Developmental regulation and complex organization of the promoter of the non-coding *hsrw* gene of *Drosophila melanogaster*

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The nucleus-limited large non-coding hsrω-n RNA product of the *93D* or the *hsrw* gene of *Drosophila melanogaster* binds to a variety of RNA-binding proteins involved in nuclear RNA processing. We examined the developmental and heat shock induced expression of this gene by *in situ* hybridization of nonradioactively labelled riboprobe to cellular transcripts in intact embryos, larval and adult somatic tissues of wild type and an enhancer-trap line carrying the $hsrw^{05241}$ allele due to insertion of a *P-LacZ-rosy*$^{+}$ transposon at – 130 bp position of the *hsrw* promoter. We also examined *LacZ* expression in the enhancer-trap line and in two transgenic lines carrying different lengths of the *hsrw* promoter upstream of the *LacZ* reporter. The *hsrw* gene is expressed widely at all developmental stages; in later embryonic stages, its expression in the developing central nervous system was prominent. In spite of insertion of a big transposon in the promoter, expression of the $hsrw^{05241}$ allele in the enhancer-trap line, as revealed by *in situ* hybridization to hsrω transcripts in cells, was similar to that of the wild type allele in all the embryonic, larval and adult somatic tissues examined. Expression of the *LacZ* gene in this enhancer-trap line was similar to that of the *hsrw* RNA in all diploid cell types in embryos and larvae but in the polytene cells, the *LacZ* gene did not express at all, neither during normal development nor after heat shock. Comparison of the expression patterns of *hsrw* gene and those of the *LacZ* reporter gene under its various promoter regions in the enhancer-trap and transgenic lines revealed a complex pattern of regulation, which seems to be essential for its dynamically varying expression in diverse cell types.

Introduction

The eukaryotic nucleus produces a large variety of transcripts, only some of which are actually translated into proteins. Many other RNA species like snRNAs are not translated but are involved in processing the nascent transcripts of other genes so that they become translatable. Besides these, an increasing number of "typical" genes are being identified, which neither code for any protein themselves nor are involved in processing of the other genes' transcripts; their biological roles have remained enigmatic (Lakhotia 1996, 1999; Erdmann *et al* 1999). Persistent efforts are now succeeding in unravelling novel roles for such non-coding RNAs. For example, the Xist and Rox non-coding nuclear RNAs in mammals and *Drosophila*, respectively, have crucial roles in regulating the activity status of the entire X-chromosome through their binding. Xist RNA "paints" one of the two X-chromosomes in somatic cells of female mammals and keeps that chromosome inactive while the Rox-family transcripts "paint" the single X-chromosome in male *Drosophila* to make it hyperactive (Kelley and Kuroda 2000; Spusta and Goldman 1999). Apparently, these transcripts mediate the binding of effector protein molecules which either repress or hyperactivate the chromosomal DNA templates.

The *93D* or the *hsrw* gene of *Drosophila melanogaster* is yet another example of a non-coding gene which is

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Abbreviations used: RISH, RNA : RNA *in situ* hybridization; PSS, Poels' salt solution; HSE, heat shock elements.

widely expressed in *Drosophila* cells and which is induced by heat shock and by amides (for a recent review, see Lakhotia *et al* 1999). Studies in our laboratory (Lakhotia *et al* 1999; Prasanth *et al* 2000) have provided interesting insights into the functions of this gene's major nuclear transcript. It has been shown that the > 10 kb nucleus-limited non-coding transcripts of the *hsrw* gene, the hsrω-n transcripts (Bendena *et al* 1989), are distributed as minute speckles in the nucleoplasm in close vicinity to chromatin areas in a cell- and tissue-specific manner; nucleoplasmic heterogeneous nuclear RNA binding proteins (hnRNPs) and other related RNPs colocalize specifically and universally with these "omega speckles" (Lakhotia *et al* 1999; Prasanth *et al* 2000). It has been suggested that nuclear hnRNPs and related proteins which at any given time are not productively engaged in the processing of hnRNAs on active chromatin regions, are sequestered for storage by the hsrω-n RNA and form the omega speckles in perichromatin space. The dynamic changes in transcriptional and RNA processing activities in relation to developmental requirements in different cell types or to continuously changing environmental conditions cause variable amounts of hnRNPs to be exchanged between an active state on the chromatin and an inactive state in omega speckles (Lakhotia *et al* 1999; Prasanth *et al* 2000). In order to efficiently sequester hnRNPs and to release them for processing activities as and when required, the levels of hsrω-n transcripts in a nucleus need to be continuously "fine tuned". For such a global and dynamic regulation of post-transcriptional processing of various nuclear transcripts to be in place, it is expected that the transcription of *hsrw* should also be tightly controlled in space and time according to tissue type, developmental stage and environmental conditions.

In view of the above, we have undertaken a detailed study of the developmental expression of *hsrw* during embryonic development and in different larval and adult tissues of *Drosophila* using RNA : RNA *in situ* hybridization as well as *LacZ* reporter gene expression. The present study extends earlier reports (Bendena *et al* 1991; Mutsuddi and Lakhotia 1995) on developmental expression of the *hsrw* gene. In addition, we have also examined promoter functions of the *hsrw* gene using transgenic lines carrying a reporter *LacZ* gene under defined promoter sequences. In the present work, we have utilized transgenic lines generated earlier in our laboratory (Mutsuddi and Lakhotia 1995). We also used the *05241 P*-element *LacZ* enhancer-trap line (Spradling *et al* 1995) whose *LacZ* reporter is under the control of the resident *hsrw* promoter. The expression of the *LacZ* reporter gene in the transgenic and enhancer-trap lines was compared to normal expression of the *hsrw* gene in different cell types by RNA : RNA *in situ* hybridization.

2. Materials and methods

2.1 *Fly stocks*

D. melanogaster cultures were reared at 22°C on standard food containing agar, maize powder, yeast and sugar. For cytological preparations, staged larvae were grown in petri plates with food, supplemented with additional yeast for healthy growth. The different fly stocks used in this study were as follows.

(i) *Oregon* R^+ – A wild type strain of *D. melanogaster*.
(ii) *l(3)05241/TM6B* – The *l(3)05241* is an enhancer-trap line with the *P-LacZ-rosy*$^+$-transposon inserted in the 93D region. This insertion line was generated by Spradling *et al* (1995) during a screen of lethal *P*-insertions. In this stock, the *P*-insertion carrying homologue is balanced with the *TM6B* balancer. The *TM6B* balancer chromosome carries the dominant *Tb* marker which allows the carriers of this chromosome to appear shorter and thicker or "tubby" (for details, see Lindsley and Zimm 1992). The *P*-insertion homologue will be referred to as *05241*.

Our studies revealed that *05241* homozygotes do not show any mutant phenotype except sterility of homozygous males (Lakhotia *et al* 1999; T K Rajendra, K V Prasanth and S C Lakhotia, manuscript in preparation). PCR amplification of genomic DNA from *05241* flies with combinations of *P*-element and *hsrw* gene-specific primers and sequencing of the amplicons showed that the enhancer-trap *P-LacZ-rosy*$^+$ transposon in *05241* chromosome is inserted at – 130 bp in the promoter region of the *hsrw* gene (details not shown). Consequently, the *hsrw* promoter region in the *05241* chromosome is split into two components with the *LacZ* reporter gene being under control of the *hsrw* upstream regulatory region, minus the proximal – 129 bp sequence of the promoter while the resident *hsrw* transcription unit is flanked on the 5′ end by only the proximal 129 bp of the promoter (figure 1).

