Accumulation of deleted mitochondrial DNA in aging

*Drosophila melanogaster*

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Cumulative damage in mitochondria by reactive oxygen species is thought to result in a decrease in mitochondrial respiratory function and to contribute to the age-related decline in the physiological function of organisms. The mitochondrial genome is also subjected to damage with age through deletions. The accumulation of deleted mitochondrial DNA (mtDNA) has been observed in various animals, but still remains unclear in insects. We examined the accumulation of deleted mtDNA in *D. melanogaster* at various ages from larvae to 65-day-old adults. When DNA extracted from whole bodies was examined by PCR and Southern hybridization, the age-related accumulation of deletions was not clear. However, when the accumulation of deleted mtDNA with age was examined separately in three parts of the body (head, thorax and abdomen), deleted mtDNA signals were detected more frequently in the thorax and the accumulation was age-dependent. Three of the deleted mtDNA were cloned, and the breakpoints of the deletions were identified. These results strongly suggest that deleted mtDNA accumulates in *Drosophila* with age in a tissue-specific manner.

**Key word:** aging, deletion, *Drosophila*, mitochondrial DNA

**INTRODUCTION**

Mitochondria are the major source of reactive oxygen species (ROS) in cells. ROS, generated as by-products of the electron-transport chain, have been shown to cause oxidative damage to lipids and proteins within mitochondria, and to mitochondrial DNA (mtDNA) throughout the lifespan of an organism. The free radical theory of aging, first proposed by Harman (1956), suggested that cumulative damage in mitochondria could result in a decrease in mitochondrial respiratory function, thus resulting in reduced ATP production and decreased cell function. These changes contribute to the age-related decline in the physiological function of an organism.

Oxidative damage to mtDNA has been investigated by the detection of deletions or point mutations within the mtDNA. Previous studies have documented the accumulation of deleted mtDNA with age in various organisms such as mouse (Tanhauser and Laipis 1995), rat (Edris et al. 1994), monkey (Lee et al. 1993), nematode (Melov et al. 1994, 1995a) and human (Cortpassi and Arnheim 1990, Melov et al. 1995b, Wang et al. 1997, Liu et al. 1998, Melov et al. 1999). The accumulation of point mutations has recently been reported to be associated with age in the control region of human mtDNA (Michikawa et al. 1999). The deletions observed thus far have been variable in size and location within the mitochondrial genome. Furthermore, the occurrence rates of deletions have been found to vary between individuals and, in particular, between different tissues or organs. In many cases, deleted mtDNA accumulates in an organ or tissue-specific manner. In some organisms, including humans, the age-related accumulation of common deletions has been reported (Corral-Debrinski et al. 1992, Soong et al. 1992, Filser et al. 1997), a phenomenon related to mitochondrial disease in humans.

*Drosophila* has provided a very useful model system for the investigation of aging. The accumulation of mtDNA deletions with age, however, has remained unclear even with the use of PCR (Schwarze et al. 1998a). An age-related and ROS-induced decline in mitochondrial enzyme activity has been indicated (Schwarze et al. 1998b). Orr and Sohal (1994) have shown that the simultaneous overexpression of superoxide dismutase and catalase extends the lifespan of *Drosophila*. These results suggest that oxidative damage accumulates in *Droso-
phila, as has been reported for other animals. It is likely that ROS induces age-related oxidative damage within mitochondrial components, but that the age-dependent accumulation of deleted mtDNA is too small to be detected by PCR analysis. Another possibility may be that the accumulation occurs in specific organs or tissues, so that the detection of deleted mtDNA is made more difficult when whole bodies of flies are examined for mtDNA deletions.

In the present study, we investigated the age-related occurrence of mtDNA deletions in *D. melanogaster* using PCR and Southern hybridization analyses. DNA was extracted from whole bodies or parts, including the head, thorax and abdomen. Furthermore, the deleted regions were confirmed by sequencing for several deletions. The results indicated that small amounts of mtDNA deletions accumulate with age, and that the accumulations are found primarily in the thorax, which is rich in muscle.

**MATERIALS AND METHODS**

**Aging of flies** The Oregon-R strain of *D. melanogaster* was used in all experiments. Virgin females and males were maintained at 25°C on standard *Drosophila* medium at initial densities of 40 and 50 flies per vial for females and males, respectively. Flies were transferred to new vials every 2 or 3 days until all the flies had died. Figure 1 shows that the survivorship curves for the flies. The survivorship began to decrease after approximately 30 days, and half of the female and male flies had died after 50 and 57 days, respectively. All of the female and male flies had died after 65 and 75 days, respectively.

