respectively. These sizes are as expected from the P transposon insertion at – 130 bp of the *hsrw* promoter. *hsrw*^{05241} genomic DNA did not produce any amplicon with RIP-L226P primer combination (not shown). The genomic DNAs from wild type (lane 4) and the four P-excision-revertant lines (*hsrw*$^{05241–R1}$ to *hsrw*$^{05241–R4}$, lanes 5 to 8) generated the expected 393-bp amplicons with the RIP-L226P primer combination in each case. None of these genomic DNAs generated any amplicons with PP-RIP or PP-L226P primer combinations (not shown) showing absence of any P-element terminal sequences in the four revertant lines.

were tested either produced PCR amplicons with primers for P element termini and *hsrw* genomic sequences or produced aberrant amplicons with primers for *hsrw* genomic sequences flanking the – 130 bp promoter region (data not shown). This suggested that in the excision lines that displayed male sterility either a part of the P transposon was still present at the site or the excision was associated with loss of some of the flanking genomic region.

Expression of hsrw in testis

Expression of *hsrw* was examined by *in situ* hybridization of dig-labelled *hsrw* riboprobe to cellular RNA in whole testes from various genetic backgrounds. The *hsrw* gene was found to be expressed abundantly in the cyst cells of testes. In intact testes, the hybridization signal in cyst cells was seen at low magnification as small dots whose intensity varied in different genotypes (figure 3). The hybridization signal in cyst cells in wild type (figure 3a) was much less pronounced than in testes from $hsrw^{05241}$ homozygotes (figure 3b). Expression in testes of $hsrw^{05241}$/*TM6B* heterozygotes was higher than in the wild type but less than in $hsrw^{05241}$ homozygotes (figure 3c). The level of expression in cyst cells of $hsrw^{05241}$/*Df(3R)GC14* testes, with only one copy (mutated) of the *hsrw* gene, was comparable with that in the wild type (figure 3d). The RISH pattern in intact testes from $hsrw^{05241–R1}$ and $hsrw^{05241–R2}$ homozygous flies (not shown) was similar to that in wild-type testes.

Among the male germ cells, the *hsrw* gene is expressed only in spermatids (Bendena *et al*. 1991). Although the spermatids were not clear in the RISH preparations of intact testes, fluorescence *in situ* hybridization of the *hsrw* riboprobes with partially squashed testes revealed that the level of *hsrw* transcripts in spermatids in wild-type and $hsrw^{05241}$ mutant testes did not differ (not shown). The $hsrw^{05241}$ mutant testes did not show expression of the *hsrw* gene in any other male germ cells (not shown).

A chance observation on the reproductive system of a wild-type male, which was processed for *in situ* hybridization, was interesting. As shown in figure 3e, the reproductive organs of this male looked normal except that one of the testes (on the right side in figure 3e) was loaded with sperm bundles and the seminal vesicle on that side was small and devoid of sperms. The other testis (on the left side in figure 3e) was normal with its seminal vesicle being typically full with sperms. The cyst cells in the testis that was associated with the empty seminal vesicle showed a stronger hybridization signal with the *pDRM30* riboprobe (the right testis in figure 3e), comparable to that in the $hsrw^{05241}$ homozygotes (figure 3b). The normal-looking left testis (figure 3e), whose associated seminal vesicle contained sperms, showed weak hybridization typical of wild-type testis.

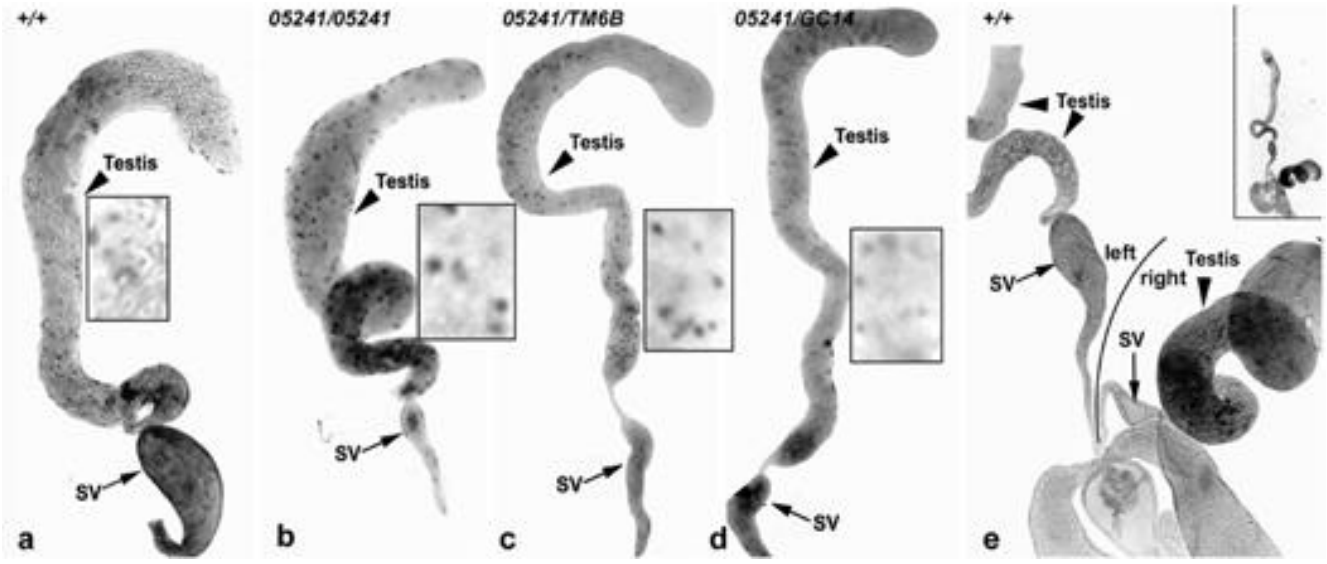

Figure 3. RISH of dig-labeled pDRM30 riboprobe in intact testes from wild-type (a and e), $hsrw^{05241}$/$hsrw^{05241}$ (b), $hsrw^{05241}$/*TM6B* (c) and $hsrw^{05241}$/*Df(3R)GC14* (d) flies. The hybridization signals (detected through AP-conjugated anti-dig antibody) on cyst cell nuclei are seen as dark specks on testes and seminal vesicles (sv); note the more prominent signal on cyst cells in b. Higher magnification images of small areas of testes are shown in insets in a–d to more distinctly reveal the cyst cells. Reproductive organs in the example in **e** are from a wild-type male in which left testis and seminal vesicle appeared normal but the right seminal vesicle was much smaller and the right testis was much larger. The cyst cells in the right testis showed a distinctly stronger hybridization signal with the *hsrw* riboprobe. Inset in upper right corner in e shows a lower-magnification image of these reproductive organs.

