Mammalian Mitochondrial Genetic Complementation and Recombination

A Dissertation Submitted to
the Graduate School of Life and Environmental Sciences,
the University of Tsukuba
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Science
(Doctoral Program in Functional Biosciences)

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ABBREVIATIONS

B6	C57BL/6J
B6-mt^{spr}	C57BL/6J-mt^{spr}
COX	cytochrome *c* oxidase
CRS	Cambridge reference sequence
ΔmtDNA	deletion mutant mitochondrial DNA
dpc	day *post coitum*
DSB	double strand break
mtDNA	mitochondrial DNA
mito-mouse (-mice)	mouse (mice) with deletion mutant mtDNA
np	nucleotide position
ROS	reactive oxygen species
SDSA	synthesis-dependent strand annealing

ABSTRACT

In Section I, I showed *in vivo* mitochondrial interaction by creating mitochondrial disease model mice, mito-mice. The mito-mice were created by using zygotes of B6-mt^{spr} strain mice carrying mtDNA from *Mus spretus* as recipients of exogenous mitochondria carrying wild-type and mtDNA with large deletion (ΔmtDNA) of *M. musculus domesticus*. In these experiments, mtDNAs from different mouse species were used for identification of exo- and endogenous wild-type mtDNAs in the mito-mice. Results showed transmission of exogenous ΔmtDNA, but not exogenous wild-type mtDNA of *M. m. domesticus* to following generations through the female germ line. Complete elimination of exogenous wild-type mtDNA would be due to stochastic segregation, whereas transmission of exogenous ΔmtDNA would be due to its smaller size leading to a propagational advantage. Tissues in mito-mice of the F_3 generation carrying exogenous ΔmtDNA showed protection from respiration defects until ΔmtDNA accumulated predominantly. This protection from expression of mitochondrial dysfunction was attained by the help of endogenous wild-type mtDNA of *M. spretus*, since mito-mice did not possess exogenous wild-type mtDNA of *M. m. domesticus*. These observations provide unambiguous evidence for the presence of interaction between exogenous mitochondria carrying ΔmtDNA and endogenous mitochondria carrying *M. spretus* wild-type mtDNA.

The existence of mitochondrial interactions shown in Section I suggested exclusion of physical barrier against the exchange of mtDNA between mitochondria and following recombination. In Section II, I showed

the presence of rare mitochondrial recombination in mice tissues. To avoid misinterpretation of PCR jumping artifacts as recombinants, purified mtDNAs without PCR amplification were used for cloning and sequencing. I examined *in vitro* recombination in human hybrid cells carrying different pathogenic mtDNAs from patients with mitochondrial diseases, and also examined *in vivo* recombination in a mito-mouse carrying exogenous ΔmtDNA of *M. m. domesticus* and endogenous wild-type mtDNA of *M. spretus*. While no mtDNA recombinants were observed in human hybrid cells, one of 163 clones of whole ΔmtDNA purified from mito-mouse tissues possessed two very small regions carrying identical sequences to *M. spretus* mtDNA. Considering the high concentration of reactive oxygen species around the mtDNA and its frequent strand breakage, a clone carrying changed sequences would correspond to a gene conversion product created by repair of nucleotide mismatches, rather than to a classic recombination product for providing sequence divergence.

GENERAL INTRODUCTION

Mammalian cells possess hundreds of mitochondria, each containing several mtDNA molecules consisting of about 16 kbp double stranded closed circular DNA. Copy number of mtDNA is 10^3 to 10^4 in somatic cells and 10^5 in mature oocytes (Michael *et al.* 1982; Piko and Taylor 1987). Mammalian mtDNA encodes 2 rRNAs, 22 tRNAs, and 13 polypeptides constituting part of the five multisubunit enzyme complexes involved in oxidative phosphorylation (Anderson *et al.* 1981; Bibb *et al.* 1981). Other components of oxidative phosphorylation complex and all enzymes involved in other mitochondrial functions are encoded in nuclear genome. Mitochondrial respiratory function, therefore, regulated by both mitochondrial and nuclear genomes.

