

Mitochondrial Single-stranded DNA-binding Protein Is Required for Mitochondrial DNA Replication and Development in *Drosophila melanogaster*

Dieter Maier,* Carol L. Farr,[†] Burkhard Poeck,* Anuradha Alahari,[†] Marion Vogel,* Susanne Fischer,* Laurie S. Kaguni,[†] and Stephan Schneuwly[‡]

*Lehrstuhl für Entwicklungsbiologie, Universität Regensburg, Regensburg, Germany D-93040; and
[†]Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, Michigan 48824-1319

Submitted August 15, 2000; Revised December 22, 2000; Accepted January 24, 2001
Monitoring Editor: Thomas D. Fox

The discovery that several inherited human diseases are caused by mtDNA depletion has led to an increased interest in the replication and maintenance of mtDNA. We have isolated a new mutant in the *lopo* (*low power*) gene from *Drosophila melanogaster* affecting the mitochondrial single-stranded DNA-binding protein (mtSSB), which is one of the key components in mtDNA replication and maintenance. *lopo*¹ mutants die late in the third instar before completion of metamorphosis because of a failure in cell proliferation. Molecular, histochemical, and physiological experiments show a drastic decrease in mtDNA content that is coupled with the loss of respiration in these mutants. However, the number and morphology of mitochondria are not greatly affected. Immunocytochemical analysis shows that mtSSB is expressed in all tissues but is highly enriched in proliferating tissues and in the developing oocyte. *lopo*¹ is the first mtSSB mutant in higher eukaryotes, and its analysis demonstrates the essential function of this gene in development, providing an excellent model to study mitochondrial biogenesis in animals.

INTRODUCTION

Mitochondria are critical for their central role in energy metabolism, but they also play important roles in aging, apoptosis, and a variety of genetically inherited human diseases (Larsson and Clayton, 1995; Grossman and Shoubridge, 1996; Zeviani *et al.*, 1997). Whereas there is a wealth of information on the structure and function of mitochondria, very little is known about the regulation of their biogenesis. Eukaryotic cells contain not only highly variable numbers of mitochondria per cell but mitochondria also differ substantially in their mtDNA copy number, where the number of mtDNA genomes varies depending on the energy requirements of a particular cell (Shadel and Clayton, 1997). A complex process of interactions between proteins encoded in the nuclear and mtDNA genomes regulates mitochondrial biogenesis. mtDNA generally encodes 22 tRNAs, 2 rRNAs, and 13 polypeptides that are all components of the respiratory chain enzyme complexes. All of the remaining mitochondrial proteins are encoded by nuclear genes and are targeted specifically to mitochondria (Schatz, 1996). In particular, enzymes required for mtDNA replica-

tion and transcription are all encoded by the nuclear genome.

The complexity of interactions between the nucleus and mitochondria is also apparent in the variety of human diseases resulting from defects in mtDNA: a large number of mutations (either point mutations or mtDNA rearrangements) have been identified as the cause of severe human diseases. There are, however, autosomal inherited genetic defects that lead to mtDNA depletion (Larsson and Clayton, 1995; Zeviani *et al.*, 1997). Obvious candidate genes for the latter are nuclear genes involved in mtDNA replication or maintenance, and among them are those encoding the mtDNA polymerase, pol γ , which consists of two subunits and is the key enzyme in mtDNA replication (Wernette and Kaguni, 1986; Shadel and Clayton, 1997). At least four other proteins that are required for proper replication and/or maintenance of mtDNA have been identified, including two DNA helicases (Lahaye *et al.*, 1993; Sedman *et al.*, 2000), the mitochondrial transcription factor A (Larsson *et al.* 1998), and mitochondrial single-stranded DNA-binding protein (mtSSB).

mtSSB is a conserved molecule of 13–18 kDa identified in a variety of eukaryotic organisms ranging from yeast to humans (Pavco and Van Tuyle, 1985; Hoke *et al.*, 1990; van Dyck *et al.*, 1992; Tiranti *et al.* 1993, Stroumbakis *et al.*, 1994;

[‡] Corresponding author. E-mail: Stephan.Schneuwly@biologie.uni-regensburg.de.

Thömmes *et al.*, 1995; Li and Williams, 1997). mtSSB binds to single-stranded DNA (ssDNA), coating the displaced ssDNA, which is the template for lagging DNA strand synthesis in mtDNA replication, and preventing it from renaturation (Van Tuyle and Pavco, 1985). mtSSB also stimulates greatly the activity of pol γ from various species (Mignotte *et al.*, 1988; Hoke *et al.* 1990; Williams and Kaguni, 1994; Thömmes *et al.*, 1995). Although the mtSSB gene was shown to be essential in yeast (van Dyck *et al.* 1992), its role in mitochondrial biogenesis in animals has not been explored, despite the fact that it represents a candidate gene for inherited human mtDNA depletion.

In this report, we present the molecular and phenotypic analysis of a new mutation in the *Drosophila* mtSSB gene, which we have called *lopo* (*low power*). We show in this first mtSSB mutant in animals that the absence of mtSSB results in the loss of mtDNA and hence a loss of respiration in mitochondria. Furthermore, *lopo* is absolutely required for proper development because flies die prematurely during the larval and pupal stages. The *Drosophila* mtSSB mutant might therefore serve as an excellent model system to study mtDNA maintenance and replication in higher eukaryotes.

MATERIALS AND METHODS

Fly Stocks, P-Element Excision, and Transgenic Rescue Analysis

The line P[mini-w+;lacW]0046/13 was originally isolated as a third chromosomal lethal mutation (Deák *et al.* 1997). Remobilization was achieved using the $\Delta 2-3$ Sb chromosome as a transposase source. Excision events were collected as white-eyed flies. From 109 established lines, 99 lines were still lethal, whereas 10 lines showed a reversion of the lethal phenotype. Rescue of *lopo*¹ lethality was evaluated at 25°C in crosses of *lopo*¹ heterozygotes carrying independent insertions of a wild-type mtSSB transgene in the second chromosome and under the control of the hsp70 promoter (lines B and D). Whereas ~25% of the progeny of a control cross of *lopo*¹ heterozygotes lacking a wild-type mtSSB transgene die in the late third larval instar, 14.9 and 9.8% of the anticipated 25% moribund larvae are recovered as adults in crosses of *lopo*¹ heterozygous lines B and D.

Cloning and Molecular Analysis of lopo

DNA flanking the P-element insertion was isolated using the plasmid-rescue method (Wilson *et al.*, 1989) and sequenced with an Alf-sequencing apparatus (Pharmacia, Piscataway, NJ). A 3.2-kb genomic *Xho*I fragment from the P1 phage DS07224 (Hartl *et al.*, 1994) was identified as the site of the *lopo* gene, subcloned in pKS and further characterized using standard molecular methods (Sambrook *et al.*, 1989). Several different methods of DNA extraction were tested for mtDNA analysis. Best results were obtained by the protocol described by Pflugfelder *et al.* (1990).