In this context, it is further interesting to note that in occasional *hsrw*^{05241}*/TM6B* heterozygous individuals one or both testes contained nonmotile bundles of sperms while the corresponding seminal vesicles were small and empty. As noted above, the overall expression of hsrω-n transcripts in the *hsrw*^{05241}*/TM6B* heterozygotes is higher than in wild-type males.

Omega speckles are differently organized in cyst cells of hsrw^{05241} *mutant testes*

We have reported earlier that the hsrω-n transcripts colocalize with the nucleoplasmic hnRNPs to form fine omega speckles (Lakhotia *et al*. 1999; Prasanth *et al*. 2000). Since the above results with RISH in intact testes showed that the hsrω-n transcripts were much more abundant in the cyst cells of *hsrw*^{05241} homozygous males, status of the omega speckles in these cells was examined by combined RISH and immunostaining with antibodies against several hnRNPs and related proteins like HRB87F, Hrb57A, Hrp40, S5, Sxl, Rb97D and SRp55 in lightly squashed preparations of wild-type and *hsrw*^{05241} mutant testes (figure 4).

The hsrω-n transcripts were distributed as numerous fine speckles in wild-type cyst cell nuclei (figure 4a) but in the *hsrw*^{05241} mutant cyst cells these transcripts were present as much larger clusters or aggregates (figure 4,c&d) comparable to those seen in wild-type cells after heat shock (figure 4b). As in the other somatic cell types (Lakhotia *et al*. 1999; Prasanth *et al*. 2000), in the wild-type cyst cells also the HRB87F (figure 4a′) and Hrb57A antibodies (not shown) produced a diffuse staining of chromatin and a fine-speckled pattern in the nucleoplasm. The fine speckles formed by the hsrω-n transcripts and the two hnRNPs were always coincident (the omega speckles, figure 4a″). In the *hsrw*^{05241} mutant cyst cells, the HRB87F (figure 4,c′&d′) and Hrb57A (not shown) formed large aggregates or clusters in parallel with the hsrω-n transcripts (figure 4,c″&d″), while the diffuse chromatin staining was less obvious. The cyst cells in *hsrw*$^{05241–R1}$ flies revealed typical fine omega speckles (figure 4,d–d″) similar to those seen in wild-type cyst cells.

To more closely compare the omega speckles in wild-type and *hsrw*^{05241} mutant cyst cells, the following four patterns were identified: (A) nuclei with many fine speckles (figure 4,a–a′); (B) nuclei with fewer and thicker speckles, (C) nuclei showing one or more clusters in addition to some thin or thick speckles (figure 4,b–b″) and (D) nuclei with one or two larger clusters and few thin or thick speckles (figure 4,c–c″). The data on relative frequencies of these different patterns in wild-type (unstressed control and heat-shocked) and *05241* mutant (unstressed) cyst cells are presented in figure 5. These reveal that most of the wild-type unstressed cyst cells showed the characteristic fine omega speckles (pattern A), while after heat shock, as in other cell types (Lakhotia *et al*. 1999; Prasanth *et al*. 2000), these formed thicker speckles or clusters (patterns B, C or D). Significantly a majority of the *hsrw*^{05241} mutant cyst cells, even without any stress, showed pattern C or D while only about 3% of them showed pattern A or fine speckles (figure 5). Thus the organization of omega speckles in unstressed *hsrw*^{05241} mutant cyst cells resembled that in heat-shocked wild-type cyst cells.

As noted earlier (Prasanth *et al*. 2000), in cyst cell nuclei in the *hsrw*-nullisomic (*Df(3R)e*^{Gp4}*/Df(3R)GC14*) testes HRB87F and Hrp57A were distributed in a uniform smear, rather than in speckles.

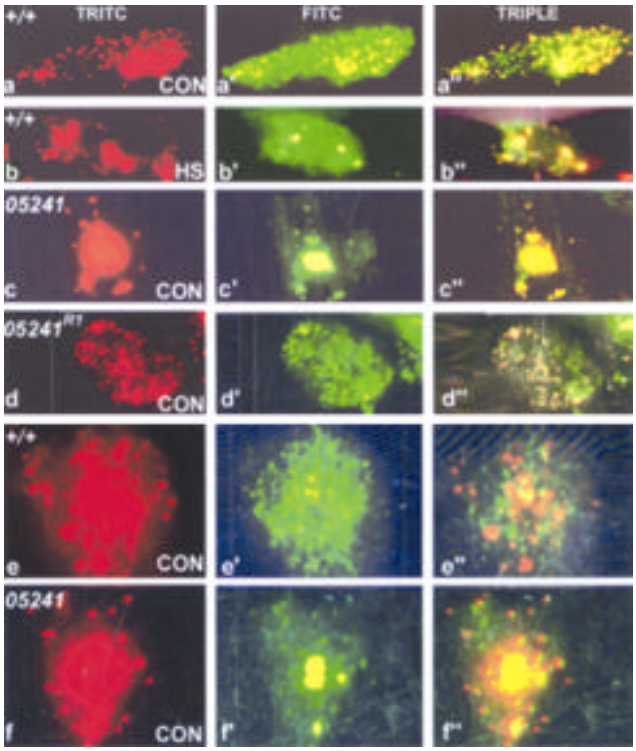

Figure 4. a–d: Colocalization of hsrω-n RNA and HRB87F protein as seen after combined RISH with pDRM30 riboprobe (a–d red fluorescence, detected with rhodamine-conjugated anti-dig antibody) and immunostaining with the HRB87F (P11) antibody (a′–d′ green fluorescence, detected with FITC-conjugated anti-mouse secondary antibody) in lightly squashed cyst cells of wild-type unstressed control (a), wild-type heat-shocked (b), *hsrw*^{05241}*/hsrw*^{05241} unstressed (c) and *hsrw*$^{05241–R!}$*/hsrw*$^{05241–R!}$ unstressed (d) testes. e, f: Absence of colocalization of hsrω-n RNA and SRp55 protein after combined RISH with pDRM30 riboprobe (e, f) and immunostaining with the B52 antibody (e′, f′) in lightly squashed cyst cells of wild-type (e) and *hsrw*^{05241}*/hsrw*^{05241} (f) unstressed testes. The last column (a″–f″) shows localization of hsrω-n transcripts and the HRB87F or SRp55 proteins as seen through a triple-pass (DFT) fluorescence filter.

