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MUSCLE-SPECIFIC OVEREXPRESSION OF THE CATALYTIC SUBUNIT OF DNA POLYMERASE γ INDUCES PUPAL LETHALITY IN *Drosophila melanogaster*

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Abstract

We show the physiological effects and molecular characterization of overexpression of the catalytic core of mitochondrial DNA (mtDNA) polymerase (pol γ - α) in muscle of *Drosophila melanogaster*. Muscle-specific overexpression of pol γ - α using the UAS/GAL4 (where UAS is upstream activation sequence) system produced more than 90% of lethality at the end of pupal stage at 25°C, and the survivor adult flies showed a significant reduction in life span. The survivor flies displayed a decreased mtDNA level that is accompanied by a corresponding decrease in the levels of the nucleoid-binding protein mitochondrial transcription factor A (mtTFA). Furthermore,

an increase in apoptosis is detected in larvae and adults overexpressing pol γ - α . We suggest that the pupal lethality and reduced life span of survivor adult flies are both caused mainly by massive apoptosis of muscle cells induced by mtDNA depletion.

Keywords

apoptosis; DNA polymerase γ ; *Drosophila melanogaster*; mitochondria; oxidative phosphorylation

INTRODUCTION

Mitochondria are ubiquitous membrane-bound organelles that contain their own genetic system. The mitochondrion is the site of the electron transport chain and oxidative phosphorylation (OXPHOS) system that provides the bulk of cellular energy in the form of ATP (Scarpulla, 2008). Animal mitochondrial DNA (mtDNA) is a circular DNA that contains only 37 genes. Only 13 genes encode polypeptides, all of which are components of the oxidative phosphorylation system (Oliveira et al., 2010); the remainder encodes the 22 tRNAs and 2 rRNAs necessary for the translation. All other proteins involved in the normal function of the mitochondria are encoded by nuclear DNA (nDNA), synthesized in the cytoplasm and imported into the organelle (Schmidt et al., 2010).

MtDNA is continuously replicating and the timing of mtDNA replication appears to proceed independently of that in the nucleus. DNA polymerase γ (pol γ) is a nuclear-encoded enzyme responsible for mtDNA replication (Stumpf and Copeland, 2011). In *Drosophila melanogaster*, pol γ is a heterodimer, with a catalytic core (pol γ - α) that contains both DNA polymerase and 3'-5' exonuclease activities (Lewis et al., 1996), and an accessory subunit (pol γ - β) that facilitates DNA binding and stimulates DNA synthesis (Fan et al., 1999).

We reported previously that overexpression in *Drosophila* of pol γ - α in the whole animal using a constitutive GAL4 driver produces a significant mtDNA depletion that causes morphological abnormalities and pupal lethality (Lefai et al., 2000). Later, we developed a system for tissue-specific depletion of mtDNA in flies by overexpression of pol γ - α in the nervous system, which showed OXPHOS defects, increased sensitivity to oxidative stress, and a mitochondrial disease-related mortality rate (Martínez-Azorín et al., 2008).

To characterize further the consequences of an excess of pol γ - α in *Drosophila*, we overexpressed the catalytic subunit of *D. melanogaster* DNA polymerase γ specifically in muscle using the GAL4-UAS (where UAS is upstream activation sequence) system. Overexpression in muscle induced lethality at the end of the pupal stage and reduced life span of survivor adult flies by causing mtDNA depletion and increased apoptosis.

MATERIALS AND METHODS

Transgenic Lines

Construction of the UAS-PolG transgenic fly lines containing the complete pol γ - α cDNA was reported previously (Martínez-Azorín et al., 2008). Briefly, the plasmid pUAST- γ 125

containing the complete pol γ - α cDNA subcloned in pUAST was injected into y^-w^- embryos to give the UAS-DNA polymerase γ subunit α (PolG) transgenic fly lines (Lefai et al., 2000). Transformants with w^+ eye color were mapped and rendered homozygous using the double balancer stock w , CyO/If , $MKRS/TM2$. We used the UAS/GAL4 system (Brand and Perrimon, 1993; Orfanos, 2008) to overexpress pol γ - α in muscle: the homozygous 24B-GAL4 (w^* ; $P\{w^+(GAL4^{24B})\}$, sb^1) line was crossed with the homozygous UAS-PolG lines. 24B-GAL4 enhancer trap line expressed Gal4p mostly in embryonic mesoderm (Brand and Perrimon, 1993; Luo et al., 1994) and third instar wing and eye imaginal discs, but it also showed a discrete expression in the larval central nervous system (CNS) (Suster et al., 2003).

