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Original Paper

Overexpression of the catalytic subunit of DNA polymerase γ results in depletion of mitochondrial DNA in *Drosophila melanogaster*

E. Lefai · M. Calleja · I. Ruiz de Mena · A. T. Lagina III · L. S. Kaguni · R. Garesse ✉)

E. Lefai · I. Ruiz de Mena · R. Garesse
Departamento de Bioquímica, Instituto de Investigaciones Biomédicas "Alberto Sols" CSIC-UAM, Facultad de Medicina, Universidad Autónoma de Madrid, c/ Arzobispo Morcillo 4. 28029 Madrid. Spain

M. Calleja
Centro de Biologia Molecular "Severo Ochoa" CSIC-UAM, Campus de Cantoblanco, Madrid, Spain

A. T. Lagina III · L. S. Kaguni
Department of Biochemistry, Michigan State University, East Lansing, MI 48824-1319, USA

✉ E-mail: rafael.garesse@uam.es
Phone: +34-91-3975452
Fax: +34-91-5854587

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Abstract. The mechanisms involved in the regulation of mitochondrial DNA (mtDNA) replication, a process that is crucial for mitochondrial biogenesis, are not well understood. In this study, we evaluate the role of DNA polymerase γ (pol γ), the key enzyme in mtDNA replication, in both *Drosophila* cell culture and in developing flies. We report that overexpression of the pol γ catalytic subunit (pol γ -α) in cultured Schneider cells does not alter either the amount of mtDNA or the growth rate of the culture. The polypeptide is properly targeted to mitochondria, yet the large excess of pol γ -α does not interfere with mtDNA replication under these conditions where the endogenous polypeptide is apparently present in amounts that exceed of the demand for its function in the cell. In striking

contrast, overexpression of pol γ -α at the same level in transgenic flies interferes with the mtDNA

replication process, presumably by altering the mechanism of DNA synthesis, suggesting differential requirements for, and/or regulation of, mtDNA replication in *Drosophila* cell culture versus the developing organism. Overexpression of pol γ -α in transgenic flies produces a significant depletion

of mtDNA that causes a broad variety of phenotypic effects. These alterations range from pupal lethality to moderate morphological abnormalities in adults, depending on the level and temporal pattern of overexpression. Our results demonstrate that although cells may tolerate a variable amount of the pol γ catalytic subunit under some conditions, its level may be critical in the context of the

whole organism.

Key words. *Drosophila melanogaster* - Mitochondrial DNA depletion - Mitochondrial DNA replication - DNA polymerase γ catalytic subunit

Introduction

Mitochondrial biogenesis is unique at the cellular level because it requires the coordinated expression of genes encoded in two different genetic systems, the nuclear and the mitochondrial (Attardi and Schatz *1988*; Scarpulla *1997*). The mitochondrial genome is a small and compact double-stranded circular DNA that in most animals contains 37 genes, encoding 22 tRNAs and 2 rRNAs that are essential for the translational machinery of the organelle and 13 polypeptides that are components of the respiratory complexes of the inner mitochondrial membrane. The rest of the mitochondrial proteins, including those involved in the replication and transcription of the mtDNA, are encoded in the nucleus, synthesized on cytoplasmic ribosomes and targeted to their final mitochondrial location (Schatz *1996*; Neupert *1997*).

mtDNA replication is a necessary step in organelle proliferation. The key component of the replication machinery, DNA polymerase γ , has been characterized biochemically in a variety of systems, and in

all of them the enzyme comprises two activities, 5’ $\rightarrow$ 3’ DNA polymerase and 3’ $\rightarrow$ 5’ exonuclease (Insdorf and Bogenhagen *1989*; Kaguni and Olson *1989*; Longley and Mosbaugh *1991*; Foury and Vanderstraeten *1992*; Gray and Wong *1992*). It was shown in *Drosophila* (Wernette and Kaguni *1986*; Olson et al. *1995*), and more recently in other animal systems, that the enzyme is a heterodimer composed of a large polypeptide of 125-140 kDa (pol γ -α), and a small accessory subunit of

35-50 kDa (pol γ -β). The genes and/or cDNAs encoding both subunits have been identified and

characterized in several organisms, including *Drosophila* and man (Lewis et al. *1996*; Ropp and Copeland *1996*; Ye et al. *1996*; Lecrenier et al. *1997*; Wang et al. *1997*; Carrodeguas et al. *1999*). Both polymerase and exonuclease activities are associated with the large subunit. The accessory subunit shares sequence and structural homology with aminoacyl-tRNA synthetases, and is likely to be involved in primer recognition and template-primer DNA binding, and in enhancing enzyme activity and processivity (Carrodeguas et al. *1999*; Fan et al. *1999*; Lim et al. *1999*; Wang and Kaguni *1999*)

There are enormous differences in the mitochondrial content of the different tissues of the organism, and a precise program of organelle biogenesis is needed during development. Recently, mitochondrial transcription factor A (Tfam) has been shown to be essential for mitochondrial biogenesis. Disruption of the Tfam gene in mouse produces a severe depletion of mtDNA, thereby abolishing oxidative phosphorylation, whereas a reduction in gene dosage reduces mtDNA copy number and produces mitochondrial defects in the heart (Larsson et al. *1998*). In *Drosophila,* the catalytic subunit of mitochondrial DNA polymerase is encoded by the gene *tamas*. Not surprisingly, pol γ-α is also

essential for mitochondrial biogenesis, and null mutations in the *tamas* gene result in lethality (Iyengar et al. *1999*). Interestingly, development continues through the larval stages, where the prominent phenotype is a locomotory deficit that alters the behavior of the wandering third-instar larva. This indicates that maternal mitochondria are sufficient to supply at least a minimal oxidative capacity that is compatible with viability during the embryonic and early larval stages. However, the muscle dysfunction observed in late larvae indicates that lack of mtDNA replication blocks the mitochondrial biogenesis program, and terminally differentiated cells, particularly those with higher oxidative load, do not receive the correct number of functional mitochondria to meet their demand for energy production.

To gain further insight into the physiological regulation of the mtDNA replication process, we have overexpressed the pol γ-α gene in cultured *D. melanogaster* cells and in flies. *Drosophila* offers

several advantages as a model for the study of mitochondrial function: the pol γ enzyme has been

characterized extensively (Wernette and Kaguni *1986*; Kaguni and Olson *1989*; Olson et al. *1995*), a combination of molecular and genetic approaches is available that allow one to study many complex biological processes (Roberts *1998*), and embryonic development has been characterized in detail at both the morphological and molecular levels (Bate and Martinez-Arias *1993*). Here we report that in Schneider cells, overexpression of the catalytic subunit of *Drosophila* pol γ alters neither mtDNA

replication nor growth rate. However, its overexpression in the fly causes a severe depletion of mtDNA that produces lethality at the end of the pupal stage.

