Biochemical and Molecular Consequences of Massive Mitochondrial Gene Loss in Different Tissues of a Mutant Strain of *Drosophila subobscura*

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In the studied mutant strain of *Drosophila subobscura*, 78% of the mitochondrial genomes lost >30% of the coding region by deletion. The mutations were genetically stable. Despite this massive loss of mitochondrial genes, the mutant did not seem to be affected. Distribution of the two genome types, cell levels of mitochondrial DNA, steady-state concentrations of the mitochondrial gene transcripts, mitochondrial enzymatic activities, and ATP synthesis capacities were measured in the head, thorax, and abdomen fractions of the mutant strain in comparison with a wild type strain. Results indicate that the deleted genomes are detected in all fractions but to a lesser extent in the male and female abdomen. In all fractions, there is a 50% increase in cellular mitochondrial DNA content. Although there is a decrease in steady-state concentrations of mitochondrial transcripts of genes affected by deletion, this is smaller than expected. The variations in mitochondrial biochemical activities in the different fractions of the wild strain are upheld in the mutant strain. Activity of complex I (involved in mutation) nevertheless shows a decrease in all fractions; activity of complex III (like-wise involved) shows little or no change; finally, mitochondrial ATP synthesis capacity is identical to that observed in the wild strain. This latter finding possibly accounts for the lack of phenotype. This mutant is a good model for studying mitochondrial genome alterations and the role of the nuclear genome in these phenomena.

Various alterations in the mitochondrial genome have been correlated with severe human pathology (1–3). Among the most substantial of these alterations, deletions, which account for a small fraction of the described cases, have always been detected at the heteroplasmic state (4–6). Once the proportion of mtDNA cell content (per nuclear genome) showed a 50% increase in the mutant strain compared with the *D. subobscura* wild strain. Determinations of steady-state concentrations of mitochondrial transcripts showed these to be identical to values measured for transcripts of genes unaffected by deletion (12 S, COIII). On the other hand, the transcript concentrations of the concerned genes showed a decrease, although this was less than expected and differed according to the genes (from 55% for ND1 to 35% for cytochrome b). Furthermore, detection of the fusion transcript (13) showed that both types of genomes were expressed. Maximum respiratory complex activities were identical in the wild type and mutant strains for complex IV. They were, respectively, 30% and 40% lower for complexes III and I (14). Nevertheless, these differences were not seen for complex III in our experimental conditions for respiratory chain observation (oxygraphy), whereas complex I consistently presented a 30% decrease in activity. The respiratory chain would thus seem to be operative as of complex III.

No ultra structural mitochondrial alteration was observed irrespective of the studied tissues. Moreover, cytochrome oxidase activity was detectable in all mitochondria. Heteroplasmy was probably intramitochondrial (15, 16), since both types of genomes coexisted in each mitochondria.

Overall, these results tend to indicate compensation for the consequences of deletion at the level of the whole fly. One possible explanation for this surprising apparent innocuousness of the mutation is that compensation may exist between the various tissues or certain (vital) tissues may be less affected than others. The results presented in this article were obtained from measurements performed in mitochondrially enriched fractions of various tissues with high energy requirements. Whereas studies described in man show that distribution of

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1 The abbreviations used are: mtDNA, mitochondrial DNA; W, wild strain; H, mutant strain; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]glycine; α-GP, α-glycerophosphate; CO, cytochrome oxidase; ND, NADH dehydrogenase.
deleted molecules and effects of deletion may differ substantially from one tissue to another (17), this is not so for the mutant D. subobscura strain we studied, in which biochemical specificities of the various tissues are upheld.

**EXPERIMENTAL PROCEDURES**

**Strains**

The studied flies belong to the D. subobscura strain and were raised on medium standard cornmeal as described (12) at 19 °C. The wild strain (W) acts as a control to study the effects of deletion on the mutant strain (H).

**Preparation of the Different Tissues**

The various studied fractions were enriched with a given mitochondrial type: the mitochondria of the nerve tissue for the head, those of the muscle tissue for the thorax, and those of male or female genital organs for the male or female abdomens. The flies were frozen for molecular biology or anaesthetized in ice for biochemistry before dissection of the three parts (head, thorax, abdomen). The obtained fractions were used as quickly as possible for extraction of nucleic acids or mitochondria.

**Total DNA Preparation**

Total DNA was obtained from 50–60 heads, thoraces, or abdomens according to the method described by F. Beziet al. (13).

