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RESEARCH ARTICLE

The *Hsp60C* gene in the 25F cytogenetic region in *Drosophila melanogaster* is essential for tracheal development and fertility

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Abstract

Earlier studies have shown that of the four genes (*Hsp60A, Hsp60B, Hsp60C, Hsp60D* genes) predicted to encode the conserved Hsp60 family chaperones in *Drosophila melanogaster*, the *Hsp60A* gene (at the 10A polytene region) is expressed in all cell types of the organism and is essential from early embryonic stages, while the *Hsp60B* gene (at 21D region) is expressed only in testis, being essential for sperm individualization. In the present study, we characterized the *Hsp60C* gene (at 25F region), which shows high sequence homology with the other three *Hsp60* genes of *D. melanogaster*. *In situ* hybridization of *Hsp60C*-specific riboprobe shows that expression of this gene begins in late embryonic stages (stage 14 onwards), particularly in the developing tracheal system and salivary glands; during larval and adult stages, it is widely expressed in many cell types but much more strongly in tracheae and in developing and differentiating germ cells. A P-insertion mutant ($Hsp60C^{1}$) allele with the P transposon inserted at –251 position of the *Hsp60C* gene promoter was generated. This early larval recessive lethal mutation significantly reduces levels of *Hsp60C* transcripts in developing tracheae and this is associated with a variety of defects in the tracheal system, including lack of liquid clearance. About 10% of the homozygotes survive as weak, shortlived and completely sterile adults. Testes of the surviving mutant males are significantly smaller, with fewer spermatocytes, most of which do not develop beyond the round spermatid stage. *In situ* and Northern hybridizations show significantly reduced levels of the *Hsp60C* transcripts in $Hsp60C^{1}$ homozygous adult males. The absence of early meiotic stages in the $Hsp60C^{1}$ homozygous testes contrasts with the effect of testis-specific *Hsp60B* (21D) gene, whose mutation affects individualization of sperm bundles later in spermiogenesis. In view of the specific effects in tracheal development and in early stages of spermatogenesis, it is likely that, besides its functions as a chaperone, Hsp60C may have signalling functions and may also be involved in cation transport across the developing tracheal epithelial cells.

[Sarkar S. and Lakhotia S. C. 2005 The *Hsp60C* gene in the 25F cytogenetic region in *Drosophila melanogaster* is essential for tracheal development and fertility. *J. Genet.* **84**, 265–281]

Introduction

Various environmental stresses including heat shock induce a group of conserved families of heat shock proteins or Hsps, which together with their developmentally expressed cognates play critical roles in a variety of essential processes, mostly through their molecular chaperoning activities (reviewed by Morimoto *et al.* 1994; Lakhotia 2001; Soti and Csermely 2002). Among the Hsp families, the Hsp60 family is a ubiquitous group of molecular chaperones with important roles in mediating folding of non-native proteins to their native state. Hsp60 proteins in animals are found primarily in the mitochondria, though 15% to 20% are also cytosolic (Cheng *et al.* 1990; Horwich and Wilson 1993).

Initial studies on heat-shock-induced proteins in *Drosophila melanogaster* (Tissieres *et al.* 1974; Lewis *et al.* 1975) did not identify Hsp60 family protein/s. However, later studies in our laboratory (Lakhotia and Singh 1989,

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Keywords. Hsp60; trachea; spermatogenesis; liquid clearance; fertility.

1996; Singh and Lakhotia 1995) revealed that a polypeptide of ~64 kDa size was induced by heat shock in Malpighian tubules of *Drosophila* larvae. Kozlova *et al.* (1997) cloned the first gene belonging to the Hsp60 family (*Hsp60A*) in *D. melanogaster*. This gene was localized at the 10A4 cytogenetic region of the X chromosome and was identical with the 'essential locus' at 10A, studied earlier by Zhimulev and group (Zhimulev *et al.* 1987). Another gene, located at 21D cytogenetic region, and whose putative product displayed properties of the Hsp60 family, was named *Hsp60B* and this gene was found to have a male germ-cell specific function, especially in the sperm individualization process (Timakov and Zhang 2001; Srivastava 2004).

The Berkeley Drosophila Genome Project subsequently revealed two more *Hsp60*-like DNA sequences on the basis of predicted properties of their putative protein products (Adams *et al.* 2000; Rubin *et al.* 2000). These two genes are localized at the 25 F and 34 C polytene cytogenetic regions, respectively, of chromosome 2. We name these two genes as *Hsp60C* and *Hsp60D*, respectively. In the present study, we have characterized the putative *Hsp60C* gene localized at the polytene band 25F02 (CG7235, GenBank accession number AE003610, www.flybase.org). In the present study, we examined the expression of the *Hsp60C* gene in embryonic stages, late third instar larval tissues and in adult reproductive tissues by RNA : RNA *in situ* hybridization. The strongest expression of this gene is seen in tracheae and in adult testes and ovaries. In addition, we also generated a P-insertion mutant allele of *Hsp60C* gene, which causes recessive lethality at first instar larval stage, although ~10% mutant homozygotes survive to adulthood. All of the mutant first instar larvae show defects in their tracheal system while the surviving mutant adult males as well as females are sterile. Our present studies show that expression of *Hsp60C* gene is essential for proper tracheal development and for development of spermatogonia and spermatocytes.

Materials and methods

Drosophila stocks, P-element mutagenesis and genetic mapping

All the fly stocks were maintained on standard *Drosophila* food medium at 22 ± 1°C. Oregon R^+ stock, maintained in the laboratory, was used as the wild type. A deficiency stock, *Df(2L)cl-h2/CyO*, carrying deficiency for the region 25D06 to 25F04–05 on the *Df(2L)cl-h2* chromosome (for details, see Lindsley and Zimm 1992), was obtained from the Bloomington Drosophila Stock Center. A P-insertion mutation in the *Hsp60C* gene was generated by mobilizing the protein trap transposon (PTT) inserted at 25E6 cytological position (a recessive lethal mutation, #G262 of Morin *et al.* 2001) using the Δ2-3 transposase source (Bier *et al.* 1989). Out of 400 individual 'jumps', 81 lines were identified as homozygous viable and 46 as recessive lethal insertions on chromosome 2. The recessive lethal insertions were balanced with *CyO* and individually crossed with *Df(2L)cl-h2/CyO* flies; 14 lines that did not complement this deficiency were selected. These 14 lines were individually subjected to single fly genomic DNA PCR as described by Gloor and Engels (1992) with *Hsp60C* gene and P-element-specific primer combinations as described later. One of these lines, which generated a positive amplicon with PP–G1 primer combination (see below), displayed reduced viability in homozygous condition with only ~10–12% surviving to adult stage. This line was putatively identified as a P-insertion recessive lethal allele of the *Hsp60C* gene and was named $Hsp60C^1$ mutant allele. The $Hsp60C^1/CyO$ flies were crossed with *G262/CyO* flies, and since the $Hsp60C^1$/G262 progeny flies showed normal survival and phenotype it was clear that the gene mutated in *G262* allele by the PTT was fully repaired when the same 'jumped' from *G262* to generate the $Hsp60C^1$ allele. Revertants of the P-insertion $Hsp60C^1$ mutant allele were obtained by remobilizing the PTT in the $Hsp60C^1$ allele with the Δ2-3 transposase source following standard genetic crosses (Bier *et al.* 1989). Six lines that had lost the P transposon (loss of red eye phenotype) were identified and named $Hsp60C^{1R1}$ to $Hsp60C^{1R6}$.

Other fly stocks were: (i) *w; CyO,* $w\{w^{+mc} = ActGFP\}$ *JMR*1, which carries a second chromosome *CyO* balancer chromosome and which ubiquitously expresses the green fluorescent protein (GFP) in all cell types (http://www.flybase.org); (ii) *Hrb98DE-GFP (ZCL 0588)/Hrb98DE-GFP (ZCL 0588)*, which is a protein-trap line generated by Morin *et al.* (2001) in which the Hrb98DE hnRNP family protein (Haynes *et al.* 1990) is GFP-tagged; (iii) *dj-GFP*/TM6B, which is a transgenic line expressing Don-Juan-GFP fusion protein in the elongated spermatids and sperms (Santel *et al.* 1997). Appropriate crosses were made to generate the following stocks—(i) *w;* $Hsp60C^1/CyO;$ $w\{w^{+mc} = ActGFP\}JMR1$; (ii) *w;* $Hsp60C^1/CyO;$ *Hrb98DE-GFP/Hrb98DE-GFP;* (iii) *w;* $Hsp60C^1/$ *CyO; dj-GFP/TM6B.*

PCR mapping of P-transposon insertion in $Hsp60C^1$ *and the excision revertant lines*

The nucleotide sequence for the *Hsp60C* gene (accession number AE003610) was obtained from Flybase (http://www.flybase.org) and the following five 20-base-long *Hsp60C*-gene-specific primers were synthesized for different regions: (i) G1, corresponding to +417 to +437 region; (ii) G2, corresponding to +2166 to +2186 region; (iii) G3, corresponding to +4446 to +4466 region; (iv) G4, corresponding to +6169 to +6189 region; and (v) G5, corresponding to +7836 to +7856 region. The P-element-specific PP primer was 31 bases long (5′-

CGACGGGACCACCTTATGTTATTTCATCATG-3′). For each of the above 14 PTT-insertion recessive lethal lines, single-fly PCR (Gloor and Engels 1992) was carried out using PP–G1, PP–G2, PP–G3, PP–G4 and PP–G5 combinations. For examining the status of DNA sequence of the *Hsp60C*^{1R1} through *Hsp60C*^{1R6} revertants, genomic DNA from each of the lines was used for PCR with the PP–G1 primer combination. The cycling parameters in each case included an initial denaturation for 3 min at 94°C followed by 30 cycles of 94°C 1 min, 62°C 1 min and 72°C 1 min, with 5 min at 72°C in the last cycle. Fifteen μl of PCR products were loaded on a 1.5% agarose gel to check for amplification and size of amplicons.

Cloning and sequencing of amplicon

One of the 14 lines (see above) generated an amplicon with the PP–G1 primer combination. This PCR amplicon was eluted from the agarose gel and cloned in pGEM-T-Vector (Promega, USA) following the manufacturer's protocol. Sequencing of the cloned PCR amplicon was done by Bangalore Genei (Bangalore, India).

