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The Expression of Heat Shock Protein HSP60A Reveals a Dynamic Mitochondrial Pattern in *Drosophila melanogaster* Embryos

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The evolutionarily conserved *hsp60* (*heat-shock protein 60*) family of molecular chaperones ensures the correct folding of nuclear-encoded proteins after their translocation across the mitochondrial membrane during development as well as after heat-shock treatment. Although the overexpression of HSP60 proteins and their localization in the cytoplasm have been linked with many human pathologies, the detailed pattern of their expression in different animal models and their subcellular localization during normal development and in stress conditions are little-known. In this report, we have used two-dimensional gel electrophoresis followed by MALDI-TOF to identify and purify heat shock protein HSP60A of *Drosophila melanogaster*. We demonstrate that it is heat-shock inducible and describe two novel antisera, specifically designed to recognize the denatured and native polypeptide, respectively, in *Drosophila*. Immunoelectron microscopy and immunostaining of *Drosophila* cells with these antibodies reveals that HSP60A is always localized to the inner membrane of mitochondria. Expression of HSP60A is post-transcriptionally regulated in a highly dynamic pattern during embryogenesis, even under heat-shock conditions. In contrast, in very stressful situations, its expression is upregulated transcriptionally over the entire embryo. These findings suggest novel roles for HSP60 family proteins during normal *Drosophila* development.

Keywords: *Drosophila* • Heat shock • HSP60A • Mitochondria

1. Introduction

The proper biogenesis of mitochondria is critical in order to satisfy the energetic demands of cells and to trigger the execution of apoptotic programs.^{1,2} Thus, mitochondrial malfunctions are hallmarks of neurodegenerative diseases, cancer and aging.³ The evolutionarily conserved *hsp60* (*heat-shock protein 60*) family of molecular chaperones⁴ plays a key role in the folding of proteins in mitochondria during normal development and in stressful situations.^{5,6} Although HSP60 proteins are located in the “cytosol” of bacteria and in the stromal compartment of chloroplasts, their overexpression outside the matrix compartment of mitochondria seems to be associated with pathological situations.⁷ Furthermore, overexpression of HSP60 in the cytoplasm is frequently used as a marker of various carcinomas.⁸ Little is known about the correct localization of the HSP60 in different animal models and the mechanisms controlling its expression during normal development and under stressful conditions.

The powerful genetic techniques available for altering gene expression in *Drosophila melanogaster* and the extensive knowledge about all stages of development make this organism an attractive model for the investigation of the *in vivo* corre-

tion between gene expression patterns and the functions of human orthologs of heat-shock proteins.⁹ Heat-shock proteins were originally identified in *D. melanogaster* as heat shock-induced proteins synthesized de novo in specific puffs of the polytene chromosomes of salivary gland cells.^{10,11} Latter, the Berkeley Drosophila Genome Project revealed the presence of four *Hsp60* genes in *D. melanogaster*, that have been named *hsp60A*, *hsp60B*, *hsp60C*, and *hsp60D*, respectively.¹² In this work, we have focused our attention on the first of these genes, *hsp60A*. This gene is located in the 10A4–5 divisions on the X chromosome and surprisingly was not identified as a heat-shock inducible gene. *hsp60A* consists of two exons and a large intron.¹³ The protein product contains a signal peptide required for its transport to mitochondria.¹⁴ *hsp60A* has been described as a protein that binds to single-stranded DNA and that recognizes specifically the putative origin of mtDNA replication.¹⁵ However, there is controversy about its subcellular localization. Some workers have reported that the HSP60 proteins are exclusively localized in mitochondria even under stressful conditions,¹⁶ while others have identified HSP60 in a variety of subcellular structures.¹⁷ The shortage of data on the localization and regulation of HSP60 proteins in different animal models and their potential role in mitochondrial biogenesis prompted us to conduct a detailed analysis of their expression and subcellular localization during development and under stress.

In this work, we show that *Drosophila* HSP60A is a heat-shock inducible protein that is normally localized to mitochon-

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dria. We report also that during embryogenesis it displays a conserved but highly dynamic pattern of expression that is controlled by post-transcriptional mechanisms, but that stress responses call for transcriptional activation of the gene. These findings suggest novel functions for HSP60A during embryogenesis, independent of stress, and provide evidence for unknown mechanisms involved in its regulation and the biogenesis of mitochondria.

2. Materials and Methods

2.1. Fly Strains and Cell Culture. All fly strains are described at <http://www.flybase.bio.indiana.edu> unless otherwise indicated. To analyze the effect of cell death on HSP60A expression during embryo development, *engrailed*-Gal4 females were crossed with UAS-*ricin^{ts}* males at 25 °C. Later, the temperature was shifted to 31 °C to activate the toxin-ricin, and induce apoptosis. Other experiments in this report were carried out at 25 °C unless otherwise indicated and employed wild-type flies from Madrid (Vallecasa). The genotype of the hypomorphic HSP60 mutant embryos was *Hsp60^{EY01572}*/*Hsp60^{GF319}*. The established cell line used was derived from wing imaginal discs of mature larvae of *D. melanogaster* (strain Oregon R), and was designated CME W2. Cells (3×10^5) were plated in multiwell plates (2 cm²) as described.¹⁸

2.2. Preparation of Samples and 2-D Gel Electrophoresis. Dissection and labeling of imaginal discs was carried out as described previously.¹⁹ Analytical 2-D was performed according to O'Farrell²⁰ with some modifications.²¹ Second-dimension separations were carried out on 15% polyacrylamide gels (24 × 24 cm) and run overnight at room temperature (18 °C). Gels were processed for fluorography,²² dried, and exposed at -70 °C for various periods of time. For preparative gels, CME W2 cells from 20 confluent 65 cm² plates were used to prepare proteins as previously described.¹⁸

2.3. Computer Analysis of 2D Patterns. Four fluorograms, each after different exposure times (4, 24, 96 and 240 h) were made from each gel and digitized at 176 × 176 μm resolution with a pdi scanning densitometer. The resulting 2D images were merged and analyzed with PDQUEST software (Version 5.0) running on a SPARC station IPC microcomputer (Sun Microsystems).