To study the effect of this *P*-insertion on *hsrw* expression, the non-tubby larvae and flies (i.e. those not carrying the *TM6B* balancer and, therefore, homozygous for the *05241* chromosome) from this stock were used.
(iii) *951LacZ* – The *951LacZ* is a homozygous viable transgenic line, generated by Mutsuddi and Lakhotia (1995). The transgene in this carries the *LacZ* reporter gene under the control of 844 bp of proximal promoter and + 107 bp of the first exon of *hsrw* (figure 1). The transposon in this line is inserted at 30B on 2L chromosome.
(iv) *498LacZ* – The *498LacZ* is also a homozygous viable transgenic line generated by Mutsuddi and Lakhotia (1995) in which the reporter *LacZ* was placed under control of – 844 to – 345 bp of *hsrw* promoter. This line differs from the *951LacZ* line by not having the region from – 346 bp to + 107 bp of *951LacZ* (figure 1). The transposon in this line is inserted at 44B on 2R chromosome.

2.2 *Preparation of anti-sense riboprobes*

The *pJG10* and *pDRM30* clones, originally received from Prof. M L Pardue's Laboratory (MIT, USA), were used for generating anti-sense RNA probes. *pJG10* contains the 1·2 kb *93D* cDNA in a pSP65 vector. *pDRM30* carries the 280 bp *Asu II* fragment of the *93D* repeat region in a *pGem3* vector. The *pDRM30* riboprobe detects only the nucleus limited hsrω-n RNA while the *pJG10* riboprobe can recognize the nuclear as well as the cytoplasmic transcripts of *hsrw* (Lakhotia and Sharma 1995). For production of dig-labelled anti-sense riboprobes, *pDRM30* and *pJG10* clones were linearized with *Hin*dIII and the linearized plasmids transcribed *in vitro* using either T7 RNA polymerase (for *pDRM30*) or SP6 RNA polymerase (for *pJG10*) with dig-UTP (Roche Biochemicals, Germany) as the labelled precursor as prescribed by the manufacturer.

2.3 *RNA : RNA in situ hybridization in embryos and larval and adult somatic tissues*

Wild type and *05241* eggs were collected at hourly intervals or for a period of 12–16 h on an agar plate and washed with sterile water on a nylon mesh. For heat shock, the nylon mesh with embryos was transferred to a moist chamber pre-warmed to 37°C for 1 h following which the control and heat shocked embryos were repeatedly washed in DEPC treated distilled water. Heat shocked embryos were washed in water prewarmed to 37°C to prevent recovery from heat shock.

Wild type and *05241* late 3rd instar larvae and flies were heat shocked at 37°C for 1 h. Both the control (maintained at 22°C) and heat shocked animals were quickly dissected in Poels' salt solution (PSS, Lakhotia and Tapadia 1998) and the desired tissues taken out. For

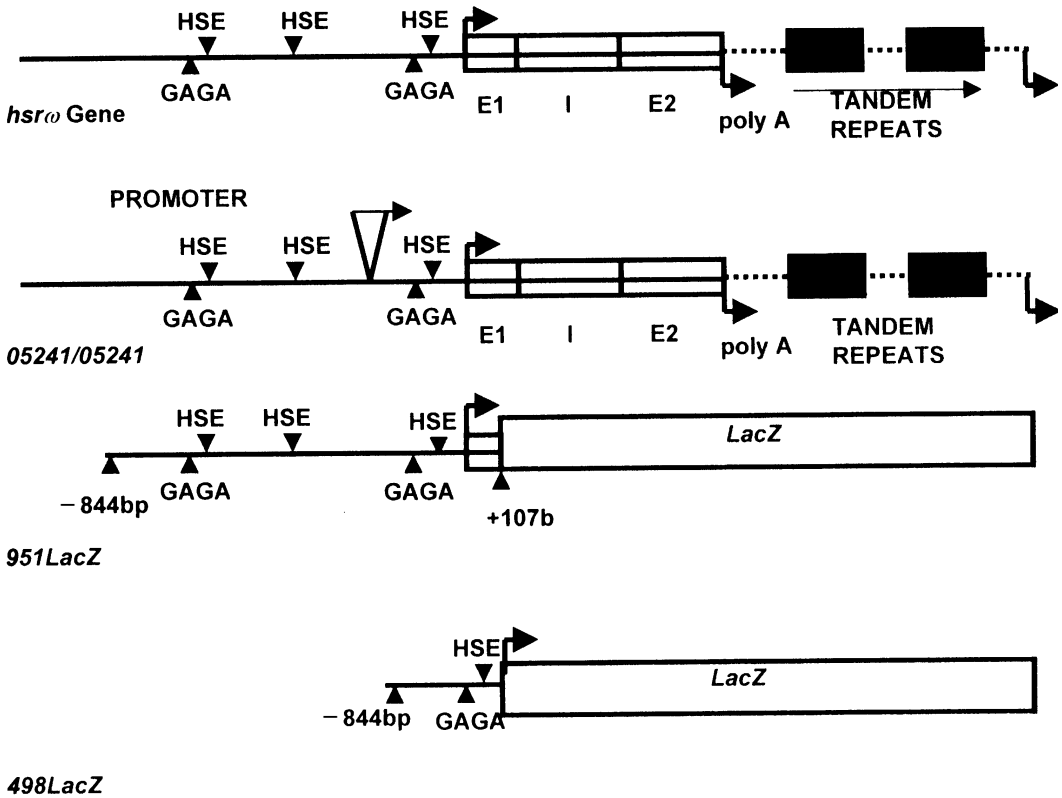

Figure 1. Architecture of *hsrw* gene in wild type and *05241* enhancer-trap line and of the two *hsrw–LacZ* fusion transgenes. Locations of the HSE and GAGA-factor binding sites (GAGA), mapped by Mutsuddi and Lakhotia (1995), relative to the *hsrw* gene or the *LacZ* reporter gene are indicated. The *P-LacZ-rosy*$^+$ transposon is inserted at – 130 bp region of the *hsrw* promoter. The *951LacZ* transgenic line carries the – 844 to + 107 bp region of the *hsrw* gene upstream of the *LacZ* reporter gene while the *498LacZ–rosy*$^+$ transgenic line carries only – 844 to – 107 bp region of the *hsrw* gene upstream of the *LacZ* reporter gene while the *498LacZ* transgene carries only – 844 to – 345 bp region of the *hsrw* promoter upstream of a minimal promoter and *LacZ* reporter gene (for details, see Mutsuddi and Lakhotia 1995). Not drawn to scale.

RNA : RNA *in situ* hybridization (RISH) on the adult thoracic structures, the control and heat shocked flies were etherized and glued to a glass slide with a small amount of 80% glycerol and frozen in liquid nitrogen. The slides were quickly placed under a stereo-binocular microscope and the thoraces were cut dorso-ventrally with the help of a sharp razor blade.

RISH on adult brains was carried out in histological sections of adult heads. For heat shock, 3 to 4 days old flies of different genotypes were kept at 37 ± 1°C for 1 h. The heat shocked and control flies were decapitated and the proboscis removed to facilitate accessibility of internal head structures to fixative. The cut heads were fixed in 4% paraformaldehyde in PBS (13 mM NaCl; 0·7 mM Na_2HPO_4; 0·3 mM NaH_2PO_4, pH 7·0–7·5) for 20 min at 4°C. Following fixation, the heads were dehydrated through ethanol grades and processed for paraffin embedding and histological sectioning following Ausubel *et al* (1994).