Pupal Survival

Flies were maintained at 25°C and 60% humidity on standard medium (Calleja et al., 1993). Pupal survival was measured by the frequency of pupal eclosion. Adult flies were removed from the vials after 12 h. After 20 days, noneclosed pupae were assumed to be dead and all pupal cases on the side of the vials were scored as either eclosed (empty) or dead. The percentage of decrease of pupal survival was calculated from the data of Table 1. In some experiments, the flies were maintained at 18°C.

Life Span Analysis

For longevity experiments, a cohort of female flies (20 per vial) were transferred to fresh medium every 3 or 4 days and scored for survivors. Several independent experiments were carried out with ~60 flies per experiment. The median life span (the age in days required to reach 50% survivorship) and the maximum life span (the age in days required to reach 10% survivorship), were calculated from the survival curves fitted to the three-parametric Gompertz equation ($y = a \times \exp(-\exp(-(x - x_0)/b))$).

Immunoblot Analyses

Whole flies or isolated thoraces (after separation of frozen heads by shaking and abdomens by cutting with scalpel) were homogenized in 2× Laemmli loading sample buffer. Extracts were cleared by centrifugation and protein content determined by using the DC Protein Assay (Bio-Rad, Alcobendas, Madrid, Spain). Finally, protein extracts were fractionated in 10% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970), transferred to polyvinylidene difluoride membranes and probed with polyclonal antibodies against *D. melanogaster* proteins: anti-pol γ - α (Wang et al., 1997), anti-mtTFA (mitochondrial transcription factor A; Goto et al., 2001), anti- α -ATPase (nuclear-encoded mitochondrial ATPase subunit α ; Martínez-Azorín et al., 2008), and anti-Grim (Claveria et al., 1998). Horseradish peroxidase labeled goat anti-rabbit antibody (Life Technologies, Alcobendas, Madrid, Spain) was used as secondary antibody and visualized with an ECL kit (GE Healthcare Life Sciences, Alcobendas, Madrid, Spain).

Southern Blot Analyses

Southern blot analyses were performed as described previously (Martínez-Azorín et al., 2008). Briefly, DNA was digested with *Nsi*I, electrophoresed in a 0.6% (w/v) agarose gel,

transferred to Zeta-Probe membrane (Bio-Rad, Alcobendas, Madrid, Spain), and hybridized with radiolabeled mtDNA probe and nDNA probe. The mtDNA probe was a PCR-amplified region of *D. melanogaster* mtDNA between nucleotide positions 12,502 and 12,943 (Garesse, 1988). The nDNA probe was a fragment of 459 bp of *D. melanogaster* 18S rDNA amplified by PCR (nucleotide positions 488–946; Tautz et al., 1988). The relative amounts of mtDNA and nDNA were determined by densitometric analysis of autoradiographs.

Visualization of Apoptosis in Imaginal Disc

Quantitation of the level of apoptosis in wing imaginal discs of *Drosophila* was performed by staining with the vital dye acridine orange that permeates dying cells to bind chromatin (Denton et al., 2008; McCall et al., 2009). Third instar larvae were dissected and wing imaginal discs were collected in phosphate buffered saline (PBS). Imaginal discs were then incubated for 2 min in a 1 µg/ml acridine orange in PBS solution and rinsed briefly in PBS before observation. Images were taken in a fluorescence microscope and subsequently processed using Adobe Photoshop.

Statistical Analysis

The results were compared using Student's *t*-test (GraphPad software: <http://www.graphpad.com>). *P* values of less than 0.05 were considered statistically significant.

RESULTS

Pattern of Expression of the 24B-GAL4 Driver

The UAS lines contain the GAL4-responsive UAS and express the protein only in the presence of Gal4 protein. Therefore, to express pol γ - α in the muscle, we used the GAL4 enhancer trap line 24B-GAL4. Initially, the functionality and pattern of expression in adult flies was confirmed in crosses with the UAS-GFP ($w^+; P\{w^+(GFP^{UAS})\}/CyO$) reporter line and as expected, GFP was expressed predominantly in the muscle tissue including the adult head, thorax, and abdomen (Fig. 1A).