Western Blots

Embryos of wild-type and heterozygous *lopo* flies were collected for 0–16 h on yeast-grape agar plates, washed with 0.01% Triton X-100 and 0.7% NaCl, and allowed to develop on grape agar in Petri dishes for 5–6 d. The heterozygous (*tubby*) larvae were separated from the homozygous (*thin*) *lopo* larvae at third instar and, in parallel with the wild-type larvae, were washed five times with 0.01% Triton X-100 and 0.7% NaCl and then frozen in liquid nitrogen and stored at –80°C. To prepare larval extracts, 300 larvae (~0.5 g) were thawed on ice for 30 min and then suspended in 1.4 ml of extraction buffer (50 mM Tris-HCl, pH 7.5, 10% glycerol, 8 mM

EDTA, 0.1% Triton X-100, 0.5 M NaCl) at 3°C and homogenized by 10 strokes in a Dounce homogenizer. The homogenate was filtered through a 75-mm Nitex screen; the retentate was rehomogenized in the same buffer (0.5 ml) and filtered. The combined homogenates were centrifuged at 17,500 \times g for 30 min at 3°C, and the resulting supernatant fraction was used as the larval extract. Larval extracts were fractionated on Blue Sepharose (Pharmacia) essentially as described by Farr *et al.* (1999), except that batch loading and elution were used. The larval extracts derived from 300 larvae were mixed with a 50% slurry of Blue Sepharose (140 ml) in buffer containing 30 mM Tris-HCl, pH 7.5/10% glycerol/100 mM NaCl/2 mM EDTA/2 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride/10 mM sodium metabisulfite/2 mg/ml leupeptin. The beads were incubated overnight at 3°C with gentle agitation and then collected by centrifugation at 3000 \times g for 30 s. The beads were then twice washed for 10 min with 2 volumes (140 ml) of the same buffer containing 800 mM NaCl and recentrifuged. The bound protein was eluted twice with 2 volumes each of buffer lacking NaCl and containing 0.5 and 1.0 M sodium thiocyanate. Finally, mtSSB was eluted twice for 2 h with 2 volumes of buffer lacking NaCl and containing 1.5 M sodium thiocyanate.

Oxygen Measurements

Five third instar larvae were immobilized in liquid nitrogen, homogenized in 100 μ l of buffer (0.9% NaCl, 20 mM Tris-HCl, pH 8.0, 20 mM glucose), and stored on ice. Oxygen consumption was determined by measuring oxygen concentration (in percentage saturation) with optoelectronic sensors kindly provided by PreSens Precision Sensing (Regensburg, Germany). These electrodes measure oxygen due to the dynamic quenching of luminescence that depends on oxygen concentration (Preininger *et al.* 1994). Measurements can be done in volumes as small as 10 μ l and were preferred over oxygen electrodes which require several milliliters of volume with large oxygen buffer capacity. Samples were adjusted to 20°C, 10 μ l of 1 M NADH was added, and oxygen consumption was measured for at least 5 min or until oxygen concentration was below 10%. The dependence of oxygen consumption on mitochondrial activity was tested by the ability to inhibit the reaction with 1 μ l of 1 mM KCN. Wild-type and mutant homogenates were measured alternatively to ensure that samples were unaffected by storage (up to 30 min on ice). Protein concentrations were determined by the Bradford (1976) method. Oxygen concentrations are given in nanomoles, assuming that 100% saturation at 20°C corresponds to 0.276 μ mol O₂/ml.

Histological Analysis

Third instar larval sections (10 μ m thick) were used for mitochondrial enzyme histochemistry. For succinate dehydrogenase (SDH) activity, sections were stained unfixed according to the method of Lojda *et al.* (1979). Nitroblue tetrazolium was used as the color reagent, and the staining specificity was evaluated by omission of the enzyme substrate (succinate). For cytochrome *c* oxidase (COX) histochemistry, sections were fixed for 15 min in 1% glutaraldehyde in phosphate-buffered saline (PBS) before staining. Staining specificity was tested by staining in the presence of 0.0065% KCN. 5-Bromodeoxyuridine (BUdR) staining was performed according to the method of Truman and Bate (1988), except for a 15 min 2% paraformaldehyde fixation instead of Carnoy.

Immunocytochemistry

Embryonic, larval or adult tissue was frozen in OCT (Tissue-tek, O.C.T. compound, Sakura Finetek Europe, Netherlands) and cut at 10 μ m in a cryostat. Tissue was fixed for 10 min in 4% paraformaldehyde in PBS, washed with PBS, and incubated with the primary antiserum (anti-mtSSB; diluted 1:100; Farr *et al.* 1999) overnight at 4°C. The secondary antibody was a goat anti-rabbit immunoglobulin

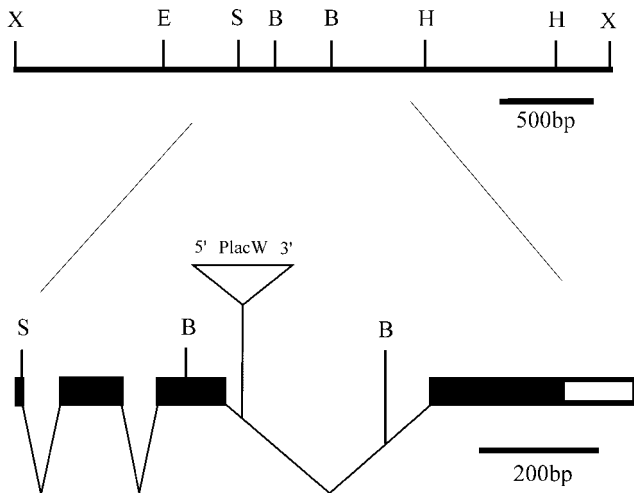


Figure 1. Molecular map of *lopo*. The gene was mapped within a 3.2-kb genomic *XhoI* fragment isolated from the P1-phage DS07224 (Hartl *et al.*, 1994). The open reading frame is indicated by the filled box. It starts at the *SphI* site and is disrupted by three introns. The P-element insertion in *lopo*¹ is located within the third intron. B, *BamHI*; E, *EcoRI*; H, *HindIII*; S, *SphI*; X, *XhoI*.

lin G-CY3 (Jackson Laboratories, West Grove, PA) used at a 1:1000 dilution for 2 h. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI).

Electron Microscopy

Midgut tissue from third instar larvae was dissected in ice-cold fixative (4% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4) and fixed overnight at 4°C in the same fixative. Tissue samples were washed in phosphate buffer, postfixed in ice-cold 1% Os O4 in phosphate buffer for 1 h, and washed again in phosphate buffer. The specimens were dehydrated in an alcohol series and embedded in Durcupan (Fluka Chemicals, Deisenhofen, Germany). Gray sections were cut on an Ultracut E (Reichert-Jung, Heidelberg, Germany), stained with uranyl acetate (2% in 50% alcohol, 15 min) and lead citrate (2 min; Reynolds, 1963), and examined with an EM10 electron microscope (Zeiss, Thornwood, NY).