2.4. In-Gel Digestion and MALDI-TOF Analysis. Coomassie protein spots were excised from gels, digested with trypsin and processed as described elsewhere.²³ Peptide mixtures were analyzed with an Applied Biosystems 4700 proteomics analyzer (Applied Biosystems, Framingham, MA) with TOF/TOF ion optics. MS spectra were obtained in reflectron mode using an acceleration voltage of 1 kV. Desorption and ionization of sample was performed with an Nd:YAG operating at 355 nm. The final mass spectra were produced by averaging 3600 laser shots and the autodigestion products of trypsin were used for internal calibration. MS/MS sequencing analysis was performed with the same equipment. Protein were identified by mass fingerprinting and the monoisotopic peptide mass fingerprinting data obtained by MALDI-TOF was used to search the Swiss-Prot/TrEMBL nonredundant protein database.

2.5. Antibodies. We made two antibodies, one against denatured HSP60A (α -HSP60A_{Den}), the other against the native polypeptide (α -HSP60A_{Nat}). In the former case, the cell contents of 14 confluent 500 cm² trays (Nunc) of CME W2 cells were used as the source of HSP60A for immunization. The cell monolayers were washed with Hanks' solution and scraped off with a rubber policeman in 10 mL of the same. After centrifu-

gation, the cells were sonicated, treated with DNase and RNase, and passed several times through a narrow-gauge needle. The sample was then lyophilized and resuspended in 5 mL of lysis buffer. [³⁵S]Methionine + [³⁵S]cysteine-labeled proteins from wing imaginal discs were added in order to identify the polypeptides by autoradiography. One hundred 2-D gels were run as described above, immediately dried (without fixation) and exposed for 4 days. The HSP60A protein was located by autoradiographs and cut out of the gels. The gel pieces were rehydrated and the protein was recovered by electroelution. Approximately 150 μg of the protein was injected into a New Zealand White rabbit, and its sera were tested by immunoblotting against HSP60A as described below. To prepare the antibody against native HSP60A, a cDNA containing the complete ORF was cloned into the pRSET vector (Invitrogen, Leek, The Netherlands), and expressed as a His₆-fusion protein in *Escherichia coli* BL21 (DE3)pLysS (Novagen, Madison, WI). Approximately 240 μg of this protein was purified by NiTA chromatography (Clontech, Palo Alto, CA) and injected into a New Zealand White rabbit.

A polyclonal antibody against *Drosophila* mitochondria was obtained by injecting 400 μg of mitochondria, purified as previously described,²⁴ into a New Zealand White rabbit. Polyclonal rabbit antibody against mitochondrial super oxide dismutase was obtained from Stressgen and the polyclonal antibody against the β-ATPase was kindly provided by Dr. Garesse.

2.6. Immunoblotting. Proteins resolved by 1-D or 2-D gels were transferred to nitrocellulose filters at 130 mA for 6 h. The filters were incubated for 120 min at room temperature in PBS containing 10% bovine serum and α -HSP60A_{Den} diluted 1:100. They were then incubated with peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) for 120 min in the same buffer and immunocomplexes were visualized with diaminobenzidine as substrate.

2.7. Immunoelectron Microscopy. Embryos were devitellinized manually as described,²⁵ and fixed in 4% PFA. Immunocytochemistry was performed after postembedding embryos in LR White resin following standard procedures.²⁶ Rabbit α -HSP60A_{Nat} was used as primary antibody (dilution 1:50) and goat α -rabbit IgG (H & L) coupled with 10 nm colloidal gold (BBInternational) as secondary antibody (dilution 1:40). The grids were observed in a JEOL 1010 electron microscope with 80 kV accelerating voltage.

2.8. Immunostaining of Embryos and Imaginal Discs. Standard procedures were used for whole-mount immunohistochemistry.²⁷ Imaginal discs were stained as described.²⁸ The α -HSP60A_{Nat} was used at a 1:500 dilution in all experiments. Rabbit anti-β-ATPase (dilution 1:100), rabbit α -superoxido dismutase (SOD) (dilution 1:500), rabbit α -mitochondria (dilution 1:100) and mouse anti-β-gal (Amersham) (dilution 1:4000) were also used as primary antibodies. Alexa Fluor-488 and Fluor-546 (Molecular Probes) were used as secondary antibodies. TOPRO-3 (Molecular Probes) was used for DNA staining.

For double immunostaining, HSP60A-RFP-conjugate was used after immunostaining for all other primary antibodies. The conjugate was prepared as follows: first, the α -HSP60A_{Nat} antibody was passed through a column of Protein G Sepharose (GE Healthcare) in order to remove serum proteins before coupling. Next, the purified antibody was coupled with Oyster-550-P-NHS (Denovo Biolabels) as recommended, and used at a 1:25 dilution.

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2.9. Microscopy and Image Treatment. Embryos for immunostaining were mounted in Vectashield (Vector Laboratories, Inc.). Images were acquired with a Microradiance or Radiance 2000 confocal microscope and analyzed with ImageJ and Photoshop CS2 (Adobe Corporation) software. To avoid problems of focus, we captured and merged at least 30 planes of the embryos. Bright field images were captured with a Zeiss Microscope.