**DNA extraction** Total DNA was extracted from larvae, pupae and adult fly parts, including the head, thorax and abdomen, collected from 10 – 50 flies. These were gently homogenized in 500 ml of homogenizing solution (0.1 M Tris pH 9.1, 0.1 M NaCl, 0.2 M sucrose, 0.05 M EDTA, 0.5% SDS) and then incubated at 65°C for 30 min. Following incubation, 120 µl of 5 M CH₃COOK was added, and the samples were incubated at 0°C for 30 min and then centrifuged at 15,000 rpm at 4°C for 5 min. The DNA was recovered after ethanol precipitation, dissolved in Tris/EDTA buffer (10 mM Tris, 1 mM EDTA, pH 7.6), extracted with phenol, phenol-chloroform, and ether, re-precipitated, and finally dissolved in 50 µl of H₂O.

Mitochondrial DNA was extracted from approximately 50 adult flies according to the method previously described (Satta et al. 1987). Extracted DNA was dissolved in 50 µl of H₂O.

**PCR analysis** Total DNA or mtDNA extracted from larvae, pupae, and adult flies at various ages were used as template DNA in the PCR analyses. PCR amplification was carried out using four sets of primers as indicated in Fig. 2. In this way, almost all of the coding regions of the mtDNA could be analyzed. The nucleotide sequences of the primers used were as follows:

- A (1867-1887): 5'-GCTGGAATTGCTCATGGTGGA-3'
- A' (4738-4759): 5'-AGGGTGATTTGAGTGTGTAGAC-3'
- B (4738-4759): 5'-GTCTACACACTCAAATCACCCT-3'
- B' (7796-7775): 5'-TAGGGTGAGATGTTTAGGACT-3'
- C (7179-7200): 5'-TGTGAATAATAGCCCCAGCACA-3'
- C' (11287-11308): 5'-GGTATGATGCCCCGCCGACTAC-3'
- D (11287-11308): 5'-GTAACACCTGCCCATATTCAC-3'
- D' (14725-14744): 5'-GCCAGCAGTCGCGGTTTTAC-3'

Nucleotide positions are referred to in parentheses according to the standard sequence (GenBank accession.

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Fig. 1. Survivorship curves of adult females and males of the Oregon-R strain. Approximately 650 flies were examined as described in Materials and Methods.

Fig. 2. Map of the *D. melanogaster* mitochondrial genome. The locations of four sets of PCR primers (A-A', B-B', C-C', and D-D') and probes for Southern hybridization (I, II and III) are indicated.
The reactions were performed using DNA thermal cyclers (Perkin Elmer 2400, TaKaRa TP240) in 50 µl of solution containing 1.5 or 2 mM MgCl₂, 0.16 mM each of dNTP mixture, 10 ng, 100 ng, or 1 µg of template DNA. The reaction conditions were determined according to Cortopassi and Arnheim (1990) with modifications: 90 s at 94°C, then 30 s at 94°C, and a single annealing and extension time at 65°C or 68°C for 30 cycles, and 5 min at 65°C or 68°C afterwards. Normal PCR products were amplified by annealing and extension for 270 s, while products from deleted mtDNA were amplified preferentially for 45 s. The midpoint of these two times was taken as 120 s. 10 µl of PCR products was separated on 1.5% agarose gels and visualized by ethidium bromide staining.

For each of the four regions indicated in Fig. 2, PCR analyses were performed at least twice for each corresponding age. DNA extracted from whole bodies, third instar larvae, pupae, a mixture of 1-day-old females and males, 20-, 40-, 43-, 56-, and 61-day-old females, and 60-, 68-, and 75-day-old males were examined. Fly parts (head, thorax and abdomen), 1-, 25- or 37-, and 55- or 56-day-old females, and 1-, 25- or 37-, 55- or 56-, and 65-day-old males were also examined.

