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ORIGINAL ARTICLE

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The *Drosophila melanogaster* homologue of the *hsp60* gene is encoded by the essential locus *l(1)10Ac* and is differentially expressed during fly development

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Abstract The *hsp60* (*heat-shock protein 60*) gene family of molecular chaperones has been a subject of study in numerous systems due to its important role in the correct folding of non-native proteins in development as well as after heat-shock treatment. Here we present the characterization of the first *Drosophila hsp60* homologue. *Drosophila* HSP60 is most closely related (72% identity across the entire protein sequence) to the mouse mitochondrial HSP60. Western blot experiments indicate that *Drosophila* HSP60 is enriched in the mitochondrial fraction. The distribution of HSP60 protein is dynamic during fly embryogenesis, suggesting that various cell types might have different HSP60 requirements. The molecular analysis of a P-element-induced mutation that affects the *l(1)10Ac* locus shows that the transposon is inserted in a 3-kb intron present in the *hsp60* gene. By genetic rescue experiments we prove that *Drosophila* HSP60 is encoded by the essential locus *l(1)10Ac* opening the possibility for detailed genetic analysis of HSP60 functions in the fly.

Key words *Drosophila* · *l(1)10Ac* locus · Development · *hsp60* gene

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Introduction

The HSP60/GroEL (heat-shock protein 60/Large gene of the GroE operon) family of molecular chaperones has an important role in mediating folding of non-native proteins to their native state (Parsell and Lindquist 1993; Ellis 1994). These ring-shaped oligomeric complexes are composed of 14 subunits with a molecular mass of 60 kDa. Subunits are arranged in two stacked heptameric rings, forming a barrel-like structure (Hutchinson et al. 1989). The function of HSP60/GroEL is distinct from that of the HSP70 class of chaperones. HSP70 maintains proteins unfolded and in a relatively extended conformation.

HSP60 proteins are found in the cytosol of bacteria, in the matrix compartment of mitochondria and in the stromal compartment of chloroplasts (Cheng et al. 1990; Horwich and Wilson 1993). The TCP1 (t-complex polypeptide-1) complex, a chaperonin located in the cytosol of some eukaryotic cells, is a related protein to the HSP60/GroEL family and has been demonstrated to have a role in the folding of some cytoskeleton proteins (Gao et al. 1992; Yaffe et al. 1992).

HSP60 functions are strongly dependent on HSP10/GroES (heat-shock protein 10/Small gene of the GroE operon) chaperonin. Like HSP60 proteins HSP10 proteins are found across all evolutionary lineages. HSP10 binds to the HSP60 complex and modulates ATPase activity as well as substrate binding (Saibil et al. 1993). The HSP60 protein is required at normal temperatures. It has been proposed that HSP60 catalyses the folding of proteins, but more precisely it modulates the correct folding of proteins (Martin et al. 1991; Ishi et al. 1994; Weissman et al. 1994). A combined model based on the crystal structures of the proteins GroEL and GroES has been proposed (Mande et al. 1996), and a three-dimensional reconstitution map of the domain movements in GroEL in the presence of nucleotides has been reported recently (Roseman et al. 1996). These two studies suggest relevant mechanisms for the high efficiency of this protein complex in mediating protein folding.

Mitochondrial HSP60 modulates folding of a large number of nuclear-encoded proteins after mitochondrial membrane translocation. A number of proteins have been characterized whose functions are required for the assembly of specific protein complexes in the mitochondrial inner membrane (Langer and Newpert 1994). Mutants that affect the mitochondrial *hsp60* gene have been described in yeast (Cheng et al. 1989; Cheng et al. 1990). These mutants lack the enzymatic activities of some imported mitochondrial proteins (Cheng et al. 1990). It has also been demonstrated that the HSP60 protein is required for its own assembly as well as for the assembly of some mitochondria-encoded proteins. Like many other HSP proteins, HSP60 has a role in the heat-shock response by apparently facilitating the correct folding of a large number of preexisting mitochondrial proteins after being denaturated by heat treatment (Mendoza et al. 1991; Parsell and Lindquist 1993).

Heat-shock proteins were originally described in *Drosophila melanogaster* by the identification of the de novo synthesis of proteins produced after heat shock (Tissiers et al. 1974; Lewis et al. 1975; McKenzie et al. 1975; McKenzie and Meselson 1977; Lindquist 1980; Lindquist and DiDomenico 1985). Surprisingly, a protein that may correspond to HSP60 was not identified in these experiments. In agreement with this observation it has been reported that the mitochondrial HSP60 protein is only induced two- to threefold by heat shock in *Tetrahymena* (McMullin and Hallberg 1987). The heat-shock response in *Drosophila* is associated with the appearance of specific puffs in salivary gland polytene chromosomes (reviewed in Ashburner and Bonner 1979). The in vivo induction of the puffs by heat shock is very rapid; it occurs within a few minutes of the increase in temperature. Various *hsp* genes that are associated with these specific puffs have been cloned and a significant amount of information about gene regulation after heat shock has been accumulated (Ashburner and Bonner 1979; Fernandes et al. 1994; Wu et al. 1994). In spite of this, a gene corresponding to any HSP60 in *D. melanogaster* has not been described thus far. Santaren et al. (1993) reported the identification of wing imaginal disc proteins by using two-dimensional gels and determining the partial amino acid sequence for some of the protein spots found. In this work, the sequence of an 11-amino acid polypeptide potentially included in a putative *Drosophila* HSP60 protein is determined. The presence of a TCP1 like protein in flies is also documented (Ursic and Ganetzky 1988)

Recently, Kozlova et al. (1994) have reported the physical map of the 10A1-2 band as well as the adjacent regions present in the *X* chromosome of *D. melanogaster*. In this work, several P-element-induced alleles that correspond to the *l(1)10Ac* locus (position 10A4-5) were mapped and several corresponding cDNA clones were isolated. Different alleles that result in embryonic and larval lethality have been described for the *l(1)10Ac* locus (Bgatov et al. 1986; Zhimulev et al. 1987; Lindsley and Zimm 1992).

In the present work, we have characterized different cDNA clones that correspond to the *l(1)10Ac* locus; from its sequence analysis we suggest that they code for a *Drosophila* homologue of the *hsp60* gene. Interestingly, our results show that the *hsp60* gene is differentially expressed during fly development. HSP60 protein distribution is very dynamic during fly embryogenesis; the protein preferentially accumulates in specific cell types. Moreover, the molecular analysis of a P-element-induced mutation and the genetic rescue experiments have confirmed that the *l(1)10Ac* locus encodes for a *D. melanogaster* HSP60 homologue.