2.10. Immunocytochemistry and *in Situ* Hybridization. *In situ* hybridization of embryos and imaginal discs with digoxigenin-RNA-labeled probes was carried out following standard protocols. Digoxigenin-labeled RNA probes were prepared from the *hsp60A* cDNA cloned between XbaI and XbaI in pBluescript II KS. Antisense mRNA probes were synthesized for the various experiments using an RNA-DIG labeling Kit (Roche Diagnostics). Whole-mount *in situ* hybridization was carried out in 70% glycerol.

3. Results

3.1. Identification of HSP60A. Figure 1A shows a representative fluorogram of a 2-D gel of acidic polypeptides from wing imaginal discs of mature larvae (wild-type, strain Vallecás). The gels were analyzed on a computer with the PDQUEST system and each polypeptide was assigned a number in the database. In this way, we compiled a database of 1492 [³⁵S]methionine + [³⁵S]cysteine-labeled polypeptides (1226 acidic, IEF and 266 basic, NEPHGE).

Previously, we reported the identification of some of the polypeptides in our database by microsequencing.²⁹ At that time, we sequenced an internal polypeptide of 11 amino acids (VIIEQSWGSPK) that enabled us to assign the corresponding spot to the Mitochondrial Matrix protein P1 precursor. It was, in this way, that what eventually turned out to be HSP60A was first seen in *Drosophila*. It was originally designated IEF16 in our manual database. The same spot in the current quantitative database corresponds to SSP 7506. In order to identify SSP 7506, we isolated 100 wing imaginal discs, and lyophilized and resuspended them in 40 µL of lysis buffer. A total of 10⁶ cpm of proteins from wing imaginal discs labeled with [³⁵S]methionine + [³⁵S]cysteine were added to the lysate and a 2D gel was run. The gel was stained with Coomassie, dried and exposed to film for 4 days. The radiolabeled proteins acted as tracers, allowing the identification of polypeptide SSP 7506, which could be located on the autoradiography and later on the stained gel. Once the location of SSP 7506 was determined and verified, a new 2D gel was run with a further sample from 100 wing discs. After Coomassie staining, the SSP 7506 spot was excised and processed for MALDI-TOF analysis. Figure 1B shows the MALDI-MS spectrum of the tryptic digest obtained. A database search identified SSP 7506 as heat shock protein 60 (HSP60A). The 24 matching peptides covered 52% of the sequence of the protein. The assignment was corroborated by MALDI-TOF-TOF of ions 1022.51, 1457.63, 1609.89 and 2560.24 whose sequences were determined and are detailed in Figure 1B.

3.2. Kinetics of Heat-Shock Induction of HSP60A. For heat-shock experiments, groups of 10 wing imaginal discs were incubated at 25 °C for 1 h (control, Figure 1C), and at 37 °C for more than 1 h. In all experiments, proteins were labeled with [³⁵S]methionine + [³⁵S]cysteine for the last hour of incubation, and analyzed by 2-DE and fluorography. HSP60A was then identified by computerized gel scanning as described in section 2.3. Figure 1D illustrates the significant changes visible on

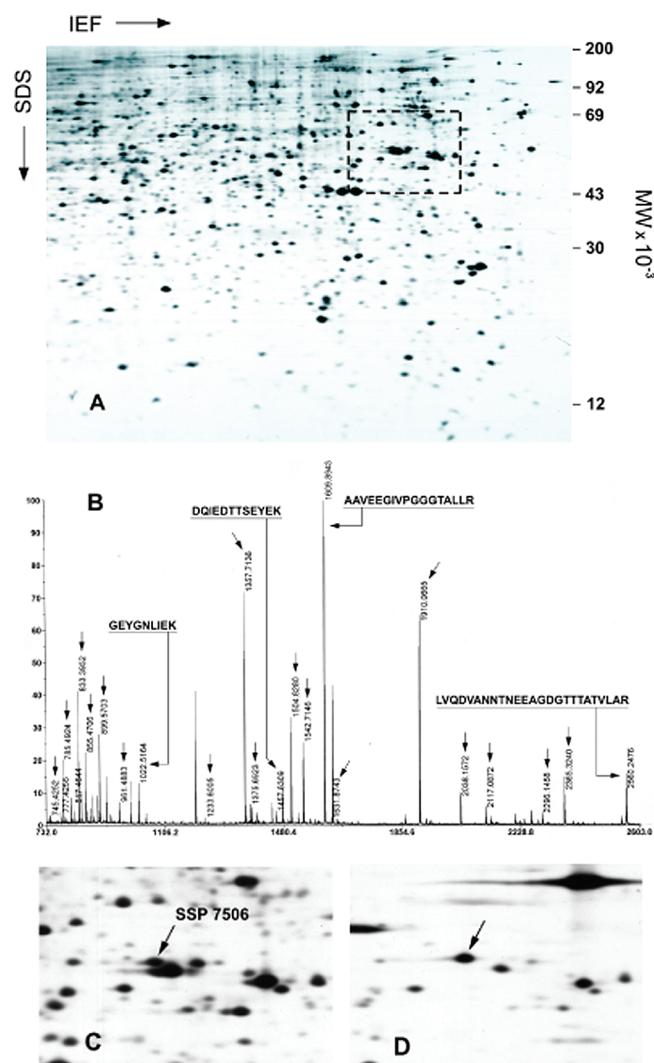


Figure 1. 2-DE (IEF) of total [³⁵S]methionine + [³⁵S]cysteine-labeled polypeptides in wing imaginal discs of late third instar larvae of *D. melanogaster*. (A) The pH ranges from 7.0 (left) to 4.5 (right). The area shown in panel C is boxed. (B) MALDI-TOF spectrum of a tryptic digest of SSP 7506. Peaks with arrows represent some of the peptides matched to *D. melanogaster* HSP60A. The MS/MS spectra of the peptides 1022.51, 1457.63, 1609.89 and 2560.24 correspond to the sequences underlined. (C) Close-up regions of 2-D gel separation showing the effect of heat shock induction of *hsp 60* in wing imaginal discs of *Drosophila*. (C) Control after 60 min of labeling with [³⁵S]methionine + [³⁵S]cysteine at 25 °C and (D) induction after 120 min at 37 °C. Polypeptide SSP 7506 is indicated by the arrow.