The antibodies against S5, Hrp40, Sxl and Rb97D did not show any binding in cyst cells, neither in wild-type nor in any of the *hsrw* mutant testes, suggesting that these proteins are not synthesized in cyst cells.

The distribution of SR proteins like SRp55, which are present in the IGCs (Misteli and Spector 1997), was comparable in wild-type, $hsrw^{05241}$, $hsrw^{05241-R1}$ and $Df(3R)e^{Gp4}/Df(3R)GC14$ cyst cells, and as known in other cell types (Lakhotia *et al*. 1999; Prasanth *et al*. 2000) these proteins did not generally colocalize with hsrω-n RNA (figure 4, e–f″).

The 7Fb antibody against the heat-shock-inducible Hsp70 (Velazquez and Lindquist 1984) did not produce any staining in the cyst cells in control testes either in wild-type or in $hsrw^{05241}$ homozygous flies but Hsp70 was clearly detectable in cyst cells after a typical heat shock (not shown).

Distribution of various RNA-binding proteins in germ cells in wild-type, $hsrw^{05241}$, $hsrw^{05241-R1}$ and hsrw-nullisomic testes

To see if the male sterility associated with the $hsrw^{05241}$ mutation involved any change in the *in situ* distribution of the various RNPs in male germ cells, fluorescence immunostaining was carried out on partially squashed testes of wild-type, $hsrw^{05241}$, $hsrw^{05241-R1}$ and $Df(3R)e^{Gp4}/Df(3R)GC14$ (*hsrw*-nullisomic) flies.

***Spermatogonia and primary spermatocytes*:** While Rb97D and Sxl were absent in gonial cells, all the other RNPs like HRB87F (figure 6a), HRB57A, S5, Hrp40 and SRp55 (not shown) showed cytoplasmic localization in gonial cells in all the four genotypes. As exemplified by the immunostaining patterns of HRB87F (figure 6), all these proteins began to move to the nucleus as the gonials differentiated into early primary spermatocytes (figure 6b), and beginning with the polar spermatocyte stage staining intensity in the nucleus became stronger (figures 6c and 7).

Distribution patterns of the various RNPs in primary spermatocytes in testes of wild-type, $hsrw^{05241-R1}$, *hsrw*-nullisomic and $hsrw^{05241}$ mutant flies were similar (figure 7).

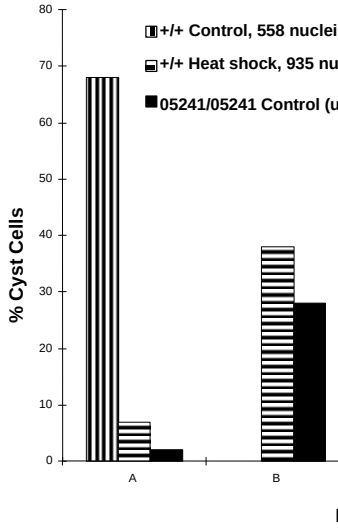

Figure 5. Histograms of frequencies (Y-axis) of the different patterns (A–D, X-axis) of speckles/clusters of hsrω-n RNA and HRB87F in cyst cell nuclei in unstressed wild-type control, wild-type heat-shocked and unstressed $hsrw^{05241}$ mutant (without heat shock) testes. Pattern A, many fine speckles; B, thicker speckles; C, a few small clusters in addition to some thin or thick speckles; D, one or two larger clusters and few thin or thick speckles. The numbers of nuclei and the numbers of testes examined in each case are indicated.

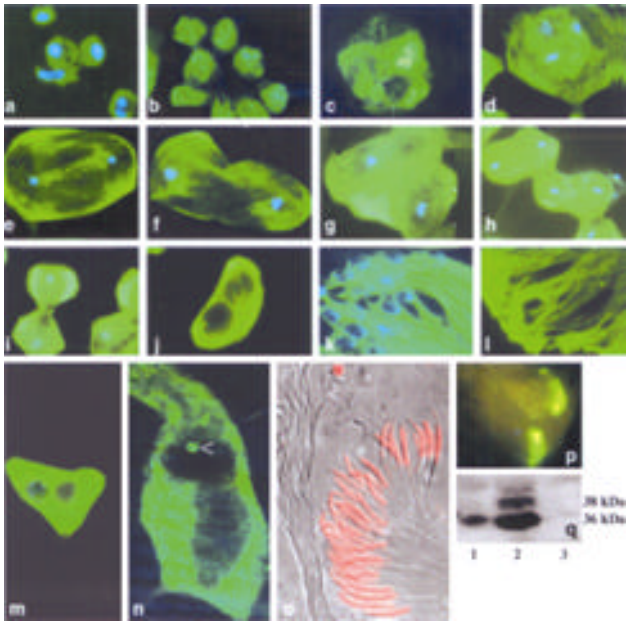

Figure 6. a–l: Distribution of HRB87F (green fluorescence) in wild-type spermatogonia (a), early spermatocytes (b), polar spermatocyte (**c**), late primary spermatocyte (d), early anaphase I (e), late anaphase I (f), telophase I (g), secondary spermatocytes (h), anaphase II (i), early spermatid (j), elongating spermatids (k) and waste bags (l). m–o: Immunolocalization of S5 (green fluorescence in m and n and red fluorescence in o in early spermatid (m), elongating spermatid (n) and maturing and mature sperms (o); the arrowhead in n points to the S5-positive round structure in spermatid nucleus; the example in o is a combined phase-contrast and rhodamine fluorescence image with a bundle of maturing sperms (positive for S5) marked with star (*) and the sperm heads (negative for S5) in a bundle of mature spermatozoa marked with arrowhead (<). p: Immunostaining (green fluorescence) of a primary spermatocyte with Sxl antibody. q: Western-blot detection of Sxl protein in adult testes (lane **1**), ovaries (lane **2**) and whole male flies excluding testes (lane **3**); the molecular sizes (in kilodaltons, kDa) of reacting bands are indicated at right. FITC-conjugated secondary antibody was used in examples in a–n and p while a rhodamine-conjugated secondary antibody was used in the example in o. Preparations in a–l were counterstained with DAPI (blue fluorescence) to reveal chromatin. All the immunostaining examples are from wild-type control testis preparations, but similar patterns were seen in the other genotypes.