Southern hybridization  PCR products were blotted onto membranes (MILLIPORE Immobilon™-Ny+) and hybridized with [α³²P] dATP-labeled probes. Probes I (1867-2800), II (7179-7796) and III (14191-14744) were used for regions A-A', B-B' and C-C', and D-D', respectively. The nucleotide positions of the probes are referred to in parentheses according to the standard sequence (GenBank accession no. U37541). Radioactive probe bound to the membrane was visualized by exposing the membranes to X-ray film (Fuji) for 1–4 days at –80°C or by using a bioimaging analyzer, FLA-2000 (Fuji Film).

DNA sequencing  Nucleotide sequences of some of the deleted mtDNA were determined in the region A-A' from 55-day-old females. For the second PCR, 1 µl of the PCR products was used as a template. The second PCR products were purified with the QIAquick PCR Purification Kit (QIAGEN), and cloned into the pGEM™-T Easy Vector using the pGEM™-T Easy Vector Systems (Promega). The cloned DNAs were sequenced with the automated sequencer DSQ-2000L (SHIMADZU) using the Thermo Sequence fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Ammersham Biosciences). The sequence of the region A-A' in normal mtDNA was also determined using young flies.

RESULTS

Accumulation of deleted mtDNA with age in whole bodies  To investigate the accumulation of deleted mtDNA, we first examined DNA extracted from the whole bodies of individuals. Using larvae and pupae, three sets of experiments, each of which included the three different PCR conditions, were employed for each of the four regions (see Materials and Methods). In the case of adult flies, 2–13 sets of experiments using three different PCR conditions were conducted for each of the four regions.

Total DNA from 50 larvae and pupae were examined for deletions, but deleted mtDNA was not detected by either PCR under all reaction conditions, or after hybridization of the PCR products under examination (Table 1). In the case of adult flies, mtDNA extracted from 13–50 flies ranging in age from 1–75 days was examined. When the reaction conditions that preferentially amplified deleted mtDNA were employed, several signals of deleted mtDNA were detected after Southern hybridization (Fig. 3, Table 1). Deleted mtDNA was detected in approximately 50% of the total PCR products for the region amplified by primers A and A' (Table 1). The results also showed that deleted mtDNA could be detected in both old and relatively young flies, especially in mtDNA from 20-day-old flies. From these

<table>
<thead>
<tr>
<th>Region</th>
<th>Larva</th>
<th>Pupa</th>
<th>(Day)</th>
<th>20</th>
<th>40</th>
<th>43</th>
<th>56</th>
<th>61</th>
<th>60</th>
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<tr>
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<td>0/3</td>
<td>4/14</td>
<td>7/14</td>
<td>5/11</td>
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<td>2/2</td>
<td>5/11</td>
<td>4/4</td>
<td>2/2</td>
<td>29/69</td>
</tr>
<tr>
<td>B-B'</td>
<td>0/3</td>
<td>0/3</td>
<td>0/11</td>
<td>0/12</td>
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<td>0/3</td>
<td>–</td>
<td>0/9</td>
<td>0/2</td>
<td>–</td>
<td>0/54</td>
</tr>
<tr>
<td>C-C'</td>
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<td>0/3</td>
<td>1/13</td>
<td>3/13</td>
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<td>0/6</td>
<td>4/12</td>
<td>1/9</td>
<td>0/3</td>
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Total number of replicates of PCR (a) and the number of PCR that detected deleted mtDNA signals (b) is indicated as b/a. –indicates that was not tested.

Table 1. Detection of deleted mtDNA using whole bodies of Drosophila.
results, however, a clear correlation between deleted mtDNA and age could not be established.

Accumulation of deleted mtDNA with age in three parts of flies To investigate the accumulation of deleted mtDNA with age in more detail, fly heads, thoraces and abdomens were separated, and the total DNA extracted from each part was subjected to PCR analyses. Two sets of experiments, each of which included the three kinds of PCR conditions, were conducted for each of the four regions (see Materials and Methods). After Southern hybridization, some signals of deleted mtDNA were observed in samples subjected to PCR conditions that preferentially amplified deleted mtDNA. Table 2 outlines the results of the two experiments.