RESULTS

Isolation and Molecular Characterization of *lopo* (Low Power)

*lopo*¹ was isolated in a screen for lethal mutants with defects in the developing visual system. It originates from a collection of P-element-induced lethal mutations on the third chromosome (Deák *et al.*, 1997). Molecular analysis of this line led to the identification of a single P-element inserted in the chromosomal region 89B12. Flanking genomic DNA was isolated by plasmid rescue and was subsequently sequenced, which placed the P-element within the third intron of the gene encoding the mtSSB (Figure 1; Ruiz de Mena *et al.*, 2000). *Drosophila* mtSSB is a small polypeptide of ~14 kDa that forms a homotetramer (Thömmes *et al.*, 1995) and is characterized by a mitochondrial presequence and a DNA-binding domain that is conserved among mtSSBs from yeast (van Dyck *et al.*, 1992) to humans (Tiranti *et al.*, 1993).

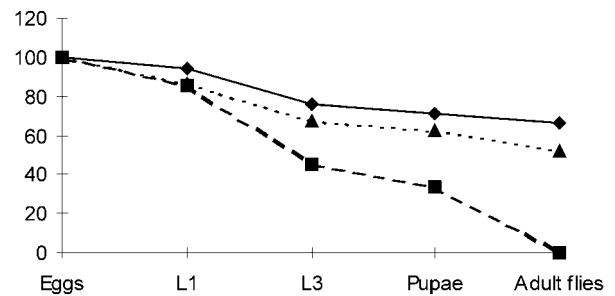


Figure 2. Effective lethal phase of *lopo*¹. Eggs of wild-type Canton S (bold line), *lopo*¹ (hatched line), and a *lopo*¹ revertant *lopo*^{1RA47} (dotted line) were collected on apple juice plates and staged accordingly. The graph shows the percentage of surviving individuals at various stages of development (L1, first larval instar; L3, third larval instar). The effective lethal phase is during late third instar and early pupation. Thirty percent of *lopo*¹ larvae enter pupation but never reach adulthood. The revertant *lopo*^{1RA47} shows a wild-type developmental profile.

To prove that the *lopo*¹ mutation is indeed induced by the P-element insertion in the mtSSB gene, the P-element was remobilized. In several independent lines, a precise excision was detected by genomic Southern blots and polymerase chain reaction, which led to a complete reversion of the lethality and all observed phenotypic effects of *lopo*¹ (Maier, Poeck, Vogel, Fischer, and Schneuwly, unpublished results). Moreover, we were able to rescue the lethal phenotype at 25°C, using transformed lines carrying wild-type mtSSB transgenes under the control of the *hsp70* promoter (see MATERIALS AND METHODS). We therefore conclude that *lopo*¹ is indeed a lethal P-element-induced mutation in the mtSSB gene.

Lopo Is an Essential Gene Required for Cell Proliferation in Development

To determine the major cause for the lethality in *lopo*¹ mutants, we pursued a phenotypic analysis to determine the lethal phase in this line. As shown in Figure 2, most flies enter the first larval instar, but only 30% are able to start pupariation and eventually die prematurely. The late lethality is perhaps surprising for an essential gene in mitochondrial function (see later) but could be explained by a strong maternal contribution. To test this hypothesis, we performed immunocytochemical analysis with rabbit antiserum against mtSSB. As expected, mtSSB is present in all tissues of adult flies (male and female) and shows a typical mitochondrial spot-like cellular distribution (Figure 3). However, it is not expressed uniformly in all tissues. One of the tissues with a high concentration of mtSSB is the CNS, and in particular the neuropil region where axons are located, whereas only limited expression is seen in neuronal cell bodies (Figure 3A). Other tissues with high mtSSB levels are the digestive tract and ovaries: mtSSB is synthesized highly in nurse cells (Figure 3B) and is transported into the oocyte (Figure 3C). This is consistent with the fact that most mtSSB cDNAs have been isolated from ovarian cDNA libraries (Stroumbakis *et al.*, 1994) and confirms the hypothesis of a strong maternal contribution.

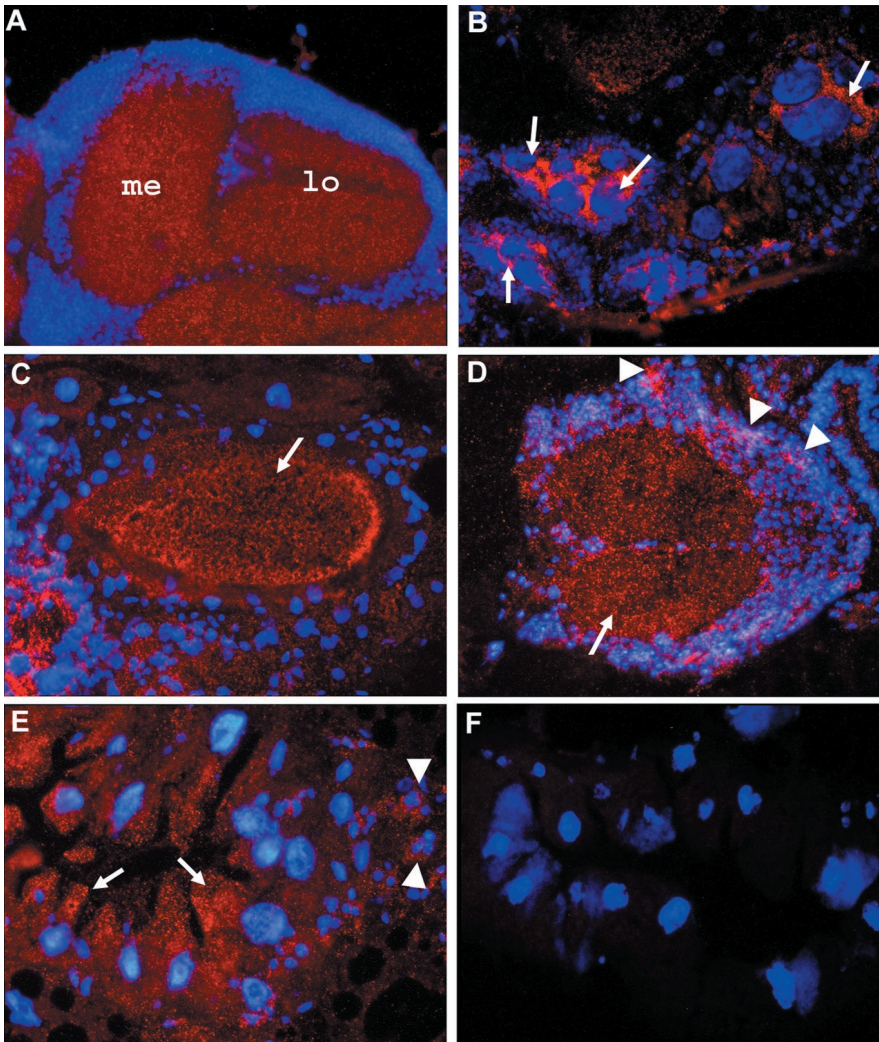


Figure 3. Immunohistochemical localization of mtSSB. Cryosections of various tissues and stages (10 μm thick) were subjected to immunofluorescence analysis using a rabbit mtSSB antiserum. The tissue was counterstained with DAPI to visualize nuclei. (A) Horizontal section through the visual system of an adult head. Strong labeling is seen in the neuropil region. (B and C) Horizontal sections through the abdomen of an adult female. In B, a high level of mtSSB is seen in nurse cells (arrows). (C) The protein is transported into the oocyte (arrow). (D) A cross-section through a third instar larval ventral ganglion shows expression of mtSSB in the neuropil region (arrow) and elevated expression in proliferating neuroblasts (arrowheads). (E) Cross-section through the midgut of a third instar larvae. A high concentration of mtSSB is found in the apical region of epithelial cells facing the gut lumen (arrows) and in islets of small imaginal cells, giving rise to the adult midgut tissue (arrowheads). (F) Comparable section as in E from a homozygous *lopo*¹ larva. No specific staining is seen.