Materials and methods

Fly strains

The wild-type stock was Oregon R. The *l(1)10Ac* mutants, and alleles $l(1)\,10Ac^{F409} m\,f$ and $l(1)10Ac^{F437}\,m\,f$ were maintained in the presence of the *FM6* balancer (Zhimulev et al. 1987; Lindsley and Zimm 1992). Flies were reared on standard yeast-dextrose medium at 25°C. 4,6-diamidino-2-phenylindole (DAPI) staining of embryos was performed following the protocol described by Robbins and Pimpinelli (1994).

Cloning and DNA manipulations

A 4.5-kb *XhoI* restriction fragment present in the clone 5D was used in the first screening of an ovarian cDNA library (10^5 pfu; Kozlova et al. 1994). The cDNA clone BP5-3 isolated in the first screening was used as a probe to identify new cDNA clones in a commercial embryonic cDNA library (0- to 24-h embryos; Stratagene) and two cDNA clones were identified. The hybridization conditions were performed as reported in Sambrook et al. (1989). cDNA clones were excised from the phage vector using the zapping procedure as recommended by the supplier and either directly sequenced or subcloned into M13 vectors. DNA sequencing was performed according to the dideoxy chain termination procedure (Sanger et al. 1977). DNA and protein analysis was performed using the GCG Wisconsin program. RNA blot hybridizations were performed after RNA separation by electrophoresis in formaldehyde-agarose gels as described (Seed 1982). Low stringency DNA hybridizations and other standard DNA manipulations were performed according to protocols of Sambrook et al. (1989).

Immunocytochemistry

Whole-mount embryos were immunostained for the HSP60 protein using a commercial monoclonal antibody (dilutions 1:100 or 1:200; Sigma) that recognizes an epitope present in all HSP60 chaperones. The immunostaining conditions were essentially as described (Lee et al. 1993) except that the fixation step was carried out in "engrailed" fixing buffer (140 mM KCl, 40 mM NaCl, 4 mM Na_3EGTA, 1 mM spermidine, 30 mM Pipes pH 6.9, 0.2% 2-mercaptoethanol) containing 4% formaldehyde. Staining was visualized using fluorescein isothiocyanate (FITC)-conjugated anti-mouse secondary antibody and analysed in a confocal microscope.

Cell fractionation and immunoblot analysis

Embryos (0–12 h) were gently homogenized with 20 strokes in a dounce homogenator in the presence of homogenization buffer (Birnie 1972). This extract was centrifuged at slow speed (600 *g*) for 10 min. The pellet contained nuclei and unbroken cells. The supernatant was recovered and centrifuged for 30 min at 10,000 *g*. The second pellet had the mitochondrial and lisosomal fractions. The supernatant was ultracentrifuged at 100,000 *g* in order to separate the microsomal fractions (pellet) from the cytosolic fraction (supernatant; Birnie 1972). The protein concentrations of the frac-

tions were normalized with the Bradford assay. Proteins from different cell fractions were separated on 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (Laemmli 1970), and the gel was blotted onto a nitrocellulose membrane for immunostaining (Burnette 1981) in a 1:100 dilution of the anti-HSP60 antibody, followed by alkaline phosphatase-conjugated goat anti-mouse secondary antibody (Gibco). The different cellular fractions were virified by specific antibodies.

Fly transformation and rescue experiments

A genomic *SalI-SalI* DNA fragment of about 15 kb that contains the *Drosophila* hsp60 transcription unit was cloned in the *XhoI* site present in the Casper AUG transformation vector (Thummel et al. 1988; Ashburner 1989). This DNA was microinjected in w^{1118} embryos and stocks with the insertion of $[Pw^{+}hsp60]$ were established and genetically mapped. Heterozygous $l(1)10Ac^{F409}$ or $l(1)10Ac^{F437}$ females balanced with *FM6* chromosome mated with *FM6/∧* males produce homozygous $l(1)10Ac^{F409}$ or $l(1)10Ac^{F437}/\wedge$ which die during embryonic development. The initial cross $l(1)10Ac^{F409}$ or $l(1)10Ac^{F437}/FM6$ with transgenic males $yw/\wedge;\ P[w^{+}hsp60]/P[w^{+}hsp60]$ (line 512 transgene on the second chromosome) or $yw/\wedge;P[w^{+}hsp60]/+$ (line 561 transgene on the third chromosome) produced non *FM6* males $l(1)10Ac^{F409}$ or $l(1)10Ac^{F437}/\wedge;\ P[w^{+}hsp60]/+$ which were viable and fertile indicating the rescue of the lethal phenotype in the males. Rescued females were obtained by crossing $l(1)10Ac^{F409}$ or $l(1)10Ac^{F437}/FM6$females with $l(1)10Ac^{F409}$ or $l(1)10Ac^{F437}/\wedge;\ P[w^{+}hsp60]/+$ males; the resultant homozygous females $l(1)10A^{cF409}/l(1)10A^{cF409}$ or $l(1)10Ac^{F437}/l(1)10Ac^{F437};\ P[w^{+}hsp60]/+$ were also viable and fertile.

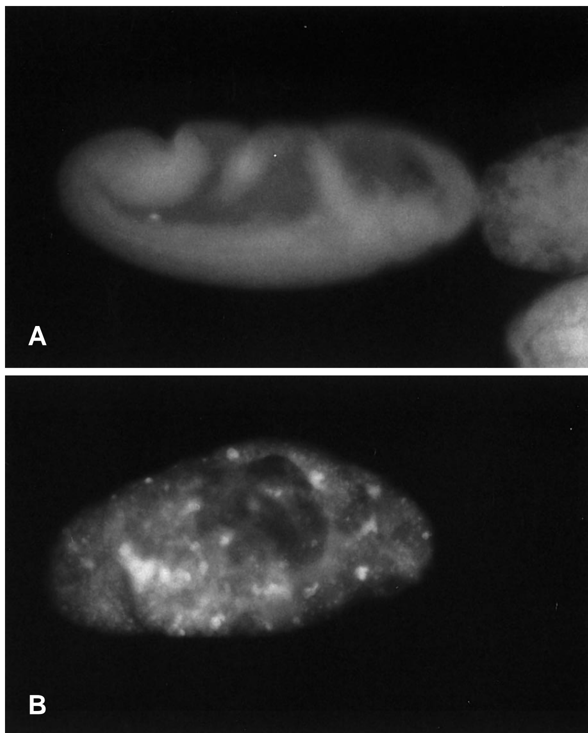

Fig. 1A, B Embryonic phenotypes of the lethal alleles affecting the *l(1)10Ac* locus. Embryos of the $l(1)10A^{F409}$ or $l(1)10A^{F437}$ alleles were stained with DAPI. **A** Wild-type (Wt) embryo at stage 8. **B** $l(1)10A^{F409}/Y$ embryo. Note the amorphous shape of the embryo just before gastrulation. No other lethal mutations are present on the *X* chromosome (see Results)