DNA : DNA in situ hybridization to polytene chromosome

DNA : DNA nonradioactive *in situ* hybridization was employed to determine the position of P-insertion site in *Hsp60C*^{1} chromosome. Squash preparations of polytene chromosomes of salivary glands from *Hsp60C*^{1}/+ larvae were processed for *in situ* hybridization of digoxigenin (Dig)-labelled 'mini white' DNA probe derived from *Hind*III-digested pCaSpeR4 vector (Thummel *et al.* 1988) to chromosomal DNA as described in Lakhotia and Sharma (1995). Hybridization signal was detected chromogenically following binding of the alkaline-phosphatase-conjugated anti-Dig antibody according to manufacturer's (Roche Molecular Biochemicals, Germany) instructions. The chromosomes were counterstained with 1% aqueous safranine and examined under bright field/phase-contrast optics (60× or 100× oil immersion objective) in a Nikon E800 microscope.

Viability of Hsp60C^1 *homozygotes*

In the progeny of *w; Hsp60C*^{1}*/CyO, w[w*$^{+mC}$ *= ActGFP] JMR1* flies, all the non-GFP-fluorescing embryos/larvae are homozygous for the *Hsp60C*^{1} mutant allele. Eggs were collected at hourly intervals and the GFP fluorescence of embryos/larvae was examined under a fluorescence microscope. Nonfluorescent embryos were segregated and monitored for further development. Almost all the nonfluorescent embryos hatched but since most of these first instar larvae appeared weak and dying, these were examined more carefully under a Nikon E800 microscope using 10× or 20× objective and DIC optics.

Adult male reproductive organs

Internal reproductive organs from 3–4-day-old male flies (wild type and *Hsp60C*^{1}*/Hsp60C*^{1}) were dissected in Poels' salt solution (PSS, Lakhotia and Tapadia 1998), mounted on a slide in PSS, and directly examined for morphology of testes and other organs. To examine the progression of spermatogenesis, testes of 3-day-old wild-type and *Hsp60C*^{1} homozygous mutant flies were dissected out and transferred to a slide in PSS. The testis was torn open and very lightly squashed under the weight of a coverslip (Kemphues *et al.* 1980) and immediately examined under a Nikon E800 microscope with phase-contrast optics. Since the GFP-tagged HRB98F protein shows strong expression in primary spermatocytes (our unpublished observations), freshly dissected unfixed testes from *y*^{1} *w*^{1118}*; Hsp60C*^{1}*/Hsp60C*^{1}*; Hrb98DE-GFP/Hrb98DE-GFP* or *y*^{1} w^{1118}*; Hsp60C*^{1}*/CyO; P{PTT-GC} Hrb98DE-GFP/Hrb98DE-GFP* flies were examined by combined phase-contrast and fluorescence microscopy using the Nikon E800 microscope to compare the relative populations of primary spermatocytes. Similarly, for examining spermatids and sperms, freshly dissected unfixed testes from *w*^{118}*; Hsp60C*^{1}*/CyO; dj-GFP/TM6B* or *w*^{118}*; Hsp60C*^{1}*/Hsp60C*^{1}*; dj-GFP/TM6B* flies were examined by combined phase-contrast and fluorescence microscopy.

To study the individualization complex (Fabrizio *et al.* 1998), paraformaldehyde-fixed wild-type and *Hsp60C*^{1} homozygous mutant testes were stained with rhodamine-conjugated phalloidin (8 U/ml in PBS; Sigma, USA) for 30 min at room temperature and diaminophenylindole (DAPI, 1 μg/ml; Molecular Probes, USA) to visualize individualization complex and DNA, respectively. The slides were mounted in antifade solution (Molecular Probes, USA) and examined with a Bio-Rad multiphoton confocal (Radiance 2100) microscope.

RNA: RNA in situ hybridization in embryos, larval and adult tissues using Hsp60C- specific riboprobe

The multiple *Hsp60* genes in *D. melanogaster* share considerable sequence homologies at the DNA level and therefore it was necessary to identify a unique region in the *Hsp60C* gene that could be used as gene-specific probe. Comparison of the *Hsp60C* gene sequence with those of the other three *Hsp60* genes of *D. melanogaster* (http://www.flybase.org) revealed that the region corresponding to 3′UTR and the adjacent 160-base-pair coding sequence of the *Hsp60C* gene were unique. The *Hsp60C* cDNA clone AT16985 (obtained from MRC Gene Services, UK) contains the entire *Hsp60C* cDNA (2.18 kb *Eco*RI–*Xho*I fragment) in pOTB7 vector (http://www.flybase.org). To get the *Hsp60C*-gene-specific riboprobe (antisense), the AT16985 DNA was linearized with *Sac*I so that when transcribed *in vitro* with T7 RNA poly-

merase a riboprobe specific for the 3′ UTR and adjacent upstream 50 bases of the *Hsp60C* gene transcript is generated. Dig-UTP (Roche Molecular Biochemicals, Germany) was used as the labelled substrate for T7 RNA polymerase driven *in vitro* transcription of the plasmid linearized using *Sac*I. Specificity of this riboprobe for the *Hsp60C* gene was confirmed by the unique hybridization (RNA: DNA) of the riboprobe at the 25F region of polytene chromosome spreads (not shown). For preparation of the *Hsp60C*-specific sense riboprobe, AT16985 DNA was linearized with *Pst*I, which cuts at +397 position (within the 5′UTR), and transcribed *in vitro* with SP6 RNA polymerase using Dig-UTP as the labelled precursor. Since the 5′UTR of *Hsp60C* gene is unique to this locus, the 397-nucleotide-long sense riboprobe is not expected to recognize transcripts from any other *Hsp60* gene.

The Dig-labelled *Hsp60C*-specific riboprobe was hybridized *in situ* with cellular RNA in wild-type embryos, different tissues of late third instar larvae and adult testes as described earlier (Lakhotia *et al.* 2001). In another set, embryos were collected from w; $Hsp60C^{1}/CyO$ or *w;* $Hsp60C^{1}/CyO$, $w\{w^{+mc}=ActGFP\}JMR1$ stocks, and testes from $Hsp60C^{1}/Hsp60C^{1}$ adult males were used for *in situ* hybridization of the riboprobe with cellular RNA. In the case of eggs collected from *w;* $Hsp60C^{1}/CyO$, $w\{w^{+mc}=ActGFP\}JMR1$ flies, the embryos that did not show GFP fluorescence (*w;* $Hsp60C^{1}/Hsp60C^{1}$) were selected and used for *in situ* hybridization. The hybridization signal was detected either chromogenically, using alkaline-phosphatase-conjugated anti-Dig antibody or by confocal microscopy following binding with FITC-conjugated anti-Dig antibody.

To ascertain the specificity of *in situ* hybridization of the *Hsp60C*-gene-specific riboprobe (antisense) to cellular RNA, the following three controls were carried out with wild-type embryos: (i) *in situ* hybridization of cellular RNA in embryos with the sense *Hsp60C* riboprobe, (ii) *in situ* hybridization with the antisense riboprobe following RNAase treatment (10 mg/ml at 37°C for 3 h) of embryos after fixation, and (iii) negative control in which no riboprobe was used. All these embryos were processed for anti-Dig antibody binding and chromogenic detection of the signal as above.

Some of the FITC-conjugated anti-Dig antibody-stained samples of adult testis were also counterstained with rhodamine-conjugated phalloidin (8 U/ml in PBS) and DAPI (1 μg/ml) to visualize individualization complex and DNA, respectively. All slides for fluorescence analysis were mounted in antifade solution.

For light (bright field/phase-contrast/DIC) and fluorescence microscopy, a Nikon E800 microscope was used and images were captured using a Nikon DXM1200 digital camera. For fluorescence-stained *in situ* hybridization preparations, a Bio-Rad multiphoton confocal (Radiance 2100) microscope was used. All images were assembled using Adobe Photoshop software.

Isolation of total RNA from adult males and Northern hybridization

Total RNA from 50 wild-type and 50 $Hsp60C^{1}/Hsp60C^{1}$ 3-day-old male flies was extracted using Trizol reagent following the manufacturer's (Gibco BRL, USA) description. Briefly, 50 adult male flies of each genotype were separately homogenized in 1 ml Trizol using a sterilized glass homogenizer for 1 min. Insoluble material was removed by centrifugation at 13,200 *g* for 10 min at 4°C. One ml chloroform was added to each supernatant fraction, and after incubation at room temperature for 5 min the samples were briefly vortexed and then centrifuged at 13,200 *g* for 15 min at 4°C. RNA in the aqueous phase was precipitated with 0.5 ml isopropyl alcohol and pelleted by centrifugation at 13,200 *g* (13,200 *g* for 10 min at 4°C). The precipitated RNA was washed with 75% ethanol, dissolved in 50 μl deionized formamide and stored at –70°C. Twenty μg of total RNA from each genotype was separated on 1% agarose gel containing 2.2 mol formaldehyde in 5× MOPS buffer (0.1 mol MOPS, pH 7.0, 40 mmol sodium acetate and 5 mmol EDTA) at 50 V for 4 h as described in Sambrook *et al.* (1989). RNA was transferred by capillary blotting onto Hybond-N nylon membrane (Boehringer Mannheim, Germany) overnight using 20× SSC (pH 7.0). The blot was baked at 70°C for 60 min and hybridized overnight at 68°C in 5 ml hybridization buffer containing 50% deionized formamide, 5× SSC, 0.1% *N*-lauroylsarcosine, 0.02% SDS, 50 μg/ml yeast tRNA, 1% blocking reagent and 500 ng Dig-labelled *Hsp60C*-specific riboprobe (same as used for RNA : RNA *in situ* hybridization, see above). After binding with alkaline-phosphatase-conjugated anti-Dig antibody, hybridization was detected by chemiluminiscence using disodium 3-(4-methoxyspiro{1, 2-dioxetane-3, 2′-(5′-chloro)tricyclo [3.3.1.13,7]decan}-4-yl) phenyl phosphate (CSPD; Roche Molecular Biochemicals, Germany) following the manufacturer's instructions. The blot was deprobed and rehybridized with Dig-labelled *Drosophila* β1-tubulin DNA probe, which served as a loading control. The β1-tubulin DNA probe was prepared by gel elution of the 1 kb *Xho*I fragment from the pET15B clone (Novagen, USA) and its random-primed labelling using Dig-dUTP as the labelled precursor.