**Total RNA Extraction**

50–60 heads, thoraces, or abdomens were ground in 1 ml of RNA-ZOL (Bioprobe system) at 4 °C. 0.1 volume of chloroform, isomyl alcohol (24.1, v/v) was added. After stirring and incubation (5 min at 4 °C), the aqueous and organic layers were separated by centrifugation at 12,000 × g, 15 min, 4 °C. The RNA was precipitated with a volume of isopropyl alcohol.

**Measurement of Heteroplasmy**

Heteroplasmy was determined by Southern blotting of DNA fragments obtained after digesting DNA withMspI and hybridization with the COII probe labeled as described (13). The signals were analyzed by densitometry.

**Measurement of Mitochondrial DNA Content**

Mitochondrial DNA content was measured by comparison with nuclear DNA by slot blot of the various DNA fractions followed by hybridization with a mitochondrial probe (12 S RNA) and a nuclear probe (18 S). The signals were analyzed by densitometry, as was heteroplasmy.

**Measurement of RNA Content**

RNA content was estimated by the Northern technique using 18 S RNA as the control RNA. The studied RNAs are 12 S RNA and COIII (genes not involved in deletion), cytochrome b, and ND1, ND5, ND4-4L (genes involved in deletion), using the probes described in Ref. 13.

**Isolation of Mitochondria**

The mitochondria were isolated by differential centrifugations of ground tissue samples in buffer containing 0.22 M sucrose, 0.12 M mannitol, 1 mM EDTA, 10 mM Tricine, pH 7.6 (18). Protein determination was performed with Bio-Rad reagent using the method of Bradford method (19).

For enzymatic assays, mitochondria (about 1 mg protein/ml) were sonicated for 6 s at 4 °C then frozen and unfrozen except for α-glycerophosphate dehydrogenase and ATP synthesis determinations.

**Measurement of Biochemical Activities**

All activities were measured at 28 °C and expressed in nmol/min·mg⁻¹.

**Complex I (NADH-ubiquinone Reductase)**

NADH oxidation was monitored at 340 nm in a pH 7.2 buffer containing 35 mM NaH₂PO₄, 5 mM MgCl₂, and 2.5 mg/ml bovine serum albumin in the presence of 2 mM KCN, 2 μg/ml rotenone, 15 μM ubiquinol, 15 μM cytochrome c, and 5 μM of mitochondrial proteins. The nonenzymatic reduction of cytochrome c with ubiquinol (in the absence of mitochondria) was deduced from this measurement.

**Complex II (Ubiquinol-Cytochrome c Reductase)**

Ubiquinol was obtained by reduction of ubiquinone with sodium dithionite followed by extraction with cyclohexane. After evaporation of the cyclohexane, ubiquinol was dissolved in ethyl alcohol and stabilized with 10 mM HCl. To measure the activity of complex II, cytochrome c reduction was monitored at 550 nm (ε = 18,500 M⁻¹·cm⁻¹) in 1 ml of pH 7.2 buffer containing 35 mM NaH₂PO₄, 1 mM KH₂PO₄, 1 mM EDTA, 56 μM cytochrome c, 5 μM of mitochondrial proteins.

**Citrate Synthetase**

The activity of citric acid cycle enzyme citrate synthetase was measured.

**Measurement of ATP Synthesis**

Mitochondrial ATP synthesis was measured using a technique derived from that described by Libby et al. (20) based on the luminescence of luciferin in the presence of luciferase, which is proportional to the ATP concentration in the test medium (21). In the presence of ATP, the photon flux emitted by the reagent (BioOrbit) remains constant for several minutes for a determined ATP concentration. Synthesis kinetics can thus be directly monitored with a BioOrbit 1251 lumimeter. Kinetics were recorded at 25 °C, pH 7.5, with 50 μM of the incubation medium containing 0.15 mM ATP and 1 μM of mitochondrial proteins. After 2 min of incubation, 5 μl of substrate and 100 μl of reagent were added simultaneously, and fluorescence was monitored for 1 min. The final concentrations of substrates were 5 mM glutamate + 2 mM malate, 15 mM proline + 2 mM malate, 1 mM palmityl carnitine + 2 mM malate, or 10 mM α-glycerophosphate. Calibration was performed at the end of each measurement by the addition of 200 pmol of ATP. A control run in the presence of oligomycin allows ATP synthesis imputable to intermembranar adenylate kinase activity to be subtracted from the result.

**Analysis of Results**

Results are compared for the various tissues of a single strain, then for tissues of the two strains (W and H). The values thus determined are considered significantly different when the statistical study (Student's t test) yields a value of p < 0.05.