Overexpression of the Catalytic Subunit of Pol γ in Muscle Induces Pupal Lethality and Survivor Adult Flies Show a Reduced Life Span

The UAS-PolG lines were crossed with 24B-GAL4 stocks to obtain the UAS-PolG $\times$ 24B-GAL4 combination in the F1 generation and to evaluate the phenotypic consequences. Overexpression of pol γ - α in muscle induces high lethality at the pupal stage (Table 1). Crosses using the UAS-PolG_{III-1} line produced 95% of pupal lethality at 25°C, which decreased to 53% at 18°C, in agreement with the lower expression of the GAL4 protein at lower temperature (Duffy, 2002). Crosses with UAS-PolG_I showed 100% lethality in males and 90% in females. Additionally we determined the life span of adult flies expressing high levels of pol γ - α subunit in the muscle. Although UAS-PolG_I flies have a median life span of 30.8 days, similar to that of the 24B-GAL4 stock (31.0 days), we found that the low percentage of flies that eclose (10% of UAS-PolG_I $\times$ 24B-GAL4 females) that is, survivor flies that overexpress pol γ - α , have a median life span of 11.3 days, which represents a decrease in the median life span of ~63% (Table 2).

In order to demonstrate that the observed phenotype resulted from the overexpression of the pol γ - α gene, the steady-state level of pol γ - α protein was determined by immunoblot analysis (Fig. 1B). Pol γ - α protein is expressed at high levels in UAS-PolG $\times$ 24B-GAL4 flies as compared to the parental lines, where pol γ - α was not detected due to the very low level of expression of the endogenous gene (Fig. 1B). The α subunit of the *Drosophila* F1-H⁺-ATPase complex (α -ATPase) was used as a control.

Pol γ - α Overexpression in Muscle Induces mtDNA Depletion and Increases Apoptosis

Because overexpression of pol γ - α in the nervous system reduced the rate of mtDNA replication that resulted in reduction of the steady-state levels of mtDNA and mtTFA protein (Martínez-Azorín et al., 2008), we evaluated the effects of muscle-specific overexpression of pol γ - α on mtTFA and mtDNA levels. An immunoblot analysis was performed using antibodies against mtTFA and α -ATPase (Fig. 2A) and the levels of mtTFA protein were normalized relative to the levels of the α -ATPase protein. We observed decreases in the level of mtTFA (45%) in the UAS-PolG_I $\times$ 24B-GAL4 transgenic line and 65% in UAS-PolG_{III-1} $\times$ 24B-GAL4 flies. As the levels of mtTFA correlate well with mtDNA content (Larsson et al., 1994; Larsson et al., 1998; Scarpulla, 2008), mtDNA content was examined by Southern blot analysis. The blot was hybridized with a radiolabeled mtDNA-specific probe and a fragment of nuclear-encoded 18S rDNA, and the mtDNA/nDNA hybridization ratio was used to quantitate mtDNA (Fig. 2B). We observed that the mtDNA/nDNA ratio decreased by 25% in the UAS-PolG_I $\times$ 24B-GAL4 lines and 50% in the UAS-PolG_{III-1} $\times$ 24B-GAL4 flies as compared to controls.

It has been described that compounds that inhibit mtDNA replication and deplete mtDNA also induce apoptosis (Mansouri et al., 2003). Hence, we evaluated the level of apoptosis in wing imaginal discs dissected from third instar larvae overexpressing the pol γ - α subunit predominantly in muscle by staining with acridine orange (Fig. 3A). Apoptotic cells are brightly fluorescent. We observed a significant level of apoptosis in cells expressing the pol γ - α protein (24B-GAL4 $\times$ UAS-PolG_I and 24B-GAL4 $\times$ UAS-PolG_{III-1}) in regions in which this driver is most strongly expressed (Roote and Zusman, 1996). To evaluate the level of apoptosis in thoraces of survivor adult flies, we examined the expression of the proapoptotic protein Grim, which is a positive downstream regulator known to interact physically with caspases (Claveria et al., 1998; Steller, 2008; McCall et al., 2009). This protein was undetectable in control flies (UAS-PolG_I, UAS-PolG_{III-1}, and 24B-GAL4), indicating a low level or absence of apoptosis in the muscle of adult flies. However, the protein is expressed highly in the thoraces of flies overexpressing the pol γ - α subunit (24B-GAL4 $\times$ UAS-PolG_I and 24B-GAL4 $\times$ UAS-PolG_{III-1}) (Fig. 3B).