Materials and methods

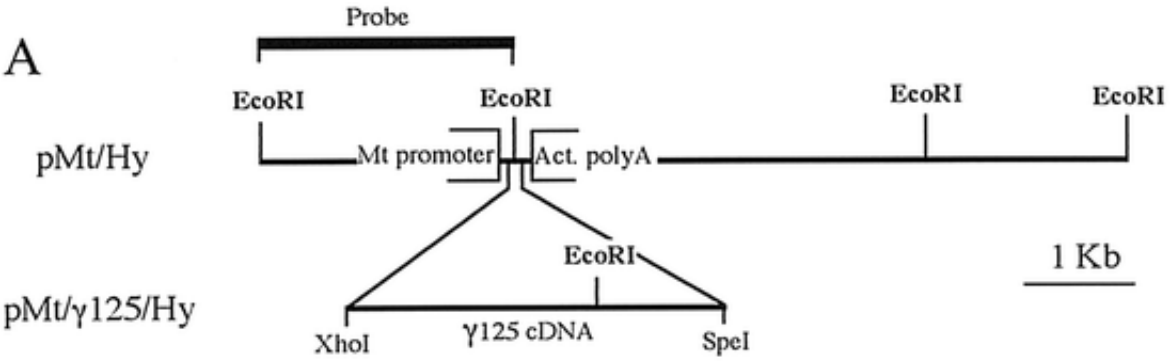
Plasmid construction

The complete pol γ-α cDNA, cloned in the pET11a vector (Lewis et al. *1996*), was obtained as a

*Nde*I fragment, which was rendered blunt and cloned in the *Eco*RV site of pBluescript (pBS, Stratagene) to generate the plasmid pBS-γ 125. For use in cell transfections, the cDNA was cloned

into the vector pMt/Hy, which contains the *Drosophila* metallothionein (Mt) promoter and a hygromycin resistance gene, allowing the selection of stable cell lines that have integrated the plasmid (Koelle et al. *1991*). pMt/γ 125/Hy was constructed by cleaving pMt/Hy with *Xho*I and *Spe*I and

inserting the *Xho*I-*Spe*I DNA fragment obtained from pBS-γ 125 (Fig. 1A).


A
Probe
EcoRI
EcoRI
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pMt/Hy
Mt promoter
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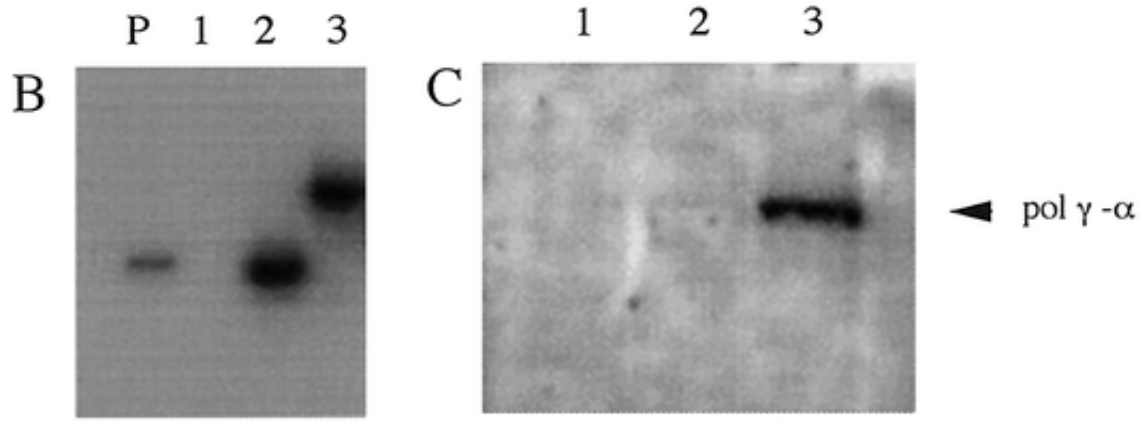
pMt/γ125/Hy
XhoI
γ125 cDNA
SpeI


P 1 2 3
B
1 2 3
C
pol γ -α

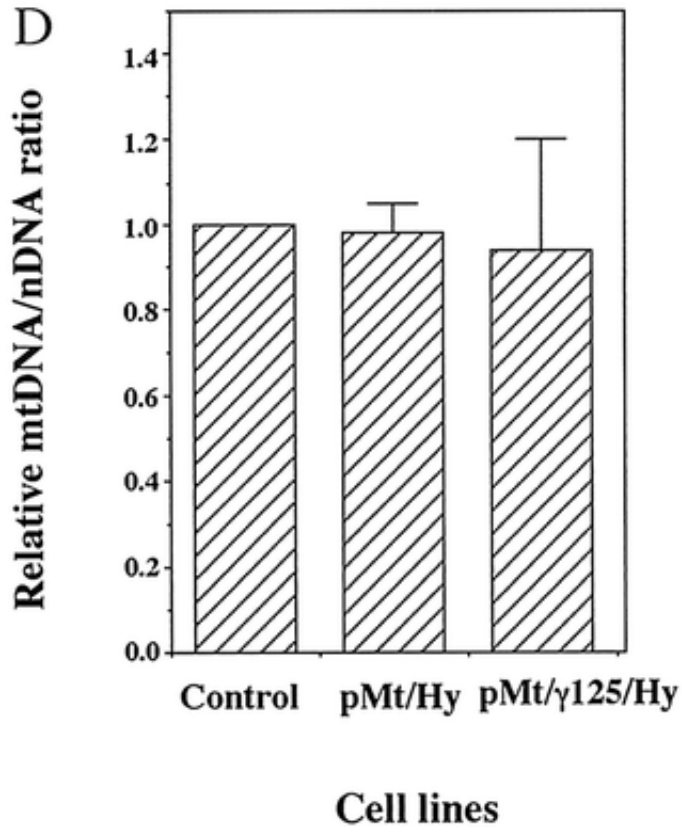

D
Relative mtDNA/nDNA ratio
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
pMt/Hy
pMt/γ125/Hy
Cell lines

Fig. 1A-D. Generation of stable Schneider cell lines that overexpress the pol γ-α gene under the control of the metallothionein promoter. **A** Schematic representation of the structures of the pMt/Hy vector and the pMt/γ 125/Hy construct containing the complete pol γ-α cDNA. A 2.3-kb *Eco*RI fragment of the pMt/Hy vector was used as a probe in Southern blot experiments. **B** Analysis of genomic DNAs of stably transformed Schneider cell lines. Total DNA (5 µg) from control Schneider cells (lane 1), and stable Schneider cells containing the pMt/Hy vector (lane 2) or the pMt/γ 125/Hy construct was digested with *Eco*RI, electrophoresed in a 1% agarose gel, transferred to nitrocellulose and probed with radiolabeled 2.3-kb *Eco*RI fragment. Lane P contains the pMt/Hy vector (30 ng) digested with *Eco*RI. **C** Immunoblot analysis of total mitochondrial protein (50 µg) prepared from control (lane 1), pMt/Hy (lane 2) and pMt/γ 125/Hy (lane 3) Schneider cells grown in the presence of 0.7 mM Cu^{++}, probed with rabbit antiserum against the *D. melanogaster* pol γ-α polypeptide. **D** Slot-blot analysis of the mtDNA content of the control, pMt/Hy and pMt/γ 125/Hy cell lines grown in the presence of Cu^{++}. The value for the control cells was taken as 1. The data represent averages of at least three independent experiments