Results

Homology of the different Hsp60 genes in D. melanogaster

A bioinformatic analysis of the predicted amino acid sequences of the four *Hsp60* genes of *D. melanogaster* reveals that the putative amino acid sequence of the Hsp60C protein shows highest (~80%) similarity with

that of the product of the *Hsp60A* gene at 10A and ~60% homology with the putative protein products of the *Hsp60* genes located at 21D and 34C regions (figure 1A). An analysis of the predicted amino acid sequence of the CG7235 gene reveals typical Hsp60-related domains (figure 1B). Interestingly, the nucleotide sequence homology between the four genes is restricted to protein-coding regions, with little similarities in the 3′ and 5′ UTR sequences, which suggests differential regulation of the four *Hsp60* genes. The *Hsp60C* gene produces three transcripts differing in the 5′ UTR exonic sequences owing to alternative splicing (figure 1C).

A P-insertion mutant allele of Hsp60C

As described in Materials and methods, P-element mutagenesis was performed using a protein trap transposon (PTT; Morin *et al.* 2001) as the mutator, and 14 independent recessive lethal insertion lines were selected on the basis of their noncomplementation with the *Df(2L)cl-h2* chromosome in which the region 25D06 to 25F04–05 is deleted. In order to identify if any of these 14 P-insertion lines carried a mutation in the *Hsp60C* gene, genomic DNAs from all the 14 lines were used for PCR with *Hsp60C*-gene-specific and P-element-end-specific primers (see Materials and methods for details). Only one of these 14 lines generated an amplicon of ~700 bases with the PP (P-element end) and G1 (corresponding to +417 to +437 region of the *Hsp60C* gene sequence) primer combination (figure 2A). PP and the other gene-specific primer (G2 to G5) combinations did not show any amplicon in this or any of the other 13 lines. Cloning and sequencing (data not shown) of the amplicon revealed this to be 719 bases long, which indicated that the P element was inserted in the promoter region of the *Hsp60C* at –251 bp position (figure 2B). *In situ* hybridization with a Dig-labelled ‘mini-white’ DNA probe with polytene chromosomes of $Hsp60C^1/Hsp60C^+$ larvae showed only two sites of hybridization, one at the white locus on the X chromosome and the other at the 25F site (figure 2C), where the P transposon carrying the ‘mini-white’ marker is inserted in the *Hsp60C* gene. No other site showed any hybridization signal. This also confirmed a single insertion of the P element in the 25F region in this line.

This line, therefore, was identified as a P-insertion recessive lethal mutant allele of *Hsp60C* and was named $Hsp60C^1$ and was maintained as $Hsp60C^1/CyO$ stock. This line was found to fully complement the original mutator line, G262 of Morin *et al.* (2001), which confirmed that the P-element insertion in the G262 line is removed with normal repair.

To confirm that the recessive lethality and other phenotypes (see below) associated with the $Hsp60C^1$ were indeed due to the PTT insertion in the *Hsp60C* promoter region, the PTT element was remobilized by crossing the $Hsp60C^1/CyO$ flies with the ‘jump starter’ Δ2-3 line (Bier *et al.* 1989). In subsequent progeny, six of the 26 excision lines, identified by loss of the mini-white marker, displayed normal viability and fertility. The genomic DNAs of these six normally viable and fertile excision lines did not generate any amplicons following PCR amplification with primers for P-element termini (PP primer) and any of the five (G1 to G5) *Hsp60C*-gene-specific primers but generated the expected wild-type amplicons with *Hsp60C*-gene-specific primers (not shown). This confirmed complete excision of the PTT in these revertant lines, which also displayed normal development, viability and fertility.

To further confirm that a second-site mutation is not responsible for the recessive lethality and other mutant phenotypes of $Hsp60C^1$, the $Hsp60C^1/CyO$ flies were crossed with w^{118} flies, which carry a wild-type chromosome 2. The $Hsp60C^1/+$ flies were allowed free interbreeding for five generations, selecting $Hsp60C^1/+$ (selected on basis of mini-white expression) flies as parents in each generation. After five generations of free-floating of the $Hsp60C^1$ chromosome, more than 300 progeny male flies carrying the P transposon (showing red eye phenotype due to the mini-white marker) were individually crossed with the original $Hsp60C^1/CyO$ stock females. No normally viable and fertile progeny fly homozygous for the P transposon insertion was obtained.

Thus we conclude that the $Hsp60C^1$ mutation is due to the PTT insertion in the promoter region of the *Hsp60C* gene and is responsible for the recessive lethality and other mutant phenotypes described below.

Phenotypes associated with $Hsp60C^1$ mutation

Since the $Hsp60C^1/CyO$ stock showed rare presence of non-curly flies ($Hsp60C^1/Hsp60C^1$), embryos were collected from the $Hsp60C^1/CyO$, $w\{w^{+mc}$ = *ActGFP}JMR1* stock to ascertain the period of death of the mutant homozygotes. This CyO balancer chromosome carries a transgene so that the green fluorescent protein (GFP) is constitutively expressed in all cells under the actin promoter (http://www.flybase.org). All the GFP-fluorescence-negative progeny in this cross would be homozygous for the $Hsp60C^1$ allele ($Hsp60C^1/Hsp60C^1$). The non-GFP-fluorescing embryos hatched later than the heterozygous sibs and all of them produced smaller and slow moving first instar larvae. The mean length of 40 non-GFP-fluorescing freshly hatched larvae was 0.63 ± 0.04 (S.D.) mm compared to the 1.13 ± 0.05 mm mean length of heterozygous sibs. The homozygous mutant larvae also displayed defects in the tracheal system (see table 1, figure 3). In wild type, the liquid present in the tracheae during embryonic development is cleared two hours before the larvae hatch (Manning and Krasnow 1993) so that the tracheal trunks appear refractile owing to the

filled air (figure 3A). In most of the mutant larvae, however, the liquid was not cleared from parts of the tracheal trunks (table 1), making them less refractile (figure 3B–D). In a few larvae, one of the two dorsal tracheal trunks failed to have any liquid clearance. In most of the mutant larvae the main dorsal trunks were, in addition, highly convoluted and/or asymmetrically organized (figure 3B,E) while in some the lateral branches were very thin and/or abnormally developed (figure C–E,H). In a few cases, one or both dorsal trunks did not extend the entire larval length but showed unusual midway block and bifurcation (figure 3G).

A

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Hsp60A   ---MFRLPVSLARSS-ISRQLAMRGYAKDVRFGPEVRAMMLQGVDVLADAVAVTMGPKGR 56
Hsp60C   MMRMFRYTNTLQRTAKISHVLWARNYAKDVRFGPEVRAMMLQGVDVLADAVAVTMGPKGR 60
Hsp60B   ---MFRSCVPKAITS---SRCFARMYSKDVRFGSGVRAMMIRGVDILADAVAVTMGPKGR 54
Hsp60D   ---MLSRLGRSGRAG-------VRTFANDIRFGAEARCLLMQGVNVLANAVATTLGPKGR 50

Hsp60A   NVIIEQSWGSPKITKDGVTVAKSIELKDKFQNIGAKLVQDVANNTNEEAGDGTTTATVLA 116
Hsp60C   NVIIEQSWGSPKITKDGVTVAKSIALKDKFQNIGAKLVQDVANNTNEEAGDGTTTATVLA 120
Hsp60B   SVIVERPWTSPKITKDGFTVARSIALKDQHMNLGAKLVQDVADNTNESAGDGTTTATVLA 114
Hsp60D   NVLIEQLLISPRITKDGITVANNVQLGNRRQDMGVQLLRQATNNTNNKVGDGTTTATILA 110

Hsp60A   RAIAKEGFEKISKG-ANPVEIRRGVMLAVETVKDNLKTMSRPVSTPEEIAQVATISANGD 175
Hsp60C   RAIAKEGFEKISRG-ASPVEIRRGVMLAIETVKDNLRRLSRPVNTPEEICQVATISANGD 179
Hsp60B   RAIAKEGFNQITMG-ANPVEIRRGVMLAVDVVKDKLKEMSKAVETREEIQQVATLSANGD 173
Hsp60D   RGIACQGMHVLRQSKVNVQLLREGILEGSRAVCDALGEMSQSVDTIGQVEAVAKVALNGD 170

Hsp60A   QAIGNLISEAMKKVGRDGVITVKDGKTLTDELEVIEGMKFDRGYISPYFINSSKGAKVEF 235
Hsp60C   KSVGNLISEAIKKVGRDGVITVKDGKTLCDELEVIEGMKFDRGYISPYFINTSKGAKVEF 239
Hsp60B   TEIGRLIGEATDKVGPRGTITVKDGKRLKDELNIIQGLRFDNGYVSPFFVNSSKGSKVEF 233
Hsp60D   ERLAELIGDIILELGDSGVILLKESHSPFDEAKIQEGITIASGYYSPFFAKQS--HTLEL 228

Hsp60A   QDALLLLSEKKISSVQSIIPALELANAQRKPLVIIAEDIDGEALSTLVVNRLKIGLQVAA 295
Hsp60C   QDALLLFCEKKIKSAPSIVPALELANAQRKPLVIIAEDLEAEALSTLVVNRLKVGLQVCA 299
Hsp60B   ANALVMISLKKITGLSQIVKGLEQSLRQRRPLIIIAEDISGEALNALVLNKLRLGLQVCA 293
Hsp60D   ENCLLLLTLAKIDQVEQILPALELARLKERPLLIIAKNFGSDLLKILVLNNLQGRVQVCA 288

Hsp60A   VKAPGFGDNRKSTLTDMAIASGGIVFGDDADLVKLEDVKVSDLGQVGEVVITKDDTLLLK 355
Hsp60C   VKAPGFGDNRKENLMDMAVATGGIVFGDEANMVRLEDIKMSDFGRVGEVVVSKDDTMLLK 359
Hsp60B   VKSPSYGHHRKELIGDISAATGATIFGDDINYSKMEEAKLEDLGQVGEAVISKDSTMLLQ 353
Hsp60D   VKAPSFGDEQCEEMEDIAFATGGHLLEDASSLADLSE---EDLGEVMEAVVDAKETHLLQ 345