typical fluorograms after 2 h of incubation at 37 °C. A general shutoff of the synthesis of proteins was observed. However, the amount of HSP60A increased slightly, reaching a maximal increase of 1.7-fold after 2 h at 37 °C. It then declined, reaching basal level by 4 h (data not shown). These data clearly indicate that HSP60A is an inducible heat-shock protein.

3.3. HSP60A Is a Glycosylated Polypeptide. To further characterize HSP60A, we generated a polyclonal antibody against the denatured polypeptide (α -HSP60A_{Den}) (see section 2). The specificity of the antibody was demonstrated by Western blotting of 2-D gels. Forty wing imaginal discs were labeled as described, separated on 2-D gels, transferred to a nitrocellulose filter and exposed to antibody. The α -HSP60A_{Den} antibody recognized two spots, a major spot at the position of SSP 7506

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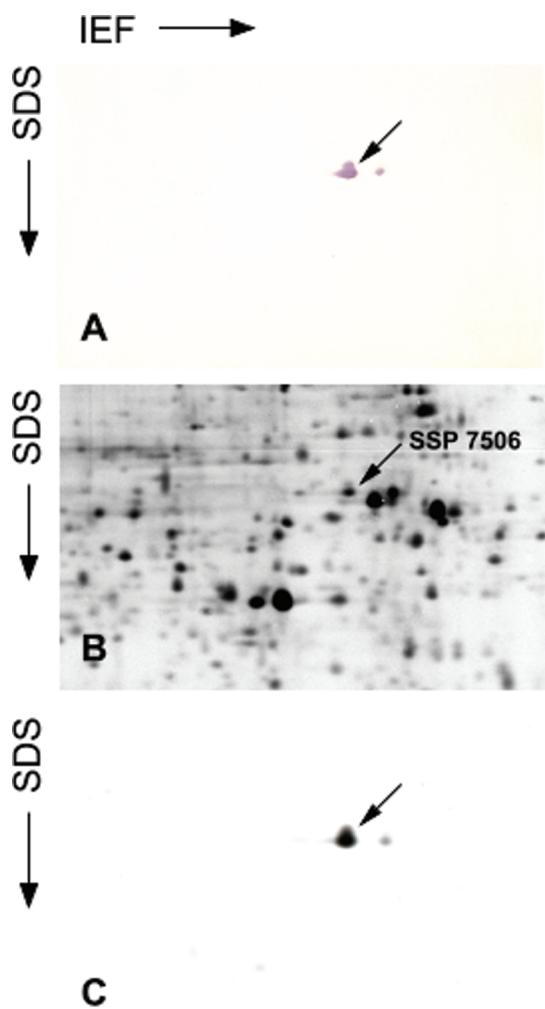


Figure 2. Specificity of the polyclonal antibodies against HSP60A polypeptide. Twenty wing imaginal discs of late third instar larvae of *D. melanogaster* were labeled over 60 min, resolved by 2-DE and transferred to nitrocellulose paper. The filter was analyzed with a polyclonal antibody against denatured SSP 7506. (B) Autoradiograph of (A). Forty wing imaginal discs were labeled as described, lysed and immunoprecipitated with a polyclonal antibody against native SSP 7506. The immunoprecipitate was resolved by 2-DE (C).

and a minor slightly more acidic one, suggesting that HSP60A can exist as a modified polypeptide (Figure 2A). This observation was corroborated by immunoprecipitation experiments using a polyclonal antibody raised against the native HSP60A polypeptide (α -HSP60A_{Nat}), which again showed a major spot and a faint more acidic spot (Figure 2C). To identify the kind of modification involved, we repeated the immunoprecipitation experiment and divided the immunoprecipitate into four aliquots. One was treated with shrimp alkaline phosphatase (Promega) for 60 min at 37 °C and another with *N*-glycosidase F from *Flavobacterium meningosepticum* (Roche) overnight at 37 °C. The other two aliquots served as controls for the two treatments. Only the treatment with glycosidase removed the faint acidic spot (data not shown), showing that HSP60A is glycosylated.

3.4. HSP60A Is Restricted to Mitochondria. We used the α -HSP60A_{Nat} antibody as a cytological marker to immunostain embryos and larval tissues. At all stages of development, staining was detected in cytoplasmic particles (red in the figure)

surrounding the nuclei of cells but never inside the nuclei (Figure 3A,B). In view of the functions reported for HSP60A, these particles seemed likely to be mitochondria. In addition to embryo cells (Figure 3A) and wing imaginal discs (Figure 3B), the antibody stained similar particles in all larval tissues (data not shown).