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To construct the pUAST-γ 125 plasmid, the complete cDNA was obtained by digesting the pBS-γ 125 clone with *Not*I and *Kpn*I, and the resulting fragment was subcloned in the vector pUAST (Brand and Perrimon *1993*), which had been digested with the same restriction enzymes. To construct the pCaSpeR-hs-γ 125 clone, the plasmid pCaSpeR-hs, containing the *Drosophila hsp70* promoter (provided by Dr. James Kadonaga, University of California, San Diego; Burke and Kadonaga *1996*), was cleaved with *Stu*I and *Not*I, and the resulting DNA fragment was gel purified. pBS-γ 125 was cleaved with *Xho*I, then end-filled with the Klenow enzyme, and subsequently digested with *Not*I. The resulting fragment containing the complete catalytic subunit cDNA was gel purified and then ligated to the *Stu*I-*Not*I fragment of pCaSpeR-hs. All the constructs were checked by standard restriction analysis and sequencing.

Generation of stable cell lines and overexpression of pol γ-α

Drosophila Schneider cells were grown at 25°C in *Drosophila* Schneider medium (GIBCO-BRL) supplemented with 10% fetal bovine serum and 0.5% penicillin-streptomycin. Cells were transfected by the calcium phosphate coprecipitation method as described elsewhere (Soeller et al. *1993*). Hygromycin-resistant cells lines carrying the plasmids pMt/Hy and pMt/γ 125/Hy were generated as described (Rio and Rubin *1985*). Cells were passaged at least five times in hygromycin-containing medium and then cultured in standard medium. Genomic integration of the constructs was confirmed by Southern analysis.

Mt/γ 125/Hy cells, as well as wild-type and Mt/Hy control cells, were grown to a density of 0.5×10^6 to 3×10^6 cells/ml and then treated with 0.7 mM $CuSO_4$ to induce expression from the

metallothionein promoter (Bunch et al. *1988*). Treatment with copper was performed for between 4 days and 1 month without significant change in the expression level of pol γ-α.

Generation of transgenic lines and fly stocks

Fly stocks were grown at 25°C unless otherwise specified. The pUAST-γ 125 and

pCaSpeR-hs-γ 125 constructs were injected into y^- w^- embryos according to standard procedures.

The hs-GAL4 and 444 lines were a generous gift of Andrea Brand and Fernando Jimenez. In pUAST-γ 125 the pol γ-α subunit was overexpressed using the GAL4 system as described by Brand and

Perrimon (*1993*). Overexpression of the pol γ-α subunit from pCaSpeR-hs-γ 125 was induced by

heat shock at 37°C for 30 min. Mature flies or pharate adults obtained from the crosses were dehydrated and mounted in Euparal.

Molecular biological methods

Total DNA from cells and flies was extracted as described (Calleja et al. *1993*), and total RNA was extracted using RNAZol (Gibco). Southern and Northern blot analyses were performed using standard procedures (Sambrook et al. *1989*). For slot-blot analysis, varying amounts of total DNA or total RNA were blotted onto Zeta-probe membrane (Biorad) using the Manifold II system (Schleicher and Schuell), and fixed following the manufacturer's instructions. The filters were probed under high-stringency conditions with specific, [α-^{32}P]dCTP-labeled clones. The intensity of the hybridization signals was determined by densitometric scanning and computer integration analysis.

Cell fractionation and immunoblotting

Nuclear extracts were prepared from cells as described by Preston et al. (*1988*). To obtain cytoplasmic and mitochondrial fractions, cells were resuspended carefully in Buffer A (20 mM HEPES-KOH pH 7.4, 1 mM EGTA) and incubated for 15 min at 4°C. After the addition of 1 vol of Buffer A supplemented with 0.5 M mannitol, homogenization was carried out in a Dounce homogenizer, and the homogenate was centrifuged twice at 1000 $\times$ *g* for 10 min. The supernatant was then centrifuged

at 7500 $\times$ *g* for 10 min, and after removal of the cytosolic fraction, the mitochondrial pellet was

washed twice and centrifuged at 7500 $\times$ *g* for 10 min, and then resuspended in Buffer A

supplemented with 0.5 M mannitol. Protein was determined as described by Bradford (*1976*) with bovine serum albumin as standard.

SDS-PAGE was performed according to Laemmli (*1970*). Proteins were transferred to PVDF membranes (Immobilon-P, Millipore), probed with rabbit antiserum against the pol γ-α polypeptide

(Wang et al. *1997*) and detected with the ECL kit (Amersham) using horseradish peroxidase-labelled goat anti-rabbit antibody.

Results

Overexpression of the *D. melanogaster* pol γ-α subunit in Schneider cells

To examine the effects of overexpression of the catalytic subunit of *D. melanogaster* mtDNA polymerase, pol γ-α, in Schneider cells, we generated stable cell lines harboring the pMt/γ 125/Hy plasmid that contains the complete pol γ-α cDNA under the control of the metallothionein promoter (Fig. 1A). We also generated stable cell lines harboring the original pMt/Hy vector as a control. Because it is very rare to recover clones in Schneider cells (Rio and Rubin *1985*), we have used mixed cultures. In each case, these were examined for correct integration by Southern analysis (Fig. 1B). No hybridization was detected with DNA isolated from control Schneider cells (lane 1), but the expected 2.3-kb and 6-kb fragments were observed in the pMt/Hy and pMT/γ 125/Hy cell lines respectively (lanes 2 and 3). By comparison with a plasmid DNA standard (lane P), we deduce that the plasmids are tandemly integrated with a similar copy number in both lines (>10). Thus, multiple copies of the pol γ-α gene are present in each cell, and numerous sites of insertion are utilized within each cell line.

To induce expression of pol γ-α, cells were grown in the presence of 0.7 mM $CuSO_4$. After 4 days of exposure to copper a mitochondrial extract was prepared, and the protein was detected by immunoblot analysis using antiserum against the *Drosophila* pol γ-α subunit (see Materials and methods). Transformed cells containing the pMT/γ 125/Hy construct show a large increase in the amount of pol γ-α polypeptide as compared to controls (Fig. 1D, lane 3). Moreover, overexpressed pol γ-α copurifies quantitatively with mitochondria, because no signal was observed in the cytosolic fraction (data not shown). While the extremely low level of endogenous pol γ-α polypeptide (Fig. 1C, lanes 1 and 2) makes it difficult to estimate the level of induction, it probably exceeds 50 fold.

To evaluate the effect of the large increase in pol γ-α expression at the cellular level, we examined several parameters in three cell lines (control, pMt/Hy, and pMt/γ 125/Hy) grown in the presence and absence of copper over a period of 3 weeks. The treatment with copper does not produce significant changes in morphology or cell growth either in the pMt/Hy or in the pMt/γ 125/Hy cell lines as compared to the control line (data not shown). To determine if overexpression of the catalytic subunit of pol γ-α has any effect on mtDNA replication, total DNA was extracted from the different cell lines and the relative mtDNA content per cell was measured by slot-blot analysis using specific mitochondrial and nuclear DNA probes. Compared to control cells, no statistically significant change

in the mtDNA/nDNA ratio was observed in the pMt/Hy or pMt/γ 125/Hy cells (Fig. 1D).