During early embryogenesis, the protein is evenly distributed, and in late third instar larvae, a high concentration of mtSSB is detected in proliferating tissues such as neuroblasts of the ventral ganglion of the CNS (Figure 3D), in islets of small imaginal cells, which give rise to the adult midgut epithelium (Figure 3E), and in imaginal discs (Maier and Schneuwly, unpublished results), documenting the higher demand for mtSSB in proliferating tissues. Subcellular localization of mtSSB shows its association with mtDNA, as indicated by double staining with DAPI (Maier and Schneuwly, unpublished results). Staining specificity was verified by examination of *lopo*¹ mutant tissue from third instar larvae, which shows no mitochondrial staining (Figure 3F).

These results together with the late onset of lethality suggest that defects in cell proliferation during metamorphosis are likely the major cause for lethality. Cell proliferation can be visualized by BUdR labeling in developing larvae as shown in Figure 4. Indeed, aberrant and drastically reduced proliferation are detected in *lopo*¹. These data also explain the disturbance in visual system development, which was the primary screen used in the identification of this mutant (Maier, Poeck, and Schneuwly, unpublished results).

*Lopo*¹ Is Defective in mtDNA Replication and Maintenance Resulting in Loss of Mitochondrial Respiration

What are the molecular causes for premature lethality and aberrant cell proliferation in *lopo*¹? *Lopo* encodes *Drosophila* mtSSB, which binds ssDNA (Stroumbakis *et al.*, 1994; Thömmes *et al.*, 1995) and stimulates dramatically the activity of mtDNA polymerase (Thömmes *et al.*, 1995; Farr *et al.*, 1999). It was therefore important to evaluate mtDNA content and integrity in *lopo*¹. Figure 5 shows a quantitative Southern blot of wild-type and *lopo*¹ DNA hybridized with an mtDNA probe and with a single-copy genomic probe as a control. These experiments reproducibly fail to detect any mtDNA and thus demonstrate severe mtDNA depletion in *lopo*¹. Similar results were obtained by performing quantitative polymerase chain reaction (Maier, Vogel, and Schneuwly, unpublished results).

To determine the level of mtSSB protein in larvae, extracts were examined by immunoblot analysis. Protein extracts were prepared from wild-type, heterozygous, and homozygous *lopo*¹ larvae at the third instar stage and subjected to

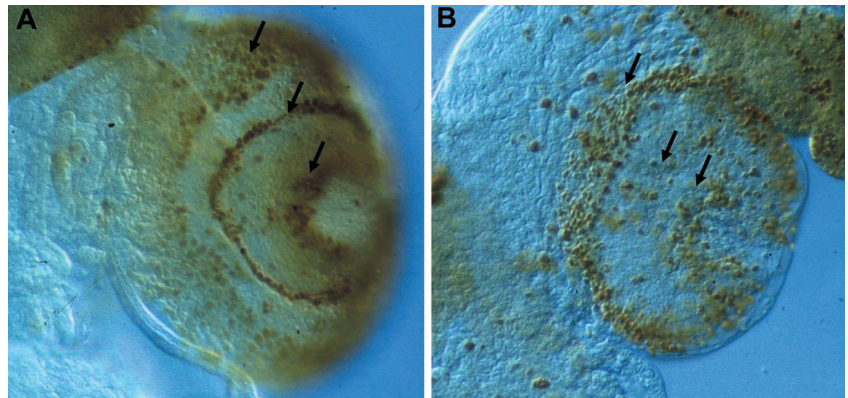


Figure 4. Proliferation defects in *lopo*¹. BUdR labeling is shown in the third instar larval CNS of wild-type (A) and *lopo*¹ (B). Proliferating cells are organized in proliferation centers (arrows). In *lopo*¹, the number of labeled cells is reduced drastically.

SDS-PAGE followed by immunoblot analysis with rabbit antiserum against native mtSSB from *Drosophila* embryos (Figure 6). The immunoblot analysis shows the presence of mtSSB migrating as a polypeptide of ~12 kDa in the wild-type and heterozygote extracts, whereas mtSSB is entirely absent in the corresponding homozygote extract (Figure 6,

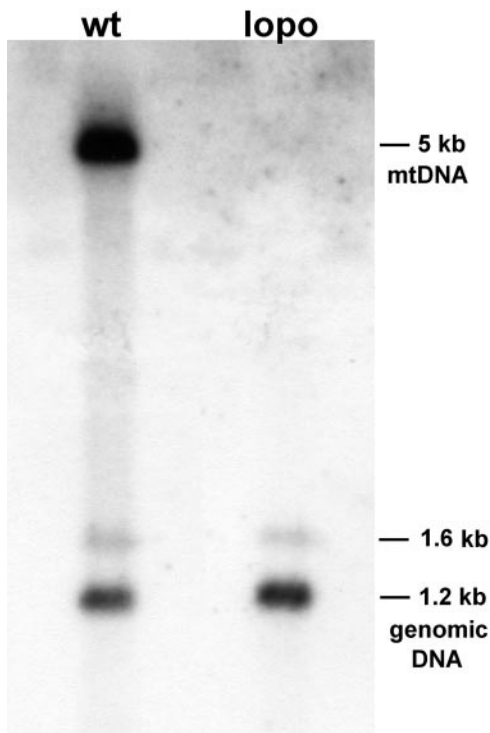


Figure 5. mtDNA depletion in *lopo*¹. A quantitative Southern blot was generated containing equal amounts of total DNA from wild-type Canton S (wt) and *lopo*¹ (*lopo*) digested with *Eco*RI (equivalent of 100 mg of third instar larvae). mtDNA was visualized using a 5-kb fragment of mtDNA (Garesse, 1988). No mtDNA is detected in *lopo*¹. Efficiency of loading and blotting was verified by hybridizing the blot with a single-copy nuclear gene (*aos* cDNA; Kretzschmar *et al.*, 1992). The corresponding 1.2-kb fragment (and a weaker 1.6-kb fragment) show equal intensities in both lanes.

lane 7). Quantitation of three independent experiments indicated that the level of mtSSB protein is ~0.5 μg/g of larvae in the wild-type and heterozygote extracts, a value comparable to that determined previously for mtSSB from wild-type embryos (Thömmes *et al.*, 1995). We then purified the mtSSB from the soluble cell extracts by a chromatographic step in which the protein is bound to Blue Sepharose under stringent conditions (in the presence of 0.8 M NaCl) and eluted with increasing concentrations of sodium thiocyanate (Farr *et al.*, 1999). The 1.5 M sodium thiocyanate eluate demonstrates clearly the enrichment of mtSSB over immunoreactive contaminants in both the wild-type and heterozygote extracts (Figure 6, lanes 2 and 3, respectively). Nonetheless, a polypeptide corresponding to mtSSB was still absent in the homozygote *lopo*¹ eluate (Figure 6, lane 4). The absence of mtDNA in the *lopo*¹ mutant and the complete absence of mtSSB support further the conclusion that *lopo* encodes a gene affecting the replication and maintenance of mtDNA.