It has been reported that mutation rate of mtDNA is extremely higher than that of nuclear genome (Wallace *et al.* 1987; Richter *et al.* 1988). One reason is that mtDNA is exposed to reactive oxygen species (ROS), such as superoxide radical or hydrogen peroxide, generated in mitochondria as by-product of oxidative phosphorylation. Another reason is that repair systems for mtDNA are poorer than that for nuclear genome. It has been shown that mitochondria possess base excision repair mechanism but not nucleotide excision repair mechanism (Clayton *et al.* 1974; LeDoux *et al.* 1992; Kang and Hamasaki 2002). Moreover, mtDNA lacks histon-like proteins, which bind tightly to mtDNA and protect DNA from various mutagens. Therefore, pathogenic mutations in mtDNA could accumulate predominantly and following energy deprivation causes mitochondrial diseases in many tissues, in particular in highly energy requiring ones, such

recombination has been shown. However, it has been generally accepted that there is no mtDNA recombination in animal species. Recently, several population genetical studies have suggested that recombination occur in mammals (Awadalla *et al.* 1999; Eyre-Walker *et al.* 1999; Hagelberg *et al.* 1999), although these studies have been criticized on several grounds (Arctander 1999; Merriweather and Kaestle 1999; Kivisild *et al.* 2000). Thyagarajan *et al.* (1996) have demonstrated that human mitochondria contain enzymes necessary for homologous recombination. Ladoukakis *et al.* (2001) provided evidence for mtDNA recombination in sea mussels (shellfish) exhibiting unique inheritance of parental mtDNA (doubly uniparental inheritance: maternal mtDNA is transmitted to male somatic cells, and female somatic cells and germ cells, but paternal mtDNA is transmitted to only male germ cells). Evidence of mtDNA recombination was provided in human mitochondrial disease patient exceptionally transmitted paternal pathogenic mtDNA (Kraytsberg *et al.* 2004). These studies in mussel and human used PCR method for detection of recombinant mtDNA, encompassing artifacts generation, although all of these observations are suggestive of the possibility of recombination in animal mtDNA. Therefore, it is still a controversial issue of whether mtDNA recombination exists or not.

The presence or absence of mtDNA recombination has an important issue for biologists studying phylogeny and evolution. Currently, all major methods for phylogenetic reconstruction assume that mtDNA is transmitted only from mother and that no mtDNA recombination occur.

Based on the evidence that many studies in molecular population and evolutionary biology are based on mtDNA (~70% of phylogenetic studies have involved analyses of mtDNA) (Avise 2000), it is essential to elucidate whether the mtDNA recombination exists in mammalian species

In Section I, I generated heteroplasmic mice possessing mtDNAs from different mouse species and carried out biochemical observations, and obtained a direct evidence for *in vivo* mitochondrial interaction in mammalian cells. In Section II, I tried to detect recombinant mtDNA in human cultured cells and mice mentioned in Section I.

SECTION I

In vivo interaction between mitochondria carrying mtDNAs from different mouse species

INTRODUCTION

In yeast and plant cells, the idea of mitochondrial interaction has received support from the evidence for recombination between two mtDNA molecules derived from both parental germ cells (Dujon *et al.* 1974; Belliard *et al.* 1979). In mammalian species, however, the opportunity of coexistence of mtDNAs from both parents is likely inhibited by their strictly maternal inheritance (Kaneda *et al.* 1995; Shitara *et al.* 1996; Shitara *et al.* 2000; Shitara *et al.* 2001). Since the mammalian mtDNA population is homoplasmic throughout individuals due to maternal inheritance, recombination between maternal mtDNA molecules with the same sequences would not be productive. Although cell fusion techniques can mix mtDNA molecules from different mammalian individuals within single somatic cell hybrids, extensive mtDNA recombination as observed in yeast and plant cells (Dujon *et al.* 1974; Belliard *et al.* 1979) was not detectable even after their long-term cultivation (Hayashi *et al.* 1985).

However, evidence for interactions between mammalian mitochondria is provided by translational complementation in cultured somatic cells (Hayashi *et al.* 1994; Takai *et al.* 1999; Ono *et al.* 2001). For example, fusion of two different types of respiration-deficient somatic cells caused by different pathogenic mutant mtDNAs from patients with mitochondrial diseases resulted in overall restoration of respiration defects in their somatic cell hybrids (Takai *et al.* 1999; Ono *et al.* 2001). Moreover, evidence for rapid merging of normal mitochondria with wild-type mtDNA

and respiration-deficient mitochondria without mtDNA was obtained by introduction of normal mitochondria into mtDNA-less HeLa cells using cell fusion techniques (Hayashi *et al.* 1994).