Polymerase chain reaction (PCR) amplification and molecular anaysis of the *S15* lethal allele

Oligonucleotide sequencing primers for *hsp60* were used in combination with P-element-specific primer in typical PCR reactions. Briefly, the template DNA was purified from heterozygous adult flies using proteinase K/phenol extractions and ethanol precipitation (Sambrook et al. 1989). Amplification reactions were carried out in a Perkin Elmer 2400 thermal cycler in 50 µl volume containing 50 mM KCl, 10 mM TRIS-HCl (pH 8.3), 2 mM $MgCl_2$, 25 pmol each primer, 200 µM dNTPs and 2.5 U Taq polymerase. Samples were held at 95°C for 50 s, 60°C for 90 s and 72°C for

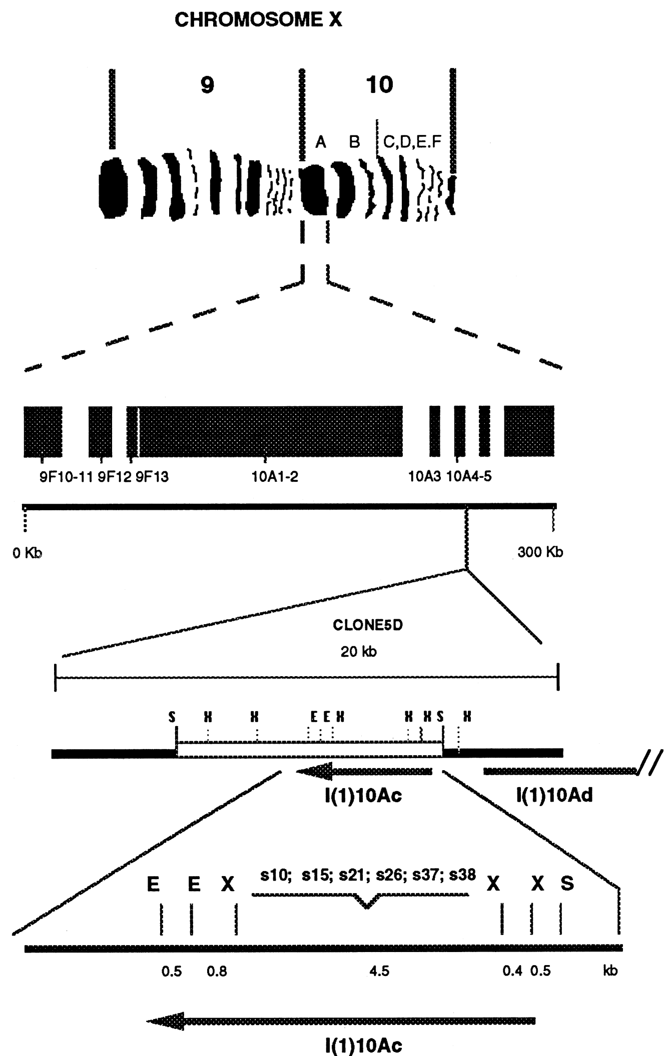

Fig. 2 Schematic representation of the localization and physical map of the hsp60 transcript. The 10A1-2 band and adjacent regions were mapped by Kozlova et al. (1994). The genomic clone 5D (about 20 kb) containing the *l(1)10Ac* locus is indicated. A 4.5-kb *XhoI* restriction fragment was identified as the region where six P-element-induced mutations (indicated as *S10*, *S15*, *S21*, *S26*, *S37* and *S38*) affect the *l(1)10Ac* locus (Kozlova et al. 1994). Two overlapping cDNA clones that encode for the HSP60 (clone BP5-3, 1.8 kb in length and BP5-31 2.0 kb in length) hybridize with 4.5-kb *XhoI* restriction fragment, as well as with the adjacent *XhoI* and *EcoRI* fragments. The transcription unit and the direction of the HSP60 gene transcription are represented by *an arrow indicated as l(1)10Ac*. The 15-kb *SalI* fragment used in the rescue experiments is indicated in the figure as a *white box* (*E EcoRI, X XhoI, S SalI*)