All other tissues were also fixed in 4% paraformaldehyde in PBS for 15–20 min on ice. The fixative was replaced with 4% paraformaldehyde + 0·6% Triton X-100 in PBS for 15–20 min. The samples were washed 3 × 5 min each in PBT (PBS + 0·1% Tween 20) and treated with 10 µg/ml proteinase K in PBT for 3 min followed by 2 × 5 min washing in chilled PBT + 2 mg/ml glycine. The tissues were further washed 3 × 5 min in PBT. The PBT was replaced with 4% paraformaldehyde + 0·2% glutaraldehyde in PBS for 15 min, followed by 5 × 5 min washing in PBT.

RISH was carried out essentially as described by Lehman and Tautz (1994). Embryos and other tissues were hybridized with a mixture of dig-labelled *pJG10* and *pDRM30* antisense riboprobes at 58°C for 12 14 h in hybridization buffer A (50% deionized formamide; 5 × SSC; 100 µg/ml yeast tRNA; 100 µg/ml Salmon sperm DNA; 50 µg/ml Heparin; 0·1% Tween 20 pH 5). Signal was detected using alkaline phosphatase (AP) conjugated anti-digoxigenin antibody as per the manufacturer's (Roche Biochemicals, Germany) instructions. After staining, the embryos and other tissues/sections were mounted in 50% glycerol.

2.4 *RISH on squash preparations of polytene chromosomes*

Salivary glands from wild type and *05241/05241* late third instar larvae were either treated with benzamide (10 mM in PSS) for 10 min at room temperature or heat shocked for 30 min at 37°C. Squash preparations of treated and control polytene chromosomes were processed for RNA : RNA *in situ* hybridization with dig-labelled riboprobes essentially as described by Sharma and Lakhotia (1995). Hybridization was detected by TRITC conjugated anti-dig antibody (1 : 10 dilution, Roche Biochemicals, Germany) The chromosomes were counterstained with DAPI and the slides were mounted in Vectashield with a 22 mm^2 coverslip and sealed with nail polish. The slides were stored at – 20°C till observation in fluorescence microscope.

2.5 *Staining for LacZ reporter b-galactosidase activity in embryos and larval and adult tissues*

Eggs of *05241 P*-transposon insertion line and of the *P-LacZ951* and *P-LacZ498* transgenic lines, with a *LacZ* reporter gene under the *hsrw* promoter (see above), were collected at hourly intervals or over a period of 12–16 h on an agar plate and washed with sterile water on a nylon mesh. For heat shock, the nylon mesh with embryos was transferred to a moist chamber pre-warmed to 37°C for 1 h. The heat shocked embryos were allowed to recover for an hour at 22°C to allow synthesis of the heat shock induced reporter *b*-galactosidase. Chorion and vitelline membranes were removed and the embryos fixed as described earlier for RISH. Finally the embryos were washed with 200 µl of 0·1 mM sodium phosphate buffer (pH 8·0) with 0·2% Triton X-100. The washing buffer was replaced with the X-gal staining solution as described earlier (Mutsuddi and Lakhotia 1995). The embryos were incubated in dark at 37°C till the colour developed sufficiently. Colour development in control and heat shocked embryos was allowed for the same time interval. The embryos were finally washed thrice in wash buffer for 5 min each and mounted in 80% glycerol.

For heat shock, the late third instar larvae and flies of the above three reporter *LacZ* lines were heat shocked at 37 ± 1°C for 1 h and allowed to recover at 22°C for 1 h. Various tissues were dissected out from control and heat shocked animals in PSS. For studying the *LacZ* reporter activity in the adult thoracic structures, the thoraces were cut dorso-ventrally with the help of a sharp razor blade as described above for RISH. All tissues were fixed in 2·5% glutaraldehyde following which they were washed three times, 10 min each, with 50 mM Na-phosphate buffer. The buffer was replaced with 50 µl of X-gal staining solution (as above) and the tissues incubated in a moist chamber in dark at 37°C till the colour developed sufficiently well. Finally, the tissues were washed twice for 10 min each in 50 mM Na-phosphate buffer and mounted in 80% glycerol.

2.6 *Microscopy and documentation*

All RISH and X-gal stained preparations of embryos, intact tissues and sections were examined under bright-

field optics using a Nikon E800 microscope. Fluorescence RISH with squash preparations of larval salivary gland chromosomes was detected using appropriate fluorescence filter blocks. Photomicrographs were taken with Fuji 200 ASA colour film and the prints were scanned at 300 to 600 dpi. The scanned images were assembled using the Adobe Photoshop 5·0 software.

3. Results

3.1 *Expression of hsrw in embryos*

3.1a *Developmental expression*: The expression of *hsrw* during embryonic development is described in table 1. The table details the expression of *hsrw* as revealed by RISH in wild type and *05241* embryos and the expression of the *LacZ* reporter in *05241*, *951LacZ* and *498LacZ* control embryos. Illustrative examples of the RISH and *LacZ* staining patterns are presented in figure 2. As revealed by RISH, there was no difference in the developmental expression of *hsrw* between wild type and *05241* embryos (not shown). The *hsrw* transcripts were mostly detected in nuclei in the form of nuclear speckles whose number varied from one in epidermal and midventral cells of the germband to 4–10 in amnioserosa cells.

Though the *hsrw* transcripts were detectable with beginning of cellularization of the blastoderm, reporter *LacZ* expression started only after the completion of germband extension. As detailed in table1 (see figure 2d′–g′), the *LacZ* expression in *05241* embryos was essentially similar to the RISH patterns. However, in the *951LacZ* and *498LacZ* reporter lines, the X-gal staining patterns were different (table 1 and figure 2d″–g″ and 2d‴–g‴).

3.1b *Heat shock*: Although the *hsrw* transcripts were normally not present in the syncitial blastoderm, they were strongly induced by heat shock at this stage (figure 2h). However, the pole cells did not show any hybridization even after heat shock (figure 2i, j). After heat shock, the expression of *hsrw* was enhanced in all the embryonic stages in all those cell types where the *hsrw* was developmentally expressed (not shown). The nuclear speckles in RISH preparations became clustered and thicker upon heat shock. While the developmental expression of *hsrw* was similar in wild type and *05241* embryos (table 1), *05241* embryos showed a comparatively stronger heat shock response than the wild type (not shown).

The X-gal staining in all regions which showed a developmental *LacZ* reporter activity, was enhanced by heat shock in all the three reporter *LacZ* lines (not shown).

3.2 *Expression of hsrw in third instar larvae*

3.2a *Developmental expression*: The detailed expression profiles of *hsrw* in different larval tissues, detected by RISH in wild type and *05241* and by X-gal staining in the *LacZ* reporter lines, are presented in table 2 and figures 3 and 4. As is clear from table 2 and the illustrations in figures 3 and 4, there was no difference in the developmental expression of *hsrw* transcripts between wild type and *05241* as revealed by RISH. The expression was mostly in the form of nuclear speckles.