Recently, the occurrence of interaction between mitochondria was extended from the *in vitro* to the *in vivo* level by the use of mito-mice (Inoue *et al.* 2000; Nakada *et al.* 2001). They were generated by introduction of respiration-deficient mitochondria carrying a predominant amount of mutated mtDNA with a large deletion (ΔmtDNA) and a residual amount of wild-type mtDNA from cultured mouse cells into mouse zygotes (Inoue *et al.* 2000). In the mito-mice, expression of mitochondrial dysfunction was not observed in any mitochondria in any cells carrying as much as 60% ΔmtDNA, suggesting the presence of interaction between exogenous respiration-deficient mitochondria with ΔmtDNA and endogenous mitochondria with wild-type mtDNA, and resultant restoration of respiratory function throughout the mitochondria (Nakada *et al.* 2001; Hayashi *et al.* 2002).

However, Attardi and co-workers (Attardi *et al.* 2002) noted that the apparent rescue of mito-mice from expression of mitochondrial defects could be explained by assuming intra-, but not intermitochondrial interaction. In this case, a proportion of residual wild-type mtDNA preexisting in exogenous mitochondria has to increase preferentially for restoration of respiratory function, followed by elimination of endogenous mitochondria carrying wild-type mtDNA from mito-mice. Although it is not obvious to assume such an exclusive increase of exogenous wild-type mtDNA

to be predominant over endogenous wild-type mtDNA, this possibility could not be excluded completely. Therefore, experiments that could distinguish exo- and endogenous wild-type mtDNAs had to be carried out for generation of mito-mice before concluding the presence of an *in vivo* interaction of mammalian mitochondria.

In this study, I created mito-mice using zygotes carrying mtDNA of different mouse species, *M. spretus*, so that both endo- and exogenous wild-type mtDNAs in the mito-mice could be distinguished. The results provided unambiguous evidence for the presence of the *in vivo* intermitochondrial interaction.

MATERIALS AND METHODS

Cells and cell culture

Cy4696 cybrids (Inoue *et al.* 2000) carrying 89.4 ± 2.3% ΔmtDNA of *M. m. domesticus* were cultivated in RPMI1640 (Nissui Seiyaku, Tokyo, Japan) containing 10% fetal calf serum, 50 μg/ml uridine and 0.1 mg/ml pyruvate. Uridine and pyruvate were supplemented, so that respiration-deficient Cy4696 cybrids caused by a predominant amount of ΔmtDNA could grow (King and Attardi 1989; Inoue *et al.* 1997).

Generation of mito-mice carrying wild-type mtDNA of *M. spretus* and ΔmtDNA of *M. m. domesticus*

Zygotes of B6-mt^{spr}, which possess the nuclear genome of *M. m. domesticus* and mitochondrial genome of *M. spretus*, were used as ΔmtDNA recipients. Cytoplasts of the Cy4696 cybrids carrying 89.4 ± 2.3% ΔmtDNA were used as mtDNA donors for generating mito-mice. Introduction of ΔmtDNA into B6-mt^{spr} zygotes was carried out as described previously (Inoue *et al.* 2000) with slight modifications. Briefly, pronuclear stage zygotes were collected from oviducts of superovulated B6-mt^{spr} females at the age of 8-10 weeks after birth, and about ten Cy4696 cytoplasts were inserted into the perivitelline space of the zygotes with a piezo-driven micromanipulator. The cytoplasts were fused with the embryos by applying an electric pulse (3,000 or 3,750 V/cm, 10 ms) with a pre- and post-pulse AC current (100 V/cm, 2 MHz, 30 sec each). After 24 h cultivation, 2-cell stage

embryos were transferred to the oviducts of pseudopregnant ICR females.

Southern blot analysis

Total DNA was extracted from cultured cells and tissues using a Pure Gene Tissue Kit (Gentra, Minneapolis, USA). DNA (1 μg) was digested with the restriction enzyme *Bgl*Ⅱ, and the resultant restriction fragments were separated in 0.6% agarose gel, transferred to a nylon membrane and hybridized with alkaline phosphatase-labeled mouse mtDNA probes, which include nucleotide positions 1,895-2,762. This region was selected as a probe because of its high sequence similarity in *M. m. domesticus* and *M. spretus* mtDNAs. Probe labeling and signal detection were carried out as described in the protocols of the AlkPhos Direct (Amersham Pharmacia Biotech, Buckinghamshire, UK). For quantitation of wild-type mtDNA of *M. spretus* and ΔmtDNA of *M. m. domesticus*, 10 replicate lanes were separately scanned and calculated by NIH image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/) together with control lanes with known amounts of mtDNAs.