**RESULTS**

**The Different Fractions**

Three fractions are readily accessible in the Drosophila. One tissue is particularly well represented in each of these three fractions. Hence, mitochondria from this tissue constitute the dominant population: the head, where the mitochondrial pellet is enriched with nerve tissue mitochondria; the thorax, with muscle mitochondria, particularly from flight muscles; and the abdomen, with mitochondria from the germ line, particularly in the abdomens of pubescent females. These samples thus allow evaluation of the characteristics of mitochondria from different tissues, all of which have high energy demands but different biochemical characteristics. The possible influence of mutation is studied on these fractions; thus, on mitochondria from different tissues.

**Measurement of Heteroplasmy and mtDNA Content**

Distribution of Heteroplasmy in the Different Fractions (Fig. 1)—Relative proportions of the two types of mitochondrial genomes are determined after incubation of total DNA with the
MspI enzyme followed by hybridization with the COIII probe as described above (13). Heteroplasmy measured in the fractions from heads and thoraces (containing somatic tissues), 74 ± 5% and 79 ± 6%, respectively, is not significantly different ($p < 0.05$) from that measured in the whole fly (78 ± 5%). On the other hand, there is a significantly lower percentage of heteroplasmy in the abdomens (containing the germ cells): 71 ± 5% in males, 63 ± 5% in females. Moreover, there is a significant difference in values for male and female abdomens.

**Measurement of mtDNA Content**—Cellular mtDNA content extracted from the various fractions of the wild and mutant strains is compared with nuclear DNA content as described previously (13) using two types of probes: a mitochondrial probe (12 S) and a nuclear probe (18 S). These cellular contents are compared for different tissues from a given strain and for each tissue, comparing the two strains.

To facilitate comparisons between the different fractions of the wild type strain, relative quantities of cellular mtDNA in the thorax and abdomen (Fig. 2) are evaluated by comparison with the content measured in the head (base 100 for the head). The cellular mtDNA content in the thorax is identical to that measured in the male abdomen and exceeds the content measured in the head by 50%. The mtDNA content is higher in the female abdomen than in the other fractions: × 3 compared with the head.

Cellular mitochondrial DNA content is thus lower in the head fraction. The difference observed between the head and thorax corroborates electron microscopy findings, where mitochondria are seen to be much more abundant in the thorax, or measurement of citrate synthetase activity, which is 2.5 times higher in the thorax than in the head. Very high levels are observed in female abdomens where maturing ova are prevalent. The same distribution of cellular mtDNA levels in the mutant strain for the various fractions is encountered (Fig. 2).

**Fig. 1.** Mitochondrial genome maps and heteroplasmy. Panel A, mitochondrial genome maps of wild type and deleted molecules. Arrows indicate the location of the COIII probe used. Filled circles atop short vertical lines, $\text{MspI}$ sites. kb, kilobases; Cyto b, cytochrome b. Panel B, Southern blot analysis of the mitochondrial genome. 10 μg of total DNA extracted from heads (1), thorax (2), male abdomen (3), and female abdomen (4). Whole flies of mutant strain (5) and wild type flies (6) were digested by $\text{MspI}$, electrophoresed on 1% agarose gel, blotted on membrane, and hybridized with a COIII probe. C, size marker. Panel C, percentage of deleted molecules in the different fractions of the mutant strain. The values represent the mean of eight measurements. Abdo, abdomen.
strains. Compared with the wild strain, the mtDNA/cell content in the mutant strain shows a 43–75% increase (respectively, 75 ± 3, 72 ± 3, 43 ± 4, and 62 ± 4 for head, thorax, male and female abdomens). However, these differences do not significantly differ from results obtained for the whole fly (50% increase; Ref. 13).

For each fraction from the mutant strain, the relative percentage of intact and deleted molecules compared with the wild strain was calculated taking two parameters into account: increased cell content and heteroplasmy (the mtDNA content in each tissue of the wild strain is 100%, Fig. 3). The percentages of intact molecules are identical in the head and thorax fractions (36%) and comparable to those measured in the whole fly (30%). The percentage is significantly higher in the male abdomen (44.5%). The female abdomen contains 54% of intact molecules. Hence, the female abdomen apparently constitutes the fraction least affected by mutation.

