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Genetics

Stable heteroplasmy for a large-scale deletion in the coding region of *Drosophila subobscura* mitochondrial DNA

(mitochondrial myopathies/fitness/absence of segregation/length variability)

ANDREA VOLZ-LINGENHÖHL*†, MICHEL SOLIGNAC‡, AND DIETHER SPERLICH*

*Populationsgenetik, Universität Tübingen, Auf der Morgenstelle 28, D-7400 Tübingen, Germany; and ‡Laboratoire de Biologie et Génétique Evolutives, Centre National de la Recherche Scientifique, F-91198 Gif-sur-Yvette, France

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ABSTRACT **Due to the extremely economic organization of the animal mitochondrial genome, large-scale deletions are rarely found in animal mtDNA. We report the occurrence of a massive deletion in the coding region of mtDNA in *Drosophila subobscura*. Restriction mapping and nucleotide sequence analysis revealed that the deletion encompasses six protein genes and four tRNAs. All individuals of an isofemale strain proved to be heteroplasmic for normal and deficient mtDNA molecules. This type of heteroplasmy resembles one observed in patients with mitochondrial myopathies but differs in that the fitness of heteroplasmic flies is not significantly reduced even though the mutant mtDNA constitutes 50–80% of total mtDNA in most of the individuals studied. The heteroplasmic strain is genetically stable: despite extensive screening not a single homoplasmic fly was observed since the foundation of the line.**

mtDNA heteroplasmy is now widely recognized in numerous groups of animals (1–16). The type most frequently observed is length heteroplasmy, where the mtDNA molecules within an individual may differ by amounts varying from a few nucleotides up to 7.5 kilobases (kb) (10). Length variability is generally located in the major noncoding region of the mtDNA molecule comprising the replication and transcriptional control sequences. In most cases differences in the copy numbers of tandemly repeated sequences account for this length variability (1, 3, 4, 7–12, 14, 16).

Although the percentage of heteroplasmic individuals in a population can vary greatly between species, they are rare in most taxa. Usually there are a few discrete mtDNA types segregating. This suggests that the mutation rate for length variants is rather low. Conversely, in some species, most or all individuals are heteroplasmic either for discrete (3, 10, 11, 14) or continuous length variations (2, 13), a situation that leads to the assumption that the mutation rate for size variants is high enough to counterbalance the stochastic purification.

Length variability involving the coding region is, however, very rare. The animal mitochondrial genome contains only a few genes, but their products are essential for the respiratory process. Consequently, they are conserved across taxa, with the exception of the ATPase 8 gene, which is missing in nematodes (17). In some species of lizards, individuals with large duplications have been observed (18, 19). However, large deletions involving the coding region are found only in the heteroplasmic state. This situation was first reported in two mice (20) but, in recent years, many similar cases have been observed in humans (21–23). These deletions mostly occur sporadically and are responsible for the development of severe diseases, the so-called mitochondrial myopathies. Certain tissues in which the deleted mtDNA molecules accumulate—mainly skeletal muscles, central nervous system, heart, kidney, and liver—are preferentially affected. The reasons of this accumulation are not yet understood.

We report the molecular characterization of a massive deletion (about 5 kb, encompassing six protein genes and four tRNAs) in the coding region of the mitochondrial genome of a strain of *Drosophila subobscura*. In spite of the high frequency of deleted genomes (50–80%), the flies are not significantly affected with respect to viability and fecundity. In addition, this strain is genetically stable: the two genomes appear in similar proportions in all individuals and this situation has persisted since the foundation of the strain—i.e., at least for 70 generations.§

MATERIAL AND METHODS

D. subobscura is a species of the *obscura* group (subgenus *Sophophora*) with a palearctic distribution. The heteroplasmic strain was established in 1986 from a single female collected in the course of population screens in the vicinity of Tübingen, Germany, and has been maintained in mass culture since then.

For physical mapping, mtDNA was isolated from adult flies according to the method of Afonso *et al.* (24). Cleavage sites of 10 restriction endonucleases were mapped by analysis of single and double digests.