PCR analysis

Total DNA (300 ng) was used for PCR amplification. Three primer sets (11,927-11,946 and 12,388-12,365; 7,587-7,611 and 12,679-12,654; 7,442-7,469 and 7,726-7,696) were used for specific amplification of wild-type and ΔmtDNA of *M. m. domesticus*, and wild-type

mtDNA of *M. spretus*, respectively. Wild-type and ΔmtDNA of *M. m. domesticus*, and wild-type mtDNA of *M. spretus* gave 462 bp, 397 bp and 285 bp fragments, respectively. The cycle times were 30 sec of denaturation at 95°C, 30 sec of annealing at 55°C, and 60 sec of extension at 72°C, for 50 cycles.

Analyses of cytochrome *c* oxidase (COX) activity

Estimation of COX activity was carried out by examining the rate of cyanide-sensitive oxidation of reduced cytochrome *c* (Seligman *et al.* 1968). In histochemical analyses, hearts were excised from mito-mice, and their 10 μm cryosections were stained for COX activity. COX electron micrographs were carried out as described (Nakada *et al.* 2001) with slight modifications. Briefly, 25 μm cryosections were fixed in 2% glutaraldehyde in PBS for 10 min at 0°C. Ultrathin sections, which were not stained with uranyl acetate and lead nitrate, were viewed directly with an H-7000 electron microscope (Hitachi, Tokyo, Japan).

Single-cell quantitative PCR

Three serial cryosections (10 μm) of heart from mito-mice possessing 85.2 ± 3.0% ΔmtDNA were used for single-cell quantitative PCR. The first and third sections were stained with dimethylaminoazobenzene for COX activity, and COX positive and negative fibers in both sections were used for quantitative PCR analysis. Each cell in the second sections was dissected with a PRO-300 Laser Scissors (Cell Robotics, New Mexico, USA).

Quantification of *M. m. domesticus* ΔmtDNA and *M. spretus* wild-type mtDNA were carried out using a TaqMan PCR reagent kit and an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, California, USA) under the conditions recommended by the manufacturer. The primer set specific for *M. m. domesticus* ΔmtDNA was nucleotide positions 7,697-7,725 and 12,528-12,508. The reporter dye FAM labeled TaqMan MGB probe (Applied Biosystems, California, USA) specific for *M. m. domesticus* ΔmtDNA was nucleotide positions 7,750-7,758 and 12,455-12,464. The primer set specific for *M. spretus* wild-type mtDNA was nucleotide positions 7,442-7,469 and 7,726-7,697. The reporter dye FAM and the quencher dye TAMRA labeled probe was nucleotide positions 7,490-7,513.

RESULTS

Generation of mito-mice by introduction of ΔmtDNA into zygotes with *M. spretus* mtDNA

Cy4696 cybrids carrying 89.4 ± 2.3% ΔmtDNA of *M. m. domesticus* were enucleated and resultant cytoplasts were used as mtDNA donors for generating mito-mice expressing mitochondrial defects. Zygotes of the mouse B6-mt^{spr} strain possessing only *M. spretus* mtDNA were used as recipients, so that endo- and exogenous wild-type mtDNAs could be identified (Fig. 1-1). The cytoplasts were fused with zygotes by electrofusion and resultant 2-cell stage embryos were transferred to the oviducts of pseudopregnant ICR females for obtaining F_0 mice.

Total DNA was prepared from tail biopsy samples of F_0 mice. Of 32 DNA samples, 20 showed PCR signals for the presence of both exogenous wild-type and ΔmtDNA of *M. m. domesticus* introduced from Cy4696, whereas the remaining 12 F_0 mice did not possess exogenous mtDNAs (Table 1-1). Then, eight F_0 females with PCR signals for the presence of exogenous mtDNAs in their tails were selected as mothers for obtaining the F_1 generation. I subsequently examined whether exogenous wild-type and ΔmtDNA of *M. m. domesticus* can be transmitted through the female germ line to the F_1 generation. Of 130 F_1 mice, 4 showed signals for ΔmtDNA by both Southern-blot and PCR analyses, whereas no mice gave signals for exogenous wild-type mtDNA of *M. m. domesticus* even by PCR analysis. These observations suggest that transmission of exogenous mtDNAs from

the F_0 to the F_1 generation was limited to ΔmtDNA (Table 1-1).