Hsp60A   GKGKKDD-VLRRANQIKDQIEDTTSEYE-KEKLQERLARLASGVALLRVGGSSEVEVNEK 413
Hsp60C   GKGQKAE-VEKRVEGLREAIKESTSSYE-KEKMQERLARLSSGVALLRVGGSSDVEVSEK 417
Hsp60B   GKPKTGL-LEMRIQQIQDELAEKQIKPEQRDRLRQRLSALTKGVAVLHIGGGSEVEVNEK 412
Hsp60D   PINVNEEQVQCRIQDIRELIDEAFTDVE-LDRLKTRLGRLQGHLATIFVGGTSELEVSER 404

Hsp60A   KDRVHDALNATRAAVEEGIVPGGGTALLRCIEKLEGVE-TTNEDQKLGVEIVRRALRMPC 472
Hsp60C   KDRVIDALNATRAAVEEGIVPGGGTALLRCIQKLNDLK-GANEDQNMGIEIIRRALRMPC 476
Hsp60B   KDRVVDALNATRAAIEEGIVPGGGTAFLRCIPYLQELK-TESADLQKGVDIVCNALRMPC 471
Hsp60D   KDRFNDALHAVRVAISDGVVPGGGTAYLRCIPVLDELPPTDIMELQVGREIVKDALRLPC 464

Hsp60A   MTIAKNAGVDGAMVVAKVENQAGDYGYDALKGEYGNLIEKGIIDPTKVVRTAITDASGVA 532
Hsp60C   LTIAKNAGVDGAMVVAKVEILDGDYGYDALKGEYGNMIERGIIDPTKVVRTAISDAAGVA 536
Hsp60B   QTIAQNAGVDGPMVVAKVLNGSEDYGYDAMGDEYCRLVEKGIIDPTKVLRTAITDAAGVA 531
Hsp60D   YTIARNAGVDPNEVLRRVLKGSGNYGYDAAAGEFGDLVVRGIVDPTKVLQSAMTSAAGIA 524

Hsp60A   SLLTTAEAVVTEIP-----------------------KEDGAPA--MPGMGGMGGMGG 565
Hsp60C   SLLTTAEAVVTELP-----------------------LEEAAAAGAAAGLGALGGMG- 570
Hsp60B   SLLSTTEVVITDSRNDDLLSKLSGAGGGMDDGLDMNMGGLEELAALSGLGGMGGMGGMGG 591
Hsp60D   SLLATTEVLITKQP----------------------TKPKIPKNQVTKDLAKLVGM-- 558

Hsp60A   MGGMGGMM---------------------------------------- 573
Hsp60C   MGGMG-M----------------------------------------- 576
Hsp60B   MGGMGGMGGGFGGMGAGGGMSASASNDGPTAEEMNEMVKAIPGMEQVEVRDIDSGMM 648
Hsp60D   ---------------------------------
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Figure 1A–C. (Caption on opposite page).

Nearly 90% of the *Hsp60C*^{1} homozygous larvae died in the first instar, but the rest continued and mostly survived to adulthood. The escapee *Hsp60C*^{1} homozygous flies displayed poor viability and sterility of males as well as females. Their sterility was confirmed by crossing the mutant males and females to wild-type females and males, respectively. In either case, no progeny could be obtained. The escapee mutant flies were weak and poor fliers, and mostly died within 10 days with none surviving beyond 15 days. The homozygous flies also showed roughening of eyes, with black patches in some cases, abnormal antennae and defects in bristle arrangements (not shown). The *Hsp60C*^{1}/*CyO* heterozygotes did not show any of the above defects.

Male-sterile phenotype of Hsp60C^1 *homozygotes*

As mentioned above, both *Hsp60C*^{1} homozygous males and females were sterile. In the present study, we examined the organization of mutant testes. Studies on ovaries of the mutant females are in progress and will be reported later.

Comparison of testes and associated internal reproductive organs of wild type and of the few surviving non-curly *Hsp60C*^{1} homozygous males in *Hsp60C*^{1}/*CyO* stock showed that testes and seminal vesicles in *Hsp60C*^{1} males were much smaller than in wild type and were devoid of sperm bundles (figure 4A,B). The accessory glands, vas deferens and ejaculatory duct, however, appeared similar in wild type and the mutant males (figure 4A,B). The number of primary and secondary spermatocytes was found to be much less in mutant testes although the packaging and initial growth of the existing germ cells appeared normal in the mutant testis up to the 16-cell cyst stage (figure 5A). However, most of the later-stage cysts from the mutant testes showed extensive degeneration (figure 5B). Unlike wild type, no individualized and motile sperms were seen in testes of any *Hsp60C*^{1}/*Hsp60C*^{1} males. Instead, a few abnormal sperm bundles, a large amount of needle-shaped debris, and degraded fragments of spermatid tails were visible (figure 5C).

The significant reduction in number of primary spermatocytes in the adult testis of *Hsp60C*^{1}/*Hsp60C*^{1} flies was confirmed using the *Hrb98DE-GFP* marker. The

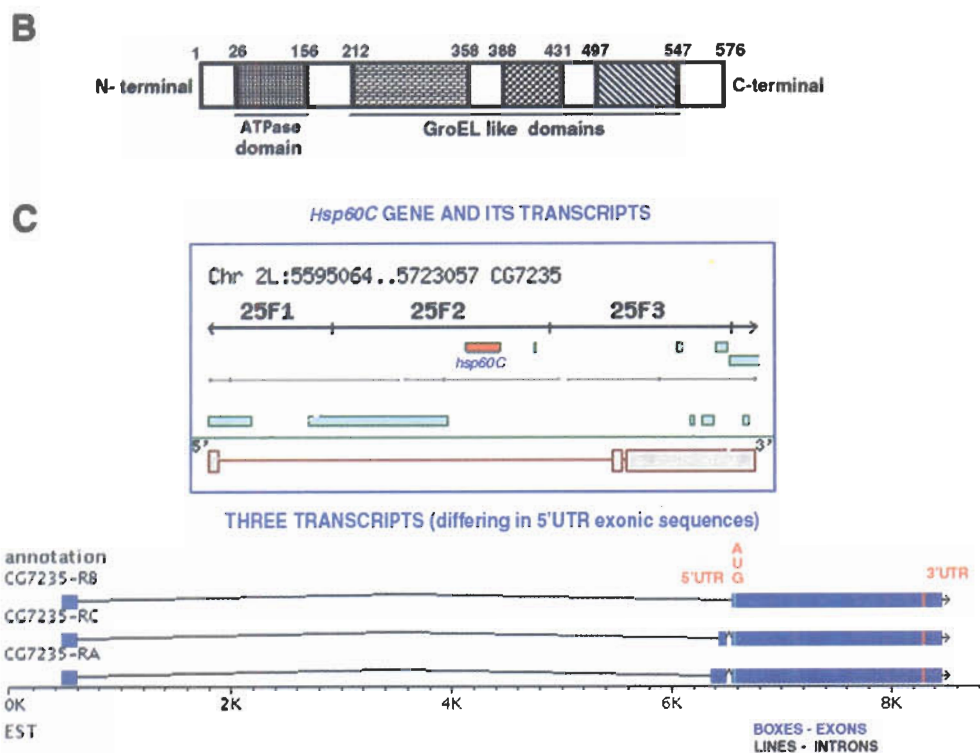

Figure 1A–C. Comparison of the amino acid sequences of the Hsp60A (AAF 47999.1), Hsp60C (AAF 52277.2), Hsp60B (AAF 51467.1) and Hsp60D (AAN 10836.1) proteins of *D. melanogaster*. The multiple sequence alignment was made using CLUSTAL W (1.82) software. **B.** Schematic representation of the different Hsp60 (GroEL)-like domains predicted by CD-Search software (Marchler-Bauer and Bryant 2004) in the putative protein product of the *Hsp60C* gene. The amino acid residue numbers are indicated. **C.** Genomic organization and the predicted transcripts of the *Hsp60C* gene of *D. melanogaster* (adapted from http:// www.flybase.org).

Hrb98DE gene encodes a member of the hnRNP family proteins (Haynes *et al.* 1990), which is expressed specifically in the primary spermatocytes in testes (our observations). Accordingly, testes from the *Hrb98DE-GFP* protein-trap line (*ZCL 0588* of Morin *et al.* 2001), homozygous for $Hsp60^{+}$ alleles, displayed strong and specific fluorescence in the primary spermatocytes (figure 5D). On the other hand, intact testes from $Hsp60C^{1}/Hsp60C^{1}$; *Hrb98DE-GFP/Hrb98DE-GFP* showed (figure 5E) much fewer brightly fluorescing spermatocytes. Likewise, the near complete absence of spermatids and sperms in the mutant testes was confirmed through the use of *don juan-GFP* fusion gene expression, which shows very strong GFP fluorescence in elongating spermatids and sperms (Santel *et al.* 1997). The wild-type testis and seminal vesicle showed strong GFP fluorescence in the numerous sperm bundles (figure 5F) but the $Hsp60C^{1}$ homozygous testis displayed very limited *don juan-GFP* fluorescence, essentially owing to degenerating spermatids (figure 5G); the mutant seminal vesicles did not show any GFP fluorescence since they did not contain any sperms.