On the basis of the mapping data, three fragments encompassing the deletion breakpoints in the deleted and the intact mtDNA molecules were cloned. For this purpose total DNA, extracted according to Preiss *et al.* (25), was digested with *Hae* III, and following agarose gel electrophoresis, the large mtDNA fragments were eluted from the gel and cut again by *Eco*RI or *Eco*RI/*Pst* I, respectively. In a second elution step the appropriate fragments were recovered from the gel. After ligation of the *Eco*RI and the two *Eco*RI/*Pst* I fragments (see Fig. 2) into the plasmid pUC19, cells of *Escherichia coli* K-12 JM 103 were transformed with the recombinant plasmids. The inserts of the three clones obtained were further cut by *Ssp* I, *Rsa* I, or *Sac* I, respectively, and subcloned in the *Acc* I or *Sac* I site of pUC19.

For sequencing, template DNA was prepared according to the manual of "Qiagen plasmid kit" (Diagen no. 41014). Sequencing was performed as described in the instruction of "T7 sequencing kit" (Pharmacia; no. 27-1682-01).

For the assessment of individual genotypes, single flies were homogenized in 0.2 M sucrose/50 mM EDTA/0.5% (wt/vol) SDS/0.1 M Tris·HCl, pH 9.2, and incubated at 65°C for 30 min. DNA was purified by phenol/chloroform and chloroform extraction, ethanol precipitated, and double-

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Abbreviations: AMPPD, 3-(2′-spiroadamantane)-4-methoxy-4-(3″-phosphoryloxy)phenyl-1,2-dioxetane disodium salt; ND, NADH dehydrogenase.
†To whom reprint requests should be addressed.
§The sequences reported in this paper have been deposited in the GenBank/EMBL data base (accession nos. X65129 and X65130).

digested with *Eco*RI/*Pst* I. Restriction fragments were separated on 0.8% agarose gels and transferred onto nylon filters (Hybond-N; Amersham). Part of the insert in clone 2, a 1.04-kb *Rsa* I/*Pst* I fragment not spanning the deleted region, was used as a probe for filter hybridizations. Labeling of the probe DNA with digoxigenin and hybridization were performed according to the instructions of the "DIG DNA labeling kit" (Boehringer Mannheim; no. 1175 033). The 3-(2′-spiroadamantane)-4-methoxy-4-(3″-phosphoryloxy)-phenyl-1,2-dioxetane disodium salt (AMPPD) chemiluminescence system (DIG luminescent detection kit; Boehringer Mannheim; no. 136514) was used for the detection of the bands (2.0-kb and 1.6-kb fragments derived from the deleted and intact molecule, respectively) following the protocol in ref. 26. X-ray films were exposed to the filters and scanned on a microdensitometer (Desaga, Heidelberg) to quantify the proportions of the two genomes.

RESULTS

In the course of surveying mtDNA variability in natural populations of *D. subobscura*, one isofemale line showed one additional band in the digestion profile for every enzyme tested (Fig. 1). In this line, the lengths of the various bands did not sum up to 15.9 kb, the standard size of the *D. subobscura* mtDNA. In addition, the fluorescence intensities of some of the bands were not proportional to the size of the corresponding fragment; in other words, there was no one-to-one molecular stoichiometry among the bands. These findings suggested heterogeneity of the mtDNA extracts, produced by the presence of two different molecular forms of mtDNA.

To confirm the mitochondrial origin of the additional bands, females showing the abnormal restriction pattern were crossed to males from a standard strain. Reciprocal crosses were also performed. All offspring of the former crosses displayed additional bands in their restriction profiles, whereas the progeny of the latter crosses showed the usual restriction pattern of *D. subobscura*. This proof of maternal inheritance made the mitochondrial origin of the additional bands highly probable.