To further investigate the subcellular location of HSP60A protein, we used two additional approaches. First, we applied the polyclonal α -HSP60A_{Nat} antibody to thin sections of *Drosophila* embryos. The gold particles revealed that HSP60A was almost exclusively localized to the inner mitochondrial membrane (Figure 3C and D); no immunoreactive material was found in other subcellular structures, such as nuclei, peroxisomes, endoplasmic reticulum, Golgi stacks and plasma membrane. A few particles (less than 1%) were however noted in the cytoplasm and probably correspond to precursor of the HSP60A subunit in transit to mitochondria. Second, Western blots of cellular fractions obtained by differential centrifugation detected HSP60A only in the mitochondrial fraction, not in the supernatant of this fraction (Figure 3E). These findings demonstrate that HSP60A is exclusively located in the mitochondria in *Drosophila*.

3.5. The Pattern of Expression of HSP60A and the Protein Composition of the Mitochondria Are Highly Dynamic during Embryogenesis of *Drosophila*. To analyze the expression of HSP60A in the different stages of *Drosophila* development, we used HSP60A antibody together with antibody to other mitochondrial markers, such as super oxide dismutase (SOD) (located in the mitochondrial matrix), the β -subunit of the ATPase (located in the internal mitochondrial membrane) and a new polyclonal antibody against whole mitochondria (α -Mit). In contrast to the uniform expression expected for this kind of protein, the α -HSP60_{Nat} antibody revealed a highly dynamic and restricted pattern of HSP60A expression (red channel, Figure 4). Expression first appeared in a small and well-defined group of dorsal cells of the embryo (Figure 4A). Later (2–4 h old embryos), expression rapidly extended to more cells of the embryo following a specific pattern (Figure 4A). HSP60A colocalized with SOD and Mit in the mitochondria, but expression of the β -subunit of ATPase was not detected during these developmental stages (data not shown). These findings suggest that there is some kind of strictly controlled heterogeneity of mitochondrial composition during development. We confirmed this hypothesis by extending the analysis to later stages of embryonic development and to the larval stages. We found that after stage 11, HSP60A is only coexpressed with SOD in a restricted population of cells (Figure 4B). In addition, it is expressed in a pattern complementary to the β -subunit of the ATPase (Figure 4D). This latter combination of proteins is particularly interesting because the ratio β -ATPase/HSP60A is a “marker” of apoptosis. During larval stages, HSP60A is uniformly expressed in all imaginal discs, although we observed variability in its coexpression with the other mitochondrial markers (data not shown). The most interesting conclusion to be derived from these results is that mitochondria have a highly dynamic, but strictly regulated, composition during development, presumably to satisfy the energetic requirements of particular cell types. From its pattern of expression during embryogenesis and larval development, we suggest that HSP60A is required only in specific cell types, after which it assumes a

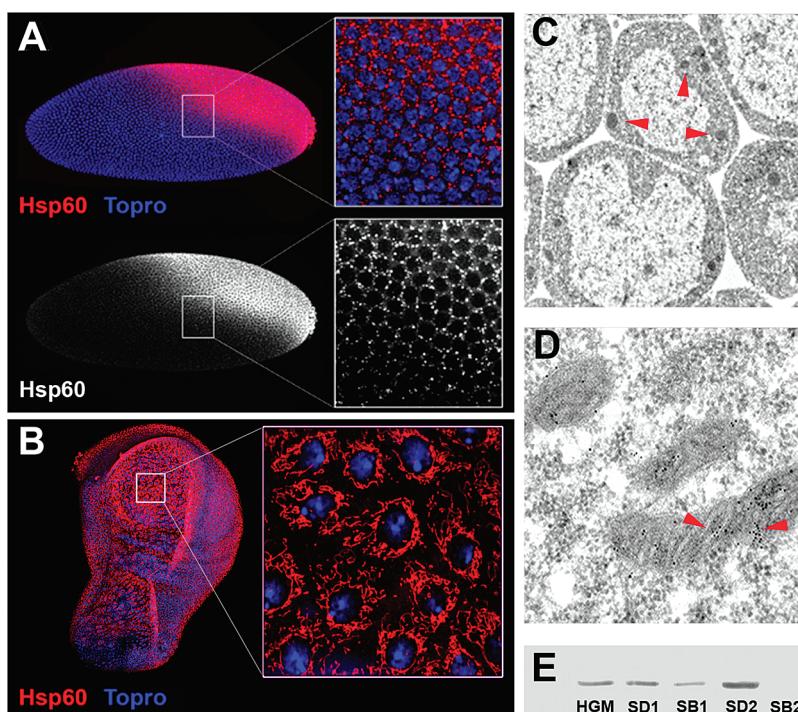


Figure 3. The expression of HSP60A is only present in certain population of embryo cells and it is localized in the mitochondria. (A) In early stages of embryonic development, the expression of HSP60A (red) is only enriched in certain population of cells. The details show that the expression of HSP60A is accumulated in dots inside the cytoplasm of the cells. (B) Expression of HSP60A (red channel) in third instar *D. melanogaster* wing imaginal discs. The nuclei of the cells is revealed in blue channel by Topro. (C and D) Immunoelectron microscopy of *Drosophila* embryo cells stained with HSP60A. Red arrowheads indicate the mitochondria (C). High magnification of electron micrograph reveals the HSP60A (arrowheads) expression as dense dots in the inner membrane of the mitochondria (D). (E) Western blot of samples of 100 µg of total protein from successive steps of mitochondria purification by differential centrifugation developed with the α-HSP60A_{Den} antibody. HGM, homogenate; SD1, sediment obtained at 800g × 10 min; SB1, first supernatant; SD2, sediment obtained at 10 000g × 15 min (mitochondrial fraction); SB2, = second supernatant.

more constant role in promoting the correct folding of mitochondrial proteins.