LacZ reporter activity in diploid tissues like brain ganglia (figure 3c) and the various imaginal disks (figure 3h) in the *05241* line was generally similar to the RISH patterns. In polytene cell types, like the prothoracic glands (figure 3c), salivary glands (figure 3m) and gut (figure 4c), *LacZ* reporter activity was almost completely absent

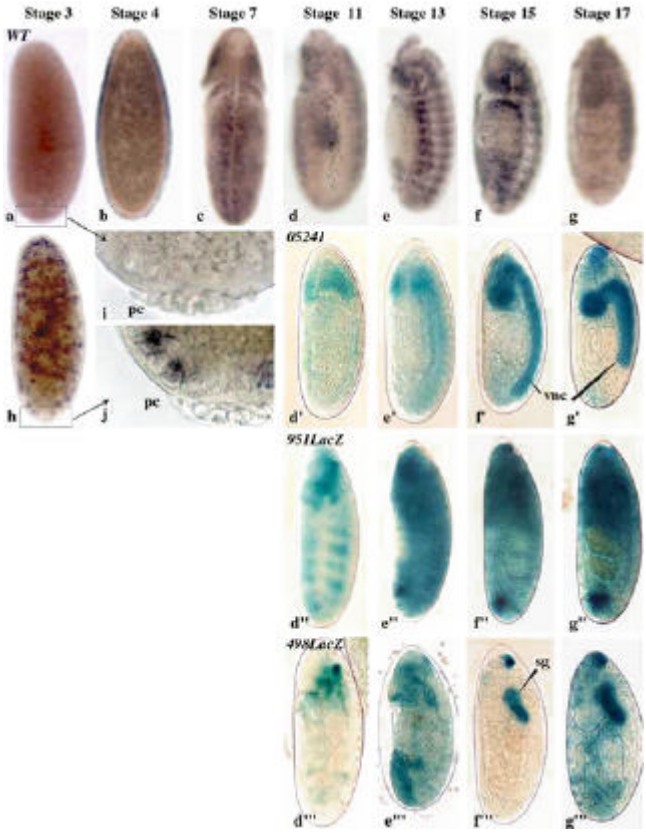

Figure 2. Expression of *hsrw* during normal embryonic development (stages 3, 4, 7, 11, 13, 15 and 17) as monitored by RISH in wild type (**a**–**j**) or by X-gal staining of the *LacZ* reporter gene activity in *05241*, *951LacZ* and *498LacZ* (**d′** to **g‴**) lines. The wild type embryo in (**h**) was processed for RISH after heat shock. A small region of the posterior end of embryos in (**a**) and (**h**) is shown at higher magnification in (**i**) and (**j**), respectively, to reveal the absence of *hsrw* transcripts in pole cells (pc). vnc, ventral nerve cord; sg, salivary glands.

Table 1. Developmental expression of *hsrw* in embryos.

Embryonic stage	RISH		LacZ staining		
	+/+	*05241*	*05241*	*951LacZ*	*498LacZ*
Syncitial blastoderm	No expression (figure 2a).	As in wild type.	No expression	No expression	No expression
Cellular blastoderm	Uniform expression except in pole cells (figure 2b).	As in wild type.	No expression	No expression	No expression
Stage 6–7	Cells bordering the cephalic and ventral furrows show nuclear speckles (figure 2c). Expression in other cell types is weak or undetectable.	As in wild type.	No expression	No expression	No expression
Stage 8–10	Cells bordering the extending germband and the cephalic region showed nuclear speckles (figure 2d). The mid-ventral cells of the extending germband and the cells that border the mid-ventral cells with weak cytoplasmic staining.	As in wild type.	No expression	No expression	No expression
Stage 11–12	Strong in the extended germband, procephalon and the amnioserosa cells in amnioproctodeal invagination (figure 2d). The amnioserosa cells showed strong nuclear signal in the form of 3 to 10 speckles with one of them being larger.	As in wild type.	Only in the procephalic neuroblasts and the ventral nerve cord (figure 2d′); none in amnioserosa.	Around the anterior midugt invagination, salivary gland primordia and the segmented mesoderm (figure 2d″); none in amnioserosa.	Around the anterior midugt invagination and salivary gland primordia, but comparatively weaker than that in *951LacZ* (figure 2d‴); none in amnioserosa.
Stage 13–14	Almost all cell types showed expression (figure 2e) with a comparatively strong expression in amnioserosa with multiple nuclear speckles. Expression in the ventral nerve cord was mainly cytoplasmic. Majority of the other cell types showed nuclear speckles.	As in wild type.	Expression increased compared to the previous stage and mostly confined to embryonic central nervous system with a very low expression in the epidermal cells (figure 2e′); none in amnioserosa; generally similar to RISH pattern.	Expression increased compared to the previous stage and almost ubiquitous; stronger expression in or around the posterior spiracles and the cephalic region; enhanced in the salivary gland primordia; none in amnioserosa (figure 2e″).	Expression increased compared to previous stage in the region in and around the anterior midgut invagination and the invaginating salivary glands; low expression in the retracting germ band; none in the amnioserosa (figure 2e‴).
Stage 14–15	No signal in amnioserosa due to disappearance of amnioserosa by dorsal closure (figure 2f); rest as in stage 13–14.	As in wild type.	Expression was further enhanced, but confined only to embryonic central nervous system with very low expression in epidermal cells (figure 2f′); generally similar to RISH pattern.	Expression was further enhanced ubiquitously; comparatively stronger expression in salivary glands, posterior spiracles and the cephalic region (figure 2f″); expression in the ventral nerve cord was comparatively weak.	Expression was enhanced but confined only to the embryonic salivary glands and a region at the anterior extremity, possibly the labial discs (figure 2f‴).
Stage 16 and later	The pattern of expression was similar to that of stage 14–15 and remained so thereafter (figure 2g).	As in wild type.	Expression further enhanced only in the embryonic central nervous system; expression in imaginal discs detectable (figure 2g′); generally similar to RISH pattern in wild type.	Expression further enhanced (figure 2g″) but similar to stage 14–15. Expression in the ventral nerve cord remained weak.	Expression further enhanced in salivary glands and labial discs; a mild expression all over except for the central nervous system (figure 2g‴).

in the *05241* line. However, the diploid imaginal cells in salivary glands and gut in these larvae showed a weak X-gal staining.

As detailed in table 2, the *LacZ* expression patterns in the various larval tissues in the *951LacZ* and *498LacZ* lines were in agreement with the earlier report by Mutsuddi and Lakhotia (1995). Expression in the *498LacZ* line was significantly weaker than in the *951LacZ* line (figures 3 and 4).

3.2b *Heat shock*: Heat shock enhanced the expression of the *hsrw* gene in a similar manner in wild type and *05241* larval tissues (see figure 3a′, b′, f′, g′, k′, l′), only in those cell types that showed developmental *hsrw* RNA. Along with an increase in the *hsrw* expression due to heat shock, the thickness of nuclear speckles also increased in both wild type and *05241* tissues.

Unlike the enhanced levels of hsrω transcripts seen after RISH in the heat shocked *05241* larval tissues, there was little induction of reporter *LacZ* in polytene cell types (prothoracic gland, salivary gland or gut cells) of these larvae (figures 3c′, 3m′ and 4c′). Heat shock caused enhanced X-gal staining in diploid cell types (figures 3c′, 3m′ and 4c′).

All the larval cells of the *951LacZ* and *498LacZ* transgenic lines that showed developmental expression of the *LacZ* reporter gene, showed enhanced X-gal staining after heat shock (figures 3d′, 3e′, 3i′, 3j′, 3n′ and 4c′, 4d′, 4e′).

The response of the 93D puff in control, heat shocked and benzamide treated salivary glands of late third instar

Table 2. Developmental expression of *hsrw* in third instar larvae.