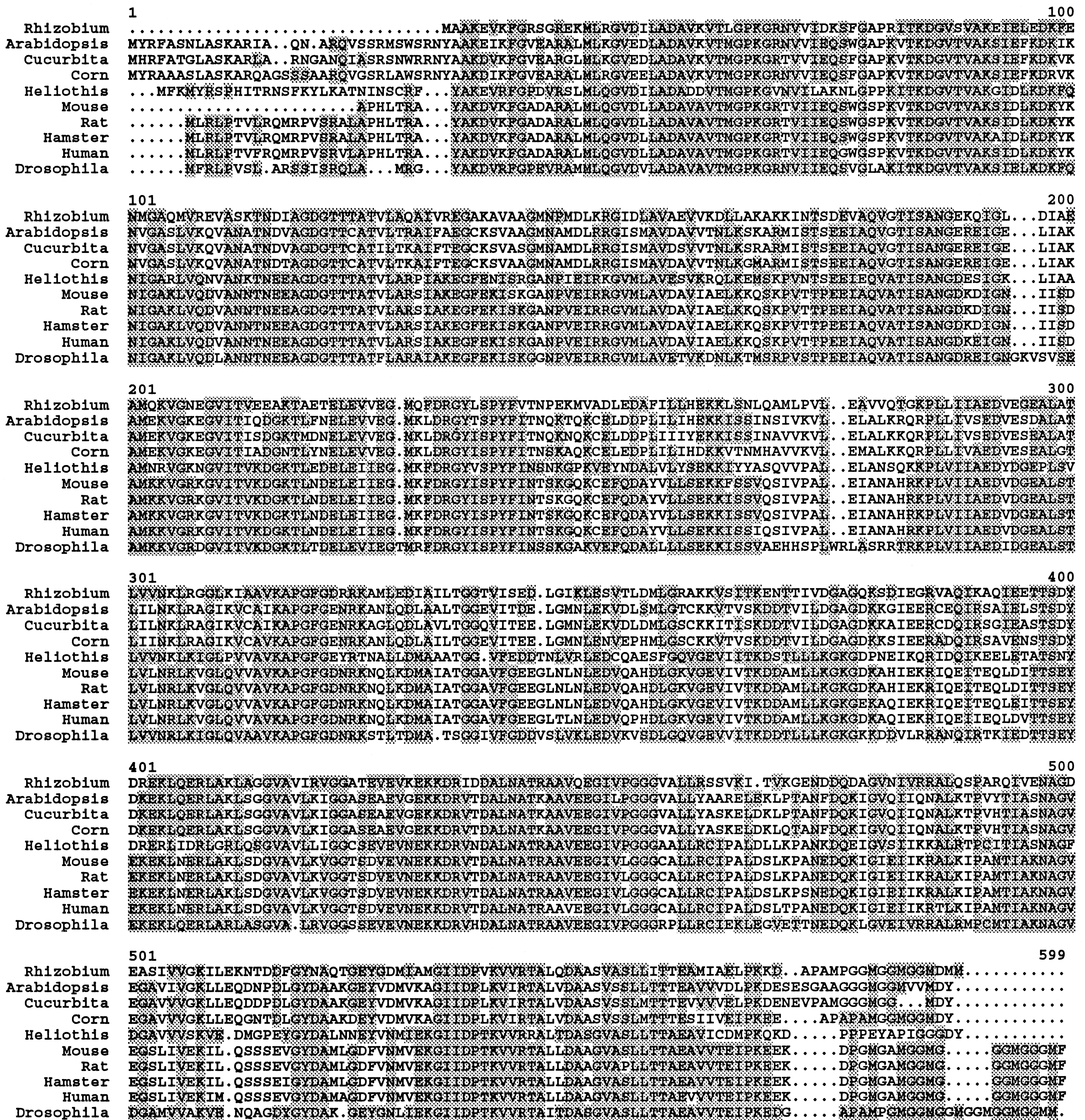

Fig. 3 Alignment of amino acid sequences for the *Drosophila* HSP60 protein and HSP60 proteins from other organisms. Only the representative HSP60 proteins from different species are shown in the figure. *Rhizobium* (Accession No: M94192; Rusanguanwa and Gupta 1993); *Arabidopsis* (Accession No.:Z11547; Prasad and Stewart 1992); *Cucurbita*; (Accession No: X70867; Tsukeyi et al. 1992); *Heliotis* (Accession No; X56034; Miller et al. 1990); Corn (Accession No: L21006; Unpublished); mouse (Accession No: X53584; Venner and Gupta 1990); rat (Accession No: X54793; Peralta et al. 1990); hamster (Accession No: M22383; Picketts et al. 1989); human (Accession No: M34664; Venner and Gupta 1990). The nucleotide sequence of the cDNA clones BP5-3 and BP5-31 is deposited with the EMBL data bank, accession number X99341

120 s for 30 cycles. Reaction products were resolved in 1% agarose gel in TRIS-acetate running buffer (Sambrook et al. 1989).

Results

Phenotypic analysis of the embryonic lethal alleles of the *l(1)10Ac* locus

Several lethal alleles of the *l(1)10Ac* locus have been described (Bgatov et al. 1986; Zhimulev et al. 1987; Lindsley and Zimm 1992), however, no detailed phenotypic analysis of these mutants has been reported. Our initial

experiments showed that $l(1)10Ac^{F409}$ and $l(1)10Ac^{F437}$ homozygous mutant animals die during embryogenesis. We proved by genetic rescue that no other lethal mutations are present on the corresponding *X* chromosome (see below). In order to determine the effect of these mutations in *Drosophila* embryogenesis, DAPI staining of 0- to 24 h embryos of these alleles was performed. In both cases the same phenotype was observed (Fig. 1). The embryos die very early in development without any signs of gastrulation, they are very amorphous and the nuclei are distributed randomly in the embryo. This result shows that the product of the *l(1)10Ac*locus is essential from the early stages of fly embryogenesis.

Cloning and characterization of the *l(1)10Ac* locus in *D. melanogaster*

In previous work, several P-element-induced mutations in the *l(1)10Ac* locus have been mapped. These mutations are all localized in a 4.5-kb *XhoI* restriction fragment within a genomic clone 5D at position 270–274.5 of the reported physical map (Kozlova et al. 1994; T. Kozlova, unpublished work). This *XhoI* restriction fragment was used as a probe to isolate cDNA clones that may correspond to the *l(1)10Ac* locus. Several clones were identified from an ovarian cDNA library (Kozlova et al. 1994) and further characterized in this report. Figure 2 shows the physical map of the region that contains the *l(1)10Ac* locus in the genomic clone 5D. Several overlapping cDNA clones that were isolated encode for the same protein product. Many clones also hybridize to a 6.5-kb zone that includes the complete *XhoI* 4.5-kb fragment and the adjacent restriction bands (Fig. 2). The cDNA library used was made with random primers; the largest clone (BP5-3) is about 1.8 kb long. Sequence data analysis revealed that a region expected to be in the 3′ end of the message was not present, indicating that the BP5-3 clone was not full length. In order to isolate new cDNAs containing the missing region, an embryonic cDNA library (0–24 h) was screened using the BP5-3 clone as a probe. A clone of about 2.0 kb that contains a complete coding sequence was isolated (clone BP5-31). The cDNA clones were sequenced and an open reading frame (ORF) of 576 amino acids was identified starting with a methionine at position 112 and ending with stop codon at the position 1840 (data not shown).

Computer analysis showed that the ORF present in the cDNA clones is homologous to chaperonin 60 from many different organisms (Fig. 3). The most closely related sequence is mouse mitochondrial HSP60 (72% of identity with the fly protein). The predicted protein product contains a putative signal peptide typical for proteins that are translocated to the mitochondrial matrix (Fig. 3), thus supporting the idea that it encodes the *D. melanogaster* mitochondrial HSP60 protein form. This signal peptide is clearly conserved between HSP60 proteins from animals (including *D. melanogaster*); it is divergent in plants and is not present in bacterial forms (Fig. 3).