3.6. The Expression of HSP60A and the Composition of Mitochondria Are Regulated by Unknown Post-Transcriptional Mechanisms during Normal Development. To further investigate how the dynamic pattern of HSP60A expression is regulated in conjunction with mitochondrial biogenesis during embryogenesis, we performed *in situ* hybridization to localize HSP60A transcripts. Surprisingly, the antisense probe mRNA-*hsp60A* revealed that the gene is uniformly expressed during the whole development (Figure 5A). We also analyzed the levels of expression of the two HSP60A isoforms (see Figure 2C) by quantitative 2-D Western blotting in synchronized 0–3 h, 3–6 h and 6–9 h embryos to see if the level of glycosylation might play a regulatory role, but we did not detect any difference in glycosylation between the three samples (data not shown). In the light of these results further strengthened by the immunostainings experiments (see above), we conclude that HSP60A expression (and by extension the mitochondrial composition) is post-transcriptionally regulated by unknown mechanisms during embryonic development. This conclusion helps to explain how the pattern of expression of HSP60A and mitochondrial biogenesis can be rapidly modified during development.

3.7. The Specific Pattern of Expression of HSP60A Is Retained under Heat Shock. Next, we investigated whether the cell-specific expression pattern of HSP60A is retained upon heat shock. *Drosophila* embryos were heat-shocked for 2 h at 37 °C to induce maximal synthesis of HSP60A, and immediately fixed and analyzed with α-HSP60A_{Nat} antibody. Interestingly, gentle

heat-shock treatments did not perturb the pattern of expression of HSP60A (Figure 5B), whereas more stressful conditions completely disrupted it (Figure 5C). In fact, strong heat-shock (40 °C) elevated the level of the protein over the entire embryo. However, despite the complete loss of the expression pattern under such conditions, the mitochondrial localization of the protein was maintained.

3.8. HSP60A Transcription Is Stimulated in Stressful Conditions. To define precisely how the expression of HSP60A is regulated in highly stressful conditions, we stimulated cell death in the embryo by overexpressing a thermosensitive ricin protein³⁰ under the control of *engrailed*-Gal4. Ricin is a potent toxin that causes cell death. We observed that expression of HSP60A was transcriptionally activated in dying cells (segmented pattern of *engrailed* cells) when larvae were grown at the restrictive temperature (31 °C) (Figure 5D). This increased transcription led to an increase in HSP60A in the dying cells (Figure 5E). Taken together with our previous results, this allows us to conclude that HSP60A expression is post-transcriptionally regulated during normal development, but that it is transcriptionally activated in very stressful situations.

3.9. HSP60A Is Essential for Maintaining the Correct Biogenesis of the Mitochondria. Mutant forms of HSP60A affect the normal arrangement of intracellular organelles, causing embryonic lethality.¹³ To explore the role of HSP60 in the biogenesis of mitochondrial while maintaining healthy embryonic cells, we analyzed the phenotype of embryos hypomorphic for HSP60. The mutant embryos (*Hsp60*^{EY01572}/*Hsp60*^{GF319}) did not show detectable levels of the protein 8 h