HRB87F and Hrp40 showed a widespread staining of the large primary spermatocyte nucleus, with some regions showing a stronger binding (figure 7,a–a″,b–b″). In contrast Hrb57A (figure 7,c–c″), S5 (figure 7,d–d″) and Rb97D (figure 7,e–e″) stained only the Y-chromosomal lampbrush loops (also see Heatwole and Haynes 1996). While Hrb57A and S5 stained the loops A and C, Rb97D stained only the loop C in wild-type as well as in *hsrw*^{05241} and *Df(3R)e*^{Gp4}*/Df(3R)GC14* spermatocytes.

Although it has been reported earlier that *Sxl* is not expressed in males and the Sxl protein is not essential for male fertility (Salz *et al*. 1987; Bopp *et al*. 1991), our immunostaining results showed a distinct presence of Sxl in the male germ cells of all the four genotypes but this was confined only to mature primary spermatocytes in the form of two comma-shaped staining bodies in the nucleus (figure 6p). As shown in figure 6q (lane 1), Sxl in testes was a full-length 36-kDa protein (Bopp *et al*. 1991) that was not seen in the extract prepared from protein samples of adult male bodies after removal of their testes (figure 6q, lane 3). The full-length 36-kDa protein in testes comigrated with that expressed in ovaries (figure 6q, lane 2). Unlike testes, which synthesized only the 36-kDa Sxl protein, the ovaries in addition synthesized 38-kDa, 40-kDa and 42-kDa forms of Sxl (also see Bopp *et al*. 1991).

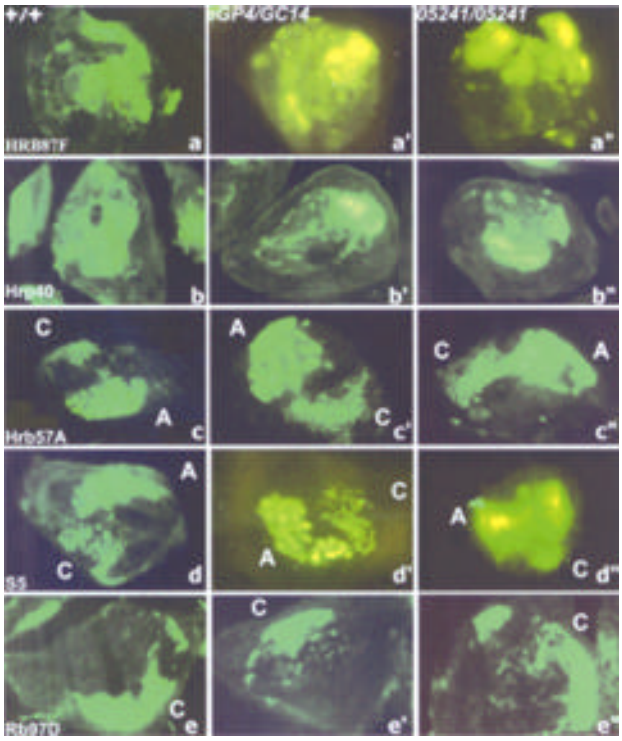

Figure 7. Similar patterns of distribution of HRB87F (a–a″), Hrp40 (b–b″), Hrb57A (c–c″), S5 (d–d″) and Rb97D (e–e″) proteins in primary spermatocytes in wild-type (a–e), *Df(3R)e*^{Gp4}*/Df(3R)GC14* (a′–e′) and *hsrw*^{05241}*/hsrw*^{05241} (a″–e″) testes. The characteristically stained A and C loops of Y chromosome are marked in c–e″.

Although the various hnRNPs (HRB87F, Hrp40, Hrb57A and S5) showed nuclear localization in the primary spermatocytes (figure 7), it is interesting that unlike in cyst cells (see above) and other somatic cell types (Lakhotia *et al*. 1999; Prasanth *et al*. 2000) none of them formed nucleoplasmic speckles comparable to the omega speckles, either in wild-type or in any of the mutant lines. Furthermore, none of the hnRNPs showed any change in their *in situ* localization patterns after a 40-min heat shock in any of the genotypes (not shown).

***Dividing spermatocytes*:** Hrp40, Hrb57A and S5 showed a general cytoplasmic staining in dividing spermatocytes in wild-type as well as the *hsrw* mutant flies (not shown). However, HRB87F was a significant exception since it showed a very characteristic dynamic distribution during the first meiotic division in all the genotypes. The generalized nuclear distribution of HRB87F in the early primary spermatocytes (figure 6b) attained a granular network architecture as the spermatocytes entered into first meiotic division (figure 6,c&d). At this stage, the condensed chromatin was observed as three DAPI-stained bodies embedded within the granular HRB87F network. During anaphase I (figure 6e), HRB87F was seen to follow the track left behind by the poleward-migrating chromosomes (figure 6,e&f). The granular path established by HRB87F following the anaphase movement started retracting away from the equator towards the poles just behind the segregating chromosomes. With completion of the segregation of chromosomes at telophase I, HRB87F became cytoplasmic and remained so thereafter all through meiosis II. No specific binding pattern was observed during meiosis II (figure 6,g&h).

***Spermatids*:** HRB87F as well as HRB57A remained cytoplasmic till the stage of elongating spermatids (figure 6,i&j) and were finally eliminated through waste bags (figure 6,k&l) in all the genotypes. S5, however, behaved differently since while it was cytoplasmic in early spermatids (figure 6m), in elongating spermatids a brightly staining nuclear signal was additionally present (figure 6n). S5 continued to show the nuclear localization till early stages of sperm development but was absent in mature sperms (figure 6o). As in the cases of other proteins, S5 distribution in spermatids and maturing sperms was not different between the different genotypes.

There was no detectable change in the localization of these hnRNPs in heat-shocked spermatids (not shown).