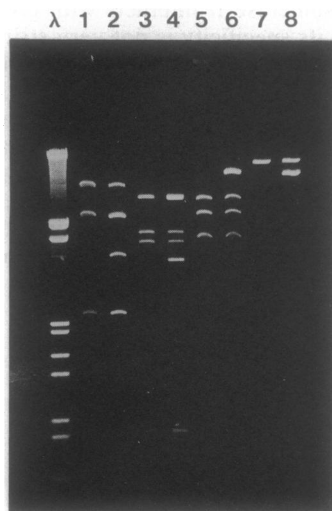

FIG. 1. mtDNA restriction patterns of homoplasmic and heteroplasmic lines obtained with the endonucleases *Hin*dIII (lanes 1 and 2), *Hpa* II (lanes 3 and 4), *Sac* I (lanes 5 and 6), and *Bam*HI (lanes 7 and 8). Lanes 1, 3, 5, and 7, mtDNA from a homoplasmic standard strain of *D. subobscura*. Lanes 2, 4, 6, and 8, mtDNA from the heteroplasmic strain. The *Eco*RI/*Hin*dIII digest of λ DNA (left lane) is used as a size standard.

The digestion profiles of enzymes cutting *D. subobscura* mtDNA only once (*Bam*HI, *Pvu* II, *Eco*RV) revealed, in addition to the linearized *D. subobscura* standard genome of 15.9 kb, a band corresponding to ≈11 kb. This suggested the existence of two length variants in the preparation. To decide whether the small molecule results from a single deletion event, the short genome was characterized by means of restriction mapping. Seven restriction sites are missing in the shorter molecular form (Fig. 2). The approximate boundaries of the deletion are marked by the lacking *Sac* I and *Eco*RI sites, which span a region of about 4.7 kb. As the size of the two molecules differs by about 5 kb, it is highly probable that the short molecule is the consequence of a single deletion event. The boundaries of the deleted region are likely to be a few hundred base pairs to the left and to the right of the *Sac* I site and the *Eco*RI site, respectively.

The genes affected by the deletion were determined by aligning the restriction map of *D. subobscura* with the known genetic map of *D. yakuba* (27). The restriction maps of both species show sufficient similarities to allow an alignment. A comparison of the maps, as shown in Fig. 2, reveals that 6 protein genes of a total of 13 and 4 tRNA genes are affected. The short molecule lacks the genes for the subunits NADH dehydrogenase 4, 4L, and 6 (ND4, ND4L, and ND6); cytochrome *b*; part of ND1 and ND5; and the tRNAs for serine, proline, threonine, and histidine. The deleted genes represent 31% of the overall length of the normal mtDNA molecule.

To determine the exact deletion breakpoints, two fragments of the intact molecule (clones 2 and 3 in Fig. 2) including the putative breakpoints were cloned and sequenced as well as the homologous fragment of the deleted molecule containing the fusion point (clone 1). The sequences of the relevant regions are given in Fig. 3. A comparison of the three sequences proves that nucleotides 6943–11,874 [numbers refer to the nucleotide sequence of *D. yakuba* (27)] are missing in the deleted molecule. Thus, the deletion corresponds to a sequence of 4932 bp present in the *D. yakuba* genome. Outside the deleted region a total of 2103 bp has been sequenced of both types of molecules. A sequence comparison failed to find any base substitutions or small insertions/deletions, indicating that no other mutations occurred after the deletion event.

The regions corresponding to the C-terminal part of ND1 and the N-terminal part of ND5 are deleted in the short molecule. Since both proteins are transcribed from the same strand, the production of a fusion protein is conceivable. It was possible to translate the nucleotide sequence into the amino acid sequence by using the genetic code known for *D. yakuba* (27). As can be seen in Fig. 3, a frame shift occurs at the breakpoint. In case the mRNA of the deleted molecule exists as a matrix for translation, only two more amino acids would be added to the N-terminal part of ND1 before the appearance of a stop codon (TAA), thus leaving a truncated ND1 protein instead of a fusion product.

For the molecular characterization of the deletion, pooled mtDNA from many individuals has been used. In addition, mtDNA from single flies of this strain was repeatedly analyzed over a period of 3 years to study the frequency distribution of the two mtDNA molecules within the strain and to look for the possible spontaneous occurrence of homoplasmic flies. However, all of the several hundred flies assayed, whatever their age and their sex, were heteroplasmic. In the vast majority of flies, 50–80% of the mtDNA came from the deleted molecule (Fig. 4) and no significant change from these proportions was observed in the course of the many generations elapsed since the foundation of the strain.