These data indicate clearly that the increased level of pol γ-α in mitochondria is not toxic at the cellular level. Furthermore, the amount of mtDNA remains unchanged, suggesting that the availability of the catalytic subunit of the pol γ is not rate limiting for mtDNA synthesis in cell culture, and that other factor(s) must be responsible for the control of mtDNA replication during cell proliferation under these conditions.

Constitutive overexpression of the pol γ-α subunit is lethal in *Drosophila*

We extended our analysis of pol γ-α overexpression to evaluate its effects in the whole organism. We reasoned that subtle changes in mtDNA replication that did not affect cellular homeostasis in culture might have deleterious consequences in the animal, especially considering the potential importance of mitochondrial biogenesis during development and cell differentiation. To achieve this, we overexpressed the pol γ-α gene constitutively using the GAL4/UAS system (Brand and Perrimon *1993*). Three independent lines carrying the UAS-pol γ-α construct in distinct chromosomal locations were obtained as described under Materials and methods. Two lines carry the construct integrated on chromosome *III* (UAS-γ *125-1* and UAS-γ *125-2*), and one has the insert on the *X* chromosome (UAS-γ *125-3*).

Initially we used a driver (line 444) that expresses the GAL4 gene at all developmental stages, as well as in the adult organism. When UAS-γ *125-3* males were crossed with 444-*GAL4*/*FM6* females, the female:male ratio in the F1 offspring was 0.5, because no females with the 444-*GAL4*/UAS-γ *125-3* genotype were recovered. We observed that a large number of late pupae failed to eclose, suggesting that pol γ-α overexpression in this line induces lethality at the end of the pupal stage. Morphological analysis of pharate adults dissected from the pupal cases showed that they had reached a similar level of development to wild-type (Fig. 2A and B), although some alteration in the cuticular pattern of the bristles in the notum was apparent (Fig. 2C and D). In particular, some macrochaetae are absent and are probably replaced by a double socket - a phenotype similar to that found in *Notch* mutants. The same lethal phenotype at the end of the pupal period was observed in parallel experiments with the remaining two UAS-γ 125 lines (UAS-γ *125-1* and *2*). These results suggest that overexpression of the pol γ-α subunit in flies, in contrast to the situation found in cell culture, alters mtDNA replication, thereby affecting energy supply and causing lethality.

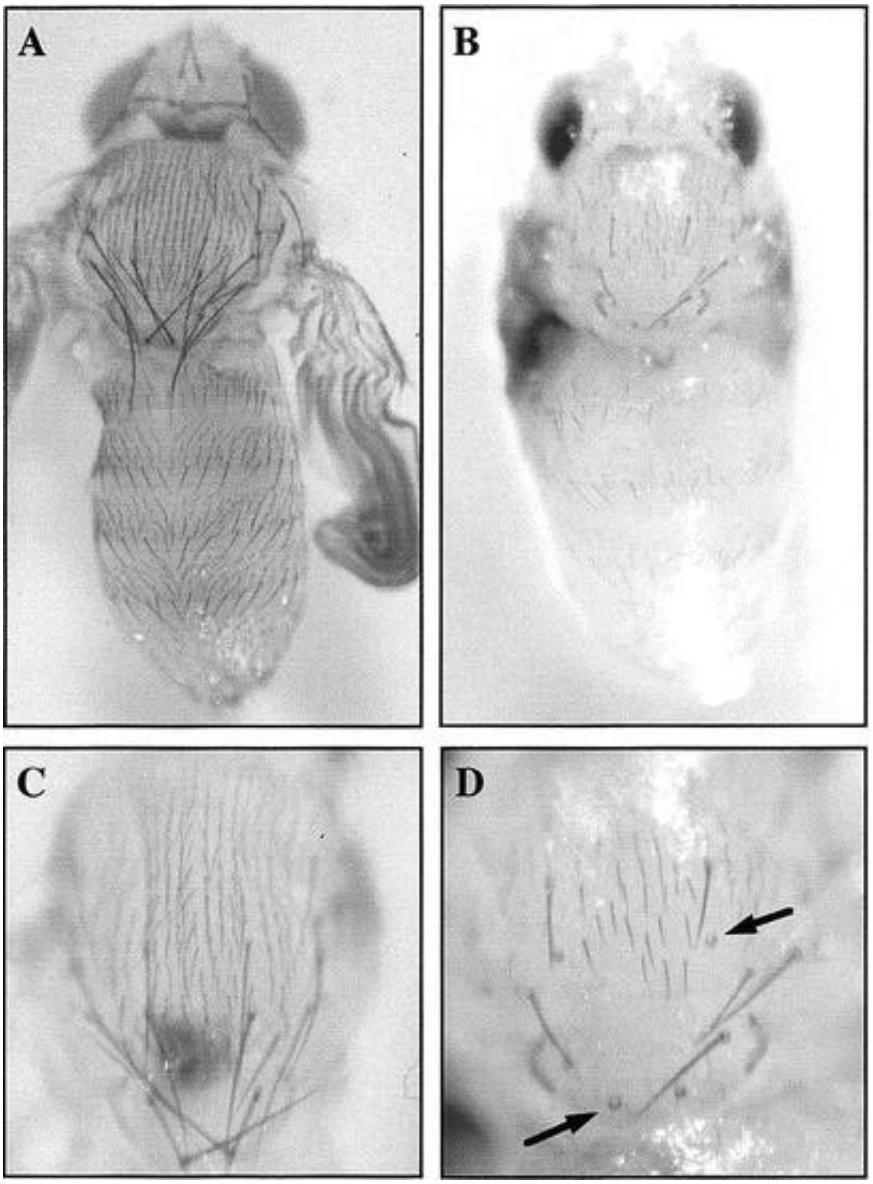

Fig. 2A-D. Pupal phenotype produced by the constitutive overexpression of the pol γ-α gene. Pharate adults were dissected from control (**A**) and 444-*GAL4*/UAS-γ *125-3* late pupae (**B**). The adult pharates that overexpress the pol γ-α gene fail to eclose. **C** Detail of the thorax of a wild-type pharate adult. **D** Detail of the same region of a 444-*GAL4*/UAS-γ *125-3* pharate adult; some cuticular defects are apparent, including the lack of several macrochaetae (*arrows*)

To demonstrate that the observed phenotypes were in fact produced by the overexpression of the pol γ-α gene, we quantitated the steady-state level of pol γ-α mRNA in late pupae by Northern analysis. From the same cross, we carefully separated 444-*GAL4*/UAS-γ *125-3* individuals from the remainder of the pupae (control pupae). It was possible to collect pupae overexpressing the transgene

just before death, because they have a slightly darker appearance and are larger than the control pupae. We detected by Northern analysis a dramatic increase in pol γ-α mRNA in the