The majority of deleted mtDNA signals were observed in DNA extracted from the thorax (Fig. 3, Table 2). Multiple deleted mtDNA signals with different sizes were detected in 1- and 55- or 56-day-old females and 1-, 55- or 56-, and 65-day-old males (Table 2). In particular, thorax DNA derived from 55- or 56-day-old females and 55- or 56- and 65-day-old males displayed more than 10 signals. From these results, the sizes of the deletions were estimated to range from approximately 1 kb to 4 kb. In contrast, only a few signals were observed in abdomen DNA samples derived from 55-day-old females (Table 2). Furthermore, head DNA samples displayed similar results to those of abdomen DNA. In this case, a few deleted mtDNA signals were detected in 25- or 37- and 55- or 56-day-old females, and 55- or 56- and 65-day-old males (Table 2).

These results clearly demonstrated two points. First, more deleted mtDNA was detected in DNA derived from the thorax than in DNA derived from other parts of the fly body. Second, deleted mtDNA signals were more frequently detected in old flies than in young ones. Furthermore, as in the case of whole bodies, signals were detected most frequently when primers A and A’ were employed in the PCR analyses (Table 2).

Nucleotide sequences of deleted mtDNA To confirm the nucleotide sequences of deleted mtDNA, some of the deleted mtDNA in region A-A’ were further analyzed. As the PCR products around 2 kb in size showed strong signals by Southern hybridization (Fig. 2), that fraction of DNA was subjected first to sequence analysis. More than ten different sequences of deletions have been obtained thus far, and three examples of the sequences are given in Fig. 4. The flanking sequences of a deleted region were matched with the corresponding mtDNA sequences from young flies without deletions. The sequencing of the PCR products is currently in progress, and the whole results will be reported elsewhere.

DISCUSSION

In the present study, we have shown the age-related accumulation of deleted mtDNA in Drosophila. The signals detected by Southern hybridization were various in size, and no common signal from young to old ages was observed (Fig. 3). In addition, a relationship between age and the size of the deletions was not particularly observed. Detection of deleted mtDNA was performed using highly specific primers for PCR amplification. The flanking sequences of a deleted region were completely
matched with the corresponding mtDNA sequences from young flies without deletions (Fig. 4). These results suggested that the detected signals were not from nuclear pseudogenes, but from mtDNA containing deletions.

Based on observations that the relationship between the accumulation of deleted mtDNA and age is not clearly shown when using whole bodies (Table 1, Schwarze et al. 1998a), we separated fly bodies into three parts (head, thorax and abdomen) so that organ- or tissue-specific accumulation of deleted mtDNA could be easily detected. Deleted mtDNA might be diluted when using whole-fly homogenate, thus making it more difficult to confirm any age-dependency. From the whole flies, the signals for deleted mtDNA were observed in relatively young flies (20-day-old) in high proportions (Table 1). The reason for this accidental increase in the signals is not clear at present.

It has been reported that deleted mtDNA accumulates with age at higher levels in neurons and muscle than in other tissues and cells in humans (Corral-Debrinski et al. 1992, Cortopassi et al. 1992, Liu et al. 1998, Melov et al. 1995b, Soong et al. 1992). As neurons and muscle require significant energy for their function, it is reasonable to expect that more ROS is produced due to elevated electron transfer, thereby damaging mitochondria more severely than those in other cell types. Our results demonstrated that deleted mtDNA can be detected at higher frequencies in the thorax than in other body parts, which is consistent with such previous studies. In Drosophila, it has been reported that no significant neuronal loss is observed in eye and antenna with age (Leonard et al. 1992, Helfand et al. 1996). Recently, it has been demonstrated that the nervous system is preserved in old age, whereas muscle gradually deteriorates with age in C. elegans (Herndon et al. 2002). It might be possible that the nervous system of insects and worms suffers from less oxidative damage than that of higher animals which have highly developed central nervous systems. We also examined the morphology of mitochondria in young and old adults, and observed that the structural changes in mitochondria were prominent in muscle in the thorax (data not shown). Although the relationship between deleted mtDNA and mitochondrial structure is not clear at present, the possible involvement of ROS is worth noting.