DISCUSSION

Large-scale length variability of mtDNA in the genus *Drosophila* was first exemplified by electron microscope analysis

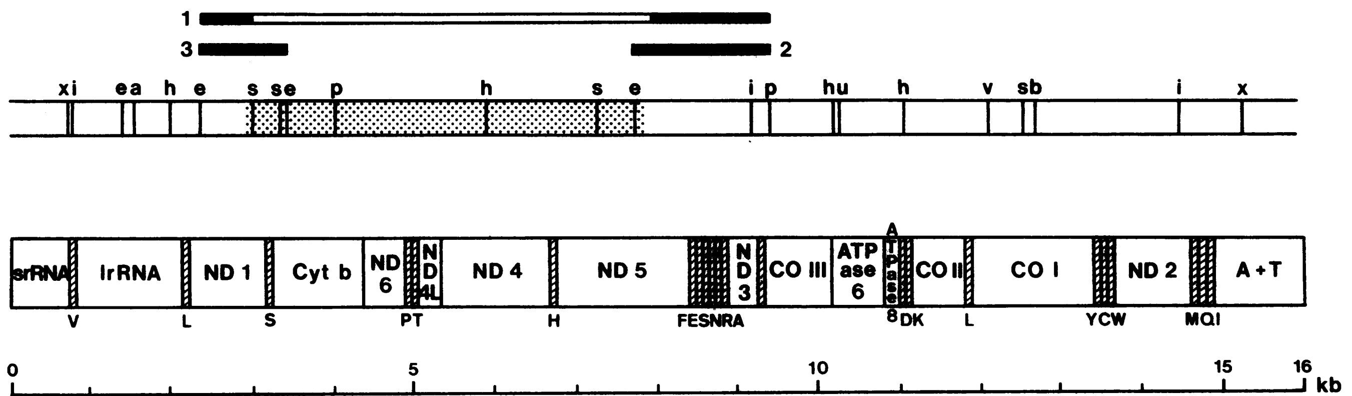

FIG. 2. Linear restriction site map of *D. subobscura* (above) aligned to the genetic map of *Drosophila yakuba* (ref. 27; below). Restriction enzyme sites are identified as follows: a, *Hae* III; b, *Bam*HI; e, *Eco*RI; h, *Hpa* II; i, *Hin*dIII; p, *Pst* I; s, *Sac* I; u, *Pvu* II; v, *Eco*RV; x, *Xba* I. The shaded area corresponds to the deleted region. tRNA genes are marked by hatched boxes. Cloned and sequenced segments of *D. subobscura* are indicated above the restriction map. Clone 1 contains a segment of the deleted molecule; clones 2 and 3 contain homologous segments of the normal molecule.

in a number of different species (28). To a lesser extent, length variability also exists within some *Drosophila* species and even in some individuals (6, 29, 30). However, length variation is always due to size differences within the (A+T)-rich region, as is, with a few exceptions (31), the case in most other species (32).

Length variability found in *D. subobscura* belongs to a different type as it involves a large-scale deletion in the coding region. Such deletions were previously known in only two mammalian species: mice (20) and humans (21). In all three cases, they occur solely in the heteroplasmic state. In mice, the deletion was molecularly characterized in two dead specimens collected in Bulgaria. In humans, deletions of that extent are found sporadically, being responsible for severe diseases. In *D. subobscura*, this deletion was detected only once, despite the large sample of flies screened during population studies (33, 34). However, the two mtDNA types within an individual, in contrast to other cases of heteroplasmy, do not segregate in the course of generations. In addition, the proportion of deleted genomes among total mtDNA is very high, though without evidence for a strong fitness reduction of heteroplasmic flies.

The large-scale deletion of the mitochondrial genome of *D. subobscura* is about 5 kb in length and involves six protein genes (four completely and two partially deleted) coding for subunits of the complexes I (ND) and III (cytochrome *c* reductase) as well as four tRNAs. In humans the sizes—1.9–8.5 kb—and locations of the deletions differ among patients and two additional complexes can be affected by the deletion: complex IV (cytochrome *c* oxidase) and V (ATP synthetase). Most of the deletions found presently in humans map to the two-thirds of the molecule potentially single stranded during mtDNA replication, which is localized between the light and the heavy strand origins of replication (22, 23). However, the genome organization (27) and the location of replication origins (35) are different in the mitochondrial genome of *Drosophila* and a direct comparison with breakpoints in human mtDNA is probably not possible.