Then, I selected an F_2 female carrying 37.1 ± 3.0% ΔmtDNA in its tail as a mother, and obtained four F_3 mice, one female and three males. All DNA samples prepared from tails of F_3 mice gave PCR signals for the presence of ΔmtDNA, while no signals for exogenous wild-type mtDNA were observed, suggesting exclusive transmission of exogenous ΔmtDNA, but not exogenous wild-type mtDNA to following generations (Table 1-1). For quantitation of the amount of ΔmtDNA, I carried out Southern blot analysis, and found that the tails of four F_3 mice contained 10.5 ± 4.4, 45.1 ± 2.2, 45.2 ± 2.5, and 65.3 ± 2.7% ΔmtDNA.

Examination of intermitochondrial interaction using mito-mice with ΔmtDNA

Hearts excised from three F_3 male mice carrying 45.1 ± 2.2, 45.2 ± 2.5, and 65.3 ± 2.7% ΔmtDNA in their tails were used for examination of their mtDNA composition by Southern blot analysis (Fig. 1-2a). The results showed that they possessed 57.5 ± 2.4, 60.6 ± 3.4, and 85.2 ± 3.0% ΔmtDNA, respectively. Moreover, complete absence of exogenous wild-type mtDNA from the F_3 hearts was observed in Southern blot (Fig. 1-2a) and PCR analyses (Fig. 1-2b).

I selected two F_3 hearts carrying 60.6 ± 3.4 and 85.2 ± 3.0% ΔmtDNA for further examination of COX activity by COX histochemistry (Fig. 1-3) and COX electronmicrographs (Fig. 1-4). Histochemical analysis of COX activity in hearts carrying 60.6 ± 3.4% ΔmtDNA showed that all

cardiac cells possessed COX activity: no COX negative cells were observed (Fig. 1-3). Moreover, COX electronmicrographs, that can identify COX activity at the individual mitochondrial level, clearly showed that no mitochondria lost COX activity, even though the heart possessed 60.6 ± 3.4% ΔmtDNA (Fig. 1-4). Considering that exogenous wild-type mtDNA was not present in hearts carrying 60.6 ± 3.4% exogenous ΔmtDNA, at least 60% mitochondria should be COX-negative in the absence of interaction between COX-negative exogenous mitochondria and normal endogenous mitochondria. Therefore, the observations in Figure 1-4 could not be obtained in the absence of mitochondrial interaction.

On the other hand, examination of a heart carrying 85.2 ± 3.0% ΔmtDNA gave apparently different features of COX histochemistry and COX electronmicrographs, but the results again supported the presence of interaction between mitochondria. The heart consisted of COX positive and COX negative cardiac cells (Fig. 1-3). Single-cell quantitative PCR analysis of the serial cross sections showed that COX positive and negative cells possessed 67.6 ± 12.9% (n=22) and 92.2 ± 3.7% (n=18) ΔmtDNA, respectively (Fig. 1-5). COX electronmicrographs showed a uniform distribution of either COX-positive or COX-negative mitochondria within single cardiac cells (Fig. 1-4). In the absence of mitochondrial interaction, 85% of the mitochondria would be COX negative in hearts carrying 85.2 ± 3.0% ΔmtDNA. However, COX electronmicrographs showed a homogeneous distribution of COX activity throughout mitochondria, and no mosaic distribution of COX positive and negative mitochondria within any single

cardiac cell was observed (Fig. 1-4).

I suggest that cardiac cells carrying less than 85% ΔmtDNA retained normal mitochondrial translation and normal COX activity by complementing tRNAs transcribed from tRNA genes missing in ΔmtDNA. On the other hand, cardiac cells carrying more than 85% ΔmtDNA progressively lost COX activity due to an insufficient amount of the tRNAs required for normal mitochondrial translation. Therefore, the translation phase may be shifted from complementation to competition of the tRNAs in cells with more than 85% ΔmtDNA, resulting in progressive inhibition of overall mitochondrial translation and resultant reduction of COX activity.

These observations consistently suggest the presence of extensive *in vivo* interaction between exogenous mitochondria carrying ΔmtDNA and endogenous mitochondria carrying wild-type mtDNA in the mito-mice.