Discussion

hsrw^{05241} is the first mutant allele of the *hsrw* gene known to have a distinct mutant phenotype, male sterility in the present case. The normal fertility of heterozygotes carrying the *hsrw*^{05241} allele and any one of the three 93D

deficiencies may apparently suggest that the genetic factor responsible for recessive male sterility of the *hsrw*^{05241} homozygotes maps outside the 93B6-7 to 93D10 cytogenetic interval, collectively covered by the three deficiencies examined. It is significant to note in this context that the *hsrw*-nullisomic (*Df(3R)e*^{Gp4}*/Df(3R)GC14*) males produce motile sperms (Lakhotia *et al*. 1999). Thus the fertility of flies with heterozygous combination of the *hsrw*^{05241} allele with any of the three deletions does not rule out the male-sterile phenotype of *hsrw*^{05241} homozygotes mapping to the *hsrw* locus itself. Existence of a second-site mutation, which may be suspected for male sterility of this line, seems to be further ruled out by our two other results. Firstly we failed to obtain any recombinant progeny that retained the P insertion but regained male fertility, and secondly, when the P element was completely excised in the four lines, as evidenced by failure to generate any PCR amplicons with primers for P element termini and flanking genomic sequences, their male fertility was also completely restored. On the other hand, all the excision lines that retained the recessive male-sterile phenotype also retained at least a part of the P element at the original site or had lost a part of the *hsrw* promoter flanking the original site of P insertion. Taking these together, we believe that the recessive male sterility exhibited by the *hsrw*^{05241} chromosome is due to P transposon insertion at – 130 bp of the *hsrw* gene. Its complementation with the three deletion chromosomes suggests that this is a gain-of-function recessive mutation.

The much higher levels of hsrω-n transcripts in cyst cells of *hsrw*^{05241} testes was associated with formation of large aggregates, characteristic of wild-type cells after thermal stress (Lakhotia *et al*. 1999). It is possible that the increased levels of hsrω-n transcripts and the clustering of omega speckles in the *hsrw*^{05241} mutant cyst cells were due to some kind of cellular stress resulting from the accumulation of nonmotile sperms in testes. However, immunostaining of *hsrw*^{05241} mutant testes with the Hsp70-specific 7Fb antibody (Velazquez and Lindquist 1984) did not show any trace of Hsp70 in the mutant cyst cells. Therefore, the increased level of *hsrw* transcripts and their aggregation into large clusters in cyst cells of *hsrw*^{05241} mutant testes does not appear to be due to a cellular stress per se.

The P transposon insertion in the promoter region of the *hsrw* gene may be expected to affect its expression. Interestingly, however, other studies (Lakhotia *et al*. 2001) have shown that the levels and distribution patterns of *hsrw* transcripts in all the examined somatic cell types of *hsrw*^{05241} individuals were similar to those in the corresponding wild-type cells. Even the heat-shock and benzamide inducibility of the 93D puff in salivary glands of the mutant larvae was not affected (Lakhotia *et al*. 2001). The normal viability and morphology of the *hsrw*^{05241} homozygotes is in agreement with the normal *hsrw* expression in various cell types. It is significant that the *hsrw*^{05241} mutation affected only male fertility and expression of this gene was also affected only in cyst cells of testes.

Earlier studies (Mutsuddi and Lakhotia 1995; Lakhotia and Tapadia 1998; Lakhotia *et al*. 2001) revealed existence of multiple regulatory elements in the *hsrw* gene promoter. It appears that the sequences around the – 130-bp position specifically affect the expression of *hsrw* in cyst cells so that insertion of a P transposon at this site resulted in its overexpression only in these cells. Overexpression of *hsrw* in the *hsrw*^{05241} mutant cyst cells also shows this to be a gain-of-function mutation.

Since expression of *hsrw* in spermatids, the only stage of male germ cells that shows presence of *hsrw* transcripts (Bendena *et al*. 1991), was not affected by the *hsrw*^{05241} mutation and also since none of the other germ-cell stages showed any expression of this gene in the *hsrw*^{05241} mutant testes, we believe that the sterility of these mutant males is somehow related to high levels of hsrω-n transcripts in the cyst cells. This is further supported by our results that the distribution patterns of several different RNA-binding proteins was very similar in meiotic cells of wild-type and *hsrw* mutant (*hsrw*^{05241}*/ hsrw*^{05241} and *Df(3R)e*^{Gp4}*/Df(3R)GC14*) testes.

A pair of head and tail cyst cells surrounds a cyst of developing male germ cells from the founder spermatogonial stage to the mature sperm bundle stage (Fuller 1993). The importance of cyst cell function for normal spermatogenesis in *Drosophila* is evident from the fact that mutation in a tubulin gene, *b3t*, which is expressed in testes only in the cyst cells, results in male sterility (Fuller 1993; Kimble *et al*. 1990). Therefore, it seems likely that the overexpression of *hsrw* in cyst cells of *hsrw*^{05241} mutant males adversely affects cyst cell functions and this results in production of nonmotile sperms and consequent sterility. In this context, the chance finding of a wild-type male in which one of the two testes showed a comparable high level of hsrω-n transcripts in cyst cells is significant. Although reasons for overexpression of *hsrw* in this apparently wild-type testis are not known, the important point is the correlation between overexpression of hsrω-n transcripts in cyst cells and the attached seminal vesicle being small and empty of sperms much like the situation in the *hsrw*^{05241} mutant testes. The occasional presence of small empty seminal vesicles attached to the testes in *hsrw*^{05241}*/TM6B* flies also seems to be related to the somewhat high level of expression of *hsrw* in their cyst cells. It is notable that the increase in *hsrw* transcripts in cyst cells was proportional to the number of copies of the *hsrw*^{05241} allele since while in the *hsrw*^{05241}*/Df(3R)GC14* heterozygotes, with only one copy of the mutated, over-expressed *hsrw* gene, the level of hsrω RNA in cyst cells was more or less comparable to that in wild-type cyst cells, which have two copies of normally expressed

genes, in the *hsrw*^{05241}/*TM6B* heterozygotes, with one overexpressed and one normally expressed *hsrw* alleles, the transcript level in cyst cells was intermediate between the wild-type and *hsrw*^{05241} homozygous mutant cyst cells. It appears that when the level of *hsrw* transcripts in cyst cells rises beyond a certain threshold sperm maturation is somehow affected.