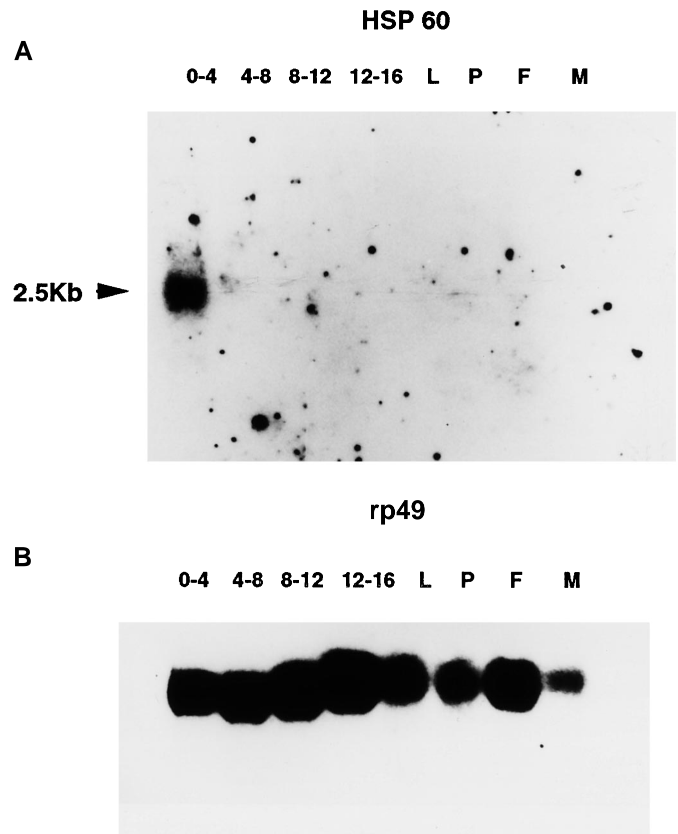

Fig. 4A, B Northern blot analysis of the hsp60 transcript during fly development. **A** A single transcript of around 2.5 kb is detected in 0- to 4-h embryos. Each lane contains 15 μg total RNA. The blots were hybridized with 1.8-kb *EcoRI* restriction fragment representing the complete insert in the BP5-3 cDNA clone. **B** The same blot was hybridized with rp49 probe (O'Connell and Rosbash 1984) as a control. Total RNA was purified from embryos 0–4, 4–8, 8–12 and 12–16 h after egg-laying, as well as larvae (*L*), pupae (*P*), adult females (*F*) and males (*M*)

This is the first characterization of the hsp60 homologous gene in *D. melanogaster* (localizing the *hsp60* gene in the position 10A4-5 on the *X* chromosome). Southern blot analysis using low stringency hybridizations indicates that there is only one copy of the *hsp60* gene in the fly genome (data not shown).

Distribution of the hsp60 mRNA and protein during *D. melanogaster* development

Heat-shock response in *D. melanogaster* is manifested by the synthesis of a specific set of proteins and the appearance of heat-inducible puffs in salivary gland polytene chromosomes. Cytogenetically heat-shock-inducible puffs correspond to the positions 33B, 63C, 64F, 67B, 70A, 87A, 87C, 93D and 95D (reviewed in Ashburner and Bonner 1979). Since no heat-shock-inducible puffs have been identified in the 10A4-5 region of the *X* chromosome, and no clear induction of HSP60 is observed after heat shock in *Drosophila* culture cells (McKenzie and

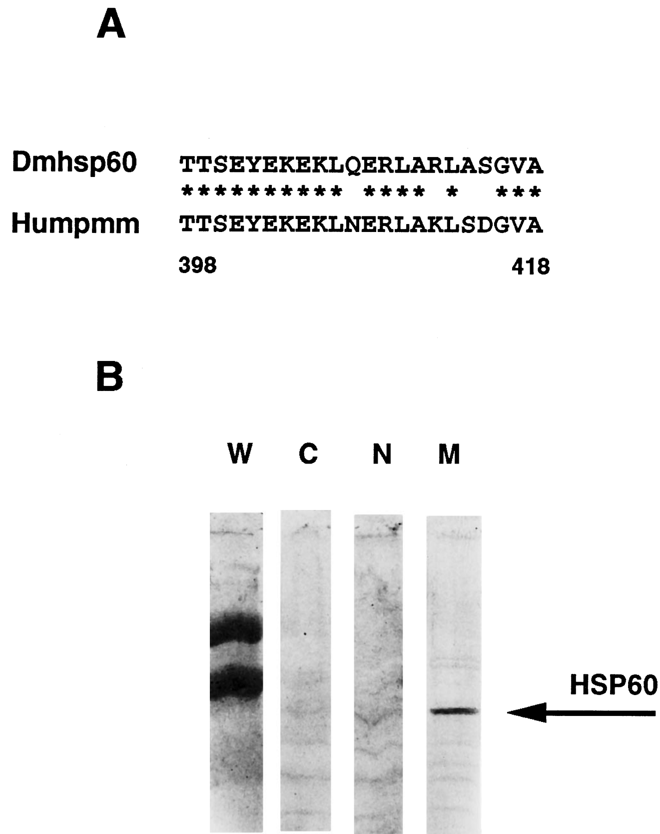

Fig. 5A, B Western blot analysis of HSP60 distribution in different subcellular fractions from *Drosophila* embryos. **A** A commercial monoclonal antibody was produced against a human HSP60 protein and recognizes an epitope present in HSP60 proteins from bacteria to humans. The equivalent sequence in the *D. melanogaster* HSP60 protein is shown. **B** Subcellular fractions were tested for the presence of the *Drosophila* HSP60 protein using this monoclonal antibody. The antibody recognizes a band of around 60 kDa enriched in the mitochondrial fraction. As positive control the same antibody was tested with the yeast HSP60 (not shown). (*W* molecular weight markers, *C* cytosolic fraction, *N* nuclear fraction and unbroken cells, *M* mitochondrial fraction)

Meselson 1977; Lindquist 1980; Lindquist and DiDomenico 1985), we thought it would be interesting to determine whether the *Drosophila hsp60* gene is expressed during fly development in normal conditions and whether it is induced by heat treatment. Developmental northern blot experiments were performed using total RNA prepared from different embryonic stages as well as third instar larvae, pupae and adults. A single message of around 2.5 kb was detected using an insert from BP5-3 cDNA clone as a probe. Unexpectedly, a difference in the amount of hsp60 mRNA during development was found (Fig. 4). In 0- to 4 h embryos the amount of hsp60 transcript is much more abundant (about thirty times) than in all other stages. In fact, only after long exposure is the transcript evident in other stages (data not shown). This result clearly shows a significant difference in the amount of hsp60 mRNA during fly development. It also opens the question as to whether the differences in the amount of mRNA are relevant at the protein level and if there is a differential distribution of the protein in various cell types. To address this problem, we took advantage of the existence of a commercial monoclonal antibody that recognizes an epitope present in many mitochondrial HSP60 proteins from different organisms. This epitope is also conserved in the *Drosophila* HSP60 protein (Fig. 5A). This antibody was tested against different embryonic (0–12 h) subcellular protein fractions in a western blot experiment. It specifically recognizes a band of around 60 kDa which is enriched in the mitochondrial fraction (Fig. 5B). Some signal is also detected in the nuclear fraction. At this point we do not know whether some HSP60 protein is localized in the nucleus or whether it is just the result of mitochondrial contamination in the nuclear fraction. It is clear, however, that the antibody specifically recognizes a *Drosophila* protein of the correct molecular weight that is located in the mitochondria.