DISCUSSION

Recent study of my colleagues (Nakada *et al.* 2001) provided evidence for the presence of intermitochondrial interaction using mito-mice (Inoue *et al.* 2000), which were generated by the introduction of COX-negative mitochondria carrying 88% ΔmtDNA and residual 12% wild-type mtDNA into zygotes carrying 100% wild-type mtDNA. All mitochondria in tissues with ΔmtDNA showed normal COX activity until it accumulated to 80%. Moreover, no coexistence of COX-positive and -negative mitochondria within single cells was observed (Nakada *et al.* 2001). These observations could be explained by the occurrence of *in vivo* inter-mitochondrial complementation by the extensive and continuous interchange of genetic materials between exogenous mitochondria carrying ΔmtDNA and host mitochondria carrying wild-type mtDNA.

However, Attardi *et al.* (Attardi *et al.* 2002) noted that observations of Nakada *et al.* (Nakada *et al.* 2001) could be explained in the absence of interaction between mitochondria by assuming simultaneous occurrence of the following two events. First, clonal expansion of exogenous COX-negative mitochondria carrying ΔmtDNA and the resultant elimination of most endogenous mitochondria. Second, an increase in the amount of wild-type mtDNA preexisting in exogenous mitochondria with the resultant recovery of COX activity in the overall mitochondria of mito-mice. In these cases, protection of mito-mice from mitochondrial defects was due to the interaction within exogenous mitochondria, but not interaction between exo-

and endogenous mitochondria. Although the occurrence of these phenomena seems unlikely, experiments that can identify exo- and endogenous wild-type mtDNA are required to exclude the possibility of clonal expansion of exogenous mitochondria, and to draw a general conclusion with respect to the *in vivo* interaction between mitochondria.

In this study, I generated mito-mice using zygotes of the mouse B6-mt^{spr} strain carrying mtDNA from a different mouse species, *M. spretus*, so that endogenous wild-type mtDNA could be distinguished from exogenous wild-type mtDNA by a restriction endonuclease. I obtained mito-mice exclusively carrying endogenous *M. spretus* wild-type mtDNA and exogenous ΔmtDNA, but not exogenous wild-type mtDNA of *M. m. domesticus*, suggesting the absence of clonal expansion of exogenous mitochondria with increased amount of exogenous wild-type mtDNA (Table 1-1). Moreover, none of the individual mitochondria in the mito-mice expressed respiration defects until exogenous ΔmtDNA accumulated predominantly, providing unequivocal evidence for the *in vivo* interaction between endogenous mitochondria with *M. spretus* mtDNA and exogenous COX negative mitochondria with ΔmtDNA.

In these experiments, transmission of exogenous ΔmtDNA to following generations was observed in F_0-F_3 generations, whereas exogenous wild-type mtDNA was not. The rapid elimination of exogenous wild-type mtDNA of *M. m. domesticus* from female germ cells in F_0 mice could be caused by stochastic segregation, since a very small amount of exogenous mtDNAs could be introduced into zygotes by electrofusion techniques.

Similar elimination of a small amount of exogenous mtDNA was observed when the transmission profile of paternal mtDNA in sperm introduced into zygotes on fertilization was examined (Kaneda *et al.* 1995; Shitara *et al.* 1998). In intraspecies crossing, paternal mtDNA was completely eliminated from zygotes within 24 h after fertilization. On the other hand, its leakage was exclusively observed in interspecies crossing (Kaneda *et al.* 1995). However, transmission of the leaked paternal mtDNA from interspecies hybrid mice to next generations through female germ cells was a rare phenomenon, if it occurred (Shitara *et al.* 1998). Probably, the proportion of sperm-derived mtDNA was extremely small (less than 0.1%), so that the leaked paternal mtDNA disappeared very rapidly by stochastic segregation. Rapid and random mtDNA segregation was also reported in mice carrying heteroplasmic mtDNAs with neutral polymorphic mutations (Jenuth *et al.* 1996).

On the other hand, transmission of exogenous ΔmtDNA through female germ cells to the following generations was observed (Fig. 1-1). The escape of exogenous ΔmtDNA from elimination may be due, at least in part, to its smaller size, which could give replication and propagational advantages over endo- and exogenous wild-type mtDNAs. Therefore, even when the amount of ΔmtDNA introduced into zygotes was extremely small, it could be transmitted and accumulate in tissues of F_3 progenies (Table 1-1).