To analyze the effect of mtDNA depletion on mitochondrial physiology, we measured the O₂ consumption of mitochondria from third instar larval tissue. Figure 7 shows a typical curve for wild-type and *lopo*¹ mitochondria, documenting that respiration is reduced by 90% in the latter.

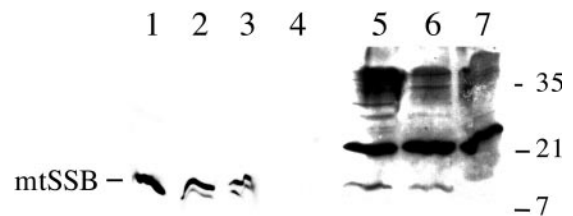


Figure 6. Larval expression and fractionation of *Drosophila* mtSSB protein. Protein fractions as described in MATERIALS AND METHODS were denatured and electrophoresed in a 17% SDS-polyacrylamide gel, transferred to nitrocellulose, and detected by immunoblotting with rabbit antiserum against *D. melanogaster* mtSSB at a 1:1000 dilution using the ECL method (Amersham Life Sciences, Arlington Heights, IL). Lane 1, recombinant *Drosophila* mtSSB (10 ng); lanes 2–4, Blue Sepharose eluates (60 μl) from wild-type, heterozygous, and homozygous *lopo*¹ larvae, respectively; lanes 5–7, larval extracts (~200 μg of total protein) from wild-type, heterozygous, and homozygous *lopo* larvae, respectively.

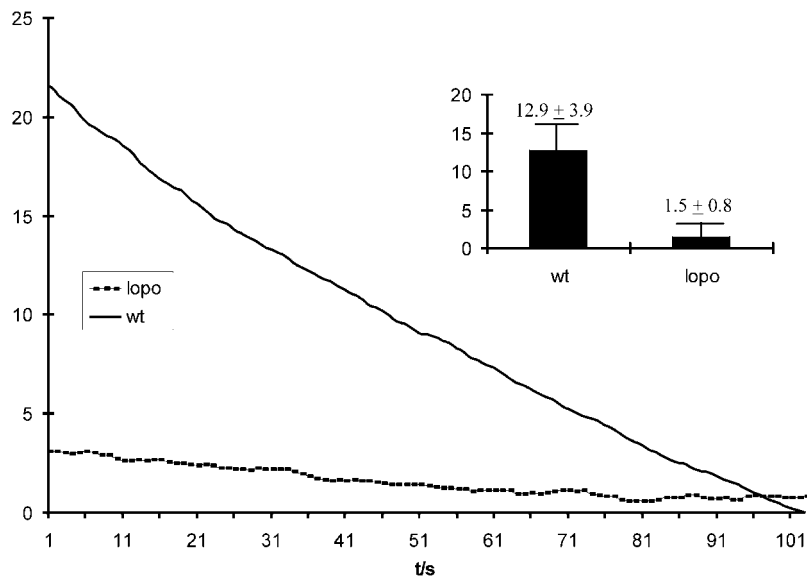


Figure 7. Oxygen consumption in larval tissues. The graph shows a typical curve of oxygen consumption in wild type (wt; straight line) and *lopo*¹ (dotted line). The oxygen consumption is given in nanomoles per microgram of protein; the x axis defines time in seconds (t/s). Typically the oxygen consumption in *lopo*¹ is reduced to ~10% of the wild-type level. The inset shows a comparison of the mean relative oxygen consumption per min (three measurements each).

Thus, whereas *lopo*¹ flies are able to survive embryogenesis most likely through a maternal contribution of mtSSB, they exhibit a nearly complete loss of oxygen consumption in late larvae. This is consistent with the developmental profile, in which *lopo*¹ larvae require almost twice as long to reach pupation, which can apparently occur under nearly anaerobic conditions. However, with the requirement for a high level of cell proliferation during metamorphosis, they eventually die.

Functionally Impaired *lopo*¹ Mitochondria Are Largely Normal in Number and Structure

The observed mtDNA depletion and the reduced physiological function of mitochondria in *lopo*¹ raise several questions about the status of mitochondria in this mutant. Is the number of mitochondria in *lopo*¹ reduced, which would indicate that there is feedback control of mitochondrial biogenesis via mtDNA replication? Alternatively, are there mitochondria present without mtDNA and hence not able to perform respiration? To answer these questions, we performed a histological analysis in *lopo*¹. One mechanism to discriminate mitochondria containing mtDNA from those that do not is differential histochemical staining for the enzymes COX and SDH. Because three subunits of COX are encoded by the mitochondrial genome, functional COX staining is observed only in mitochondria containing mtDNA, whereas, because SDH is encoded exclusively by nuclear genes, it should be functional. Figure 8 shows the digestive tract of third instar larvae, where high numbers of mitochondria are found, especially in the midgut (Figure 8, A and C). As expected, COX staining is absent in *lopo*¹, indicating either the absence of mtDNA or the absence of mitochondria per se (Figure 8, B and E). In contrast, SDH staining in larval gut tissue (Figure 8, G and H) shows the presence of SDH enzyme activity in wild type and *lopo*¹. This indicates that there are indeed mitochondria in *lopo*¹ mutants, but they are not able to perform respiration because of the absence of mtDNA. Only a very limited number of cells (Figure 8F) still show

fully functional mitochondria, probably because of the mtDNA remaining from the maternal contribution. Finally, in an ultrastructural analysis we found that, for the most part, mitochondria in *lopo*¹ exhibit normal morphology (Figure 9) and number, although some variation might occur among different tissues. No changes at all are seen in the CNS, whereas an increase in mitochondria can be seen in the midgut (Figure 9), where we observed some cells in which a large number of aberrant mitochondria appeared to be in various stages of disintegration or engulfment by lysosomes. These cells are most likely apoptotic.