This antibody was used in immunostaining experiments in whole-mount embryos and visualized in optical sections in a confocal microscope. Figure 6 shows a summary of the staining results. During fly embryogenesis many cell types are positively stained. However, it is clear that some cells contain greater amounts of HSP60 protein than others. In early embryos at the syncitial blastoderm stage there is a significant amount of staining all around the embryo (Fig. 6A). At the cellular blastoderm stage, the staining in the embryo has been dramatically reduced. No signal is detected in the yolk and cells are preferentially stained in the basal region. The amount of HSP60 in the pole cells, however, is higher than in the rest of the embryo (Fig. 6B). The high amount of HSP60 protein in the posterior part of the embryo could be either of maternal origin or due to de novo expression. At the beginning of germ band elongation (Fig. 6C), the staining is more homogeneous but some cells in the embryo have a stronger signal (cells of the cephalic furrow and mesodermal cells). In stages 11–14 (Fig. 6D) the amnioserosa cells are particularly rich in HSP60 protein.

Although the HSP60 protein is present in many cell types during fly embryogenesis, our results show that some cells have more HSP60 protein than others and that the quantity and distribution of DmHSP60 protein during fly embryogenesis is dynamic.

The levels of hsp60 mRNA and protein were not significantly increased after heat treatment of embryos, when assayed by northern and western blot experiments (data not shown). This result may explain why no heat-shock-inducible puffs are observed in the 10A4-5 region on the *X* chromosome and why the HSP60 protein is not observed in *Drosophila* culture cells after heat shock (McKenzie and Meselson 1977).

A genomic DNA fragment containing the hsp60 transcription unit rescues the lethal phenotype of mutants in the *l(1)10Ac* locus

Molecular characterization of the *l(1)10Ac* locus in the 10A4-5 region on the *X* chromosome allowed us to identify a gene homologous to *hsp60* from other organisms.

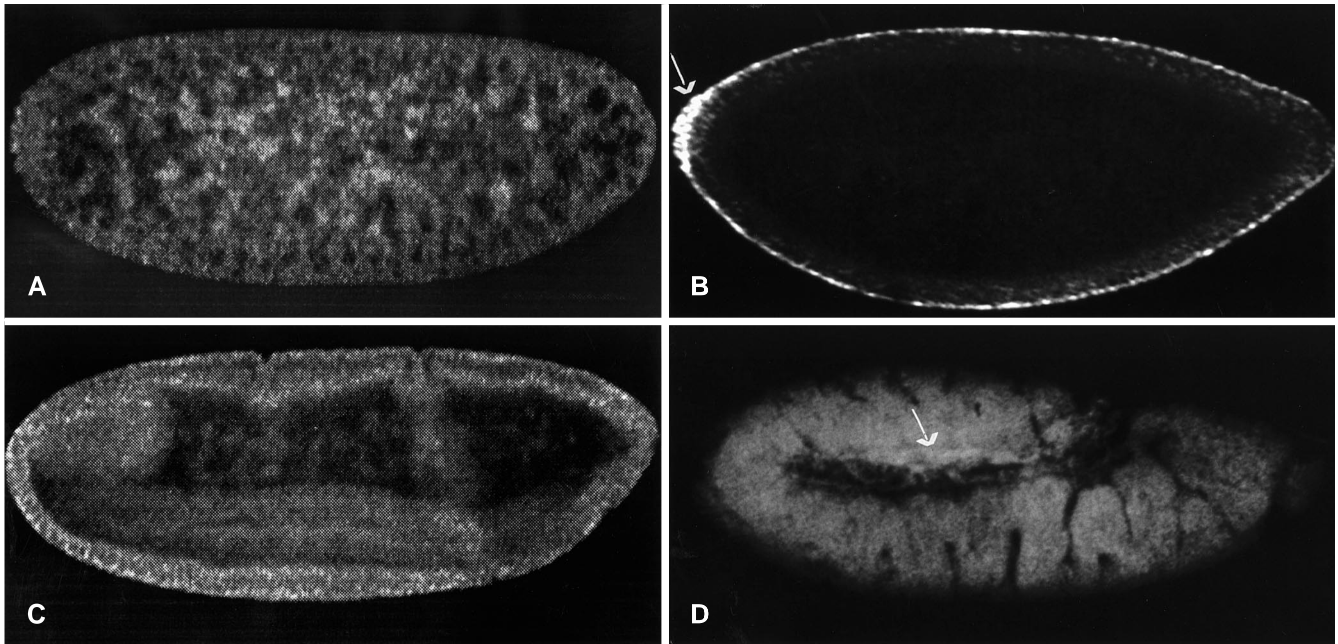

Fig. 6A–D HSP60 protein distribution in *Drosophila* embryos. **A** Syncitial blastoderm embryo; **B** cellular blastoderm embryo, stage 5; **C** stage 6 embryo; **D** stage 11 embryo. The *arrows* indicate the regions were the HSP60 protein is enriched (see the text for details)

However, it was important to demonstrate that this particular locus indeed encodes for the protein described in this work. Many alleles reported for the *l(1)10Ac* locus are lethal when homozygous, some during embryogenesis and others during larval and pupal development. Conditional and hypomorphic mutations have also been reported (Zhimulev et al. 1987; Lindsley and Zimm 1992). In order to determine if the genomic region containing the *Drosophila* hsp60 transcription unit is able to rescue alleles of *l(1)10Ac* locus, transgenic lines that contain a 15-kb *SalI-SalI* fragment in which the entire hsp60 coding region is present were generated. The position of the transcriptional unit for the *hsp60* gene in the *SalI-SalI* fragment is indicated in Fig. 2. It is important to mention that no other transcripts were detected in northern blot experiments downstream of *hsp60* that could be present in this construct (unpublished data). The *l(1)10Ad* locus is the closest gene mapped proximally to *l(1)10Ac* (Kozlova et al. 1994). We have cloned cDNAs that correspond to this gene and we know that it is not present in the rescue construct (unpublished data).