444-*GAL4*/UAS-γ *125-3* pupae, relative to controls (Fig. 3A). We extended these results by

examining the steady-state level and subcellular localization of the pol γ-α subunit in both control

and 444-*GAL4*/UAS-γ *125-3* pupae. Total protein extracts of nuclear (Fig. 3 B and C, lanes 1 and 2),

cytoplasmic (lanes 3 and 4) and mitochondrial (lanes 5 and 6) fractions were prepared as described in Materials and methods, and equivalent amounts of protein were examined by immunoblotting using pol γ-α antiserum. The data indicate clearly that the level of pol γ-α accumulated in the

444-*GAL4*/ UAS-γ *125-3* pupae is very much higher than that in control pupae. Furthermore, the

protein is detected in the mitochondrial fraction (lane 6), indicating that it is properly targeted to the organelle. As a control we probed the same membrane with a specific antiserum directed against the β subunit of the *D. melanogaster* H^+ ATPase complex (Pena and Garesse *1993*). Similar signals

were obtained in the mitochondrial fractions of both 444-*GAL4*/UAS-γ *125-3* and control pupae

(Fig. 3C), suggesting that the numbers of mitochondria present in both types were similar.

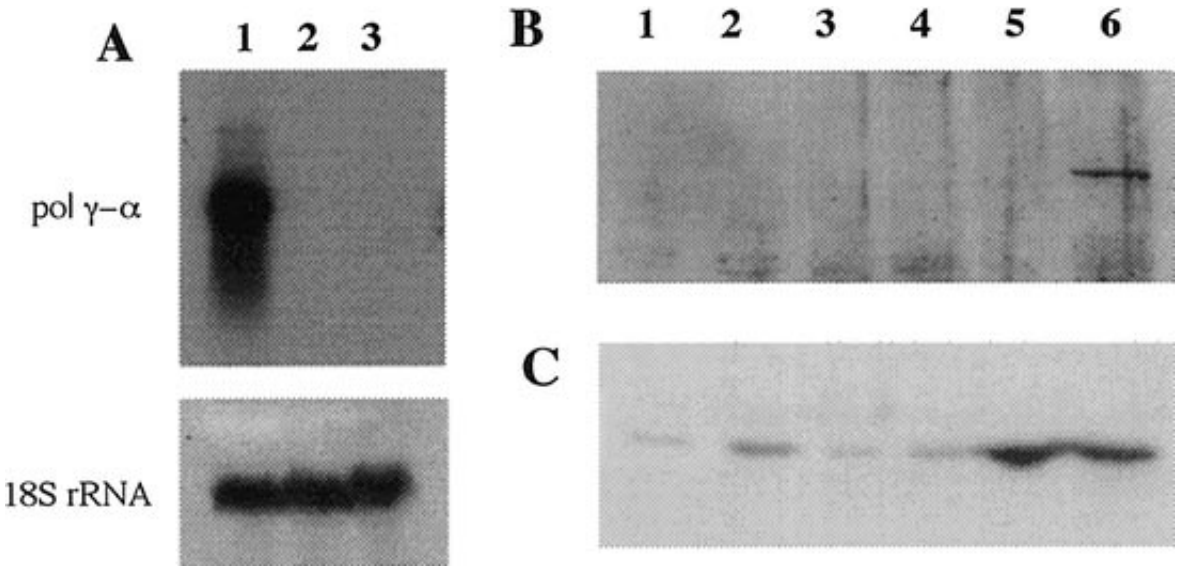

Fig. 3A-C. The Pol γ - α subunit is properly targeted to mitochondria when overexpressed in transgenic flies. **A** Total RNA (20 µg) from 444-*GAL4*/UAS-γ *125-3* pupae (lane 1), control pupae (lane 2) and control adults (lane 3) was electrophoresed in a denaturing formaldehyde-agarose gel, transferred to nitrocellulose and probed with radiolabeled pol γ - α cDNA. After washing, the filter was rehybridized using a cytoplasmic rRNA probe. **B, C** Protein extracts prepared from nuclear (lanes 1 and 2), cytosolic (lanes 3 and 4) and mitochondrial (lanes 5 and 6) fractions were denatured and electrophoresed in a 10% SDS-polyacrylamide gel. Proteins were detected by immunoblotting using rabbit antiserum directed against the *D. melanogaster* pol γ - α subunit at a 1:1000 dilution (**B**) or *D. melanogaster* β -ATPase subunit at a 1: 5000 dilution (**C**). Lanes 1, 3 and 5 contained extracts from control pupae; extracts in lanes 2, 4 and 6 were prepared from 444-*GAL4*/UAS-γ *125-3* pupae. The nuclear and soluble fractions also react slightly with the antibody due to the contamination of these fractions with mitochondrial proteins

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Finally we compared by immunoblot analysis the level of expression of the pol γ - α subunit in pMt/γ 125/Hy cells and 444-*GAL4*/UAS-γ *125-3* pupae (Fig. 4). In the pMt/γ 125/Hy cells, the amount of pol γ - α is higher than in control cells even in the absence of copper treatment, owing to the leaky expression of the construct (lane 1), whereas the level of pol γ - α in wild type pupae (lane 3) or control Schneider cells (data not shown) is undetectable by this method. The level of pol γ - α is elevated in pMt/γ 125/Hy cells after treatment with copper, and is similar to, or slightly higher than, that in the 444-*GAL4*/UAS-γ *125-3* pupae (lane 4), indicating that the lethality in the latter is not likely to be due to excessive overexpression in flies as compared to that in Schneider cells.

Furthermore, although the amount of pol γ-α polypeptide in transgenic pupae and pMt/γ 125/Hy cells is much higher than in wild-type pupae and control cells, we estimate that it does not make up more than 0.01% of the total mitochondrial protein.

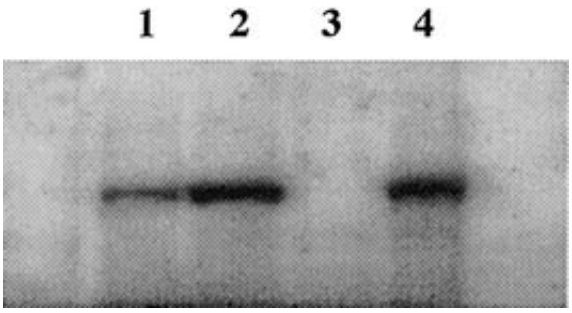

Fig. 4. Levels of Pol γ-α in pMt/γ 125/Hy cells and 444-*GAL4*/UAS-γ *125-3* pupae. Amounts of mitochondrial extracts equivalent to 50 µg of protein prepared from control pMt/γ 125/Hy cells (lane 1), pMt/γ 125/Hy cells treated with Cu^{++} (lane 2), control pupae (lane 3) and 444-GAL4/UAS-γ 125-3 pupae (lane 4) were analyzed by immunoblotting using a rabbit antiserum against the *D. melanogaster* pol γ-α subunit at a dilution of 1:1000