The presence of intermitochondrial interaction was supported by previous reports of my colleagues (Hayashi *et al.* 1994; Takai *et al.* 1999; Ono *et al.* 2001), which provided evidence for the *in vitro* interaction between

mitochondria by the fusion of cultured cells. Therefore, it can be generalized that extensive and continuous interchange of genetic materials occurs between mitochondria in cells both *in vivo* and *in vitro*, resulting in metabolic complementation of mitochondria to avoid direct expression of mtDNA mutations as respiration defects.

Therefore, these observations do not support the conventional "mitochondrial theory of aging" (Linnane *et al.* 1989; Wallace 1992; Shigenaga *et al.* 1994; Nagley and Wei 1998), which proposes that age-associated mitochondrial dysfunction appears as the consequence of age-associated accumulation of somatic mutations in the mtDNA population. This hypothesis was supported by the evidence that various pathogenic mtDNA mutations accumulated with age in mitotic (Michikawa *et al.* 1999) and postmitotic tissues (Soong *et al.* 1992; Corral-Debrinski *et al.* 1992). However, previous reports of my colleagues provided direct evidence for the functional integrity of mtDNAs from mitotic (Isobe *et al.* 1998) and postmitotic tissues (Ito *et al.* 1999) of aged subjects using mtDNA transfer techniques. For example, nuclear transfer experiments from mtDNA-less HeLa cells to human skin fibroblasts showed that nuclear-recessive mutations, but not mtDNA mutations are responsible for the age-associated mitochondrial dysfunction observed in the fibroblasts (Isobe *et al.* 1998). Moreover, introduction of mtDNA of autopsied brain tissues from aged human subjects into mtDNA-less HeLa cells resulted in complete restoration of mitochondrial respiratory function in mtDNA repopulated HeLa cells (cybrids), although brain donors possessed mtDNAs with various pathogenic

mutations, which were transferred to the cybrids (Ito *et al.* 1999). These observations could be explained by intermitochondrial complementation, which rescues aged tissues from direct expression of mtDNA mutations as age-associated mitochondrial dysfunction.

Table 1-1

Examination of exogenous wild-type and ΔmtDNA of *M. m. domesticus* in tails of F_0-F_3 mice by PCR analysis

		Presence of exogenous *M. m. domesticus* mtDNA		
Generation	Total number	Both types	Only ΔmtDNA	Neither type
F_0	32	20	0	12
F_1	130	0	4	126
F_2	8	0	8	0
F_3	4	0	4	0

Figure 1-1 Identification of *M. spretus* wild-type mtDNA, *M. m. domesticus* wild-type and ΔmtDNA.

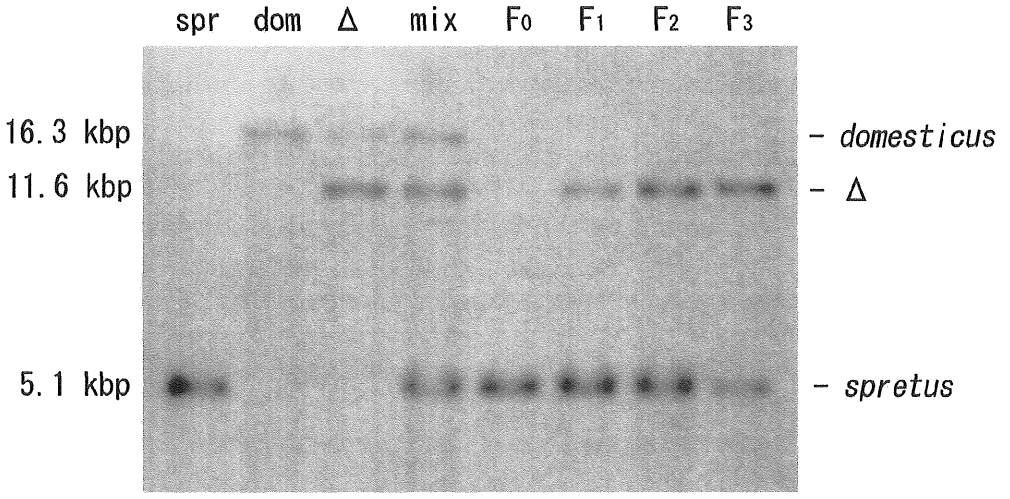
a, Southern blot analysis of *Bgl*Ⅱ fragments. Wild-type mtDNA of *M. spretus* gave three *Bgl*Ⅱ fragments (8.3, 5.1, and 2.9 kbp), and only one fragment with 5.1 kbp was detectable by the probe (nucleotide positions 1,895-2,762) used in this experiment. Wild-type mtDNA and ΔmtDNA of *M. m. domesticus* gave 16.3 and 11.6 kbp fragments, respectively.