**Measurement of Relative Concentrations of Mitochondrial Transcripts**

Total RNA extracted from the different fractions of the mutant and wild strains (see “Experimental Procedures”) is analyzed by Northern blot and hybridization with different probes. The results presented are obtained from six different extractions and Northern blot analyses. The fact that different probes with different specific activities were used makes it very difficult to exactly estimate steady-state concentrations for each transcript in the various fractions of the two strains. Steady-state concentrations are thus determined by comparing hybridization signals obtained with a single probe for transcripts extracted from head and thorax fractions and from male and female abdomens in the two strains. A control of RNA extraction and hybridization is obtained with the 18 S probe. The Rf ratios express hybridization signal ratios (corrected with 18 S) for each transcript in the various fractions of a given strain.

The same membranes were used to compare the different transcript concentrations in each fraction between mutant and wild type. The Rs ratios express hybridization signal ratios (corrected with 18 S) for each transcript in the various fractions for the two strains.

**Comparison of Transcript Concentrations in the Wild Strain**

(Fig. 4)—Head and thorax concentrations of the 12 S transcripts are identical (Rf = 1.1 ± 0.5). The COIII transcript shows a lower concentration (p < 0.05) in the head (Rf = 0.7 ± 0.2). Transcripts of complex I genes constitute identical quantities in both tissues (Rf ND1 = 0.9 ± 0.3, ND4-4L = 0.9 ± 0.2, ND5 = 1 ± 0.5). On the other hand, the concentration for
cytochrome b transcript is much higher ($p < 0.05$) in the head ($r = 3.3 \pm 1.2$).

Male and female abdomen comparison gives results similar to those described for head and thorax except for cytochrome b transcript, which is in identical concentration in both abdomens ($Rf = 0.7 \pm 0.4$, $p > 0.05$).

Comparisons of Transcript Concentrations in the Mutant Strain (Fig. 4)—Results indicate that the $Rf$ obtained for transcripts 12 $S$, ND1, and cytochrome b (head/thorax or male/ female abdomens) are identical to those obtained for the wild type. In the head and thorax, the COIII transcripts are in identical concentration ($Rf = 1.4 \pm 0.4$; the difference is not significant). ND4-4L and ND5 transcripts ($Rf = 1.3 \pm 0.3$ and $Rf = 1.5 \pm 0.1$) show levels in male abdomens to be significantly higher than those observed in female abdomens ($p < 0.05$). The most remarkable finding is the fact that the head/thorax $Rf$ for the cytochrome b transcript is very high ($Rf = 2.6 \pm 1.3$), as in the wild strain. This tissue specificity was thus unaffected by the mutation involving this gene.

Comparisons of the Transcript Concentrations in the Different Fractions of the Two Strains ($Rs = Rf \text{ H}/Rf \text{ W}$) (Fig. 5)—Results indicate that the concentrations of gene transcripts (12 $S$, COIII) unaffected by deletion are lower in the head (20–30%); COIII transcript concentration also decrease in thorax. In contrast, they are higher (20–30%) in the abdomens of male or female.

Concentrations of gene transcripts affected by mutation are lower in each fraction. Those of complex I decreased by 70% in the head and thorax. The decrease is less important in the abdomens (50% in male, 10–50% in female). The cytochrome b transcript concentration shows a 50% decrease in the different fractions.

In each fraction, the mutation has affected the transcript concentrations of the genes involved by the deletion, but as observed in the whole fly, the observed decreases are very often not directly proportional to the quantities of intact genomes.

**Fusion Transcript**—The ND1–ND5 fusion transcript (1300 base pairs) was detected in RNA extractions from the whole fly, where its relative concentration was very similar to that of ND1 (13). This transcript is detected in all fractions (results not show), indicating an expression of the deleted genomes in all tissue. The concentration in all fractions is not significantly different from that of the ND1 transcript.

**Mitochondrial Biochemistry**

Mutation affects genes implicated in respiratory chain function. Biochemical activities of respiratory complexes I, III, and IV, certain subunits of which are coded by the mitochondrial genome, were tested in various fractions of the wild and mutant strains.

The activity of citrate synthetase, an enzyme of the Krebs cycle of nuclear origin, was also measured. Enzymatic systems known to be active in insect mitochondria (28) and which possibly play a role in electron transfers toward respiratory chain complexes such as the $\alpha$-glycerophosphate dehydrogenase and $\alpha$-glycerophosphate oxidase couple, were likewise measured. Finally, ATP synthesis capacity by isolated mitochondria was evaluated.