	RISH		LacZ staining		
Tissues	+/+	*05241*	*05241*	*951LacZ*	*498LacZ*
Brain	Strong expressin in the brain hemispheres, presumably in the proliferating neuroblasts (figure 3a). There was little or weak expression in the ventral ganglion.	As in wild type (figure 3b).	Ubiquitous (figure 3c).	Strong expression on the brain hemispheres; very weak or no expression in the ventral ganglion (figure 3d).	Similar to but weaker than *951LacZ* (figure 3e).
Prothoracic glands	Very strong expression with many nuclear speckles (figure 3a).	As in wild type (figure 3b).	No expression (figure 3c).	Strong expression (figure 3d).	Weaker than in *951LacZ* (figure 3e).
Wing discs	Ubiquitous expression (figure 3f) mainly as nuclear speckles. The cells of the disc proper showed a single nuclear speckle, while two to three speckles were seen in the peripodial cells.	As in wild type (figure 3g).	Strong ubiquitous expression (figure 3h).	Ubiquitous with a more pronounced expression in the pouch region (figure 3i).	Weaker than in *951LacZ* (figure 3j).
Salivary glands	Strong expression (figure 3k) in the form of many nuclear speckles.	As in wild type (figure 3l).	No expression except in the imaginal cells near the proximal region (figure 3m).	Strong expression except at the base of the glands (figure 3n).	Similar to but weaker than in *951LacZ* (figure 3o).
Gut	Strong expression in all regions of the gut including Malpighian tubules (figure 4a) with multiple nuclear (~ 2–15) speckles in most cells.	As in wild type (figure 4b).	Almost absent in all regions of the gut including the Malpighian tubules (figure 4c).	Strong ubiquitous expression in gut and Malpighian tubules (figure 4d).	Similar to but weaker than in *951LacZ* (figure 4e).
Other larval tissues	No detectable expression in the larval musculature. Expression seen in the larval gonads, fat bodies, epidermis and the tracheal system (not shown) in the form of multiple nuclear speckles, with one of them being larger than the others.	As in wild type.	No expression in larval musculature, fat bodies and epidermis but expressed in the tracheal system.	No expression in larval musculature, fat bodies and epidermis. Weak tracheal expression.	Similar to *951LacZ* but with very weak or no expression in the tracheal system.

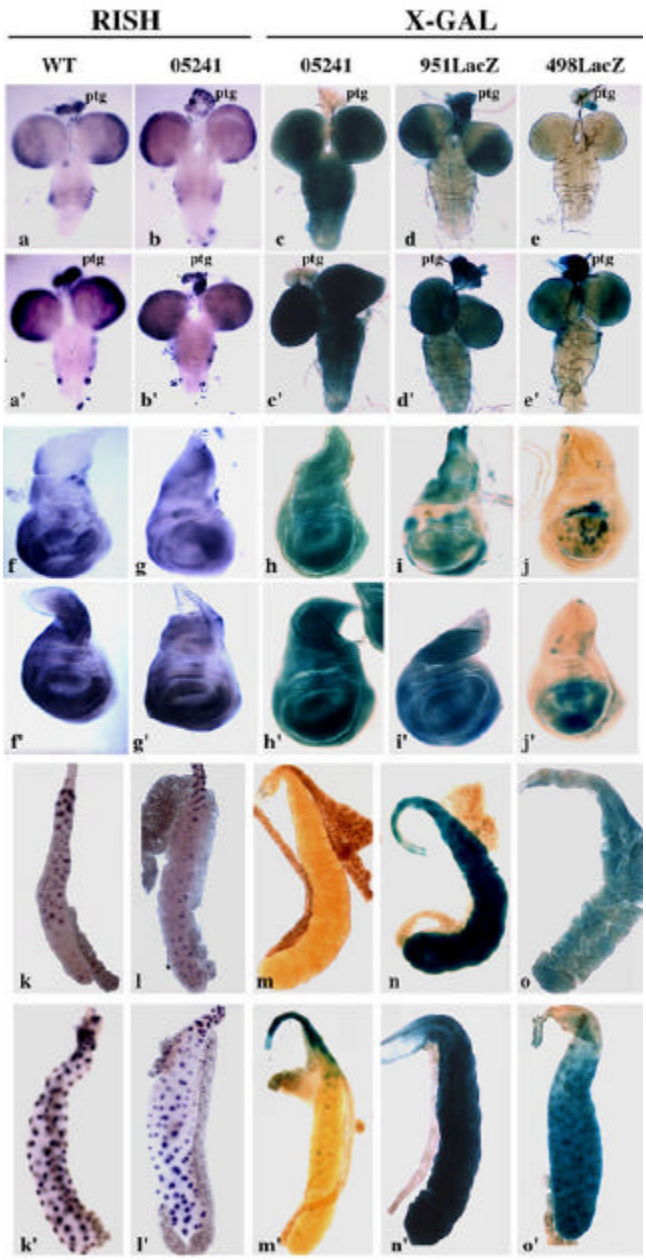

Figure 3. Expression of *hsrw* in brain ganglia (**a**–**e**), wing imaginal discs (**f**–**j**) and salivary glands (**k–o**) of wild type (**a**, **a′**, **f**, **f′**, **k**, **k′**), *05241* (**b**, **b′**, **c**, **c′**, **g**, **g′**, **h**, **h′**, **l**, **l′**, **m**, **m′**), *951LacZ* (**d**, **d′**, **i**, **i′**, **n**, **n′**) and *498LacZ* (**e**, **e′**, **j**, **j′**, **o**, **o′**) late third instar larvae under normal developmental condition (**a–o**) or after heat shock (**a′–o′**) as detected by RISH (columns 1–2) or by X-gal staining (columns 3–5) as indicated. Note the absence of any *LacZ* activity in prothoracic glands attached to the brain ganglia (ptg) and in polytene cells of salivary glands of *05241* larvae (column 3).

3.3a *Developmental expression*: The expression of *hsrw*, as detected by RISH was comparable between different somatic tissues of wild type and *05241* flies (table 3 and figures 6 and 7). Reporter *LacZ* expression in the *05241* line was generally similar to the RISH patterns, except in thoracic muscles, trachea and the gut (see table 3 for details). The other two transgenic lines containing the *LacZ* reporter gene showed differences in the X-gal staining patterns as described in table 3 (see figures 6 and 7).

3.3b *Heat shock*: The wild type and *05241* somatic tissues showed a similar increase in the *hsrw* transcript levels in response to heat shock (figures 6 and 7).

Heat shock led to an enhanced *LacZ* expression only in those cell types which showed a developmental expression (figures 6c′, 6d′, 6e′ and 7b′, 7c′, 7d′), with the exception of the brain in *498LacZ* and the gut in *05241* flies. While no reporter *LacZ* expression was seen in the control *498LacZ* brain (figure 6e), it was strongly induced in the optic lobes after heat shock (figure 6e′). Likewise, although there was no expression of the reporter *LacZ* in

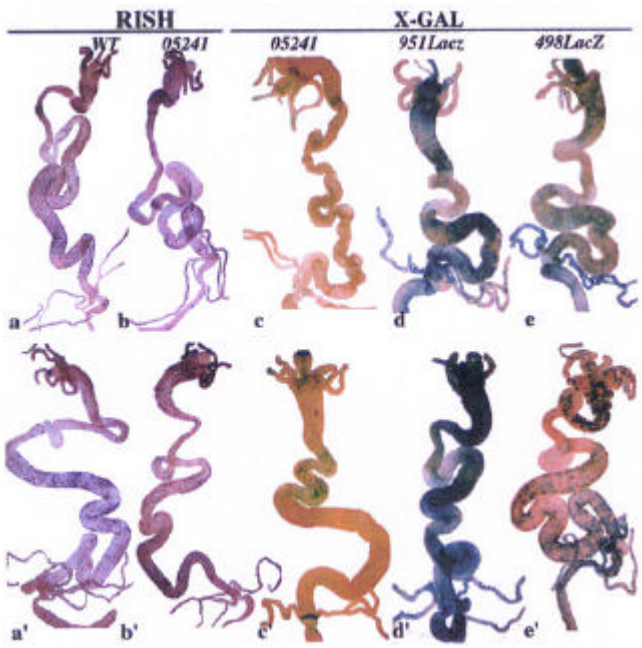

Figure 4. Expression of *hsrw* in gut of wild type (**a**, **a′**) *05241* (**b**, **b′**, **c**, **c′**), *951LacZ* (**d**, **d′**) and *498LacZ* (**e**, **e′**) late third instar larvae under normal developmental condition (**a**–**e**) or after heat shock (**a′–o′**) as detected by RISH (columns 1–2) or by X-gal staining (columns 3–5) as indicated.