Transgenic flies *P[w⁺Dmhsp60]* were crossed with two different embryonic-lethal alleles analysed phenotypically in this report (see Materials and methods for the description of rescue crosses). Genetic rescue has been achieved for both males and females homozygous for the lethal alleles of *l(1)10Ac* locus and heterozygous for the transgene construct. Homozygous *l(1)10Ac* flies in these crosses were recovered at expected numbers (Table 1). The rescued flies are viable and fertile; the *miniature* (small wings) and *forked* (defects in macrochaete) pheno-

Table I Rescue of the *l(1)10Ac* phenotype by a hsp60 transgene.

The miniature (small wings) and forked (defects in macrochaete) phenotypes are used as markers in mutated X chromosome (Zhimulev et al. 1987). Similar results were obtained with the *l(1)10Ac* $^{l(1)437}$ allele

Males rescue

♀ *l(1)10Ac* $^{l(1)409}$ *m f* / *FM6* x ♂ *w* / ⇁ ; *[P.hsp60]* / *[P,hsp60]*

Genotype	number of adults
l(1)10Ac $^{l(1)409}$ *m f* / *FM6* ; *[P.hsp60]* / +	50
+ / *FM6* ; *[P.hsp60]* / +	58
l(1)10Ac $^{l(1)409}$ *m f* / ⇁ ; *[P.hsp60]* / +	31
FM6 / ⇁ ; *[P.hsp60]* / +	39

Females rescue

♀ *l(1)10Ac* $^{l(1)409}$ *m f* / *FM6* x ♂ *(1)10Ac* $^{l(1)409}$ *m f* / ⇁ ; *[P.hsp60]* / +

Genotype	number of adults
l(1)10Ac $^{l(1)409}$ *m f* / *l(1)10Ac l(1)409 m f* ; *[P.hsp60]* / +	18
l(1)10Ac $^{l(1)409}$ *m f* / *FM6* ; *[P.hsp60]* / + / ± / +	78
l(1)10Ac $^{l(1)409}$ *m f* / ⇁ ; *[P.hsp60]* / +	17
FM6 / ⇁ ; *[P.hsp60]* / + / ± / +	46

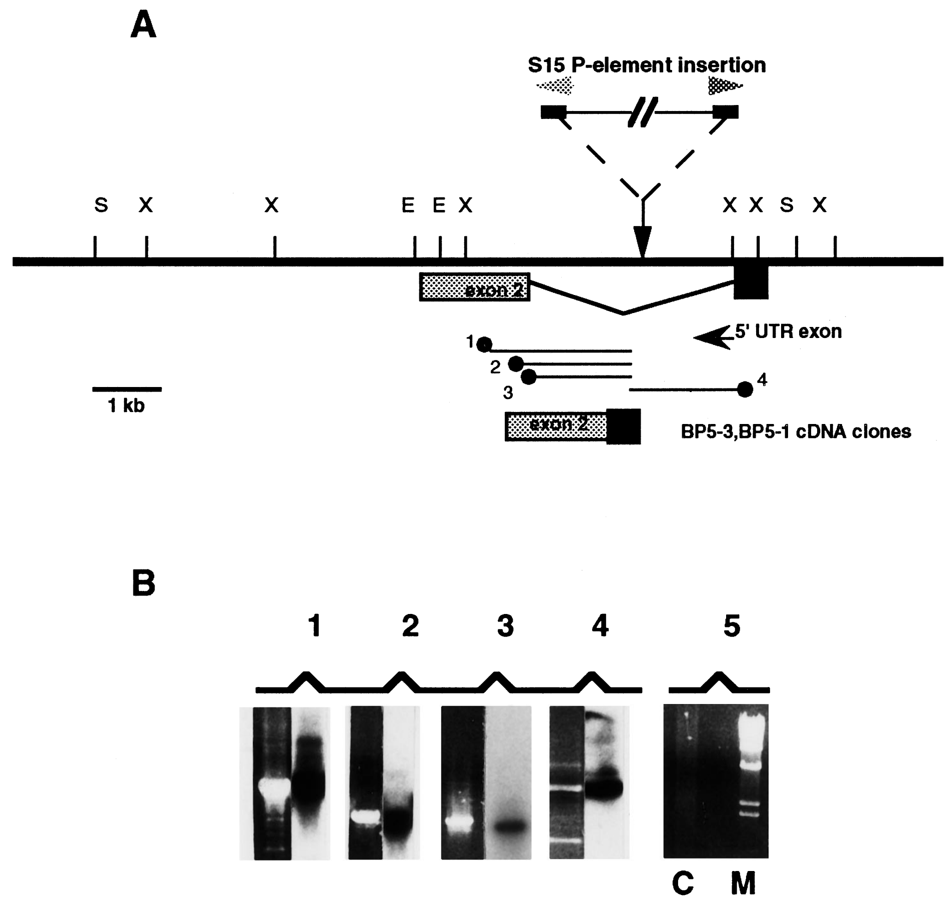

Fig. 7A, B Molecular analysis of the *S15* P-element-induced lethal allele and genomic organization of the *Drosophila Hsp60* gene. **A** Oligonucleotide primers deduced from the sequence of the BP5-3 and BP5-31 cDNA clones (shown as *grey dots* in the figure) used in combination with an inverted repeat primer from the P-element-produced specific PCR DNA fragments represented as *1*, *2*, *3* and *4*. The position of the *S15* P-element insertion almost in the middle of the intron is indicated. **B** The PCR-generated fragments hybridized with the BP5-3 cDNA clone are shown. Panels 1, 2, 3 and 4 correspond to the extended DNA indicated in Fig. 7A. Panel 5 shows the control genomic DNA from wild-type flies and molecular weight markers

types used as markers on mutated *X*chromosome (Zhimulev et al. 1987) were evident in the rescued flies (data not shown). Therefore, is clear that the 15-kb *SalI-SalI* genomic fragment containing the hsp60 transcription unit is sufficient to rescue the lethality in *l(1)10Ac* locus.

Molecular analysis of the *l(1)10Ac S15* allele and genomic organization of the *hsp60* gene

Molecular analysis of one of the P-element-induced alleles was performed to confirm that mutations in the *l(1)10Ac* locus affect the hsp60 transcription unit. Genomic DNA from the S15 allele was the substrate in PCR reactions with different primers used to sequence the *hsp60* cDNA clones in combination with an inverted repeat P-element primer. Four of the *hsp60* primers produced specific PCR products in combination with the P-element primer (Fig. 7A). Similar reactions with control DNA did not produce any PCR product (Fig. 7B). The amplified DNA fragments were hybridized with the BP5-3 cDNA clone to prove that they contain sequences homologous to *Drosophila hsp60* (Fig. 7B). The size of the obtained PCR bands indicated the presence of an intron in the 5′ region of the *Drosophila hsp60* gene and that the P-element is inserted in this intervening sequence. The amplified PCR products were sequenced around the P-element insertion site and intron/exon boundaries were defined as well. From this analysis we determined the exact position of an intron of about 3 kb located just before the initiation codon (data not shown). These results as well as additional PCR mapping and sequencing (data not shown) indicate that the *hsp60* gene consists of two exons, one containing the 5′ untranslated region (5′UTR) and a second containing the HSP60 coding sequence separated by a 3 kb intron (Fig. 7A). The analysis of the sequence of the intron DNA (600 bp around P-element insertion site) showed that no large ORF in the six possible frames is present (data not shown), confirming that the lethality of the S15 allele is due to the lesion in the *hsp60* transcription unit.