DISCUSSION

The isolation of a new mutant in the mtSSB gene allowed us to study the function of mtSSB in the context of the complex process of mitochondrial biogenesis in higher eukaryotes. Rather surprisingly, *lopo*¹ is a late third instar larval-lethal mutation, despite the fact that mtSSB is thought to be an essential mitochondrial protein. The late larval lethality can be explained by a strong maternal contribution, which was identified through an immunocytochemical analysis of adult ovaries. This maternal source of mtSSB appears to be sufficient to allow development through embryogenesis, where mtDNA replication begins 10–12 h after egg laying (Rubenstein *et al.*, 1977). However, the maternal source of mtSSB is insufficient to allow the larvae to develop through metamorphosis, where extensive cell proliferation is required and, indeed as shown by BUdR labeling, cell proliferation is disturbed drastically and appears to be the major cause of lethality. This is consistent with the immunocytochemical experiments in wild-type larvae, where we showed that, in addition to a constitutive level of mtSSB protein in all tissues, there is a high level of expression of mtSSB in proliferating cells, especially in imaginal discs, in the CNS and in the gut. The proliferation defect also explains the aberrant development of the visual system in *lopo*¹ (Maier, Poeck, and Schneuwly, unpublished results), a phenotype that is very

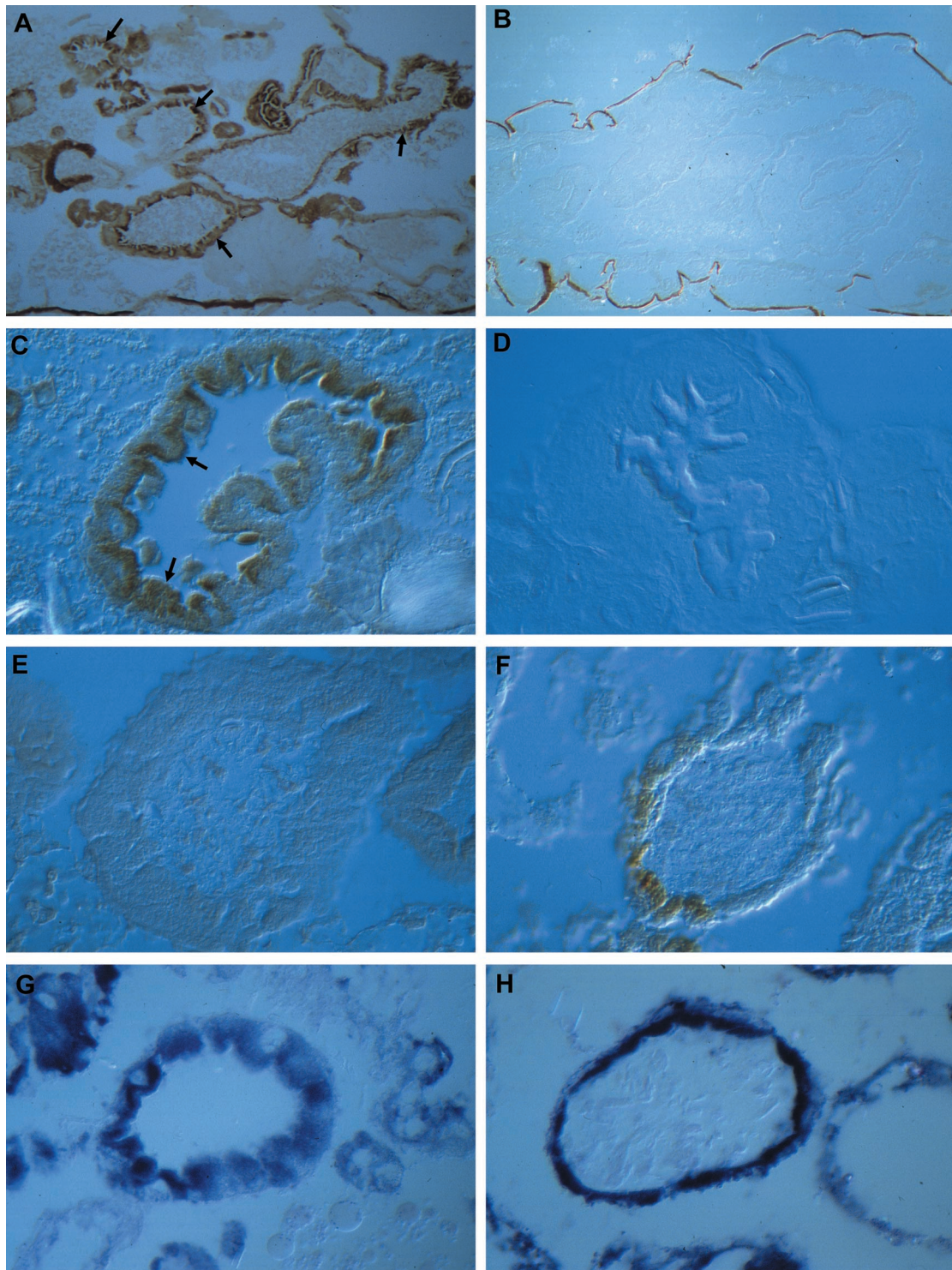


Figure 8. Mitochondrial histochemistry on third instar larvae. (A–F) Histochemical staining for COX. (A) Wild-type larvae: note the strong staining in all parts of the digestive tract (arrows). (B) Similar plane of section from *lopo*¹. No staining is detected. (C) Cross-section through the midgut. COX staining is enriched highly in endodermal cells facing the gut lumen (arrows). (D) Control of staining specificity. No COX staining is visible in the presence of KCN. (E and F) Cross-sections through the midgut of *lopo*¹. No COX staining is visible in E but partial staining can be detected in some cells of the midgut (F). (G and H) SDH staining on cross-sections through the midgut of wild-type (G) and *lopo*¹ (H) larvae. Both strains show equally strong staining.