Comparison of Biochemical Activities of the Different Fractions in the Wild Strain (Fig. 6)—Activity of NADH dehydrogenase (complex I) by comparison with the head is lower in the abdomen and higher in the thorax. Activity of complex III is higher in the head than in the other fractions: three times the activity measured in the abdomen fractions (the difference between the male and female abdomen is not significant) and five times the measured activity in the thorax. In the head fraction, this activity is superior to that of complex I ($\times 8$) and complex IV ($\times 2$). Such activity in mitochondria of the head fraction correlated with the high concentration of Cyto b transcript theoretically corresponds to higher demand on this complex and a different respiratory metabolism ensuring direct electron supply to ubiquinone and to complex III.

Complex IV activity shows no significant differences for the
studied fractions. This is the highest activity of the respiratory chain: 7–9 times the activity of complex I in the various fractions, except in the head where complex III is dominant.

The activity of citrate synthetase is identical in the head, the male abdomen, and the female abdomen. On the other hand, it is very high (× 2.5) in the thorax, showing a much greater Krebs cycle potentiality in the muscles, which are highly solicited in flight (28).

Two enzymes, the α-glycerophosphate dehydrogenase cytosolic enzyme and α-glycerophosphate oxidase, located on the outer surface of the internal membrane, allow direct reduction of ubiquinone through cytosolic redox potential (α-GP shunt). Activities of these enzymes are clearly higher in the thorax: thoracic α-glycerophosphate oxidase is 3 times more active than α-glycerophosphate oxidase in the head or male abdomen (p < 0.001), and thoracic α-glycerophosphate dehydrogenase is 4–5 times higher than α-glycerophosphate dehydrogenase in these fractions. Activity of these enzymes is low in the female abdomen. Male and female activities are significantly different (p < 0.05). This metabolic shunt, which plays a major role in insects (28), should be more effective in the muscles, since α-GP is a substrate rapidly used for flight. Hence, mitochondrial metabolism in the fly shows tissular specificity: enzymatic capacities vary and logically reflect different metabolic flux for the different tissues.

Comparison of Biochemical Activities in Fractions of the Mutant Strain—Comparison of activities in the various studied fractions leads to the same conclusions as above. Mutation did not modify tissular specificities of the studied metabolisms; hence, normal metabolic flux was theoretically upheld.

Comparison of Biochemical Activity of the Two Strains—We compared activity for each fraction of the mutant strain with that of the corresponding wild strain: Ra (activity H/activity W) is shown in Fig. 7.

Activity of complex I clearly decreases in all fractions: Ra = 0.5 in the head, 0.6 in the thorax, and 0.7 in the abdomen. In the whole fly (Ra = 0.6), this activity decreases as observed in the thorax. The smallest decrease in activity, in the abdomen, is probably attributable to the fact that heteroplasmy is only 62% in females and 71% in males (but the differences between the male and female abdomen is not significant).

Activity of the complex III is not affected in the head and male abdomen but drops in the thorax (Ra = 0.8) and in the female abdomen (Ra = 0.7). This decrease is identical to that observed in the whole fly (Ra = 0.8). Hence, mutation does not affect the very important activity of this complex in the head.

There is no significant difference in complex IV activity for the two strains irrespective of the studied fraction. Results thus bear out those observed for the whole fly (14): mutations affecting complex I and complex III activities do not affect complex IV activity. This indicates that in the mutant strain, the protein synthesis in all mitochondria is normal, as observed previously (15).

Activity of citrate synthetase is identical in the two strains for the head and thorax but is higher in the mutant abdomens (p < 0.05). This possibly indicates increased Krebs cycle activity.

The metabolic α-GP shunt seems to best account for compensation for the decrease in respiratory chain activity, since it allows functioning of the latter from complex III, which is less affected by mutation. In fact, α-glycerophosphate oxidase and α-glycerophosphate dehydrogenase activities are not modified in the head, thorax, and female abdomen (nor in the whole fly), yet they increase (Ra = 1.4) in the male abdomen.

Mutations lead to an important (but non-uniform) decrease of complex I activity in each fraction. Complex III activity is less or not affected. Hence, mutation seems to have different effects on studied activity as a function of the observed fractions. This phenomenon is doubtless related to the different metabolisms. Nevertheless, as was seen for the whole fly, decreases in activity are surprisingly limited compared with the massive loss of mitochondrial genes caused by deletion.