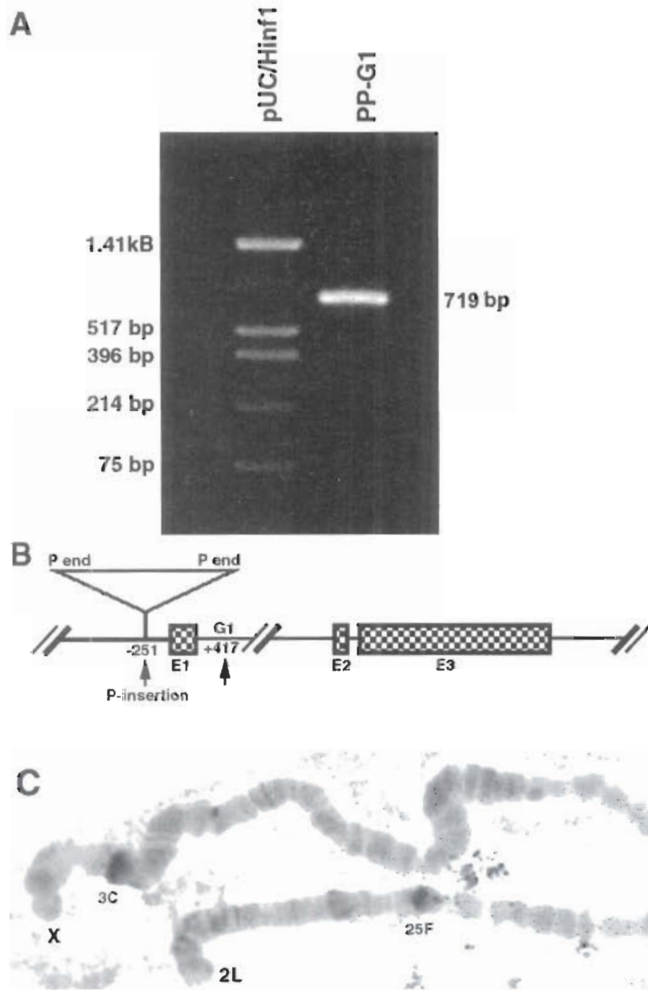

Figure 2. The P element in $Hsp60C^{1}$ is inserted at –251 base upstream of *Hsp60C* gene. **A.** The *Hsp60C*-specific G1 and the P-element-specific P-end primer combination produced the ~719-base-pair-long amplicon (lane PP-G1). The first lane in this ethidium bromide stained gel is the molecular size marker pUC DNA digested with *Hinf*1. **B.** A schematic drawing of the P insertion in relation to the predicted structure of the *Hsp60C* gene (gene CG7235; http://www.flybase.org) with the three exons (E1, E2, E3) and two introns (also see figure 1B). **C.** *In situ* hybridization of the Dig-labelled 'mini-white' DNA probe with polytene chromosomes from salivary glands of late third instar $Hsp60C^{1}/+$ larvae: note the signal on 3C (white locus) of X chromosome and on 25F region on 2L; as expected the wild-type (+) homologue does not show hybridization at the 25F region with the 'mini-white' DNA probe and, therefore, the signal does not spread across the entire width of the paired homologues. No other region in the nucleus showed any hybridization.

The individualization of the rare bundles of developing spermatids in $Hsp60C^{1}$ mutant testis was examined by phalloidin staining. In wild type, typical individualization complexes (Fabrizio *et al.* 1998), with aligned 64 cone-shaped components moving from heads to tails of sperm bundles were seen in large numbers (figure 5H). However, testes from $Hsp60C^{1}/Hsp60C^{1}$ males did not show any individualization complex, except highly malformed DAPI–phalloidin complexes in rare cases (figure 5I), and these were always nearer the junction of testis and seminal vesicles, suggesting that these failed to reach the ends of sperm tails.

Developmental expression of Hsp60C in embryonic and third instar larval cell types

In wild-type embryos, *Hsp60C* transcripts were not detectable till stage 14 of embryonic development (not shown). The first detectable expression of *Hsp60C* was specifically seen in the posterior part of the dorsal tracheal trunk at stage 14–15 (figure 6A), which marks the beginning of terminal tracheation (Manning and Krasnow 1993). During subsequent stages of embryonic development, the tracheal expression continued to be the most noticeable. As development progressed, *Hsp60C* expression extended to other portions of the tracheal tree (see figure 6C–F). Expression in the transverse connectives and primary branches of tracheal trunks was first detected at stage 16 (figure 6C,D). Beginning with stage 15, a paired structure at the posterior terminal region, which presumably represents the tips of posterior spiracles, also showed an increasingly strong presence of *Hsp60C* transcripts (figure 6B) till stage 16 after which hybridization with this structure was not distinguishable from neighbouring areas (figure 6E). From stage 16 onwards (figure 6C), strong expression was seen in almost the entire tracheal network. From stage 16 onwards, salivary glands also showed a strong signal for *Hsp60C* transcripts (figure 6C). In addition, low levels of *Hsp60C* transcripts were detectable in most of the embryonic cells from stage 16 onwards.

Specificity of the *in situ* hybridization of the Hsp60C riboprobe with cellular transcripts in tracheae in stage 14 and later embryos was checked by three controls (see Materials and methods). Most of the RNAase-treated (figure 6K) or sense-riboprobe-hybridized (figure 6L) embryos did not show any hybridization signal in tracheae or other tissues. Compared to the near-100% hybridization signal in antisense-riboprobe-hybridized stage-14 and later wild-type embryos (table 2), only about 2.5% embryos in the sense-riboprobe-hybridized or RNAase-treated samples showed detectable signals in tracheae. In both the latter cases, the signals were much weaker (not shown) than with RNAase-untreated antisense-riboprobe-hybridized embryos. Furthermore, the colour signal in RNAase-treated embryos was patchy along the tracheal tubes, probably reflecting incomplete digestion of cellular RNA. In the negative control (without any riboprobe) only a general pale blue colour was seen all over the embryos. These observations confirmed that the intense signal in tracheae and some other structures seen in stage-14 and later embryos after hybridization with the antisense Hsp60C riboprobe reflects specific hybridization with cellular RNA in the target tissues.

To determine the level of *Hsp60C* transcripts in $Hsp60C^1$ homozygous embryos, eggs were collected at hourly interval from a cross of $Hsp60C^1$/+ flies and the embryos were processed for RNA : RNA *in situ* hybridization with Dig-labelled *Hsp60C*-specific riboprobe. The general pattern of hybridization in these embryos was similar to that in the wild-type embryos. However, about 20–30% of the embryos in the population displayed significantly lower levels of hybridization in developing tracheal trunks and lateral branches, the salivary glands, as well as in the presumptive posterior spiracle tips. All these embryos that showed poorer hybridization signal also showed defects in the tracheal system, like highly convoluted and/or discontinuous dorsal trunks and irregular lateral branches, reminiscent of the defects in first instar larvae described earlier (figure 3). We believe that these embryos were homozygous for the $Hsp60C^1$ mutant allele,

Table 1. Frequencies of different types of defects in tracheae of first instar $Hsp60C^1$ homozygous mutant larvae.

Total number of larvae with tracheal defects	Partial liquid clearance	Liquid clearance only in one of the dorsal trunks	No liquid clearance in trachea	No liquid clearance and morphological defects
207	59.4%	3.4%	2.9%	34.3%

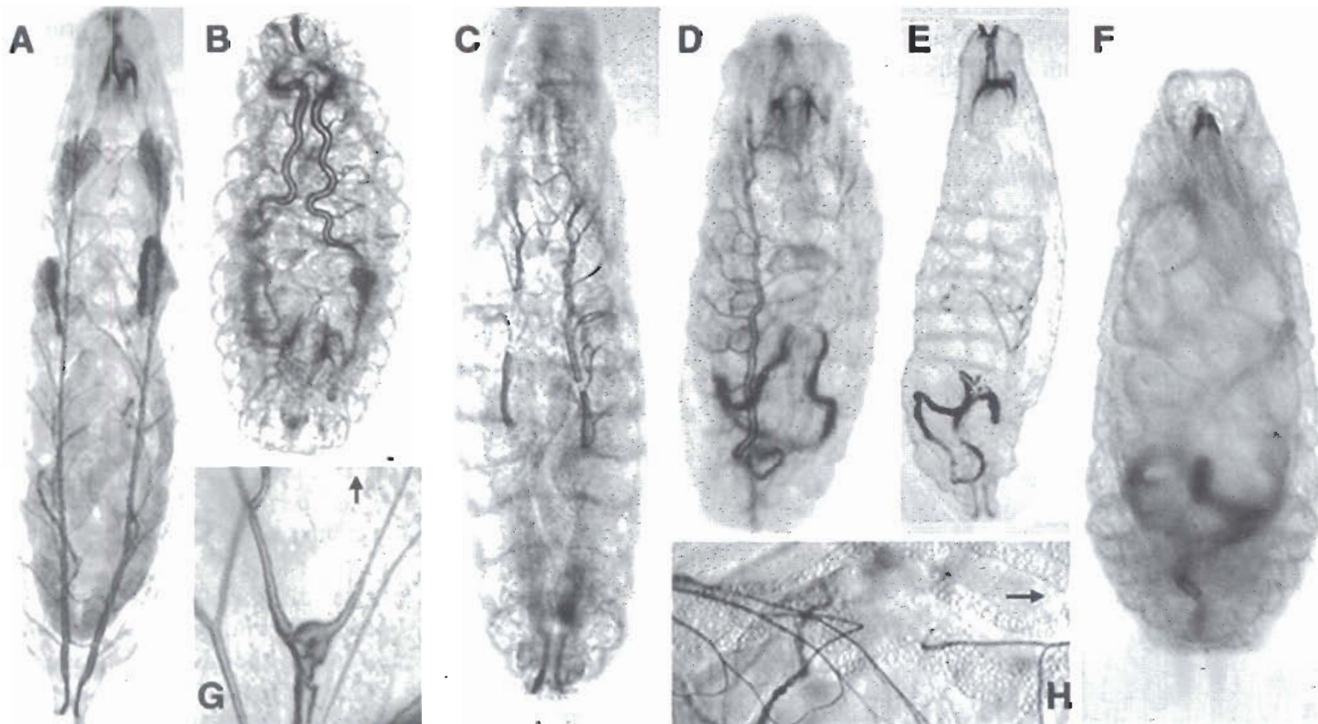

Figure 3A. Wild-type first instar larva showing normal tracheal morphology and gas filling. **B–H.** $Hsp60C^1/Hsp60C^1$ first instar larvae showing variety of abnormal tracheal morphology and incomplete gas filling. Arrows in **G** and **H** point to the anterior end.

which showed significantly reduced expression of the *Hsp60C* gene in tracheal system and the developing salivary glands. This was further confirmed by selecting non-GFP-fluorescing late-stage embryos (> stage 14) from *Hsp60C*^{1}*/CyO, w{w*$^{+mc}$ *= ActGFP}JMR1* flies and processing them for *in situ* hybridization with the Hsp60C riboprobe. All these *Hsp60C*^{1}*/Hsp60C*^{1} embryos showed tracheal defects and poorer hybridization signal (figure 6F–J).