Sequencing of the deletion breakpoints in several patients revealed two direct repeats at the edges of most of the deletions, one of them being retained in the deleted molecule (36–38). The direct repeats ranging in size from 5 to 13 bp are believed to mediate the deletion either by intragenomic recombination or by slipped mispairing. However, some of the deletions observed fall into a second class. The definition of class II (37) is imprecise and encompasses deletions either flanked imprecisely by perfect direct repeats or showing no obvious repeat elements adjacent to the deletion breakpoints. The type observed in *D. subobscura* belongs to this latter class: two perfect 6-bp repeats or imperfect 11-bp repeats, respectively, were found a few nucleotides downstream of the deletion breakpoints.

Among other mechanisms, the role of topoisomerase II in the deletion event has been suggested (37). The enzyme is likely to mediate some form of illegitimate recombination. Although topoisomerase II interacts with preferred nucleotide sequences, its specificity is not very stringent and only a consensus sequence for the cleavage site can be given (39). A search for this sequence in the *D. subobscura* mtDNA shows, in fact, some homology of the breakpoint region located in ND5 to the *Drosophila* topoisomerase II recognition site (5′-GTNWAYATTNATNNR-3′), with only three mismatches. The second breakpoint, however, in ND1 has no similarity to a topoisomerase II site but, since the recognition sequence is degenerate, this possibility cannot definitely be excluded.

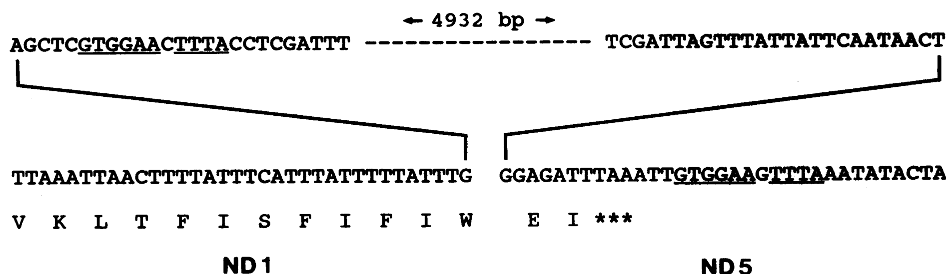

FIG. 3. DNA sequences (reading 5′ → 3′ on the coding strand) in the region of the deletion breakpoints. The lower line shows the DNA sequence of the deleted genome, with the upper line depicting the intervening deleted segment. The direct repeats nearby the deletion breakpoints are underlined. The amino acid sequence deduced from the ND1/ND5 fusion gene is shown below the DNA sequence. Amino acid residues are indicated: E, Glu; F, Phe; I, Ile; K, Lys; L, Leu; S, Ser; T, Thr; V, Val; W, Trp. bp, Base pairs.

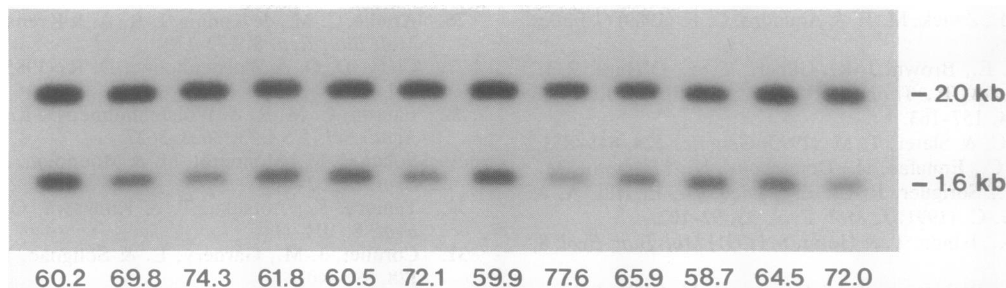

FIG. 4. mtDNA genotype of individual flies taken from the mass culture after about 60 generations. Total DNA of single flies was digested by *Eco*RI/*Pst* I and subjected to filter hybridizations. Using a 1.04-kb mtDNA fragment as a probe yields two bands: the upper band (2.0 kb) and the lower band (1.6 kb) are generated by the deleted and the normal genome, respectively. The percentage of the deleted genome is indicated below each lane.