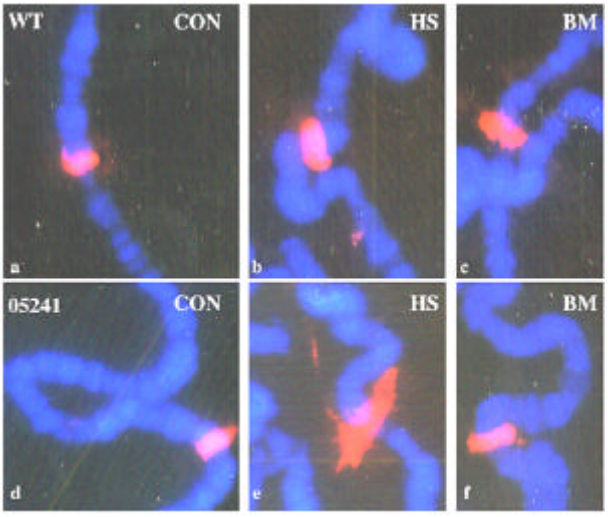

Figure 5. Expression of *hsrw* in salivary gland polytene chromosomes of wild type (**a–c)** and *05241* (**d–f**) late third instar larvae under normal developmental condition (**a**, **d**) or after heat shock (**b**, **e**) or after benzamide treatment (**c**, **g**) as detected by fluorescence RISH. The hsrω transcripts at the 93D puff site are seen in red while the chromosomal DNA is blue.

05241 adult gut (figure 7b), X-gal staining was increased to a limited extent in proventriculus, anterior midgut and the Malpighian tubules after heat shock (figure 7b′).

4. Discussion

The expression and regulation of the non-coding gene *hsrw* has been studied earlier by Bendena *et al* (1991), Mutsuddi and Lakhotia (1995) and Lakhotia and Mutsuddi (1996). The present work extends these earlier studies and provides more comprehensive information about the developmental and heat shock-inducible expression patterns of *hsrw* gene. The results with different *LacZ* reporter constructs provide additional information about the complex regulation of this widely expressed non-coding gene.

4.1 *Cell and stage-specific expression of hsrw*

Our results show that *hsrw* is not expressed to the same extent in all cell types. While it is expressed in most cell

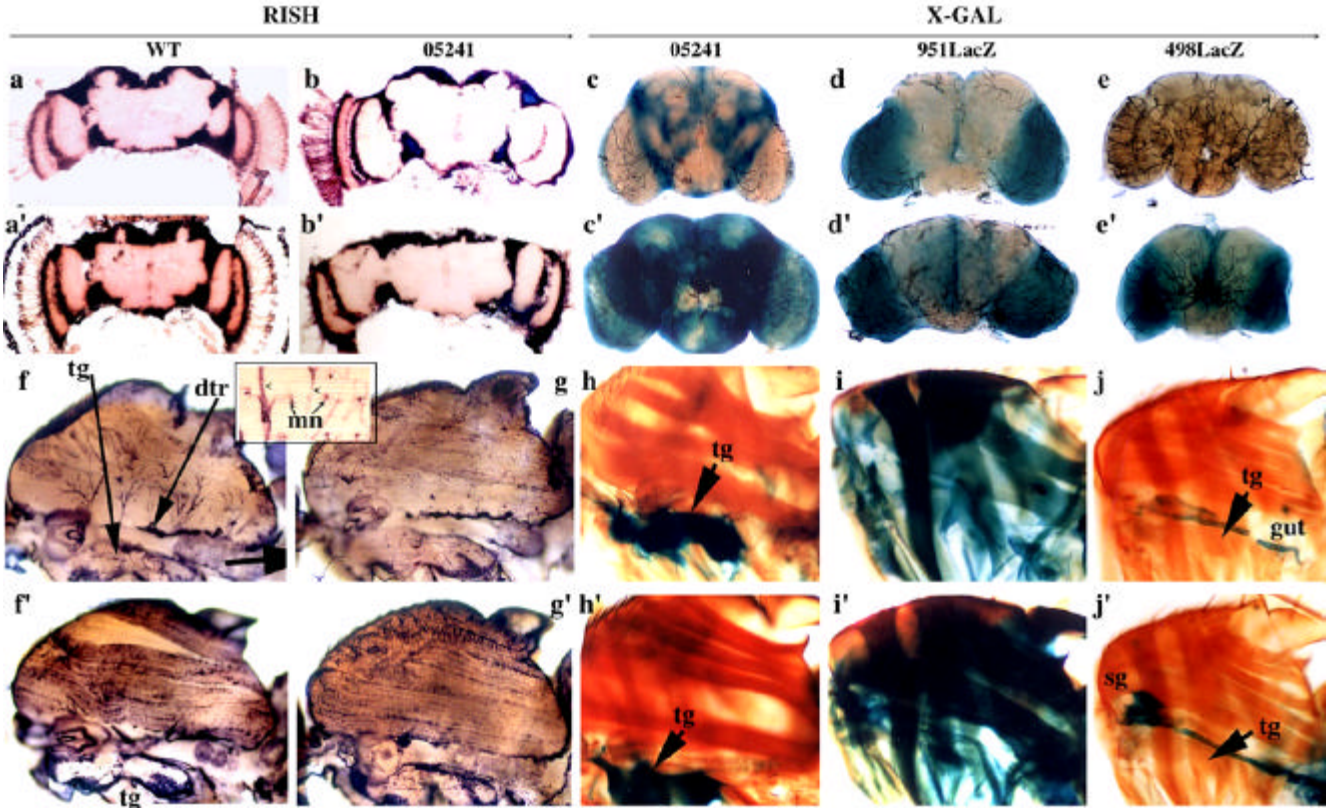

Figure 6. Expression of *hsrw* in head (**a–e** and **a′–e′**)and thoracic regions (**f–j** and **f′–j′**) of wild type (**a**, **a′**, **f**, **f′**), *05241* (**b**, **b′**, **c**, **c′**, **g**, **g′**, **h**, **h′**), *951LacZ* (**d**, **d′**, **i**, **i′**) and *498LacZ* (**e**, **e′**, **j**, **j′**) adult flies under normal developmental condition (**a–e** and **f–j**) or after heat shock (**a′–j′**) as detected by RISH (columns 1–2) or by X-gal staining (columns 3–5) as indicated. The heads in figures (**c–e**) and (**c′–e′**) are intact brains while all other figures show histological sections of head or thoracic regions. The inset in (**f–g**), shows a part of thoracic section in (**f**) at higher magnification to show the presence of hsrω transcripts in the rows of muscle nuclei (mn) and in cells of tracheal branches (small arrow heads). Dtr, dorsal trachea; sg, salivary glands; tg, thoracic ganglia [in (**j**) and (**j′**) the

types, there are distinct quantitative variations in the abundance of its transcripts in different cell types. The most common hybridization signal was nuclear, indicating a greater abundance of the hsrω-n transcripts (although in several cell types, cytoplasmic hybridization was also clearly seen). Nuclear hybridization, was in the form of a variable number of speckles distributed in the nucleoplasm of all cell types in which *hsrw* was expressed. Due to the generally low resolution of our chromogenic detection method, these "omega speckles" did not appear to be as fine and numerous as seen after fluorescence *in situ* hybridization (Prasanth *et al* 2000). Nevertheless, the speckled pattern was unmistakable. Furthermore, as noted with fluorescence *in situ* hybridization (Lakhotia *et al* 1999; Prasanth *et al* 2000), the present chromogenic detection also revealed that after heat shock, most of the nucleoplasmic speckles aggregated into a single big cluster. This further confirms the universality of omega speckles and their aggregation after heat shock in different cell types of *Drosophila*.