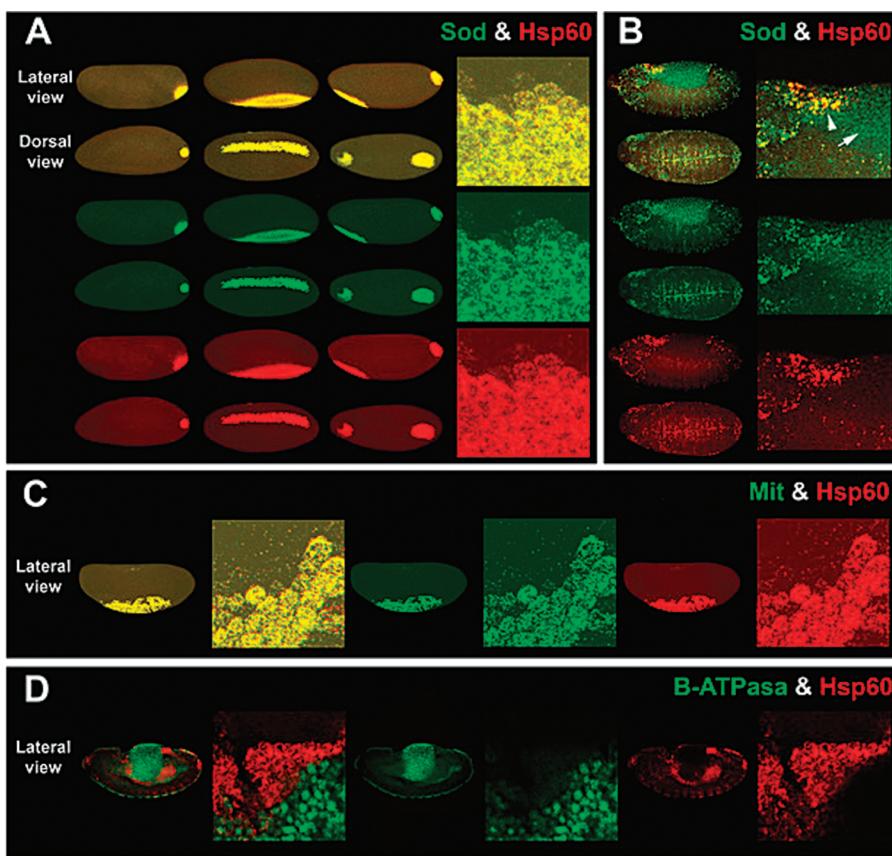


Figure 4. Immunostainings with different mitochondrial markers indicate that the mitochondrial composition is cell-specific in embryo cells and changes during the development. (A) The specific mitochondrial cell markers Superoxidase dismutase (SOD) (green) and HSP60A (red) are coexpressed in early stages of embryo development. High magnifications show the coexpression of SOD and HSP60A in detail (right panels in A). Lateral and ventral views of embryos show that the expression pattern of SOD and HSP60A changes quickly during embryo development (A). (B) In late stages of embryo development, SOD (green) and HSP60A (red) are only coexpressed in specific cell types. The coexpression SOD–HSP60A is indicated by white arrowhead in right panel of B, while other SOD-expressing cells are indicated by the white arrow. (C) Mitocondrial antibody (MIT) (green) and HSP60A (red) are coexpressed during embryo development. (D) The specific mitochondrial cell markers β -ATPase (ATPase) (green) and HSP60A (red) display a complementary pattern of expression (detail at right) in some stages of embryo development. Note that the distribution of mitochondria in cells depends on the cell type; the expression of HSP60A is dotted, while β -ATPase is compacted around the nucleus of the cells (detail in D).

409 AEL but had a viability of 95%, and interestingly, did not express
 410 SOD (data not shown). These results demonstrate that the
 411 HSP60A expression is required for the correct biogenesis of
 412 mitochondria in order to control the folding of mitochondrial
 413 proteins.

414 4. Discussion

415 This study began in our laboratory as an attempt to monitor
 416 changes in polypeptide synthesis involved in cell proliferation
 417 and differentiation in *Drosophila* using the wing imaginal disk
 418 as a model system.³¹ In this report, we have focused on
 419 HSP60A, a protein whose presence in *Drosophila* was proposed
 420 by us some years ago.²⁹ HSP60A is included in an evolutionary
 421 conserved family of molecular chaperones that ensure the
 422 correct folding of nuclear-encoded proteins after mitochondrial
 423 membrane translocation during development as well as after
 424 heat shock. Our interest in this protein was prompted by its
 425 crucial functions in mitochondria, its relationship with various
 426 human pathologies, many unanswered questions about its
 427 subcellular localization and its unknown pattern of expression
 428 during development in different model systems.

429 Once the polypeptide was identified in our database (catalogued as SSP 7506), we determined whether it was expressed
 430

431 during normal fly development and induced by heat treatment.
 432 The *hsp60* promoter region does not include binding sites for
 433 heat-shock factors, and this gene has been described as not
 434 being heat-shock inducible, or induced at low levels.³² In the
 435 present work, we analyzed and quantified its heat shock
 436 induction and observed a maximum induction of less than
 437 2-fold in wing imaginal discs after 2 h of heat shock at 37 °C.
 438 This result is not uncommon, since there are other examples
 439 of *hsp60* genes that are only activated 2- to 3-fold or less by
 440 heat shock. For example, the *Tetrahymena* HSP60 protein is
 441 only induced 2- or 3-fold by heat shock.³³ We have confirmed
 442 the *hsp60A* heat shock response *in vivo*, observing that the
 443 expression of the protein is uniformly upregulated in the whole
 444 embryo after strong heat-shock induction (Figure 5B). However,
 445 we observed that expression of HSP60A in the embryo disap-
 446 peared when the temperature was increased to above 40 °C,
 447 so explaining why HSP60A protein was not previously observed
 448 in heat-shocked cultured cells³⁴ and was mistakenly described
 449 as not heat-shock inducible.³²

450 The antibodies raised against the native and denatured
 451 HSP60A of *Drosophila* revealed that the polypeptide is constitu-
 452 tively expressed in a glycosylated form. They also showed
 453 that it is restricted to mitochondria in agreement with its

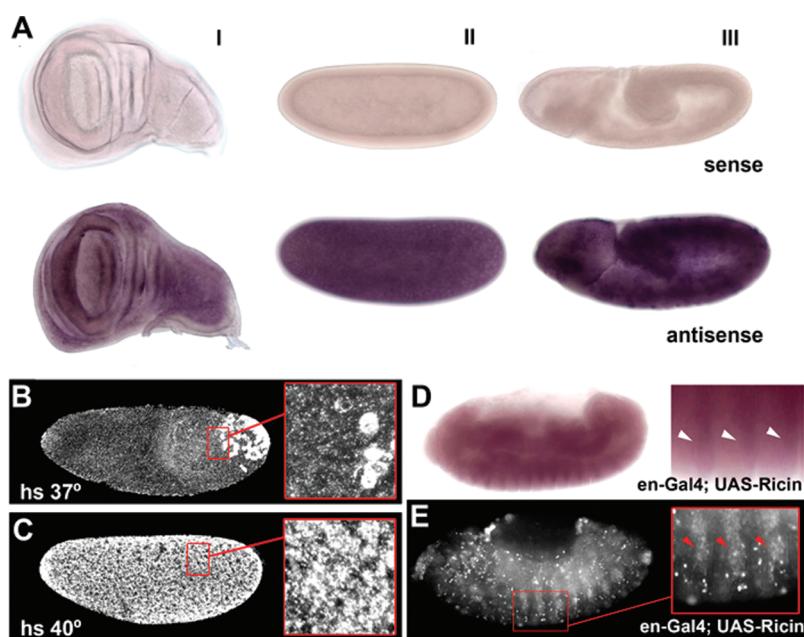


Figure 5. The expression of HSP60A is post-transcriptionally regulated during normal embryo development; however, it is transcriptionally activated in response to cell stress. (A) *In situ* hybridization reveals that *hsp60A* is homogenously expressed in wing imaginal discs (I) and early (II) and later (III) stages of embryo development. Upper panels show sense staining and lower panels antisense staining in A. (B) The expression of HSP60A (gray) is slightly promoted in cells of the embryo (low expression in embryo cells of the inset) around the normal patch of HSP60A (high levels of expression in embryo cells of the inset) at 37 °C. (C) The HSP60A (gray) is homogenously expressed in embryos cultured at 40 °C. (D) *In situ* hybridization demonstrates that the transcription of *hsp60A* is increased in the band of cells expressing engrailed in the embryo (white arrowheads in the detail) when they are killed by ricin-toxin (maximal condition of cell stress). (E) Immunostaining demonstrates that the translation of *hsp60A* is also increased in the band of cells expressing engrailed in the embryo (red arrowheads in the detail) when they are killed by ricin-toxin (maximal condition of cell stress).