Constitutive overexpression of the pol γ-α subunit results in depletion of mtDNA in *Drosophila.*

To study the consequences of pol γ-α overexpression in mitochondria, we first analyzed the steady-state level and integrity of mtDNA. Total DNA extracted from 444-*GAL4*/UAS-γ *125-3* and control pupae was digested with *Xho*I, which cuts only once in *D. melanogaster* mtDNA; after electrophoresis and blotting, the membrane was probed with a radiolabeled, mtDNA-specific probe (Fig. 5A). Only a single fragment of the expected size (~20 kb) was observed, indicating that the integrity of the mtDNA molecule is not altered. However, a significant decrease in the steady-state level of the mtDNA is detected in the 444-*GAL4*/UAS-γ *125-3* pupae (Fig. 5A, lane 1) as compared to the control (Fig. 5A, lane 2). The relative amount of mtDNA was quantitated by slot blot analysis, using serial dilutions of total DNA and specific mitochondrial and nuclear DNA probes; the ratios of mtDNA to 18S rDNA determined for two independent lines, 444-*GAL4*/UAS-γ *125-3* and 444-*GAL4*; UAS-γ *125-2*, were compared to their respective controls (Fig. 5B). In pupae overexpressing the pol γ-α subunit, the relative amount of mtDNA is only about 40% of that present in control pupae, suggesting a severe impairment in the mtDNA replication process due to overexpression of pol γ-α .

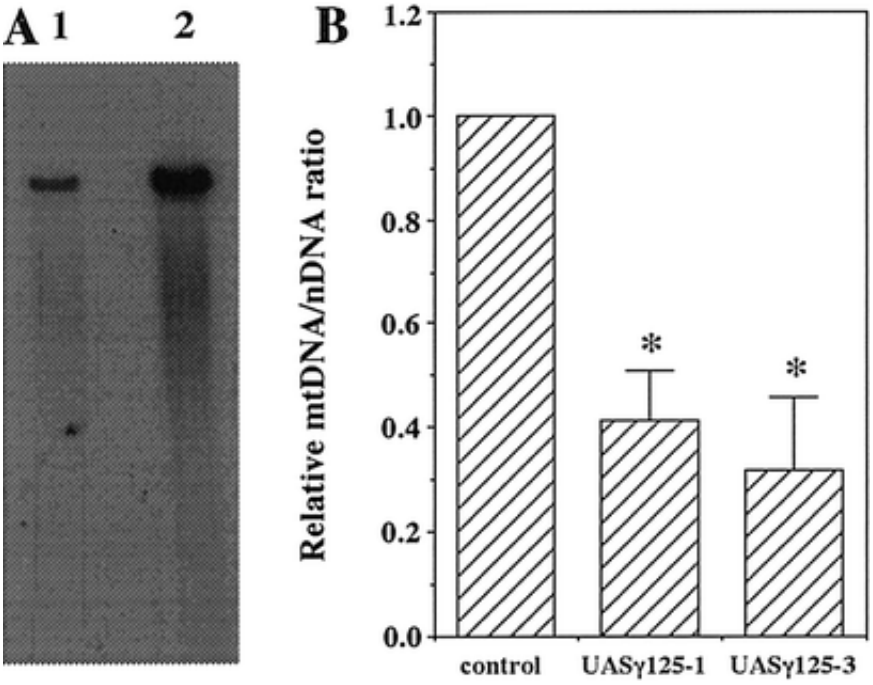

Fig. 5A, B. Overexpression of the pol γ-α gene results in depletion of mtDNA in transgenic flies. **A** Equivalent amounts of total DNA (10 µg) from 444-GAL4/UAS-γ 125-3 pupae (lane 1) and control pupae (lane 2) were digested with *Xho*I and electrophoresed in a 1% agarose gel. After blotting, the filter was probed with a specific *D. melanogaster* mtDNA probe (probe B, described by Calleja et al. *1993*) labeled with [α-^{32}P]dCTP. Because *D. melanogaster* mtDNA contains a single *Xho*I site, the fragment detected corresponds to the complete mtDNA molecule. **B** The mtDNA:nDNA ratio as determined by slot-blot analysis. Total DNA (50, 250 and 500 ng) from control pupae and pupae from two independent 444-*GAL4*/UAS-γ *125* lines was blotted onto a nylon membrane and hybridized sequentially with the mtDNA B probe and a nuclear probe (18S rDNA) labeled with [α-^{32}P]dCTP. The ratio obtained in the control pupae was taken as 1. The values represent the averages of three independent experiments (*P<0.05)

We evaluated the effect of the depletion of mtDNA on the mitochondrial transcription process by quantitating the steady-state levels of two RNAs encoded in the mitochondrial genome, 16S rRNA and CO I, using as a control the cytoplasmic 18S rRNA. In pupae overexpressing the pol γ-α gene, a substantial decrease in the steady-state level of both mtRNAs is detected, relative to control pupae (Fig. 6A and B), that is consistent with the decrease detected in the amount of mtDNA. Dot-blot analysis and densitometric scanning indicates that both mitochondrially encoded RNAs are reduced fivefold (Fig. 6D). Thus, the depletion of mtDNA produced by the overexpression of the catalytic subunit of pol γ has important consequences for mitochondrial physiology.