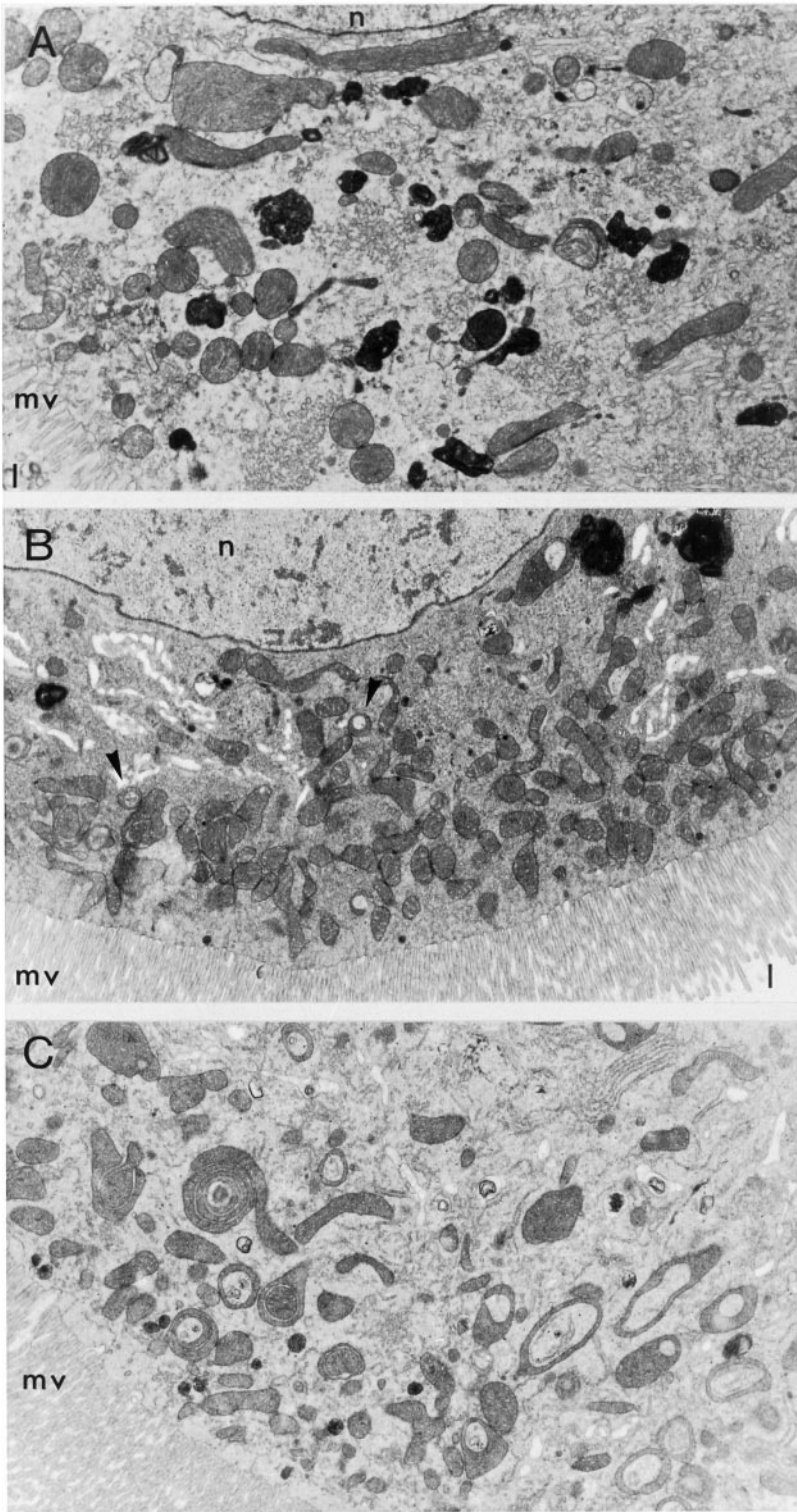


Figure 9. Ultrastructure of mitochondria in *lopo*¹. Third instar larval midgut tissue from wild-type (A) and *lopo*¹ (B and C) is shown. *lopo*¹ mutants show at least equal numbers of mitochondria as compared with wild type. The morphology of mutant mitochondria is normal (B) except for a few degenerating mitochondria (arrows). However, some cells (C) show an extremely high number of degenerating mitochondria. L, gut lumen; N, nucleus; mv, microvilli.

similar to that of the recently isolated mutant *tamas*, which encodes the catalytic subunit of mtDNA polymerase (Iyengar *et al.*, 1999). This highlights the functional interactions of

mtSSB and pol γ that have been demonstrated in biochemical studies (Farr *et al.*, 1999). Biochemical analysis of *Drosophila* mtSSB and the analysis of a yeast mtSSB mutant (van

Dyck *et al.*, 1992) document the important role of mtSSB in mtDNA replication and maintenance. Our study of *lopo*¹ demonstrates that, in the absence of mtSSB, severe mtDNA depletion occurs and, as a consequence, the respiration of mitochondria as measured by oxygen consumption is reduced drastically to <10%. Thus, the third instar larvae appear to survive nearly anaerobically during the last days of this developmental phase.

One of the fundamental problems in mitochondrial biogenesis is the regulation of the number of mitochondria per cell and the mtDNA copy number per mitochondrion. To examine this in *lopo*¹, a detailed histochemical and electron microscopic analysis was performed. Our results show that the number and the ultrastructural morphology of mitochondria are not altered in *lopo*¹ mutants, although we found that the majority of mitochondria have lost respiratory function due to the absence of mtDNA and hence functional COX. However, there are tissue-specific differences; at least in the midgut of third instar larvae, we can see alterations both in morphology and in number, whereas no changes can be detected in the CNS. However, one of the major causes of altered mitochondrial morphology might be the status of these cells, which undergo cell death. These results would exclude mtSSB as a major determinant in controlling mitochondrial biogenesis and indicate no link between mtDNA synthesis or copy number and mitochondrial biogenesis. In contrast, we have shown recently that mtSSB gene expression provides a link between nuclear and mtDNA replication in *Drosophila*: the mtSSB gene is regulated coordinately with a number of nuclear genes encoding key proteins in nuclear DNA replication (Ruiz de Mena *et al.*, 2000).

Interestingly, whereas no phenotypic or molecular defects were observed in *lopo*¹ heterozygotes, mitochondrial transcription factor A mutants show a strong dose dependence, which leads to reduced numbers of functional mitochondria in heterozygotes (Larsson *et al.*, 1998). This difference is potentially important, and both mutants may now be used as model systems to study mtDNA replication and maintenance. Furthermore, the availability of *lopo*¹, together with the potential for a combined approach of molecular, biochemical, genetic, and developmental analyses in *Drosophila*, should allow a thorough evaluation of mtSSB function in vivo.

ACKNOWLEDGMENTS

We thank P. Deak (University of Cambridge, Cambridge, UK) for the fly stock 46-13, P. Toliás (International Center for Public Health, Newark, NJ) for the *lopo* cDNA, M. Młodzik (EMBL, Heidelberg, Germany) for the P1-phage, J. Stangelmayer (University of Regensburg, Regensburg, Germany) for providing the oxygen electrodes and advice regarding measurements, Ingolf Koch for the in situ localization of the P-element insertion on chromosomes, and R. Garesse (Consejo Superior de Investigaciones Científicas-Universidad Autónoma, Madrid, Spain) for the mtDNA probe. Also many thanks to U. Roth and G. Thiess for excellent technical assistance. This work was supported by funding from the DFG (to S.S.) and the National Institutes of Health (grants GM45295 and HL59656; to L.S.K.).