454 proposed biological function.³ Evidently, it is only overexpressed in other cellular compartments in pathological conditions such as in certain human tumors.⁷

455 The most striking result obtained was that HSP60A displays
456 a stereotypical and highly dynamic pattern of expression during
457 embryogenesis. A previous study using a commercial mono-
458 clonal antibody against a 22 aminoacids epitope of human
459 HSP60 (18 residues are conserved in *Drosophila*) reported a
460 heterogeneous distribution of the polypeptide in *Drosophila*
461 embryos.¹³ Surprisingly, some signal was detected in the
462 nuclear fraction. However, our specific antibody, although
463 confirming a heterogenous pattern of expression during
464 embryogenesis, yielded a different pattern of expression from
465 the one previously reported. We observed that HSP60A ac-
466 cumulated in specific patches of cells during embryogenesis,
467 whereas the surrounding cells expressed low levels of the
468 protein. Later (0–4 h AEL), the expression of the protein is
469 rapidly extended into other dorsal cells of the embryo and
470 finally decays in stage 10 of embryogenesis. We showed also
471 that the dynamic distribution of the protein was correlated with
472 another mitochondrial markers (SOD) in the early stages of
473 embryogenesis, whereas it was complementary to it in late
474 stages of development. The most interesting conclusion from
475 our data is that HSP60A is variably coexpressed with other
476 mitochondrial markers during certain stages of embryogenesis,
477 thus, supporting the notion that it is essential for the function
478 of this organelle in specific cell types. These findings once more
479 challenge the traditional definition of heat-shock protein, by
480 providing evidence of differential expression of heat-shock
481 proteins during *Drosophila* development.³⁵ Moreover, the
482 difference between the expression pattern of HSP60A and those
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484 of other mitochondrial markers (SOD, β -ATPase) in the late
485 stages of embryonic development (Figure 4D) and in larval
486 stages indicates once more that the protein composition of
487 mitochondria is specific to each cell state and environmental
488 condition.³⁶ These results provoke two additional questions:
489 How is the dynamic pattern of HSP60A regulated and what is
490 its biological significance?

491 Interestingly, we found that the expression of HSP60A during
492 embryogenesis is post-transcriptionally regulated: the buildup
493 of *hsp60A* during this period occurs in the absence of changes
494 in the abundance of its mRNA, which is uniformly distributed
495 throughout the early embryo. Our findings point to unknown
496 mechanisms that post-transcriptionally regulate the levels of
497 HSP60A in the mitochondria and hence the biogenesis of this
498 organelle, thus, providing a novel explanation for the rapid
499 changes of this organelle that occur during development and
500 in response to environmental changes. It is important to
501 remember that the biogenesis of mitochondria is a complex
502 cellular event requiring the concerted expression of two physi-
503 cally separated genomes, nuclear and mitochondrial.³⁷

504 We also investigated the regulation of HSP60A under stress
505 conditions, by promoting cell death in the segmental patterning
506 of engrailed. The ectopic expresión of the toxic protein ricin
507 in the pattern of engrailed cells stimulated cell death and
508 increased levels of HSP60A. Transcription of the gene was
509 upregulated in parallel with the increase in the protein. These
510 results reveal that different mechanisms regulate levels of
511 HSP60A during normal development and during stressful
512 conditions. It seems possible that increased transcription of
513 the gene is necessary to reach the high protein levels required
514 in stressful situations.

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Finally, we have tried to obtain some clue about the physiological significance of the specific accumulation of mitochondria in particular cells of the embryo. The mitochondrial network is a dynamic structure permanently subjected to remodelling³⁸ in order to fulfill the energy demands of different cell types⁴⁴ in response to both cellular and environmental factors.^{39–43} In addition, mitochondria play a central role as executioners of apoptotic cell death, triggering the activation of caspase-9.^{2,45} Our data suggest that the pattern of expression of HSP60A is not correlated with the pattern of cell death in the wild-type embryo.⁴⁶ Instead, we believe that accumulation of HSP60A is essential to maintain the protein composition and rate of biogenesis of mitochondria, able to satisfy the specific energetic requirements of particular cell types during embryogenesis.

In summary, we have demonstrated that HSP60A is a heat-shock inducible protein located in the inner membrane of the mitochondria whose regulation during normal development depends on post-transcriptional events. However, it is transcriptionally regulated by other mechanisms in very stressful situations. In addition, our data support the notion that mitochondrial biogenesis during embryogenesis is cell-specific and controlled by post-transcriptional mechanisms, probably to satisfy the specific energetic demands of the various cell types. Finally, *Drosophila* development emerges as an attractive *in vivo* experimental system to study the mechanisms that control the biogenesis of mitochondria.⁹

Abbreviations: 1-D, one-dimensional; 2-D, two-dimensional; 2-DE, two-dimensional gel electrophoresis; HSP, heat shock protein; MALDI-MS, matrix-assisted laser desorption/ionization-mass spectrometry; ORF, open reading frame; PBS, phosphate-buffered saline; TOF, time of flight.

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