Discussion

While characterizing the *l(1)10Ac* locus at the molecular level we have found that it encodes for a member of the HSP60 family of molecular chaperones. In spite of the fact that the chaperonin family has been the subject of particular attention due to the essential role of these proteins in mediating the folding of non-native proteins to their native state, this is the first report about a member of this family in *D. melanogaster*.

The HSP60 protein described in this work is unequivocally homologous to the mitochondrial HSP60 chaperones found in different organisms. The highest identity is

with mouse chaperonin. This protein also contains a signal peptide sequence that is required for the transport of proteins to the mitochondria that are synthesized in the cytoplasm, and is only present in HSP60 proteins from animal origin (reviewed in Gupta 1995). The *Drosophila hsp60* gene is located in the 10A4-5 division on the *X* chromosome. It is apparently a single copy gene since no other copies have been identified in genomic southern blot experiments.

No heat-shock-inducible puffs have been described previously in the 10A region in the *X* chromosome (reviewed in Ashburner and Bonner 1979). HSP60 protein is not detectable in *Drosophila* in vitro culture cells stimulated with heat shock (Lewis et al. 1975; McKenzie and Meselson 1977; Lindquist 1980). These results, combined with our own observations, suggest that the *Drosophila hsp60* gene is not heat-shock inducible or induced at low levels. This result is not uncommon, however, since there are more examples of *hsp60* genes that are only activated 2–3 times or less by heat shock (Parsell and Lindquist 1993).

The hsp60 mRNA is much more abundant in total RNA preparations from 0- to 4-h embryos compared with all other stages analysed. In fact, the hsp60 transcript can be detected only after a long exposure in other RNA preparations besides the 0- to 4-h embryonic sample. This indicates that the level of hsp60 mRNA is differentially regulated during fly development. Zygotic gene transcription in *D. melanogaster* embryos starts after 80 min of development, just before the pole cells are formed (Foe et al. 1993). Since very few transcripts are observed in total female RNA preparations, it is plausible that the amount of RNA observed in northern blots is the result of zygotic transcription that occurs in the first hours of development rather than from maternal contribution. It is important to note that after 4 h development the amount of hsp60 mRNA decreases dramatically. This suggests that after this time the requirements of the *Drosophila hsp60* function might be less than in early development. Alternatively it is feasible as well as in agreement with our findings that the protein produced during early embryogenesis is maintained and distributed differentially throughout the next embryonic stages. It has been shown that other HSPs are expressed in *Drosophila* embryogenesis suggesting that they function in normal development in addition to their participation in the classical heat-shock response (Hass et al. 1990). The dramatic effect of *l(1)10Ac* lethal alleles on early embryonic development presented in this work shows that zygotic expression of the *hsp60* gene is fundamental since the embryos are derived from heterozygous mothers. Unfortunately this phenotype makes the analysis of the *hsp60* gene expression in the homozygous mutant alleles difficult.

The amount of HSP60 protein is dynamic throughout fly embryogenesis. At this point we do not have a straightforward explanation for the different amount of HSP60 protein in various cell types. Some of the cells that have higher amounts of this protein (eg. pole cells, cells of the cephalic furrow; Fig. 6) are migrating or in the process of movement. Not all the cells in movement, however, are particularily rich in HSP60 and therefore it is difficult to relate the abundance of HSP60 protein to developmental cell migration. It has been previously demonstrated that mitochondrial HSP60 is an essential protein for the correct function of some mitochondrial proteins (Cheng et al. 1990). It is possible that the functional mitochondrial requirements are not the same during development and that the different amounts of HSP60 in different cells are related to a particular metabolic stage of each cell type. Interestingly, it has been reported that a decrease in the synthesis of human mitochondrial HSP60 is the primary defect in mitochondrial encephalomyopathy syndrome (Huckriede and Agsteribbe 1994). This shows that defects in the function of this protein have a more dramatic effect in some tissues than others and furthermore, it suggests that HSP60 requirements are not the same for all cell types in humans. Clonal analysis of *hsp60* function in *Drosophila* development can be used to address this point in flies.

In the present work we established that the *l(1)10Ac* locus encodes for the *D. melanogaster* HSP60 protein. We performed genetic rescue experiments in which the embryonic-lethal phenotype associated with two *l(1)10Ac* alleles was rescued with a genomic fragment containing the hsp60 transcription unit. Moreover, the reported P-element insertions that affect this locus were all localized in a 4.5-kb fragment which contains most of the hsp60 transcription unit (Kozlova et al. 1994; T. Kozlova, unpublished work). One of these mutants (*S15*) was characterized in detail confirming that the P-element is inserted in an intron located in the 5′ region of the *hsp60* gene. Our molecular analysis also shows that *Drosophila hsp60* gene consist of two exons and a large intron. The first exon contains the 5′UTR and the second exon contains the complete HSP60 coding region beginning with the translation initiation codon. *hsp60* functional gene in humans is intronless (there are many pseudo-genes in humans) and it is one of a few genes of this kind in vertebrates which might be related to its possible prokariotic origin (Venner and Gupta 1990). In the case of *D. melanogaster*, however, the 5′ untranslated region is separated from the rest of the gene by a 3-kb intron.

The genetic rescue experiments as well as *S15*allele molecular analysis confirm that *D. melanogaster hsp60* gene is encoded by the essential locus *l(1)10Ac*. The use of mutants that affect the *hsp60* gene in yeast has been relevant to the study of the function of this protein under varying physiological conditions. The genetics of the *l(1)10Ac* locus is very complex including interallelic complementation and the existence of both cold- and heat-sensitive alleles (Zhimulev et al. 1987; Lindsley and Zimm 1992; Pokholkova et al. 1991). The availability of various mutations in *Drosophila hsp60* gene might greatly facilitate genetic screens for interacting proteins such as putative HSP10 and opens the possibility for a detailed genetic analysis of the HSP60 functions in vivo.

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