REFERENCES

- Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 7, 248–254.
- Deák, P., Omar, M.M., Saunders, R.D.C., Pál, M., Komonyi, O., Szidonya, J., Maróy, P., Zhang, Y., Ashburner, M., Benos, P., Savakis, Ch., Siden-Kiamos, I., Louis, Ch., Bolshakov, V.N., Kafatos, F.C., Madueno, E., Modolell, J., and Glover, D.M. (1997). P-element insertion alleles of essential genes on the third chromosome of *Drosophila melanogaster*: correlation of physical and cytogenetic maps in chromosomal region 86E–87F. *Genetics* 147, 1697–1722.
- Farr, C.L., Wang, Y., and Kaguni, L.S. (1999). Functional interactions of mitochondrial DNA polymerase and single-stranded DNA-binding protein: template-primer DNA binding and initiation and elongation of DNA strand synthesis. *J. Biol. Chem.* 274, 14779–14785.
- Garesse, R. (1988). *Drosophila melanogaster* mitochondrial DNA: gene organization and evolutionary considerations. *Genetics* 118, 649–663.
- Grossman, L.I., and Shoubridge, E.A. (1996). Mitochondrial genetics and human disease. *Bioessays* 18, 983–991.
- Hartl, D.L., Nurminsky, D.I., Jones, R.W., and Lozovskaya, E.R. (1994). Genome structure and evolution in *Drosophila*: applications of the framework P1 map. *Proc. Natl. Acad. Sci. USA* 91, 6824–6829.
- Hoke, G.D., Pavco, P.A., Ledwith, B.J., and Van Tuyle, G.C. (1990). Structural and functional studies of the rat mitochondrial single strand DNA binding protein P16. *Arch. Biochem. Biophys.* 282, 116–124.
- Iyengar, B., Roote, J., and Campos, A.R. (1999). The *tamas* gene, identified as a mutation that disrupts larval behavior in *Drosophila melanogaster*, codes for the mitochondrial DNA polymerase catalytic subunit (DNApol- γ 125). *Genetics* 153, 1809–1824.
- Kretschmar, D., Brunner, A., Wiersdorff, V., Pflugfelder, G.O., Heisenberg, M., and Schneuwly, S. (1992). Giant lens, a gene involved in cell determination and axon guidance in the visual system of *Drosophila melanogaster*. *EMBO J.* 11, 2531–2539.
- Lahaye, A., Leterme, S., and Foury, F. (1993). PIF1 DNA helicase from *Saccharomyces cerevisiae*: biochemical characterization of the enzyme. *J. Biol. Chem.* 268, 155–161.
- Larsson, N.G., and Clayton, D.A. (1995). Molecular genetic aspects of human mitochondrial disorders. *Annu. Rev. Genet.* 29, 151–178.
- Larsson, N.-G., Wang, J., Wilhelmsson, H., Oldfors, A., Rustin, P., Lewandoski, M., Barsh, G.S., and Clayton, D.A. (1998). Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. *Nat. Genet.* 18, 231–236.
- Li, K., and Williams, R.S. (1997). Tetramerization and single-stranded DNA binding properties of native and mutated forms of murine mitochondrial single-stranded DNA-binding proteins. *J. Biol. Chem.* 272, 8686–8694.
- Lojda, Z., Gossrau, R., and Schiebler, T.A. (1979). *Enzyme histochemistry. A laboratory manual.* Berlin, Heidelberg, New York: Springer-Verlag.
- Mignotte, B., Marsault, J., and Barat-Gueride, M. (1988). Effects of the *Xenopus laevis* mitochondrial single-stranded DNA-binding protein on the activity of DNA polymerase gamma. *Eur. J. Biochem.* 174, 479–484.
- Pavco, P.A., and Van Tuyle, G.C. (1985). Purification and general properties of the DNA-binding protein (P16) from rat liver mitochondria. *J. Cell Biol.* 100, 258–264.
- Pflugfelder, G.O., Schwarz, H., Roth, H., Poeck, B., Sigl, A., Kersch, S., Jonschker, B., Pak, W.L., and Heisenberg, M. (1990). Ge-

- netic and molecular characterization of the optomotor-blind gene locus in *Drosophila melanogaster*. *Genetics* 126, 91–104.
- Preininger, C., Klimant, I., and Wolfbeis, O.S. (1994). Optical fiber sensor for biological oxygen demand. *Anal. Chem.* 88, 1841–1846.
- Reynolds, E.S. (1963). The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J. Cell Biol.* 17, 202–212.
- Rubenstein, J.L., Brutlag, D., and Clayton, D.A. (1977). The mitochondrial DNA of *Drosophila melanogaster* exists in two distinct and stable superhelical forms. *Cell* 12, 471–482.
- Ruiz de Mena, I., Lefai, E., Garesse, R., and Kaguni, L.S. (2000). Regulation of mitochondrial single-stranded DNA-binding protein gene expression links nuclear and mitochondrial DNA replication in *Drosophila*. *J. Biol. Chem.* 275: 13628–13636.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). *Molecular Cloning, A Laboratory Manual*, 2nd ed., Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Schatz, G. (1996). The protein import system of mitochondria. *J. Biol. Chem.* 271, 31763–31766.
- Sedman, T., Kuusk, S., Kivi, S., and Sedman, J. (2000). A DNA helicase required for maintenance of the functional mitochondrial genome in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 20, 1816–1824.
- Shadel, G.S., and Clayton, D.A. (1997). Mitochondrial DNA maintenance in vertebrates. *Annu. Rev. Biochem.* 66, 409–435.
- Stroubakis, N.D., Li, Z., and Toliás, P.P. (1994). RNA- and single-stranded DNA-binding (SSB) proteins expressed during *Drosophila melanogaster* oogenesis: a homolog of bacterial and eukaryotic mitochondrial SSBs. *Gene* 143, 171–177.
- Thömmes, P., Farr, C.L., Marton, R.F., Kaguni, L.S., and Cotterill, S. (1995). Mitochondrial single-stranded DNA-binding protein from *Drosophila* embryos: physical and biochemical characterization. *J. Biol. Chem.* 270, 21137–21143.
- Tiranti, V., Rocchi, M., DiDonato, S., and Zeviani, M. (1993). Cloning of human and rat cDNAs encoding the mitochondrial single-stranded DNA-binding protein (SSB). *Gene* 126, 219–225.
- Truman, J.W., and Bate, M. (1988). Spatial and temporal patterns of neurogenesis in the central nervous system of *Drosophila melanogaster*. *Dev. Biol.* 125, 145–157.
- van Dyck, E., Foury, F., Stillman, B., and Brill, S.J. (1992). A single-stranded DNA binding protein required for mitochondrial DNA replication in *S. cerevisiae* is homologous to *E. coli* SSB. *EMBO J.* 11, 3421–3430.
- Van Tuyle, G.C., and Pavco, P.A. (1985). The rat liver mitochondrial DNA-protein complex: displaced single strands of replicative intermediates are protein coated. *J. Cell Biol.* 100, 251–257.
- Wernette, C.M., and Kaguni, L.S. (1986). A mitochondrial DNA polymerase from embryos of *Drosophila melanogaster*: purification, subunit structure, and partial characterization. *J. Biol. Chem.* 261, 14764–14770.
- Williams, A.J., and Kaguni, L.S. (1994). Stimulation of *Drosophila* mitochondrial DNA polymerase by single-stranded DNA-binding protein. *J. Biol. Chem.* 270, 860–865.
- Wilson, C., Kurth-Pearson, R., Bellen, H.J., O’Kane, C.J., Grossniklaus, U., and Gehring, W.J. (1989). P-element-mediated enhancer detection: an efficient method for isolating and characterizing developmentally regulated genes in *Drosophila*. *Genes Dev.* 3, 1301–1313.
- Zeviani, M., Petruzzella, V., and Carrozzo, R. (1997). Disorders of nuclear-mitochondrial intergenomic signaling. *J. Bioenerg. Biomembr.* 29, 121–130.