Wild-type third instar larval tissues

Almost all the internal tissues of wild type third instar larvae expressed the *Hsp60C* gene as evidenced by distinctly detectable hybridization signals with the Hsp60C riboprobe. The entire tracheal network, however, showed a much stronger expression (figure 7A,B).

In the larval gut, expression in proventriculus was stronger than in midgut and hindgut (figure 7A,B). Malpighian tubules (figure 7A) showed low expression. Late third instar larval imaginal discs (not shown) and brain (figure 7C) showed moderate expression, which was more pronounced in the optic lobes. The prothoracic glands showed stronger hybridization with the *Hsp60C*-gene-specific riboprobe (figure 7C). Salivary gland polytene cells showed low level of expression (figure 7D), with the transcript localized mostly immediately outside the nuclear envelope (figure 7E). In larval ovary (figure 7F) and testis (figure 7G), hybridization was absent in the anteriormost regions, which harbour the gonial cells; the posterior regions in both cases showed strong cytoplasmic signal.

Expression in adult male reproductive system

Wild type: To localize the *Hsp60C* transcripts in different cell types of testis, fluorescent RNA : RNA *in situ* hybridization preparations of adult testes were examined by scanning confocal microscopy. The tracheae associated with testes showed very strong hybridization signal (not shown). Within the testes, *Hsp60C* transcripts were present at low levels in hub and spermatogonial cells and in spermatocytes (figure 8A). Later stages of spermatogenesis (spermatids and sperm) showed much stronger hybridization signal (see figure 8A–C). Intense granular signal was seen along the length of elongating spermatids and sperms. *In situ* hybridization preparations stained with phalloidin and DAPI showed that the *Hsp60C* transcripts were present at the anterior tip of the un-individualized sperm bundles and also along the sperm tail in a punctate manner while the phalloidin-positive individualization complex was relatively free of these transcripts (figure 8D). It may be noted that in an earlier computational and microarray analysis, Andrews *et al.* (2000) also found this gene to be transcribed in testis.

No expression was detected in cells of accessory glands and vas deferens, while moderate expression was detected in cells of the ejaculatory duct (not shown).

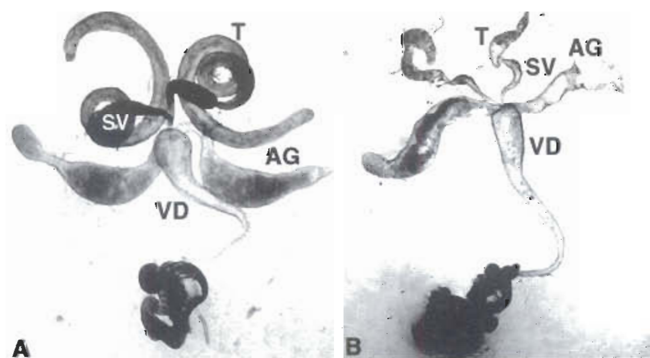

Figure 4. Internal male reproductive organs in wild type (A) and *Hsp60C*^{1}*/Hsp60C*^{1} (B) adult flies. T, testes; SV, seminal vesicles; VD, vas deferens; AG, accessory glands.

Hsp60C*^{1} *mutant testes: With chromogenic detection of RNA : RNA *in situ* hybridization, we failed to see a detectably distinct signal for *Hsp60C*-gene specific transcripts in testes of the *Hsp60C*^{1}*/Hsp60C*^{1} escapee males, although some hybridization signal was detected in tracheae surrounding the testes (not shown). Confocal-microscopic detection of RNA : RNA *in situ* hybridization following staining with FITC-conjugated anti-Dig antibody revealed very low level expression of the *Hsp60C* gene in testes of the mutant males (figure 8E,F). The hybridization signal in the limited population of spermatocytes present in anterior end of *Hsp60C*^{1} mutant testes (figure 8E) was in the form of larger clusters rather than fine specks seen in this part of the wild-type testes (compare figures 8A and 8E). The rare bundles of unindividualized and degenerating spermatids/sperms also showed hybridization signal but since the incidence of sperm bundles was rare, the overall signal in the mutant testes was very low (figure 8E,F). While the seminal vesicles of wild-type testes were full of sperms and showed abundant presence of *Hsp60C* transcripts along the length of sperms (figure 8C), no sperm and consequently no signal was seen in the seminal vesicles of mutant testes (figure 8G).

Northern hybridization of the Hsp60C riboprobe with total RNA isolated from adult wild-type and *Hsp60C*^{1} mutant male flies revealed that the level of these transcripts in the mutant flies was much less than in wild-type flies (see figure 9, upper panel)). As loading control, the same blot was reprobed with β1-tubulin DNA probe which showed comparable levels of hybridization signal (figure 9, lower panel).

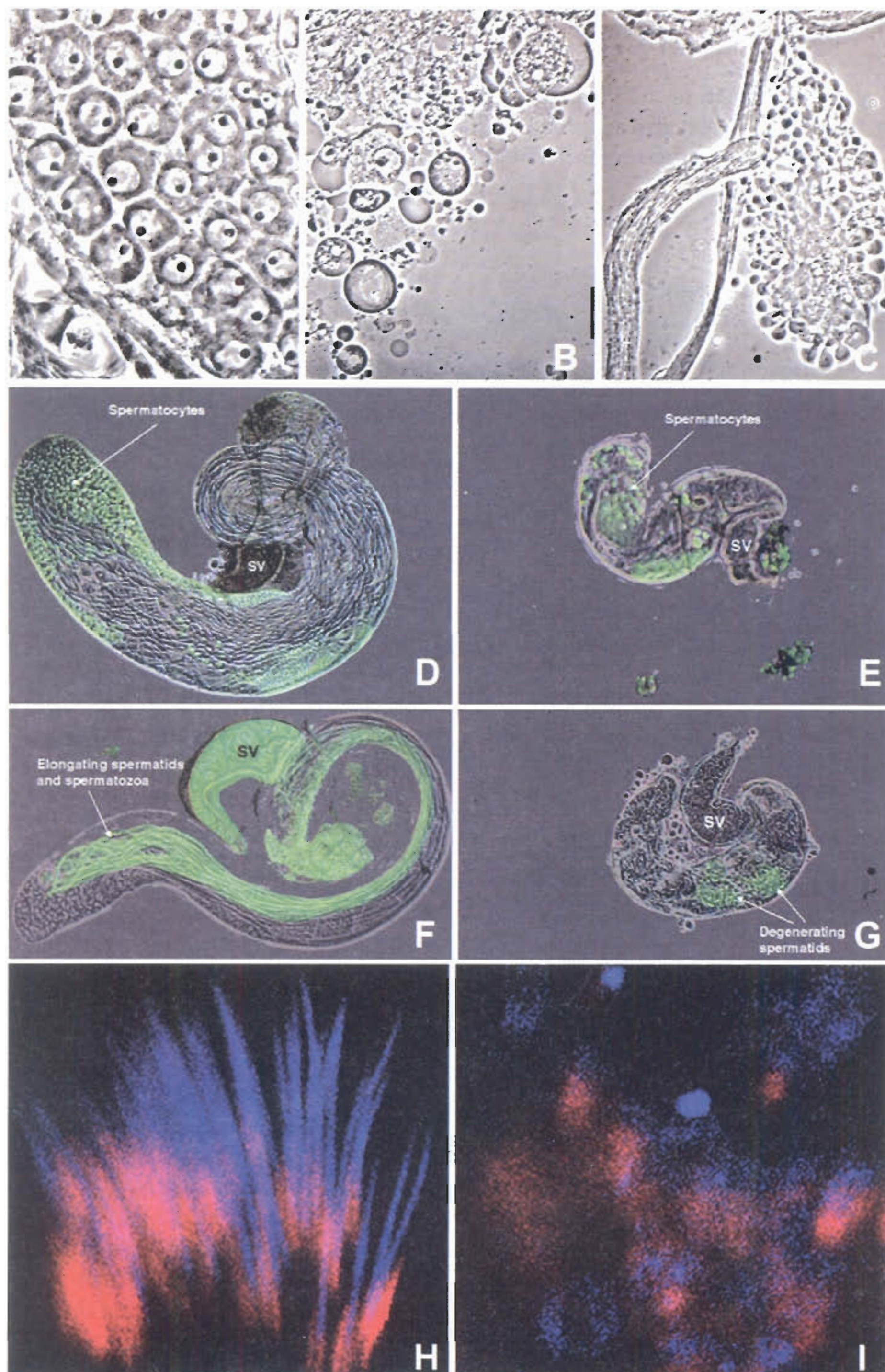

Figure 5. Testes from *Hsp60C¹* mutant flies contain very few spermatocytes and little or no spermatids/sperms. Phase-contrast images of cysts of spermatocytes (**A**), degenerating cysts (**B**) and bundles of nonmotile unindividualized sperms (**C**) in lightly squashed unfixed testes of *Hsp60C¹/Hsp60C¹* flies. GFP fluorescence in testes from *Hsp60C⁺/Hsp60C⁺; Hrb98DE-GFP/Hrb98DE-GFP* (**D**) and *Hsp60C¹/Hsp60C¹; Hrb98DE-GFP/Hrb98DE-GFP* (**E**) flies shows that compared to *Hsp60C⁺* the testes from *Hsp60C¹* mutant flies are much smaller and contain very few spermatocytes (marked by Hrb98DE-GFP synthesis). The *dj-GFP* transgene, which is expresse of in spermatids and sperms (Santel *et al.* 1997), reveals abundant spermatids and sperms in *Hsp60C⁺* (**F**) testis and seminal vesicle while those from the *Hsp60C¹* mutant flies (**G**) show almost complete absence of the GFP fluorescence except for some degenerating spermatids in testis. Unfixed testes and seminal vesicles (SV) were imaged using phase-contrast optics in conjunction with GFP fluorescence. Individualization complex in wild-type (**H**) and *Hsp60C¹/Hsp60C¹* (**I**) testes visualized by confocal microscopy following staining with rhodamine-conjugated phalloidin (red); DNA is counterstained with DAPI (blue).