We think that the severe consequence (production of nonmotile sperms and consequent male sterility) of overexpression of *hsrw* in *hsrw*^{05241} cyst cells is related to the fact that the hsrω-n transcripts are involved in regulating the availability of hnRNPs and related proteins in the nucleus (Lakhotia *et al*. 1999; Prasanth *et al*. 2000). It is interesting to note in this context that the majority of the known fertility factors are RNA-binding proteins, which are involved in one or the other processing event during post-transcriptional regulation of other target genes (Venables and Eperon 1999). We have suggested earlier (Lakhotia *et al*. 1999; Prasanth *et al*. 2000) that the heat-shock-induced increase in the levels of hsrω-n RNA and consequent clustering of omega speckles in somatic cells of *Drosophila* is a cellular mechanism to sequester the hnRNPs that are no more required to be engaged in RNA processing. Therefore, it is likely that the misregulated increased synthesis of hsrω-n transcripts in *hsrw*^{05241} mutant cyst cells and the consequent clustering of the omega speckles soaks up more of the hnRNPs, analogous to the situation in heat-shocked wild-type cells. In this context, it is notable that complete absence of *hsrw* transcripts in cyst cells does not affect sperm differentiation since the *hsrw*-nullisomic males show typical motile sperms (Lakhotia *et al*. 1999). This correlates with the fact that normal chromosomal binding of the various hnRNPs in unstressed somatic cells is also not adversely affected by *hsrw*-nullisomy (Prasanth *et al*. 2000). However, overabundance of *hsrw* transcripts in cyst cells would deplete the required hnRNPs at active chromosomal sites for processing of their transcriptional products. Titration of certain RNA-processing proteins by overabundance of some transcripts and consequent cellular pathologies are already known in mammalian cells (Philips *et al*. 1998; Singer 1998; Lu *et al*. 1999). Thus disruption in RNA processing activities in cyst cell nuclei would compromise their normal functions and consequently sperm maturation.

In agreement with the fact that the *hsrw* transcripts are not present in primary spermatocytes (Bendena *et al*. 1991 and present observations), distribution patterns of the various hnRNPs in these cells were not at all affected by *hsrw*-nullisomy or the *hsrw*^{05241} mutation. Unlike somatic cells, spermatocytes also do not mount a typical heat-shock response (Bendena *et al*. 1991); also, the male germ cells did not show any heat-shock-induced change in distribution of the hnRNPs. It appears, therefore, that the clustering of the hnRNP-containing omega speckles as seen in heat-shocked somatic cells, including the cyst cells of testes (Lakhotia *et al*. 1999; Prasanth *et al*. 2000; and present results), is a component of the heat-shock response of *Drosophila* cells. The absence of a typical heat-shock response and *hsrw* transcripts in spermatocytes could be a survival strategy. It is possible that the damages inflicted on a cell by thermal stress may not be completely repaired by the cellular responses and this could add serious genetic load on the population if germ cells transmitted these to the next generation. Since spermatogenesis is a continuing process and results in a very large number of gametes, temporary sterility caused by thermal stress may not be greatly disadvantageous. If this be so, unlike somatic cells the male meiotic cells may not need the *hsrw* transcripts to function as a 'sink' for the various nuclear RNA-processing proteins to protect them against thermal damage and/or to prevent them from remaining active during conditions of stress. Furthermore, the different functions of the various hnRNPs in meiotic cells may also require an absence of *hsrw* transcripts in spermatocytes. The propensity of *hsrw* transcripts to bind with the hnRNPs may adversely affect spermatocyte development if this gene was expressed in spermatocytes.

Although our results showed that *hsrw* mutations do not affect the *in situ* distributions of the various RNA-binding proteins in male germ cells, some other interesting aspects of distributions of these proteins during spermatogenesis revealed by the present study are significant.

All the RNPs, except S5, examined in the present study showed nuclear localization only in spermatocytes, remaining cytoplasmic in gonial cells and in spermatids. The cytoplasmic localization of the RNPs in spermatogonia and spermatids seems to be related to the low transcriptional activity of these cell types. It is known that during early embryogenesis in *Drosophila* many of the RNPs remain excluded from the nucleus until the onset of zygotic transcription (Buchenau *et al*. 1997). As the gonial cells progress to the transcriptionally more active spermatocyte stage (Lindsley and Tokuyasu 1980; Fuller 1993), the various RNPs enter the nucleus.

Unlike the other hnRNPs, S5 showed a nuclear localization as a spherical body in elongating spermatids. Although the *hsrw* gene is also expressed at the spermatid stage (Bendena *et al*. 1991) and in somatic cells S5 binds with hsrω-n RNA (Prasanth *et al*. 2000), involvement of *hsrw* transcripts in the nuclear localization of S5 in spermatids is unlikely since its nuclear localization in spermatids was not affected in *hsrw*-nullisomic testes. Significance of the nuclear import of S5 in spermatids remains to be understood. It will be interesting to see if the S5-positive nuclear structure in spermatids is the same as the aniline-positive structure reported earlier (Hime *et al*. 1998) in these nuclei.

Although all the RNPs tested showed nuclear localization in primary spermatocytes, their specific binding

patterns differed. Thus, while HRB87F, Hrp40 and SRp55 showed a more generalized nuclear staining, Hrb57A, S5 and Rb97D were associated with specific nuclear structures. The differences in binding patterns of HRB87F, Hrp40, Hrb57A and S5 in spermatocytes are interesting in light of their more or less similar binding patterns in somatic cell nuclei where all these hnRNPs generally remain associated with, besides the active sites on chromatin, hsrω-n transcripts in the omega speckles (Lakhotia *et al*. 1999; Prasanth *et al*. 2000). It is further interesting that while in primary spermatocytes Hrb57A and S5 shared common binding with the Y-chromosomal loops A and C, the distribution of S5 in larval salivary gland nuclei was somewhat different than the other hnRNPs (Dangli and Bautz 1983). The present observations on the distribution of the various nuclear RNPs, together with earlier reports (Heatwole and Haynes 1996; Lakhotia *et al*. 1999; Prasanth *et al*. 2000), clearly establish that there are major differences in their organization in somatic and germ cells. Such differences in organization imply pleiotropic functions and reiterate the common theme in developmental genetics that the same gene products, particularly those that have regulatory roles, are utilized by the genome again and again in different contexts and with differing consequences.

It is known that the binary switch gene *Sex-lethal* (*Sxl*) must be on in females and off in males for appropriate sexual development in *Drosophila melanogaster* (Baker 1989). Females express several forms of *Sxl*, of which the 36-kDa species is the functional form required for normal ovarian development (Bopp *et al*. 1991). Though full-length Sxl proteins are not detectable in males, low-abundance Sxl proteins of 33–35 kDa have been reported in the head and thorax of both male and female flies (Bopp *et al*. 1991). In view of these, the present finding of a full-length Sxl protein of 36-kDa size in the primary spermatocytes was rather unexpected. This suggests that Sxl may have some function in spermatocyte development although this may not be essential since males are fertile even in the absence of Sxl (Salz *et al*. 1987). It also remains possible that the protein detected here is a spermatocyte-specific novel 36-kDa Sxl-like protein that cross-reacts with the Sxl antibody. Further genetic and molecular studies to characterize this protein are required.