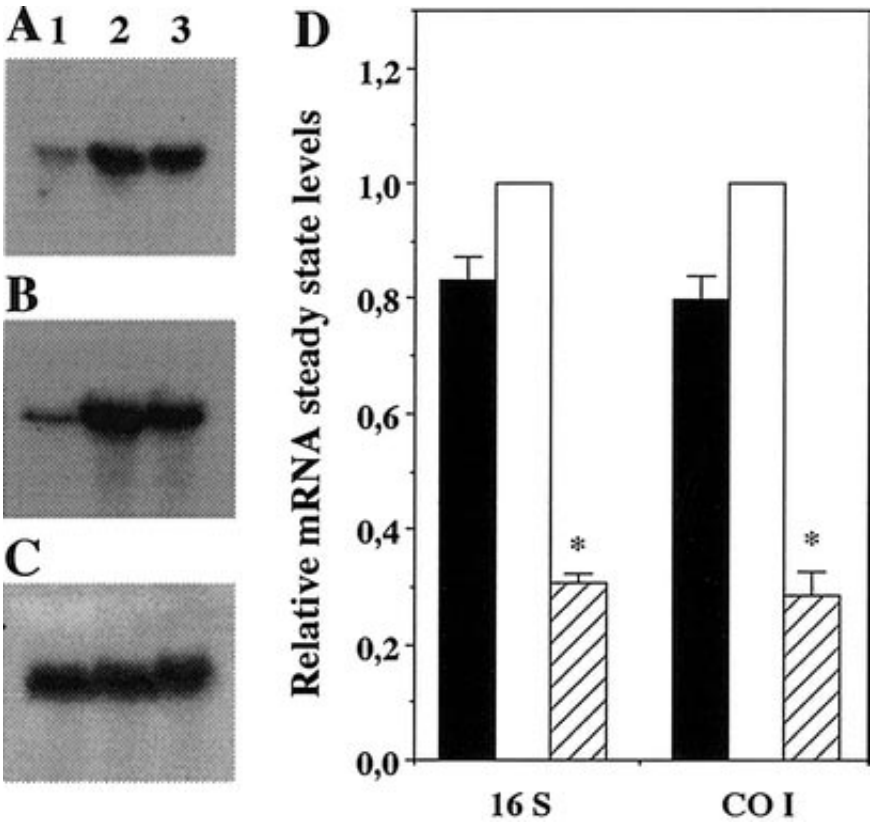

Fig. 6A-D. Overexpression of the pol γ -α gene in transgenic flies causes a significant reduction in the steady-state level of mitochondrially encoded transcripts. **A** Total RNA (20 µg) from 444-*GAL4*/UAS-γ *125-3* pupae (lane 1), control pupae (lane 2) and control adults (lane 3) was electrophoresed in a denaturing formaldehyde-agarose gel, transferred to nitrocellulose and probed with a specific radiolabeled mtDNA fragment encoding part of the 16SrRNA gene. **B, C** After washing, the filter was sequentially rehybridized using mtDNA-specific COI (**B**) and cytoplasmic rRNA (**C**) probes. **D** Relative steady-state levels of mtRNA were determined by slot-blot analysis. Total RNA (0.5, 1 and 5 µg) was blotted onto a nylon membrane and sequentially hybridized using mtDNA-specific (16S rRNA and COI) and nuclear (18S rRNA) probes. The ratios 16S rRNA:18S rRNA and COI:18S rRNA were calculated, and the value for control pupae was taken as 1. The *filled bars* correspond to control adults, *open bars* to control pupae and *hatched bars* to 444-*GAL4*/UAS-γ *125-3* pupae. Each value represents the average of at least three independent experiments (*P<0.05)

Transient overexpression of the pol γ -α subunit during *Drosophila* embryogenesis produces morphological defects

We used a hsGAL4 line to induce controlled and transient expression of the pol γ -α transgene during embryogenesis. We administered heat-shock pulses at different temperatures for variable

periods to hs-*GAL4*/UAS-γ *125-3* embryos harvested 0-20 h after egg laying (AEL), and no lethality

was observed. However, careful morphological analysis detected defects in the cuticularized structures of the emerging adults, including the eye, notum, wing, and leg, that became more apparent and more frequent as the severity of the heat-shock treatment was progressively increased. Although we did not analyze the defects quantitatively, we observed that about 10-20% of the emerging adults exhibited some cuticular alteration after a 30 min heat-shock pulse at 37°C. Some examples of the observed phenotypes are shown in Fig. 7. Abnormalities in the number and position of some bristles are apparent in the eye (Fig. 7B). Several alterations are produced in the notum, including loss of macrochaetae (Fig. 7D); disruptions in the pattern of the bristles are also frequently detected along the wing margin (Fig. 7F). We have confirmed these results in independent transgenic lines harboring the pol γ-α gene under the direct control of a hsp70 promoter (pCaSpeR-hs-γ *125*, see Materials and

methods). When a 30-min heat shock pulse at 37°C is given to pCaSpeR-hs-γ *125* homozygous

embryos at 0-20 h AEL, similar phenotypes showing subtle alterations of the cuticular pattern are also observed. Figure 7H shows as an example the absence of bristles found in the leg of one of the pCaSpeR-hs-γ 125 transgenic flies. These data are compatible with the important contribution of

maternal mitochondria during the process of embryogenesis, but suggest further that de novo mtDNA synthesis during development is important for the execution of the correct cell differentiation program.

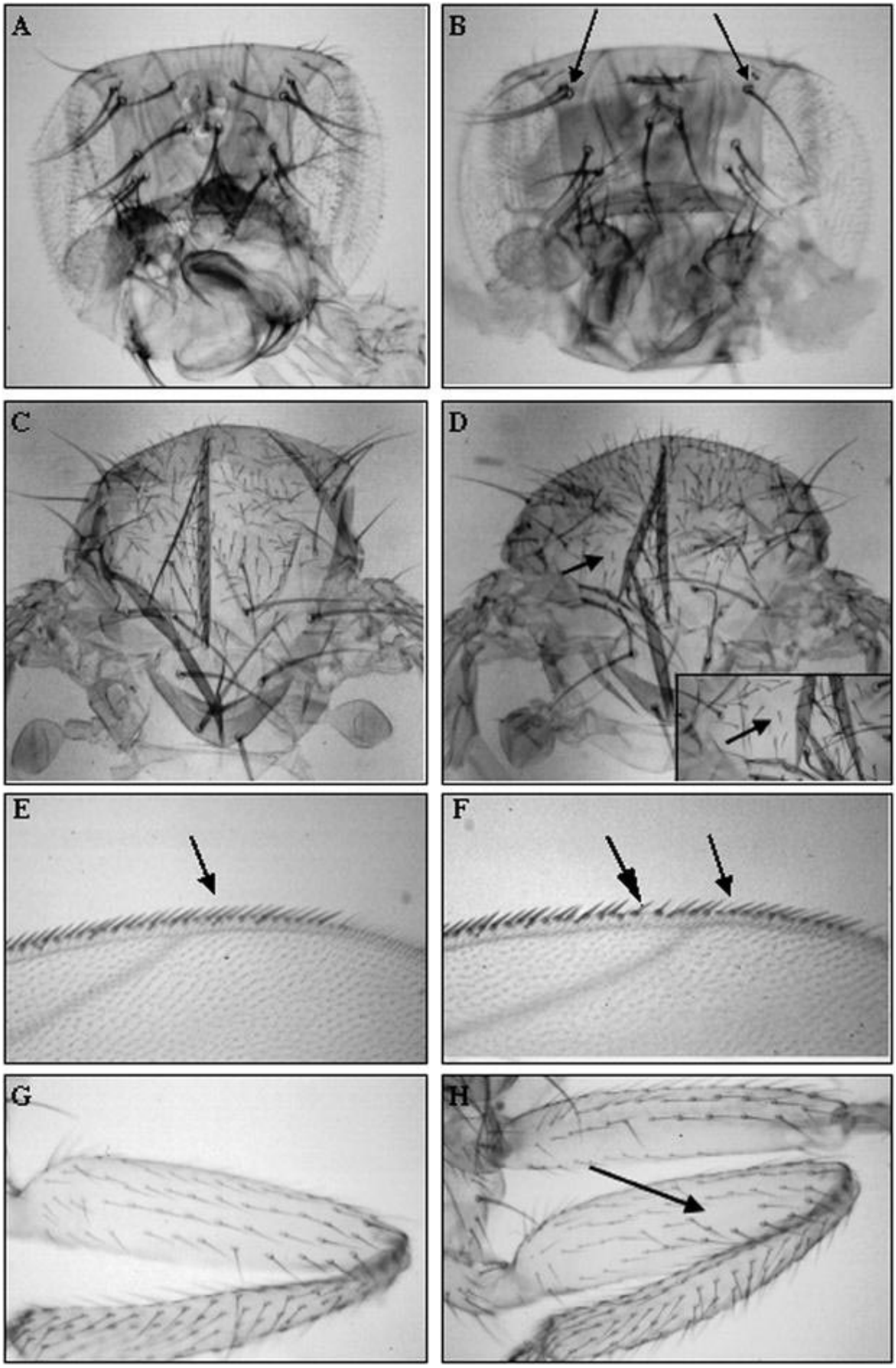
A
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Fig. 7A-H. Developmental abnormalities produced by the overexpression of the pol γ-α gene after heat-shock treatment. Short pulses of heat shock (30 min at 37°C) were administered to 0-24 h AEL transgenic HS-*GAL4*/UAS-γ *125-3* (**B, D, F**) or pCaSpeR-hs-γ *125* embryos (**H**) as described in Materials and methods. Transient overexpression of the pol γ-α subunit produces a series of alterations that are apparent in the adult cuticle. These abnormalities affect different parts of the fly including eye (**B**), thorax (**D**), wing (**F**) and leg (**H**). The equivalent wild-type phenotypes are also shown (**A**, **C**, **E** and **G**). Some of the changes detected in the number and position of the bristles are indicated by *arrows*