ATP Synthesis Capacity

ATP synthesis kinetics are measured in isolated mitochondria incubated in the presence of ADP and various substrates such as the glutamate-malate couple, which is characteristic of direct electron supply to complex I of the respiratory chain, or palmityl carnitine, which is used to represent mitochondrial capacity to employ fatty acids as a source of energy. At least some substrates used are characteristic for insect metabolism during flight, e.g., proline in the presence of pyruvate and α-GP. Their oxidation does not require the participation of complex I electron carriers to reduce ubiquinone; proline dehydrogenase is a flavoprotein localized as α-glycerophosphate oxidase on the outer surface of inner membrane, which ensures direct electron supply to ubiquinone.

Comparison of Substrate Utilization by the Various Fractions for ATP Synthesis in the Wild Strain (Fig. 8)—Results show that the synthesis capacities change with the different substrates and with the different fractions. In mitochondria isolated from the thorax, the highest synthesis is measured in the presence of α-GP. This value is 4.8 times that observed with the proline + pyruvate couple. For the other substrates, particularly palmityl carnitine and the glutamate + malate couple, the ATP synthesis rate is low and less than that observed in the other tissues. Mitochondria from the head show less efficient use of α-GP. The highest ATP synthesis capacities in this fraction are obtained in the presence of substrates glutamate + malate and pyruvate + proline. Fatty acids are better used in the head than in the thorax, but the capacity with this substrate is not very important despite the high activity of complex III. Abdominal mitochondria show good ATP synthesis capacity with all tested substrates, particularly mitochondria from the female abdomen. The highest rate is obtained with the glutamate + malate couple. Moreover, the high synthesis rate in the presence of palmityl carnitine corresponds to more active breakdown of fatty acids by abdominal mitochondria than by those of the other tissues.

ATP Synthesis Capacities in the Different Fractions of the
FIG. 8. ATP synthesis capacity in mitochondria of the different fractions of the wild strains (W). The method is described under “Experimental Procedures.” Results are expressed in nmol/min · mg−1 and represent the mean of 10 experiments. Measurements were performed in the presence of 0.15 mM ADP and 5 mM glutamate and represent the mean of 10 experiments. Measurements were performed in the presence of 0.15 mM ADP and 5 mM glutamate, 2 mM malate, 15 mM proline + 5 mM pyruvate, 10 mM α-glycerophosphate, or 1 mM palmityl carnitine + 2 mM malate. a, α.

Mutant Strain—The same experiments performed with mitochondria isolated from these different fractions show that tissue-type specificities for the test substrates are not affected by mutation. α-GP constantly remains the best substrate for mitochondria extracted from the thorax fraction, whereas mitochondria from the head fraction preferentially synthesize ATP with the pyruvate + malate or proline + pyruvate couples. Finally, abdominal mitochondria show a marked preference for glutamate and malate.

Comparison of the Wild and Mutant Strains (Fig. 9)—Results show that in each fraction, the ratio of ATP synthesis in the mutant strain to ATP synthesis in the wild type strain is not significantly different from one with substrate such as α-GP, palmityl carnitine, or glutamate + malate. On the other hand, this ratio is more important in thoracic mitochondria of the mutant with proline + pyruvate (r = 1.9, p < 0.05).

Hence, under our measurement conditions, the mutation did not affect substrate use by the different fractions. If there is a decrease of the maximal activity of the complex I in each fraction and a slight (or not) decrease in that of complex III, it did not cause any drop in ATP synthesis capacity. Consequently, no energy imbalance is encountered in the various fractions of the mutant strain.

**DISCUSSION**

The results presented in this article allow the analysis of different parameters first studied on the whole adult fly (13) and of ATP synthesis capacity in mitochondrially enriched fractions from different tissues endowed with different metabolisms.

Deleted mtDNA molecules are detected in all studied fractions. In mitochondrially enriched fractions of the nervous system (head) or in muscle mitochondria (thorax), i.e. in mitochondria of the somatic tissues, the heteroplasmy level is identical to that measured in the whole fly (about 80%). On the other hand, in fractions enriched with germ cells, namely male or female abdomens, heteroplasmy is slightly lower (respectively, 71% and 63%). The germ line is apparently less rich in deleted mtDNA, as observed previously (15). These results indicate that the deleted molecules are already present in germ cells, as it has been shown in man (29). More precise measurements by **in situ** hybridization on ovaries are currently under way in our laboratory. Heteroplasmy thus increases during development and stabilizes at 80% no latter than imaginal emergence. A preferential replication of the deleted molecules (which undergo faster replication than intact molecules) could explain this increase. Published works (30) tend to counter the hypothesis of preferential replication of deleted molecules. Another possibility is that the mechanism generating deleted molecules is still present and functional. The mechanism generating deleted molecules must nevertheless be very tightly controlled. Indeed, no more than 80% of molecules are ever deleted from emergence of the imagos until death of the flies.