In agreement with earlier observations (Bendena *et al* 1991; Buchenau *et al* 1997) no *hsrw* transcripts were found in preblastoderm embryos, presumably a reflection of the lack of general transcriptional activity and the absence of any maternal contribution. As also reported by Bendena *et al* (1991), the developmental expression of *hsrw* starts with the formation of cellular blastoderm. It is interesting to note that the activation of general transcription in early embryos coincides with the activation of *hsrw* and the nuclear recruitment of many of the RNA

Table 3. Developmental expression of *hsrw* in adult somatic tissues.

Adult tissues	RISH		LacZ staining		
	+/+	*05241*	*05241*	*951LacZ*	*498LacZ*
Brain	Strong expression in the cellular cortex of as revealed by RISH on brain sections (figure 6a) with no signal in the neuropil. Retina also showed a strong nuclear expression in the cell body rich basal region (figure 6a). In general, all cells of the cellular cortex showed a single nuclear speckle except for a group of large median cells, presumably neurosecretory, that showed multiple nuclear speckles. Weak expression also seen in the cytoplasm around the nucleus.	As in wild type (figure 6b).	Generally similar to the RISH pattern (figure 6c).	Confined only to optic lobes (figure 6d).	No expression (figure 6e).
Thoracic tissues	Strong in thoracic ganglia (figure 6f). Only the external thoracic ganglionic mass showed nuclear expression; no expression in the thoracic neuropil. Expression was more prominent in the ganglionic cells of the ventral side than on the dorsal side (figure 6f). The thoracic ganglionic mass also showed a weak cytoplasmic staining around the nucleus. Strong nuclear expression in dorsal tracheal trunk and its branches (figure 6f) and also in muscle nuclei that are arranged in rows (inset in figure 6f–g). No expression detected in the salivary glands.	As in wild type (figure 6g).	Unlike the RISH pattern, X-gal staining seen only in the ventral thoracic ganglion (figure 6h).	Expression in whole of thorax except the ventral thoracic ganglion (figure 6i).	Expressed only in gut and salivary glands (figure 6j).
Gut	Expression in whole of the digestive system except the Malpighian tubules (figure 7a). One to eight speckles per nucleus were observed in gut cells.	As in wild type (not shown).	Weak or no expression in gut and Malpighian tubules (figure 7b).	Excluding the posterior region of the midgut, expression was seen in all other regions of gut including Malpighian tubules (figure 7c).	Similar to but significantly weaker than in *951LacZ* (figure 7d).

processing factors (Dequin *et al* 1984; Segalat and Lepesant 1992; Buchenau *et al* 1997). As reported earlier (Lakhotia *et al* 1999; Prasanth *et al* 2000), the hsrω-n transcripts specifically associate with a variety of nuclear hnRNPs to form the nucleoplasmic omega speckles which may regulate the availability of nuclear RNA processing factors (Lakhotia *et al* 1999; Prasanth *et al* 2000). In view of this, the above noted coupling of activation of general chromosomal transcription with the activation of *hsrw* and the nuclear import of the various hnRNPs and other RNPs becomes very significant. It is also interesting that the timing of *hsrw* activation in the embryonic stage coincides with the third phase of Sxl expression (Salz *et al* 1989). The Sxl protein also binds to the 93D puff and, as with the hnRNPs, the binding becomes exclusive to this site after heat shock (Samuels *et al* 1994). The coincidence of the timing of *hsrw* expression and the third phase of Sxl expression may suggest a role for hsrω-n transcripts in regulating the Sxl activity, perhaps by affecting the sex-specific splicing of its transcripts. Significantly, among the *hsrw*-nullosomic survivors, the number of females is generally much higher than males (Lakhotia *et al* 2000). It will be interesting to examine if this is related to aberrant splicing of Sxl transcripts due to distribution in hnRNP metabolism caused by the absence of hsrω transcripts.

The pole cells, which are the exclusive precursors of future germ cells (Foe *et al* 1993) did not show any expression of *hsrw* even after heat shock. In the definitive germ cells also, *hsrw* is not expressed, except in spermatids and ovarian nurse cells (Bendena *et al* 1991; Mutsuddi and Lakhotia 1995). Expression of *hsrw* in germline cells may be kept repressed so that even heat shock cannot activate its transcription. We note that many hnRNPs are expressed in germ cells, but in the absence of hsrω-n transcripts none of them form nucleoplasmic speckles like those in somatic cells (our unpublished observations).

4.2 *Long range* cis*-acting multiple regulatory elements in* hsrω *promoter*

The comparative study of the expression of *hsrw* and the reporter *LacZ* gene expression driven by different regions of the *hsrw* promoter allows a better understanding of the organization of the regulatory sites controlling this gene's spatio-temporal expression in normal development and following heat shock. The *05241* line was particularly interesting because of the insertion of the *LacZ* reporter within the *hsrw* promoter. This not only split the promoter controlling the *hsrw* gene but also brought the *LacZ* reporter under control of a part of its promoter. Thus, a comparison of the expression of the *hsrw* gene (through RISH) with that of the *LacZ* reporter gene (through X-gal staining) in *05241* and the comparison of these two patterns with the RISH patterns in the wild type may allow identification of critical regulatory sites. A further comparison with the two transgenic lines (*951LacZ* and *498LacZ*) allows additional delineation of promoter components.

It is remarkable that in spite of the > 8 kb *P*-transposon insertion at the – 133 bp site of the *hsrw* promoter in *05241*, its expression, as assayed by RISH, was similar to that of the wild type allele in all somatic tissues that were examined. The only cells that showed a substantial difference in expression of *hsrw* transcripts in wild type and in *05241* were the cyst cells in the testis; and this seems to correlate with sterility of males, the only mutant phenotype shown by this *P*-insertion allele (Lakhotia *et al* 1999; other unpublished observations). However, the overall similarity in patterns indicates that either all the essential regulatory elements required for developmental expression are located within the proximal 132 bp region of the *hsrw* promoter, or more distal elements are able to exert

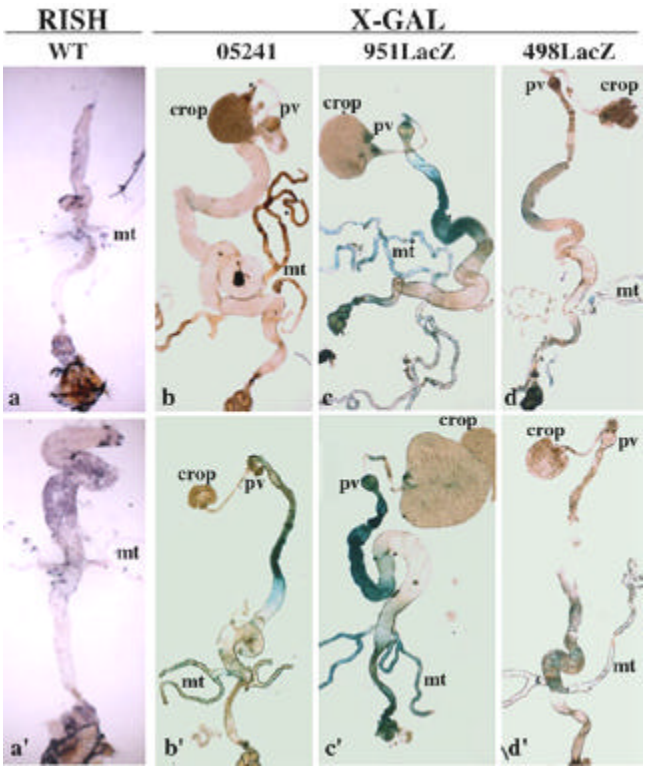

Figure 7. Expression of *hsrw* in gut of wild type (**a**, **a′**), *05241* (**b**, **b′**), *951LacZ* (**c**, **c′**) and *498LacZ* (**d**, **d′**) adult flies under normal developmental condition (**a**–**d**) or after heat shock (**a′**–**d′**) as detected by RISH (column 1) or by X-gal staining (columns 2-4) as indicated. mt, Malpighian tubules; pv, proventriculus; the region between proventriculus and origin of the Malpighian tubules from the gut is the mid gut while the region further behind is the hind gut.