Discussion

Of the four *Hsp60* genes identified in the genome of *D. melanogaster*, only two have been characterized so far: the one located in the 10A cytogenetic region (*Hsp60A* gene) is widely expressed in nearly all cell types of *Drosophila* and is essential for viability beginning from early embryonic stages (Kozlova *et al.* 1997; Perezgasga *et al.* 1999), while the *Hsp60B* gene, located in the 21D cytogenetic region is expressed only in the male germ line and is essential for sperm individualization and therefore for male fertility (Timakov and Zhang 2001; Srivastava 2004). We show that the *Hsp60C* gene has critical roles in tracheal development and in fertility of male as well as female flies. In addition, this gene may also have roles in formation of other body structures like antennae, eyes, etc but these have not been characterized yet.

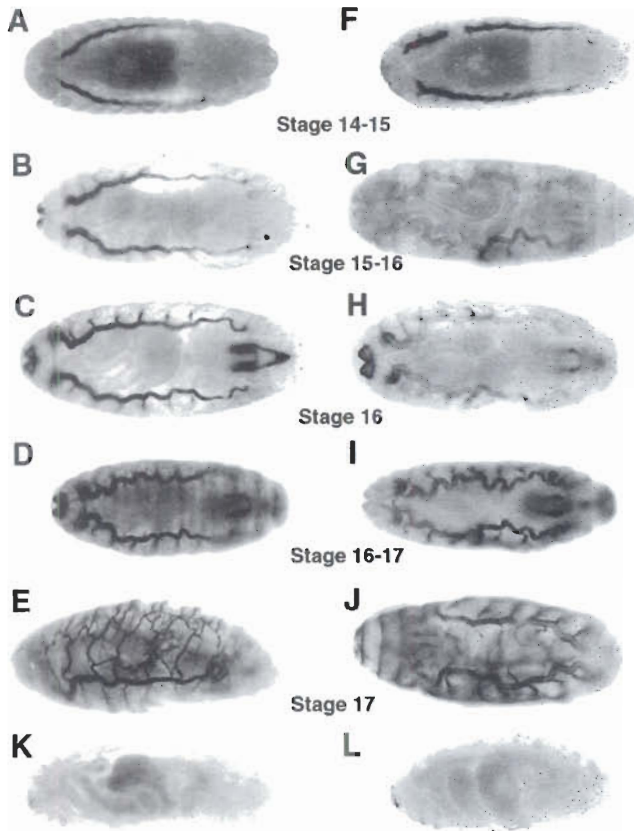

Figure 6. *In situ* hybridization of Hsp60C riboprobe with cellular transcripts at different embryonic stages (stages 14–17) in wild-type (**A–E**) and $Hsp60C^1/Hsp60C^1$ embryos (**F–J**). Examples of negative controls are shown in **K** and **L**: no signal is detectable in tracheal networks in wild-type late-stage embryos hybridized with the antisense riboprobe after RNAase treatment (**K**) or hybridized with sense riboprobe (**L**). In all cases (except in **E**, **K** and **L**) dorsal views of embryos are shown with the anterior end to right.

The present results clearly establish that the $Hsp60C^1$ mutation is due to insertion of the PTT element in the promoter region of the *Hsp60C* gene. Since the P element is inserted in the promoter region, this mutant allele is expected to show misexpression of the gene rather than make a mutated product. Our RNA : RNA *in situ* and Northern hybridization studies clearly show that the expression of this mutated allele is indeed significantly reduced in target tissues like tracheae and testis and, therefore, it may behave as a recessive loss-loss-of-function (at least partial) allele. We presume that in parallel with the lower levels of transcripts, Hsp60C protein levels are also reduced in the $Hsp60C^1$ homozygotes.

In agreement with the late onset of *Hsp60C* gene activity (embryonic stage 15), the PTT-insertion mutant $Hsp60C^1$ allele generated in the present study causes recessive first instar larval lethality rather than the early-embryonic lethality caused by most of the *Hsp60A* mutant alleles (Kozlova *et al.* 1997). The most obvious phenotype in the $Hsp60C^1$ mutant larvae was defective tracheal development, which correlates well with the significant levels of the *Hsp60C* transcripts in developing tracheae from stage 14 onwards in wild-type embryos. Starting at stage 12 the tracheal precursor cells begin their migration and differentiation, and by stage 14–15 the main dorsal trunks are identifiable (Manning and Krasnow 1993; Samakovlis *et al.* 1996). Since the *Hsp60C* transcripts are detected after the tracheal trunks have begun to form, this gene is not essential for the initial steps in differentiation of the tracheae but seems to have roles in subsequent stages of tracheal morphogenesis. Since the *Hsp60C* gene is not as

Table 2. Frequencies of detectable *in situ* hybridization signals in tracheae of stage 14–15 and later wild-type embryos following different hybridization conditions.

Antisense riboprobe (no RNAase treatment)	Sense riboprobe (no RNAase treatment)	RNAase treatment preceding antisense riboprobe	No riboprobe (negative control)
97.3% (446)	2.5% (471)*	2.5% (525)**	0.0% (> 400)

*The tracheal signal in all of the 2.5% embryos was significantly weaker than in antisense-riboprobe-hybridized (no RNAase treatment, column 1) embryos.
**The tracheal signal was patchy and weak in all of the 2.5% embryos.
Numbers in parentheses indicate the total number of embryos examined in each case.

highly transcribed in other embryonic tissues, it appears that the high lethality at first instar $Hsp60C^1/Hsp60C^1$ larval stage is due to abnormal tracheal structure and function, which would impair the basic respiratory activity. As far as we are aware, this is the first report of tracheal development defects arising directly from reduced expression of an *Hsp60* gene.

The expression pattern of *Hsp60C* gene in late embryonic stages shows striking similarities with several genes involved in tracheal morphogenesis, like *DEG/Enac pickpocket* genes (Liu *et al.* 2002) and *d-VHL* (Adryan *et al.* 2000). The different *Drosophila DEG/Enac pickpocket* genes are involved in transepithelial cation transport for clearance of liquids from the airways (Liu *et al.* 2002). The *d-VHL* gene is a homologue of a human tumour suppressor gene responsible for von Hippel–Lindau syndrome and is suggested to halt the cell movement at the end of vascular tube outgrowth with the FGF receptor gene *breathless* being its target (Adryan *et al.* 2000). Some of the tracheal phenotypes of $Hsp60C^1$ mutants, e.g. absence of liquid clearance, convoluted and narrower tubules, missing or improper lateral branches, etc., are also seen in mutants for *nrv2* (Paul *et al.* 2003) and *colt* (Hartenstein *et al.* 1997) genes. The *nrv2* gene codes for a Na^+/K^+-ATPase and has an active involvement in tracheal tube size control and proper functioning of the septate junctions (Beitel *et al.* 2003). The *colt* gene produces a mitochondrial carrier protein localizing at the inner membrane, which contributes to the selective transport of small molecules required for gas-filling of the tracheal system (Hartenstein *et al.* 1997). In view of the similarities in expression patterns of *Hsp60C* gene and/or phenotypes associated with its mutation with those of genes like *DEG/Enac pickpocket*, *d-VHL*, *nrv2*, *colt*, etc., we think that Hsp60C functions in conjunction with products of these genes. The ATPase activity of Hsp60C (see figure 1) may also have a role in cation transport across the tracheal membrane. Improper functioning of cation transport channel is known to result in severe phenotypes in mammals also, e.g. mice with delayed lung liquid clearance die shortly after birth (Barker *et al.* 1998), and in humans defect in liquid clearance causes neonatal respiratory distress syndrome (O'Brodovich 1996).

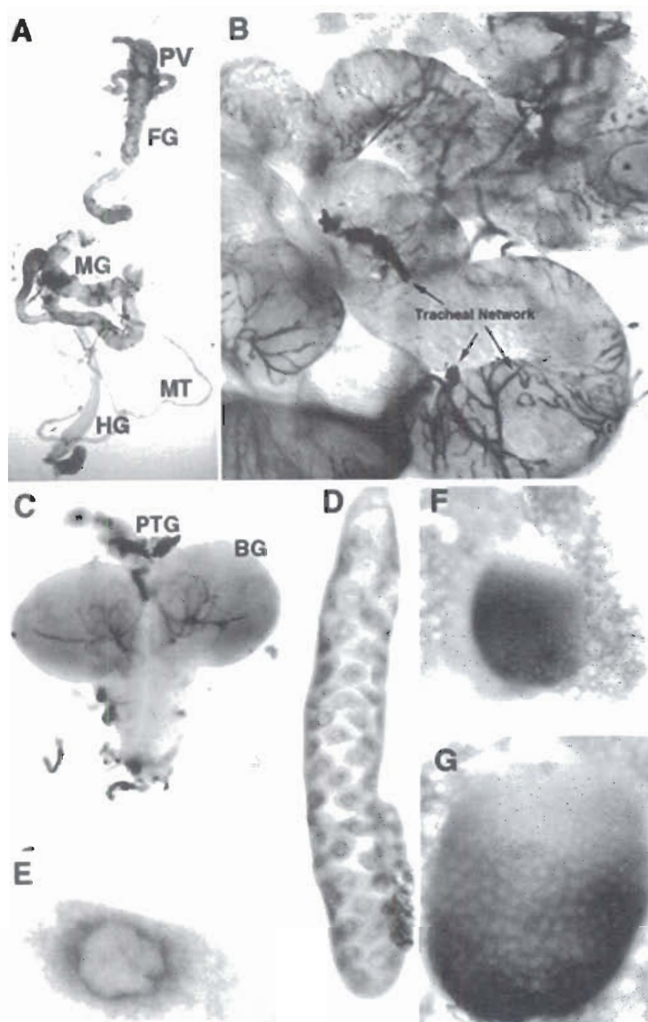

Figure 7. Expression pattern of *Hsp60C* in late third instar larval gut and surrounding tracheal network (**A** and **B**), brain ganglia (**C**), salivary gland (**D**), ovary (**F**) and testes (**G**) as revealed by RNA:RNA *in situ* hybridization followed by chromogenic detection. **E** shows a magnified view of a single cell of salivary gland showing perinuclear accumulation of *Hsp60* transcripts. BG, brain ganglia; FG, foregut; HG, hindgut; MG, midgut; MT, Malpighian tubules; PTG, prothoracic gland; PV, proventriculus.