The effect of the deficient mtDNA on the phenotype is quite different in *Drosophila* and man. In humans large-scale deletions of mtDNA cause serious diseases, called mitochondrial myopathies, with severe neuromuscular symptoms leading ultimately to death. The sporadic occurrence of these diseases suggests that the deletions originate by new mutations, possibly occurring during oogenesis or fetal development (40). Biochemical assays show decreased activity of complexes of the respiratory chain. On the individual level, unlike in humans, no obvious effect of the mutation could be observed in the flies. This isofemale strain has been cultured in the laboratory without noticeable difficulties. In addition, fitness studies (details of which will be published elsewhere) suggested only a slight, if any, reduction of their selective value. In summary, the relative fitness of heteroplasmic and homoplasmic flies was ascertained through competition experiments, using a visible nuclear encoded marker (*net*: a wing mutation, autosomal recessive; ref. 41) as tracer to infer the proportion of the two cytoplasmic constitutions in the progeny. The heteroplasmic female flies in the competition experiments were alternatively *net* (n/n) or wild type (n/+), and the homoplasmic flies were vice versa, to neutralize the effect of *net* on fitness. The selection coefficient was equal to 0.083 and was not found significantly different from zero. There might exist a compensating mechanism in the oxidative metabolism of insects that could account for the different phenotypical consequences in *Drosophila* and man.

We are largely ignorant on many aspects of mitochondrial genetics in animals. The three classical evolutionary forces—mutation, selection, and drift—are likely to be involved in the transmission and evolution of mtDNA. The least complex situation considers only drift, which will lead to fixation of one of the coexisting variants at a rate depending on the number of segregating units. In *Drosophila mauritiana* 30 generations after the initiation of a line derived from a single heteroplasmic female half of the offspring were homoplasmic for one of two mtDNA types (42). Studies on Holstein cows that are heteroplasmic for restriction site variants suggest that mitochondrial genotypes may be fixed in mammals even more rapidly than in insects, in some instances even within a single generation (43). Mainly stochastic processes are supposed to be responsible for the sorting out in these cases.

In *D. subobscura*, on the other hand, the two mtDNA types were found to coexist for at least 70 generations. Since the foundation of the strain several hundred flies have been tested for their mitochondrial genotype and all of them proved to be heteroplasmic. The proportion of the deleted molecule usually varied between 50% and 80%. Similar proportions were found in mice (78–79% in ref. 20). The apparent equilibrium of the two molecular forms might be the result of two opposing selective forces acting on the mitochondrial genome. Mitochondria containing only deleted mtDNA molecules are most probably incapable of energy production. Not only are the protein genes encompassed by the deletion affected but also the lack of indispensable tRNAs renders the organelles unable to translate all genes encoded by mtDNA. The presence of normal-sized molecules is a prerequisite for the maintenance of normal protein synthesis in mitochondria. Consequently, mitochondria with a very high proportion of deleted mtDNA might be degraded. On the other hand, the deleted form might have an advantage in replication. Supposing that the rate of replication is directly proportional to the length of the DNA molecule, the deleted molecules will replicate more rapidly than the intact ones and increase in number in every cell generation. In fact, in a patient with Kearns–Sayre syndrome, the proportion of the deleted molecule was shown to increase over the life of the individual (44). These two counteracting forces might eventually lead to a balanced coexistence of the two molecular forms.

Recurrent mutations could also be considered as a mechanism for the maintenance of heteroplasmy in *D. subobscura*. The deleted form might be continuously produced by recombination. A similar mechanism has been proposed in frogs (2) and rabbits (13), among which heteroplasmic individuals are predominant. A high rate of mutational events might counterbalance the stochastic purification in these species. Still other intracellular or even intramitochondrial mechanisms that remain to be detected may be responsible for the persistence of the intact and deleted mtDNA within a cell.

To our knowledge, *D. subobscura* is so far the only animal model of a stable system for heteroplasmy involving a massive deletion of the mtDNA. Our deletion strain could well serve as an experimental model for a better understanding of the genetics of a mutation that can cause such severe disturbances in man.

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