In all studied fractions, cellular mtDNA contents increase compared with those measured in the wild strain, and this increase is evenly distributed, accounting for about 50% no matter what fraction is involved, including female abdomens. As observed previously (13), there is probably no increase in the number of mitochondria per cell. This implies an intramitochondrial increase of mtDNA molecules, which would partially minimize the loss of genes due to deletion. The mechanism thus implemented might constitute a first (but only very partial) “compensation” step in response to the intracellular energy deficit or to an abnormally low redox state.

Steady-state concentrations of transcripts from the mitochondrial genes affected by mutation are lower in all fractions of the mutant strain, as could be expected in light of the massive loss of genes and as indeed has already been observed for the whole fly. However, this decrease is not uniform from one transcript to another and in the different fractions. At least for the cytochrome b transcript, the transcript/gene ratio increases in the mutant strain in comparison with the wild strain. Our determination methods (steady-state concentrations) do not allow discernment of any variations in transcription kinetics or any increases in half-lives. Nevertheless, given the specific transcription system for mitochondrial genes (31),
the degradation kinetics are probably modified. This might be due to increased protection by mitoribosomes, which show identical concentrations in the two strains (15). Detection of the fusion transcript in all studied fractions indicates general expression of the deleted genomes. Nevertheless, the measured concentration is low (similar to ND1 transcript concentration) and not related to representation of the deleted genomes. This may indicate subexpression of these genomes or a lower stability of this transcript; the two fused regions are out of phase, and stop codons are evident as of the first nucleotides beyond the fusion point.

The measurement of enzymatic activity of the respiratory complexes reveals that in the wild strain, the mitochondrial biochemistry is not equivalent in the different fractions. In the head, for example, the activity of complex III is surprisingly high as is the concentration of the cytochrome b transcript and exceeds that of complex IV. In the thorax, substrates such as α-glycerol phosphate, capable of directly reducing ubiquinone, and complex III seem to be more efficient. The mutation does not lead to any metabolic disturbance in the different fractions.

Massive loss of genes coding for subunits of complexes I and III has a significant impact on the complex I activity in the various fractions with less effect on complex III. However, the decreases are smaller than expected given the various levels of heteroplasmy. Hence, there is probably over-translation of the transcripts leading to possible protection of these transcripts by the mitoribosomes.

Complex IV, which is not concerned by mutation, shows high, generally identical activity in all tissues. There is no significant difference between the two strains. This indicates that loss of 80% of the genes coding for four tRNAs, essential for mitochondrial protein synthesis, does not affect this activity. Studies we have carried out using cytochemistry (15) or hybridization in situ (16) indicate that heteroplasmy is probably intramitochondrial (coexistence within each mitochondrion of two types of genomes) and that “functional complementation” may occur between the wild and deleted genomes.

The most important result is obviously the observation that, despite differences in steady-state concentrations of transcripts of genes concerned by deletion and despite lowered maximal activity of complexes I and III, there are no significant differences in ATP synthesis capacity for the two strains irrespective of the given mitochondrial incubation substrate or tissue. These capacities remain identical even with complex I substrates. Differences are observed with substrate couples more specific for complex III (proline + pyruvate) but are more favorable for the mutant strain. This could indicate metabolic adaptation or higher solicitation in terms of substrate use.

These different hypotheses are being tested in our laboratory.

This finding may indicate that the activities of complexes I and III in the mutant strain, despite their decrease, suffice to ensure ATP synthesis that is identical to that of the wild strain. These results should be related to the apparent innocuousness of the mutation. It is genetically stable generation after generation and does not disrupt essential functions of the strain, particularly reproductive capacity in terms of egg-laying (number of eggs), larval or imago emergence rates, and duration of the various development stages from the egg to the imago. As reported in these results, all tissues apparently conserve their metabolic particularity and energy balance. Indeed, this balance seems to be upheld at the cellular level. Electron microscopy indicates no structural mitochondrial anomalies, and all mitochondria show a cytochrome oxidase activity for any type of tissue (15). This mutant thus survives substantial alterations to most of its mitochondrial genomes, a phenomenon that gives rise to severe pathological consequences in man or has been correlated with premature death in Podospora anserina (32, 33).