HRB87F is a homologue of hnRNP A1, and therefore it is generally believed that its function requires an RNA substrate (Weighardt *et al*. 1996). In this light, the association of large granules of HRB87F with the path left behind by segregating chromosomes during anaphase I is intriguing. Since no other RNP examined in this study showed a similar distribution, HRB87F seems to have a novel extranuclear function during anaphase–telophase stages of meiosis I in males. The association of this protein with the path of retracting spindle fibres may suggest that HRB87F associates at this stage with some components of the spindle. Weighardt *et al*. (1999) showed that an hnRNP A1 associated protein (HAP) is identical to a previously identified scaffold attachment factor B (SAF-B). Interestingly, in HeLa cell interphase nuclei HAP behaves like HRB87F in somatic cells of *Drosophila* in terms of its nuclear localization and dynamics during heat shock. Another hnRNP, hnRNP-U, has been also described (Facklemayer *et al*. 1994; Eggert *et al*. 1997; Gohring and Facklemayer 1997) as a scaffold attachment factor A (SAF-A). Likewise, hnRNPs F and H were found to be associated with the nuclear matrix (Holzmann *et al*. 1997). It has also been reported that most hnRNPs are found in nuclear matrix preparations (Mattern *et al*. 1996). Since HAP, which is identical to SAF-B, can associate with hnRNP A1 in a nucleic-acid-independent manner in HeLa cells (Weighardt *et al*. 1999), it is possible that HRB87F, being a homologue of hnRNP A1, may also associate with some nuclear scaffold/skeletal proteins specifically during meiotic anaphase. This novel property of HRB87F needs further study.

Overall, our results show that like crucial roles of the unusually large and noncoding Y-chromosomal transcripts for male fertility in *Drosophila* (Hennig 1999), the non-coding hsrω-n transcripts also have important, although indirect, functions in male fertility. It is interesting that, like the hsrω-n transcripts (Lakhotia *et al*. 1999; Prasanth *et al*. 2000), the Y-chromosomal transcripts are also suggested to function essentially through sequestering of various nuclear proteins (Hennig 1999). Since the nuclear proteins involved in RNA processing/transport etc. are highly conserved (Krecic and Swanson 1999), and since many of the fertility factors are known to be coding for RNA-binding proteins ((Venables and Eperon 1999), it is likely that hsrω-like transcripts are present in other organisms as well. It will be interesting to examine if some forms of male sterility in man are related to analogous situations.

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References

Baker B. S. 1989 Sex in flies: the splice of life. *Nature* **340**, 521–524.

Bendena W. G., Ayme-Southgate A., Garbe J. C. and Pardue M. L. 1991 Expression of heat-shock locus *hsr-omega* in non-stressed cells during development in *Drosophila melanogaster*. *Dev. Biol*. **144**, 65–77.

Bier E., Vaeddin H., Shepherd S., Lee K., McCall K., Barbel S., Ackerman L., Carretto R., Uemura T., Grell E., Jan L. Y. and Jan Y. N. 1989 Searching for pattern and mutation in the *Drosophila* genome with a P–*LacZ* vector. *Genes Dev*. **3**, 1273–1287.

Bonaccorsi S., Pisano C., Puoti F. and Gatti M. 1988 Y chromosome loops in *Drosophila melanogaster*. *Genetics* **120**, 1015–1034.

Bopp D., Bell L. R., Cline T. W. and Schedl P. 1991 Developmental distribution of female-specific *Sex-lethal* proteins in *Drosophila melanogaster*. *Genes Dev*. **5**, 403–415.

Buchenau P., Saumweber H. and Arndt-Jovin D. J. 1997 The dynamic nuclear redistribution of an hnRNP K-homologous protein during *Drosophila* embryo development and heat shock. Flexibility of transcription sites in vivo. *J. Cell Biol*. **137**, 291–303.

Champlin D. T., Frasch M., Saumweber H. and Lis J. T. 1991 Characterization of a *Drosophila* protein associated with boundaries of transcriptionally active chromatin. *Genes Dev*. **5**, 1611–1621.

Dangli A. and Bautz E. K. F. 1983 Differential distribution of nonhistone proteins from polytene chromosomes of *Drosophila melanogaster* after heat shock. *Chromosoma* **88**, 201–207.

Eggert M., Michel J., Schneider S., Bornfleth H., Baniahmad A., Facklemayer F. O., Schmidt S. and Renkawitz R. 1997 The glucocorticoid receptor is associated with the RNA-binding nuclear matrix protein hnRNP U. *J. Biol. Chem*. **272**, 28471–28478.

Facklemayer F. O., Dahm K., Renz A., Ramsperger U. and Richter A. 1994 Nucleic-acid-binding properties of hnRNP-U/SAF-A, a nuclear matrix protein which binds to DNA and RNA *in vivo* and in vitro. *Eur. J. Biochem*. **211**, 749–757.

Fuller M. 1993 Spermatogenesis. In *The development of Drosophila melanogaster* (ed. M. Bate and A. Martinez), pp. 71–147. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Garbe J. C., Bendena W. H. and Pardue M. L. 1986 A *Drosophila* heat shock gene with a rapidly diverging sequence but a conserved structure. *J. Biol. Chem*. **261**, 16889–16894.

Gohring F. and Facklemayer F. O. 1997 The scaffold/matrix attachment region binding protein hnRNP-U SAF-A is directly bound to chromosomal DNA in vivo: a chemical cross-linking study. *Biochemistry* **36**, 8276–8283.

Heatwole V. M. and Haynes S. R. 1996 Association of RB97D an RRM protein required for male fertility with a Y chromosome lampbrush loop in *Drosophila* spermatocytes. *Chromosoma* **105**, 285–292.

Hennig W. 1999 Y chromosomal fertility genes in *Drosophila*. *Curr. Sci*. **77**, 550–552.

Hime G. R., Brill J. A. and Fuller M. T. 1998 Assembly of ring canals in the male germ line from structural components of the contractile ring. *J. Cell Sci*. **100**, 2779–2788.

Holzmann K., Korosec T., Gerner C., Grimm R. and Sauermann G. 1997 Identification of human common nuclear-matrix proteins as heterogeneous nuclear ribonucleoproteins H and H′ by sequencing and mass spectrometry. *Eur. J. Biochem*. **244**, 479–486.

Hovemann B. T., Dessen E., Mechler H. and Mack E. 1991 *Drosophila* snRNP associated protein P11 which specifically binds to heat shock puff 93D reveals strong homology with hnRNP core protein A1. *Nucl. Acids Res*. **19**, 4909–4919.