Discussion

Mitochondrial content and mtDNA copy number vary greatly among the different tissues of an organism but are maintained relatively constant within specific cell types, implying that mtDNA replication is strictly controlled at the cellular level. In this report we have shown that overexpression of the DNA polymerase γ catalytic subunit in Schneider cells does not affect cell physiology. Cells containing a large excess of the pol γ-α subunit grow well, as the generation time of the culture was unchanged, and no alterations in the amount or integrity of the mitochondrial genome were detected. Furthermore, we determined that the pol γ-α polypeptide was properly targeted to mitochondria, indicating that the absence of an effect is not due to the mislocalization of the enzyme. In *Drosophila* mitochondria, and probably in most animals, DNA polymerase γ is a heterodimer composed of a large catalytic subunit and a small accessory subunit, in a 1:1 stoichiometry (Olson et al. *1995*). The high level of pol γ-α expressed in the induced Schneider cells (increased by more than 50 fold) alters dramatically the stoichiometry usually found in vivo; yet, interestingly, the mtDNA replication process is not perturbed. This result also suggests that the level of the pol γ-α subunit in Schneider cells is normally in excess of that needed to replicate mtDNA, and thus its level does not control mtDNA copy number. This is consistent with previous data obtained in mammals, which show that the steady-state levels of pol γ-α mRNA present in different cell types do not correlate with their mtDNA content (Schultz et al. *1998*).

On the other hand, we found that pol γ-α overexpression has major physiological consequences in the whole organism. Using the UAS/GAL4 system we constitutively overexpressed the pol γ-α gene in *Drosophila* at a level similar to that achieved in Schneider cells. In striking contrast to the lack of effect in cell culture, the increased cellular level of the pol γ-α catalytic subunit is lethal in flies. In several such *Drosophila* lines, development reaches the late pupal stage, and the pharate adults are apparently well formed except for some cuticular defects. Although we do not know the detailed mechanism that produces lethality, it is clear that the excess of pol γ-α subunit interferes with the

process of mtDNA replication in the fly. In molecular terms, it produces a 2.5-fold decrease in the mtDNA:nuclear DNA ratio, with a corresponding five-fold decrease in the level of mitochondrially encoded transcripts. We have ruled out the possibility of a mislocalization of the pol γ-α subunit, as

virtually all of the overexpressed protein is detectable in the mitochondrial fraction. Moreover, the possibility that the decrease in mtDNA content is a consequence and not a cause of pupal lethality has also been excluded because the mtDNA depletion phenotype is already apparent in larval stages (data not shown). What is the mechanism by which pol γ-α overexpression interferes with mtDNA

replication? This question would be very difficult to address in vivo, but our biochemical studies of *Drosophila* pol γ offer some insight. We have demonstrated that pol γ-α alone binds

template-primer DNA and exhibits both DNA polymerase and 3' $\rightarrow$ 5' exonuclease activities (Lewis et al. *1997*), but has a catalytic efficiency 50-fold lower than that of the native two-subunit holoenzyme (Wang and Kaguni *1999*). In the transgenic flies, it is plausible to assume that the excess catalytic core participates in mtDNA replication, yet its lower activity and processivity result in stalled replication complexes that fail to complete the replication cycle. Alternatively, the excess catalytic core may sequester other mtDNA replication proteins, such as the mitochondrial single-stranded DNA-binding protein with which we have shown it to interact during the initiation and elongation of DNA strands (Farr et al. *1999*), to inhibit their participation in the mtDNA replication process. Whatever the mechanism, our data demonstrate clearly that an imbalance in the amount of a key mtDNA replication protein has significant physiological consequences in the developing organism. Although the lack of a corresponding effect of a similar excess of pol γ-α subunit on cultured cells

is perhaps puzzling, our data suggest differing protein and/or regulatory requirements for mtDNA replication under relatively relaxed conditions such as cell proliferation in culture, as compared with the rapid and dynamic process of cell proliferation and differentiation that takes place in the animal. This hypothesis correlates with previous results describing different topological forms of mtDNA in *Drosophila* embryos, adults and Schneider cells (Rubenstein et al. *1977*). Interestingly, recent data suggest that two different modes of mtDNA replication may operate in mammalian cells, and that changes in mtDNA copy number may involve an alteration in the mode of replication (Holt et al. *2000*). Experiments are currently in progress in our laboratory to induce transient mtDNA depletion in Schneider cells by ethidium bromide treatment, in order to determine if the overexpression of the pol γ-α subunit interferes with the restoration of normal mtDNA levels.

Iyengar and colleagues recently described a similar lethal phenotype produced by mutations in the *Drosophila tamas* gene that encodes the pol γ-α subunit (Iyengar et al. *1999*). *tamas* null mutants

die during the larval stages, indicating that the maternal supply of mitochondria allows the process of embryogenesis to proceed, but slows growth and impairs the correct development of larval tissues such as muscle or eye structures. Our data also argue that mitochondrial function plays an important role during *Drosophila* cell differentiation, because transient manipulation of mtDNA replication during embryogenesis produces a variety of morphogenetic alterations. Heat-shock pulses administered to embryos harboring the gene(s) hs-*GAL4*/UAS-γ *125* or hsp70-γ *125* produce several

effects that are detectable as alterations in the adult cuticular pattern. In both fly types we observed alterations affecting different parts of the body, including the eye, notum, leg and wing. The bristle pattern on the notum is slightly disorganized and in some cases dorsocentral chaetae are lacking, a result consistent with that found in pharate adults dissected from pupal cases. A temporally and spatially regulated may be necessary for various developmental processes, and a defect in

mitochondrial function, although not lethal at the cellular level, could produce important alterations in the developmental and cell differentiation program. In this regard, the use of *Drosophila* lines that express the pol γ -α transgene in specific cellular contexts provides us with a powerful approach

with which to examine the effects of mtDNA depletion during cell and tissue differentiation.

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