Though our subject is therefore not a pathological model, the survival rates and genetic stability of this mutation nevertheless make it a good model for studying how the mechanisms involved in deleting mitochondrial DNA succeed in maintaining this mutation in such a stable fashion. Thanks to the various tools developed for the Drosophila, this animal model allows study of the molecular mechanisms at the origin of deletion and identification of the genes involved, which are very probably of nuclear origin. It should also allow analysis of the mechanism whereby heteroplasmic incidence is maintained at a remarkably stable and high level in adults. A number of candidate genes possibly implicated in this mechanism are currently being studied in our laboratory.

REFERENCES

1. DiMauro, S., and Moraes, C. T. (1993) Arch. Neurol. 50, 1197–1208
2. Wallace, D. C. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8739–8746
3. Wallace, D. C., Shoffner, J. M., Trousseau, I., Brown, M. D., Ballinger, S. W., Corraldebrinski, M., Horton, T., Jun, A. S., and Lott, M. T. (1995) Biochim. Biophys. Acta 1271, 141–151
4. Holt, I. J., Harding, A. E., and Morgan-Hugues, J. A. (1988) Nature 331, 717–719
5. Holt, I. J., Harding, A. E., Petty, R. K. H., and Morgan-Hugues, J. A. (1990) Am. J. Hum. Genet. 46, 428–433
6. Lesione, P., and Ponzetto, G. (1988) Lancet i, 885
7. Hayashi, J.-H., Ohta, S., Kikuchi, A. M. T., Goto, Y.-I., and Nonaka, I. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 10614–10618
8. Attardi, G., Yoneeda, M., and Chomyn, A. (1995) Biochim. Biophys. Acta 1271, 241–248
9. Zeviani, M., Servidei, S., Gellera, C., Bertini, E., DiMauro, S., and DiDonato, S. (1989) Nature 339, 309–311
10. Sumalainen, A., Kaukonen, J., Amati, P., Timonen, R., Halila, M., Weissenbach, J., Zeviani, M., Somer, H., and Peltonen, L. (1995) Nat. Genet. 9, 146–151
11. Kaukonen, J. A., Amati, P., Sumalainen, A., Rotig, A., Piscaglia, M. G., Salvi, F., Weissenbach, J., Frattia, G., Comi, G., Peltonen, L., and Zeviani, M. (1996) Am. J. Hum. Genet. 58, 763–769
12. Voz-Lingenhöhl, A., Salignac, M., and Sperlich, D. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 11528–11532
13. Béziat, F., Morel, F., Voz-Lingenhöhl, A., Saint-Paul, N., and Alzari, S. (1993) Nucleic Acids Res. 21, 387–392
14. Débise, R., Touraille, S., Durand, R., and Alzari, S. (1993) Biochem. Biophys. Res. Commun. 196, 355–362
15. Lecher, P., Béziat, F., and Alzari, S. (1994) Biol. Cell 80, 25–33
16. Lecher, P., Petri, N., Béziat, F., and Alzari, S. (1996) Eur. J. Cell. Biol. 71, 423–427
17. Ponzetto, C., Bresolin, M., Bordini, A., Moggio, M., Meola, G., Bet, L., Prell, A., and Scarlato, G. (1990) J. Neurol. Sci. 96, 207–210
18. Alzari, S., Stepien, G., and Durand, R. (1981) Biochem. Biophys. Res. Commun. 99, 1–8
19. Bradford, M. (1976) Anal. Biochem. 72, 248–254
20. Hartfi, Y. (1978) Methods Enzymol. 53, 11–14
21. Hartfi, Y. (1978) Methods Enzymol. 53, 35–40
22. Errede, B., Kamen, M. D., and Hatefi, Y. (1978) Methods Enzymol. 53, 40–47
23. Sheperd, D., and Garland, S. (1969) Methods Enzymol. 13, 11–16
24. Gardner, R. S. (1974) Methods Enzymol. 35, 445–472
25. Hohorst, H. J. (1971) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.), 4th Ed., pp. 215–219, Academic Press, London
26. Sacktor, B. (1974) in The Physiology of Insects (Rockstein, M., ed) Vol. 4, pp. 407–407, Academic Press, New York
27. Chen, X., Prosser, R., Simonetti, S., Sadeloff, J., Jagiello, G., and Schon, E. A. (1995) Am. J. Hum. Genet. 57, 239–249
28. Moraes, C. T., and Schon, E. A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 209–215
29. Montoya, J., Gaines, G. L., and Attardi, G. (1989) Cell 54, 151–159
30. Dequard-Chablat, M., and Sellem, C. H. (1994) J. Biol. Chem. 269, 14951–14956