DISCUSSION

We found previously that constitutive overexpression of a high level of the pol γ - α subunit induces pupal lethality (Lefai et al., 2000), but overexpression in the nervous system induces only a moderate decrease in pupal eclosion (Martínez-Azorín et al., 2008). These data suggested that the high pupal lethality was due to disruption of mitochondrial function in other tissues. Therefore, we characterized the molecular and physiological effects of

overexpression of *Drosophila* pol γ - α in muscle (another tissue particularly dependent on mitochondrial function), using the same UAS-PolG lines in crosses with the 24B-GAL4 line (Brand and Perrimon, 1993); the enhancer trap 24B-GAL4 is a viable insertion in the *held out wings* locus, that encodes a putative RNA-binding protein involved in the control of muscular and cardiac activity (Zaffran et al., 1997), and expresses Gal4p in all embryonic and larval somatic muscles (Brand and Perrimon, 1993). However, the expression of 24B-GAL4 is not restricted to muscle and also shows a discrete expression in the larval CNS. Examination of the expression patterns of the 24B-GAL4 line in third instar larva revealed discrete sets of neurons (~200 neurons) distributed throughout the larval CNS (Suster et al., 2003). Nevertheless, if we take into consideration the total number of neurons in larval CNS (~15,000 neurons; Scott et al., 2001), then less than 1.5% of neurons are affected, and the effect in the nervous system can be considered marginal.

As was reported earlier, the physiological behavior of the homozygous UAS-PolG transgenic lines was similar to a wild-type line with regard to fertility, pupal lethality, and median life span (Martínez-Azorín et al., 2008). However, in transgenic lines overexpressing the pol γ - α subunit predominantly in muscle, we observed a similar phenotype as compared to that obtained upon constitutive overexpression of the enzyme, and most animals died at the end of pupal stage (Lefai et al., 2000). In contrast, when pol γ - α was overexpressed in the nervous system, pupal lethality was only moderately increased over controls (Martínez-Azorín et al., 2008). Our results suggest strongly that the pupal lethality observed previously was mainly due to disruption of muscle function, which may be critical at the end of pupation and for eclosion.

Moreover, to determine if the phenotype of shortened life span was specific to adult flies expressing high levels of pol γ - α subunit in the nervous system (Martínez-Azorín et al., 2008), we evaluated the survival of escapers upon overexpression in muscle. As we found in the nervous system, overexpression of γ - α in muscle reduced significantly the median life span by increasing the mortality rate of the population without variation in the rate of aging, indicated by the absence of significant changes in the maximum life span. Thus, these flies also showed a relevant mortality rate as for those overexpressing pol γ - α in the nervous system (Martínez-Azorín et al., 2008), indicating that the phenotype of shortened life span is a general phenomenon.

Overexpression of the pol γ - α protein in the nervous system decreases mtDNA replication rate, causing mtDNA depletion and a corresponding decrease in the level of mitochondrial transcription factor A (mtTFA; Martínez-Azorín et al., 2008). Similarly, transient expression of a polymerase-deficient human pol γ - α in cultured cells caused a reduction in the level of mtDNA (Spelbrink et al., 2000). In the present study, the pol γ - α protein was expressed at high levels predominantly in the muscle of transgenic flies, and this overexpression induced a similar mtDNA depletion as compared with depletion induced in flies expressing the pol γ - α subunit in the nervous system (Martínez-Azorín et al., 2008). Clearly, an excess of pol γ - α subunit interferes with the process of mtDNA replication. Two possible hypotheses have been proposed previously to explain this interference: (i) excess pol γ - α sequesters other mtDNA replication proteins and inhibits their function in mtDNA replication complexes (Farr et al., 2004); and (ii) the overexpressed catalytic alone participates in mtDNA

replication (Lewis et al., 1996) with low activity and processivity resulting in a reduced rate of replication (Wang and Kaguni, 1999). Whatever the biochemical mechanism is, it is clear that an imbalance in the amount of pol γ - α protein in muscle has significant physiological consequences.