A number of signalling pathways, like TGF, FGF and EGF, have been identified to be involved in tracheal morphogenesis (Zelzer and Shilo 2000; Cabernard *et al.* 2004). Since, besides defective liquid clearance, the $Hsp60C^1$ mutation is also associated with defects in morphogenesis of tracheal system and consequent reduction in the larval length, it is likely that the Hsp60C protein has roles in signalling pathways in tracheal morphogenesis. Further genetic interaction studies, presently in progress in our laboratory, are expected to provide insight into the role of this classical chaperone protein in the signalling pathways that control morphogenesis of the tracheal system.

Spermatogenesis in *Drosophila* has been a subject of intense investigations over the past few decades (reviewed in Lifschytz 1987; Fuller 1993). Chemical as well as P-element mutagenesis suggest that male sterility represents ~10–15% of the total number of recessive mutations (Wilkinson *et al.* 1974; Lifschytz 1987; Cooley *et al.* 1988). In most of the 'classic' male-sterile mutants in *Drosophila* (Fuller 1993), the first observed defects in spermatogenesis are seen during late stages, e.g. spermatid individualization, resulting in either the complete absence of mature sperm or the presence of highly disorganized, nonmotile sperm (Fuller 1993). The *Hsp60B* gene of *D. melanogaster* belongs to this category since its mu-

tation results in production of unindividualized sperm due to improper assembly and movement of the individualization complex (Timakov and Zhang 2001; Srivastava 2004). Our present results show that the *Hsp60C*^{1} mutation causes a more severe defect in the assembly of the individualization complex. However, this aggravated phenotype seems to result from defects that originate earlier in the formation of spermatids themselves. It is notable that although the 16-cell cysts of primary spermatocytes that were present in these mutant testes appeared normal, their frequency was much less than in wild-type flies. This suggests that unlike the other 'classic' male sterile mutations, the *Hsp60C*^{1} mutation affects earlier stages of development of spermatogonia and spermatocytes. Northern and *in situ* hybridizations revealed that the overall levels of *Hsp60C* transcripts in adult male flies and in testes, respectively, were significantly lower. In this context, the presence of larger clusters of *Hsp60C* transcripts in the surviving spermatocytes in homozygous mutant testes is intriguing. Since the *Hsp60C* gene produces three transcripts (figure 1C), which differ in their 5′UTRs, it remains to be examined if the P insertion in the promoter region results in production of transcripts with different 5′UTRs owing to alternative splicing.

Our observations on the distribution of *Hsp60C* transcripts in developing spermatids and mature sperms, including those stored in seminal vesicles, suggest that the Hsp60C protein may have roles in later stages of spermiogenesis and sperm function as well. In bovine sperms, Hsp60 has been shown to be present in the sperm midpiece as an important factor for sperm viability and motility (Boilard *et al.* 2004). Hsp60 is also present on sperm head and is essential for the sperm's competence to fertilize an egg (Asquith *et al.* 2004). Our finding that the *Hsp60C* transcripts are present on sperm head as well as along the length of the sperm suggests that in *Drosophila* also Hsp60 has roles in sperm function. Like the few *Hsp60C*^{1} homozygous embryos surviving to adulthood, it is possible that a proportion of the mutant spermatocytes survive to spermatid stage but most of them do not develop further and degenerate without undergoing individualization. This may be related to the fact that the

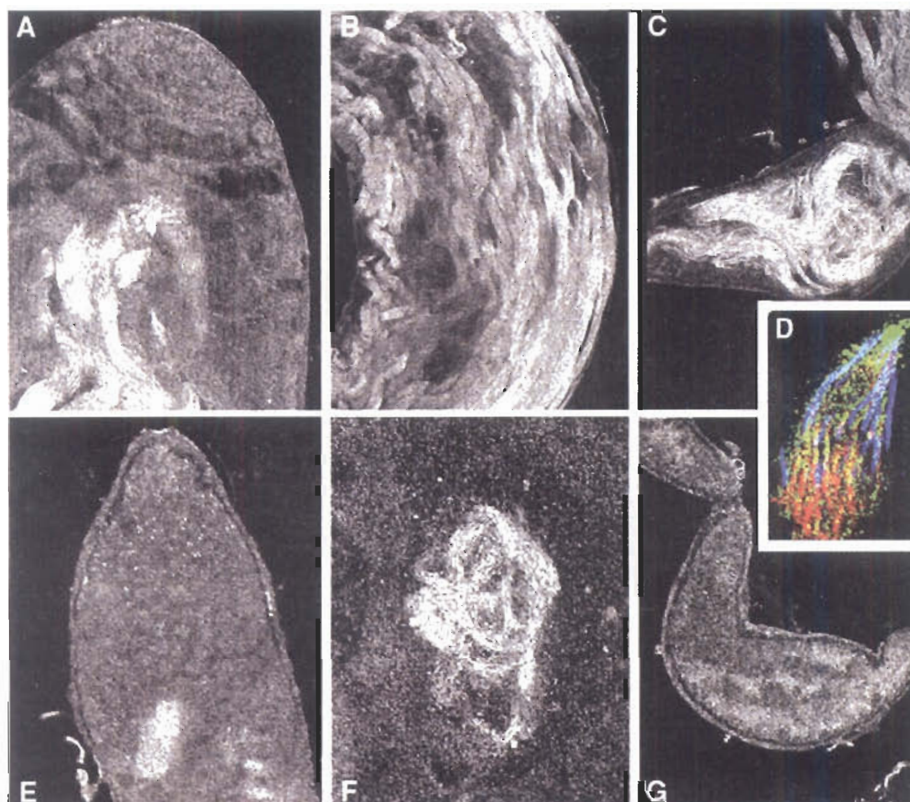

Figure 8. Distribution patterns of *Hsp60C* transcripts in wild-type (A–D) and *Hsp60C*^{1}/*Hsp60C*^{1} (E–G) adult testes as revealed by fluorescence RNA : RNA *in situ* hybridization followed by confocal microscopy (**A** and **E** show the tip of testes, **B** and **F** the middle part of testes, and C and **G**, the seminal vesicles). In **D**, the head region of a bundle of sperms from wild-type testis is shown (green, ***Hsp60C*** transcripts; blue, DNA; red, rhodamine-conjugated phalloidin individualization complexes). In all cases single optical sections are shown.

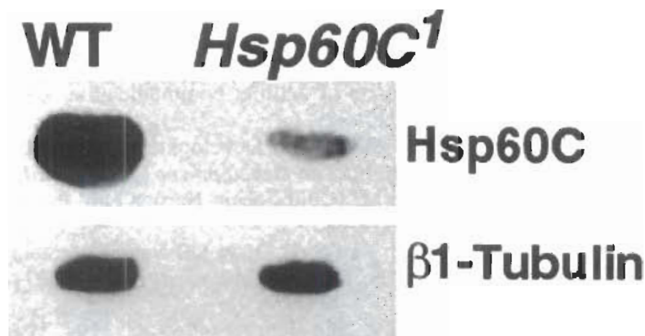

Figure 9. The level of *Hsp60C* transcripts is significantly reduced in *Hsp60C*^{1}/*Hsp60C*^{1} flies. Northern hybridization of *Hsp60C*-specific Dig-labelled riboprobe with total RNA from wild-type (WT) and *Hsp60C*^{1}/*Hsp60C*^{1} (*Hsp60C*^{1}) flies (upper panel). The same blot was deprobed and rehybridized with Dig-labelled *Drosophila* β1-tubulin DNA probe (lower panel) to confirm equal loading of RNA in both lanes.

wild-type elongating spermatids and mature sperms show high levels of the *Hsp60C* transcripts. In the *Hsp60C*^{1} mutants, the few spermatids that are produced cannot differentiate further in the absence of these transcripts and consequently degenerate.

In an interesting analysis of evolution of new genes out of the X chromosome through retroposition in *Drosophila*, Betran *et al.* (2002) found that most of the X-derived autosomal retrogenes are expressed in testis. The *Hsp60B* and *Hsp60C* genes also seem to have evolved from the X-linked *Hsp60A* gene (Betran *et al.* 2002) and both of them now have important but somewhat different functions in testis. The present results show that the *Hsp60C* gene is essential for early spermatocyte development while the *Hsp60B* gene is essential for individualization of sperm bundles (Timakov and Zhang 2001; Srivastava 2004). Apparently, following retroposition to chromosome 2, these two *Hsp60* genes acquired distinct function in spermatogenesis.

The variety of phenotypes seen in *Hsp60C*^{1} mutants (tracheal defects, male and female sterility, and eye/antennal defects) clearly suggest that the Hsp60C protein has multiple tissue-specific or cell-type-specific roles, which may not be limited to chaperoning activities alone. In recent years, the Hsp60 proteins have been reported to be present on cellular organelles and on the surface of different cell types (Soltys and Gupta 1997) and have been implicated in several cellular activities outside the mitochondria, e.g. in presentation of peptides on surface of cells, signal transduction, and amino acid transport (Ikawa and Weinberg 1992; Jones *et al.* 1994; Wells *et al.* 1997; Woodlock *et al.* 1997; Maguire *et al.* 2002; Zhang *et al.* 2004a,b). Further genetic and molecular studies on the Hsp60C protein will help us understand these novel functions of a classical chaperonin family protein.

Acknowledgements

This work is supported by a grant from Department of Biotechnology (DBT), Government of India, New Delhi, to S. C. L. and by the Department of Science and Technology (DST), Government of India, New Delhi, through its support for the Multiphoton Confocal Microscope National Facility. S. S. is supported by a research fellowship from the Council of Scientific and Industrial Research (CSIR), New Delhi. We thank Bloomington Drosophila Stock Center, Dr Xavier Morin (Cambridge) and Dr Christopher Bazinet (Switzerland) for fly stocks.

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Received 2 June 2005; in revised form 2 July 2005