their effects in spite of the intervening transposon. It is unlikely that the proximal region up to –132 bp contains all the required regulatory sequences since the *498LacZ* transgenic line, which does not carry this region of the promoter, is still able to activate the *LacZ* reporter gene in different tissues in a pattern that is not all that different from that of the *hsrw* gene (Mutsuddi and Lakhotia 1995; present observations). Lakhotia and Tapadia (1998) have also shown that the regulatory regions for amide inducibility of the *hsrw* locus are located far upstream (> 21 kb). Since the 93D puff in *05241* salivary glands remained inducible with benzamide as in wild type, it is clear that distal regulatory sequences are able to exert their effects on the *hsrw* gene, notwithstanding the *P*-transposon in between. Therefore, it is likely that the other upstream regulatory elements that have been displaced by the *P*-transposon, are also able to exert their control over the TATA box and other proximal regulatory elements.

An additional, or alternative, possibility to explain the similar expression patterns of the *hsrw* gene in wild type and 05241 flies is that some of the developmental regulatory elements are located downstream of the *hsrw* gene's transcription start point so that they remain intact even after insertion of the *P-LacZ-rosy*$^{+}$ transposon in the promoter region. However, since *LacZ* expression in the 05241 chromosome was generally similar (except in larval polytene and adult thoracic muscles and gut cells) to the *hsrw* expression on this chromosome, the downstream regulatory elements will have to be imagined to act not only upon the *hsrw* gene promoter but also upon the further upstream *LacZ* reporter gene. In some new *P*-transposon lines generated in our laboratory by mobilizing the 05241 insertion, the *P-LacZ-rosy*$^{+}$ transposon is inserted a few bases away from the original insertion site and in these cases, the expression of *hsrw* gene is altered in specific cell types (S Sengupta and S C Lakhotia, unpublished). Since the downstream region is not expected to have changed in these "jump" lines, it appears that, like other genes, the developmental regulatory elements of the *hsrw* gene are most likely in the upstream promoter region in the usual manner. However, these seem to have long-range *cis* actions. Further analysis of the mechanism of such long-distance interactions will be interesting.

4.3 *Differential regulation of* hsrω *in polytene and non-polytene cells*

Expression of the *LacZ* reporter gene in the *05241* chromosome in most cell types, except the larval and adult polytene cells, was generally similar. This strengthens our inference that the promoter elements that regulate the *hsrw* gene's expression in somatic cells are beyond – 130 bp, the site of insertion of the *P*-transposon in this enhancer-trap line. A feature of the *05241* chromosome was the singular non-expression of the *LacZ* reporter gene in all polytene larval and adult cells, although the *hsrw* gene itself expressed typically in all polytene cells. If this was because the sequences located in the proximal promoter (i.e. up to – 129 bp) were not available to activate the *LacZ* reporter in polytene nuclei, due to their being downstream of the reporter gene, one would expect the *498LacZ* line also to not show the reporter gene expression in polytene cells since in this line too, the proximal promoter region up to – 346 bp is absent (see figure 1). However, as reported earlier by Mutsuddi and Lakhotia (1995) and also seen in the present study, the reporter *LacZ* expression in this line was distinctly seen in different larval and adult polytene cells under normal developmental conditions which was enhanced by heat shock. Nevertheless, the proximal promoter region does have some role in expression in polytene cells since the polytene cells in *951LacZ* transgenic line show much stronger X-gal staining (Mutsuddi and Lakhotia 1995; present results). It appears that multiple promoter elements need to interact and because of the *P*-transposon insertion in the *05241* chromosome, some of the interactions that drive polytene cell expression are not available to the *LacZ* reporter gene. On the basis of certain differences in the response of polytene and non-polytene cells to drugs like benzamide or colchicine, it was suggested earlier (Lakhotia and Sharma 1996) that *hsrw* gene expression in polytene and non-polytene cells is differently regulated. Although the present set of data do not permit specific identification of the regulatory sequences, the differences in the reporter *LacZ* expression in polytene and non-polytene cells in the *05241* chromosome strengthens this view.

Three heat shock elements (HSE) have been identified in the *hsrw* promoter (Lakhotia and Mutsuddi 1995, see figure 1). Of these, the two distal HSEs are separated from the *hsrw* transcription unit by the *P*-transposon insertion in the *05241* chromosome. Analysis of heat shock promoters in several other genes of *Drosophila* indicated that multiple HSEs are required for a strong induction of the heat shock genes and that the HSEs that are more than a few hundred base pairs away do not seem to be effective (Hellmund and Serfling 1984; Lindquist 1986). The present results show that in spite of the two distal HSEs getting removed far away from the *hsrw* transcription unit due to the *P*-transposon insertion in the *05241* chromosome, the *hsrw* transcripts were very strongly induced in the *05241* cells (in several cell types actually more strongly than in wild type) by heat shock. Either the single proximal HSE is enough for the heat shock response of the *hsrw* gene or, like the developmental and amide response regulatory elements, the more distal HSEs are still able to interact across the *P*-transposon.

The already known independent levels of this gene's transcriptional activity in response to heat shock (Mukherjee and Lakhotia 1979) and the transient binding of the heat shock transcription factor to the *hsrw* gene promoter (Wu *et al* 1994) also shows that the response of the *hsrw* gene to heat shock is regulated in a manner different from that operating in other heat shock genes.

4.4 *Promoter complexity required for fine-tuning of* hsrω *transcript levels*

The fact that expression patterns of the *LacZ* reporter gene in the three lines with different *hsrw* promoter contexts (figure 1) were different is clearly indicative of a complex organization of the *hsrw* promoter. Apparently, the *hsrw* promoter has many *cis*-acting sites that interact over long-distances and if they are not together in *cis* in the correct context, appropriate regulatory interactions do not take place, resulting in altered expression patterns. Because of the absence of clear-cut correlation between presence of a given promoter region and expression in specific cell type, the data do not allow specific delineation of the sites responsible for expressions in a given cell type.

As suggested elsewhere (Lakhotia *et al* 1999; Prasanth *et al* 2000), an important function of the *hsrw* transcripts could be binding with hnRNPs and related proteins in the nucleus and the consequent regulation of their availability for processing of the nascent transcripts of other genes. Since different cell types show dynamic changes in their RNA processing activities, the pools of the RNA processing proteins are also likely to be regulated accordingly. This in turn would require the levels of *hsrw* transcripts to be coordinately and precisely regulated. The complexities of the *hsrw* promoter seem to be related to the necessity for fine-tuning of the levels of hsrω-n transcripts in relation to specific nuclear needs.

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