Biochemical analysis of flies with nervous system specific mtDNA depletion induced by overexpressing the pol γ - α subunit showed OXPHOS defects, increased sensitivity to oxidative stress and increased cell death by apoptosis in the nervous system of larvae but not in adult flies (Martínez-Azorín et al., 2008). By comparison, the overexpression of pol γ - α in muscle produced a significant level of apoptosis in cells of wing imaginal discs from third instar larvae, suggesting that pathological programmed cell death in the muscle in the larval stage may contribute to the high level of pupal lethality. However, unlike the results observed in the nervous system, the muscle from thoraces of survivor adult flies overexpressing pol γ - α showed a substantial level of apoptosis. This difference is not surprising because it reflects the differential tissue-specific apoptotic properties observed previously during normal *D. melanogaster* aging (Zheng et al., 2005). These results establish that under normal conditions, fly aging is coupled with a lifelong gradual increase of apoptosis within muscle cells, whereas different apoptotic properties are observed in the nervous system, where no signs of apoptosis could be detected during normal aging.

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Figure 1.

Overexpression of pol γ - α protein in muscle of *Drosophila melanogaster*. (A) Expression pattern of the 24B-GAL4 line revealed by crossing with UAS-GFP. The domain of overexpression directed by the 24B-GAL4 driver was monitored by GFP fluorescence (green). (B) Immunoblot analysis of nuclear-encoded mitochondrial proteins. Mitochondrial proteins isolated from the thorax of adult flies were detected by immunoblotting using rabbit antiserum directed against *D. melanogaster* pol γ - α subunit or α -ATPase subunit.

Figure 2.

Pol γ - α overexpression in muscle induces mtDNA depletion. (A) Immunoblot analysis of nuclear-encoded mitochondrial proteins. Upper panel, mitochondrial proteins isolated from the thorax of adult flies were detected by immunoblotting with antibodies against *Drosophila melanogaster* pol γ - α , mitochondrial transcription factor A (mtTFA) and nuclear-encoded mitochondrial ATPase subunit α (α -ATPase). Lower panel, steady-state levels of mtTFA protein were normalized to the α -ATPase subunit. Statistical comparison was performed using Student's *t*-test, **P* < 0.028, ***P* < 0.007. (B) Southern blot analysis. Upper panel, total DNA was extracted from thoraces of adult flies, digested with NsiI, electrophoresed, transferred to a nylon membrane and hybridized with an mtDNA fragment (mtDNA) and a fragment of the 18S rRNA gene (nDNA). Lower panel, quantitation of mtDNA/nDNA levels. Student's *t*-test, **P* < 0.004, ***P* < 0.001. Data are means $\pm$ SD.

Figure 3. mtDNA depletion in muscle cells increases the level of apoptosis. (A) apoptosis in larvae. Wing imaginal discs were dissected from third instar larvae overexpressing pol γ - α (24B-GAL4 $\times$ UAS-PolG_I and 24B-GAL4 $\times$ UAS-PolG_{III-1}) and control larvae (24B-GAL4, UAS-PolG_I, and UAS-PolG_{III-1}). Apoptosis was detected by acridine orange staining. (B) apoptosis in thoraces of adult flies. The *Drosophila melanogaster* α -ATPase subunit and the apoptotic protein Grim were detected by immunoblotting of proteins isolated from thoraces of adult flies.

Table 1**Pupal Lethality**

		Temperature	n	Pupal lethality (%)
UAS-PolG _I x	(–)	25°C	209	25
	24B-GAL4	25°C	234	100 (M); 90 (F)
UAS-PolG _{III-1} x	(–)	25°C	247	34
	24B-GAL4	25°C	230	95
	24B-GAL4	18°C	327	53

Pupal lethality in transgenic flies overexpressing pol γ - α under the control of the 24B-GAL4 driver. Percentages of pupae that failed to eclose are shown. Measurement of pupal lethality is described in “Materials and Methods;” *n* is the total number of pupae; (–) indicates not crossed; sex: M—males, F—females.

Table 2

Life Span Analysis

	n	Median life span (days)	Decrease (%)	Maximum life span (days)	Decrease (%)
UAS-PolG ₁ x (-)	181	30.8 ± 1.5	63 (<i>P</i> < 0.001)	39.1 ± 3.5	9 (<i>P</i> ~ 0.177)
24B-GAL4	213	11.3 ± 3.2		35.6 ± 1.2	

Increased mortality in transgenic flies overexpressing pol γ - α under the control of the 24B-GAL4 driver. Maximum (10% survival) and median (50% survival) life span are shown. The mean $\pm$ SD (in days) is calculated from female flies' survival curves; *P* value is determined by Student's *t*-test; *n* is the total number of flies from four independent experiments.