Hovemann B. T., Reim., Werner S., Katz S. and Saumweber H. 2000 The protein Hrb57A of *Drosophila melanogaster* closely related to hnRNP K from vertebrates is present at sites active in transcription and coprecipitates with four RNA-binding proteins. *Gene* **245**, 127–137.

Kimble M., Dettman R. W. and Raff E. C. 1990 The *b*3-tubulin gene of *Drosophila melanogaster* is essential for viability and fertility. *Genetics* **126**, 991–1005.

Krecic A. M. and Swanson M. S. 1999 hnRNP complexes: composition, structure and function. *Curr. Opin. Cell Biol*. **11**, 363–371.

Lakhotia S. C. and Mukherjee A. S. 1970 Activation of a specific puff by benzamide in *Drosophila melanogaster*. *Dros. Infor. Ser*. **45**, 108.

Lakhotia S. C. and Sharma A. 1995 RNA metabolism *in situ* at the 93D heat shock locus in polytene nuclei of *Drosophila melanogaster* after various treatments. *Chromosome Res*. **3**, 151–161.

Lakhotia S. C. and Sharma A. 1996 The 93D *hsr-omega* locus of *Drosophila*: non-coding gene with house keeping functions. *Genetica* **97**, 339–348.

Lakhotia S. C. and Singh B. N. 1993 A simple and inexpensive "Western-blotting" apparatus. *Indian J. Exp. Biol*. **31**, 301–302.

Lakhotia S. C. and Tapadia M. G. 1998 Genetic mapping of the amide response element/s of the *hsrw* locus of *Drosophila melanogaster*. *Chromosoma* **107**, 127–135.

Lakhotia S. C., Ray P., Rajendra T. K. and Prasanth K. V. 1999 The non-coding transcripts of *hsr-omega* gene in *Drosophila*: Do they regulate trafficking and availability of nuclear RNA-processing factors? *Curr. Sci*. **77**, 553–563.

Lakhotia S. C., Rajendra T. K. and Prasanth K. V. 2001 Developmental regulation and complex organization of the promoter of the non-coding *hsrw* gene of *Drosophila melanogaster*. *J. Biosci*. **21**, 25–38.

Lindsley D. L. and Tokuyasu K. T. 1980 Spermatogenesis. In *The genetics and biology of Drosophila*, 2nd edition (ed. M. Ashburner and T. R. Wright), pp. 225–294. Academic Press, New York.

Lindsley D. L. and Zimm G. G. 1992 *The genome of Drosophila melanogaster*. Academic Press, San Diego.

Lu X., Timchenko N. A. and Timchenko L. T. 1999 Cardiac elav-type RNA-binding protein ETR-3 binds to RNA CUG repeats expanded in myotonic dystrophy. *Hum. Mol. Genet*. **8**, 53–60.

Mattern K. A., Humbel B. M., Muijsers A. O., de Jong L. and van Driel R. 1996 hnRNP proteins and B23 are the major proteins of the internal nuclear matrix of HeLa S3 cells. *J. Cell. Biochem*. **62**, 275–289.

Matunis E. L., Matunis M. L. and Dreyfuss G. 1992 Characterization of the major hnRNP proteins from *Drosophila melanogaster*. *J. Cell Biol*. **116**, 257–269.

Misteli T. and Spector D. L. 1997 Protein phosphorylation and the nuclear organization of pre-mRNA splicing. *Trends Cell Biol*. **7**, 135–138.

Mohler J. and Pardue M. L. 1984 Mutational analysis of the region surrounding the 93D heat shock locus of *Drosophila melanogaster*. *Genetics* **106**, 249–265.

Mutsuddi M. and Lakhotia S. C. 1995 Spatial expression of the *hsr-omega 93D* gene in different tissues of *Drosophila melanogaster* and identification of promoter elements controlling its developmental expression. *Dev. Genet***. 17**, 303–311.

Philips A. V., Timchenko L. T. and Cooper T. 1998 Disruption of splicing regulated by a CUG-binding protein in myotonic dystrophy. *Science* **280**, 737–740.

Prasanth K. V., Rajendra T. K., Lal A. K. and Lakhotia S. C. 2000 Omega speckles—a novel class of nuclear speckles

containing hnRNPs associated with non-coding hsr-omega RNA in *Drosophila*. *J. Cell Sci*. **113**, 3485–3497.

Ryseck R.-P., Walldorf U., Hoffmann T. and Hovemann B. 1987 Heat shock loci 93D of *Drosophila melanogaster* and 48B of *Drosophila hydei* exhibit a common structural and transcriptional pattern. *Nucl. Acids Res*. **15**, 3317–3333.

Salz H. K., Cline T. W. and Schedl P. 1987 Functional changes associated with structural alterations induced by mobilization of a P element inserted in *the Sex-lethal* gene of *Drosophila. Genetics* **117**, 221–231.

Samuels M. E., Bopp D., Colvin R. A., Roscigno R. F., Garcia-Blanco M. A. and Schedl P. 1994 RNA binding by Sxl proteins *in vitro* and *in vivo*. *Mol. Cell. Biol*. **14**, 4975–4990.

Saumweber H., Symmons P., Kabisch R., Will H. and Bonhoeffer F. 1980 Monoclonal antibodies against chromosomal proteins of *Drosophila melanogaster*. *Chromosoma* **80**, 253–275.

Singer R. H. 1998 Triplet-repeat transcripts: A role for RNA in disease. *Science* **280**, 696–697.

Spradling A. C., Stern D. M., Kiss I., Roote J., Laverty T. and Rubin G. M. 1995 Gene disruptions using P transposable elements: an integral component of the *Drosophila* genome project. *Proc. Natl. Acad. Sci. USA* **92**, 10824–10830.

Velazquez J. and Lindquist S. 1984 Hsp70: nuclear concentration during environmental stress and cytoplasmic storage during recovery. *Cell* **36**, 655–632.

Venables J. P. and Eperon I. C. 1999 The roles of RNA-binding proteins in spermatogenesis and male infertility. *Curr. Opin. Genet. Dev*. **9**, 346–354.

Weighardt F., Balmonti G. and Riva S. 1996 The role of heterogeneous nuclear ribonucleoproteins hnRNP in RNA metabolism. *BioEssays* **18**, 747–756.

Weighardt F., Cobianchi F., Cartegni L., Chiodi I., Villa A., Riva S. and Biamonti G. 1999 A novel hnRNP protein HAP/SAF-B enters a subset of hnRNP complexes and relocates in nuclear granules in response to heat shock. *J. Cell Sci*. **112**, 1465–1476.

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