The present results contrast with the presence of a “common deletion” that is closely linked to disease in humans (Wallace 1992). The deleted mtDNA signals we detected varied in size, which suggested that the mtDNA deletions had occurred at various sites within the genome. The mechanism by which deletions are generated has not been well demonstrated. Given that direct repeats are positioned on both sides of a deletion, a slip-replication mechanism has been proposed to account for generation of the deletion (Shoffner et al. 1989, Melov et al. 1995a). Another possible mechanism leading to the generation of a deletion is the participation of 8-hydroxy-2-deoxyguanosine (8OH-dG), a biomarker of oxidative DNA damage. 8OH-dG has been known to induce a G→T transversion and a double-strand break, a process that contributes to the induction of deletion (Hayakawa et al. 1992). In the present results, direct repeats were detected in three deletions (Fig. 4). However, to determine the mechanisms for generating deleted mtDNA in Drosophila, it would be important to examine both the sequences of deleted mtDNA and the accumulation of

### Table 2. Numbers of deleted mtDNA signals detected in three parts of adult flies of Drosophila.

| Region | Female | | Male | |
|--------|--------|---|---|---|---|---|---|
|        |        | Days |        | Days | |
|        |        | 1  | 25/37 | 55/56 | 1  | 25/37 | 55/56 | 65 | Total |
| Head   |        |    | 2  | 2  | 1  | 2  | 1  | 2  | 2  |
|        | A-A'   | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  |
|        | B-B'   | 2  | 2  | 1  | 2  | 2  | 2  | 2  | 2  |
|        | C-C'   | 2  | 1  | 4  | 4  | 4  | 4  | 4  | 2  |
|        | D-D'   | 2  | 1  | 1  | 1  | 1  | 1  | 1  | 1  |
| Thorax |        | 1  | 1  | 21 | 2  | 2  | 2  | 2  | 2  |
|        | A-A'   | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  |
|        | B-B'   | 2  | 1  | 1  | 1  | 1  | 1  | 1  | 1  |
|        | C-C'   | 2  | 1  | 1  | 1  | 1  | 1  | 1  | 1  |
|        | D-D'   | 2  | 1  | 1  | 1  | 1  | 1  | 1  | 1  |
| Abdomen|        | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  |
|        | A-A'   | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  |
|        | B-B'   | 2  | 1  | 1  | 1  | 1  | 1  | 1  | 1  |
|        | C-C'   | 2  | 1  | 1  | 1  | 1  | 1  | 1  | 1  |
|        | D-D'   | 2  | 1  | 1  | 1  | 1  | 1  | 1  | 1  |
| Total  |        | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  |

– indicates that the signals were not detected after hybridization.
It is interesting that the age-related accumulation of deleted mtDNA was observed more frequently in the A-A' region than in the other regions examined. The reason for this is not clear at present. Five protein genes, COI, COII, COIII (the subunit proteins of cytochrome c oxidase), ATP8 and ATP6, and three tRNA genes are located in this region. It has been reported that cytochrome c oxidase activity in *Drosophila* declines with age (Schwarze et al. 1998b). Our results, showing that the deletions occurred preferentially in the A-A' region, are not inconsistent with this observation. However, this region may be more amenable to PCR amplification than other regions. This possibility should be investigated carefully.

In the present study, deleted mtDNA signals were not detected by electrophoresis of PCR products alone, but by Southern hybridization analyses, indicating that the amount of deleted mtDNA was very small, although the proportion of deleted mtDNA to total mtDNA was not determined. It has been shown that in humans, the deletion level in aged brains ranges from 0.00023 to 0.034 (Corral-Debrinski et al. 1992). The deleted mtDNA suggests the presence of oxidative damage in cells, but how such small amounts of deleted mtDNA contribute causally to the decline in physiological function is unclear at present. Moreover, the effects of such deletions on mitochondrial function might differ amongst different organisms. In *D. subobscura*, it is known that flies containing mtDNA deletions of up to 50–80% of their total mtDNA are not significantly affected (Volz-Lingenhöhl et al. 1992). Investigations focusing on the precise sequences deleted, the proportions of deleted mtDNA, and the distribution of damaged mitochondria would be useful.

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**Fig. 4.** Three examples of the nucleotide sequences of deleted mtDNA. Continuous sequences of the deleted mtDNA are shown in bold. The upper and lower sequences are corresponding sequences from normal mtDNA. Direct repeats are boxed. Asterisks indicate nucleotide sites at which the nucleotide is identical between the deleted mtDNA and normal mtDNA upstream and downstream of the direct repeat for the upper and lower sequences, respectively. Nucleotide positions are according to the standard sequence (GenBank accession no. U37541). The sizes of the deletions are 2,777 bp (A), 2,267 bp (B), and 2,195 bp (C).
in contributing to our understanding of the relationship between damage to mtDNA and the physiological decline of mitochondria.

REFERENCES