b, Specific detection of wild-type mtDNA of *M. spretus,* wild-type and ΔmtDNA of *M. m. domesticus* by PCR analysis. Due to high sensitivity of PCR amplification, small amount of wild-type mtDNA of *M. m. domesticus* observed by PCR analysis (b) was not detected by Southern blot analysis (a).

spretus, wild-type mtDNA of *M. spretus*; domesticus, wild-type mtDNA of *M. m. domesticus*; Δ, ΔmtDNA of *M. m. domesticus*. Lanes spr, dom, Δ, and mix represent DNA samples prepared from tails of B6mtspr strain mice, B6 strain mice, Cy4696 cybrids with 89.4 ± 2.3% ΔmtDNA, and their mixtures, respectively. Lanes F_0, F_1, F_2, and F_3 represent mtDNA prepared from tails of F_0, F_1, F_2, and F_3 progenies, respectively.

Figure 1-1

a


spr
dom
Δ
mix
F0
F1
F2
F3
16.3 kbp
11.6 kbp
5.1 kbp
- domesticus
- Δ
- spretus

b

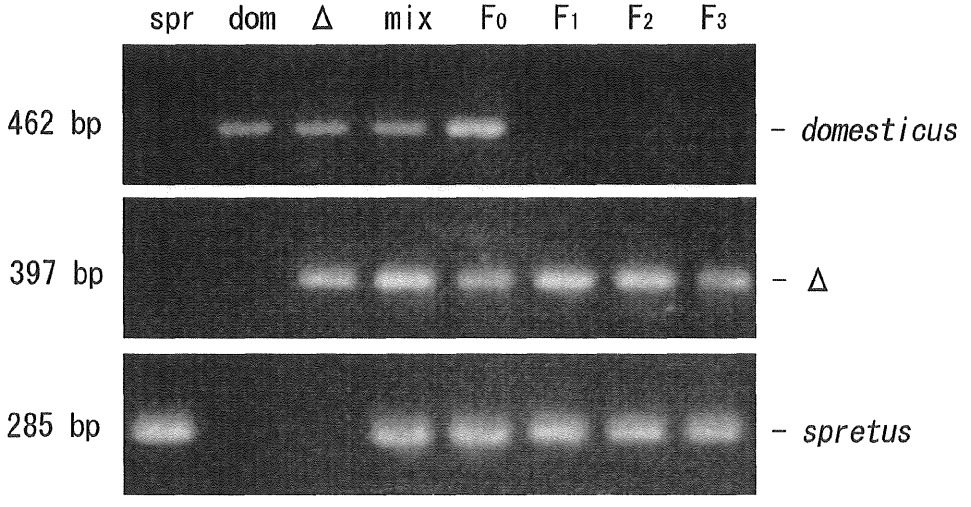

spr
dom
Δ
mix
F0
F1
F2
F3
462 bp
397 bp
285 bp
- domesticus
- Δ
- spretus

Figure 1-2 Transmission of exogenous ΔmtDNA, but not wild-type mtDNA of *M. m. domesticus* in hearts from F_3 mice.

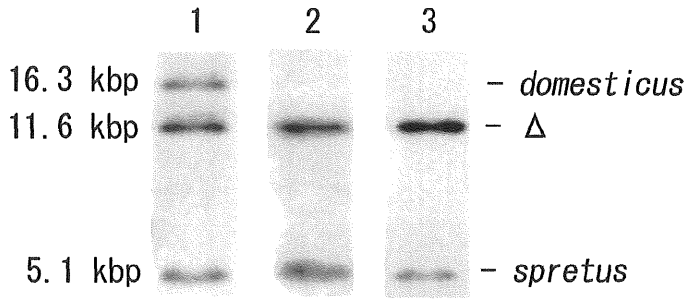
a, Southern blot analysis of *Bgl*Ⅱ fragments.

b, Specific detection of wild-type mtDNA of *M. spretus,* wild-type and ΔmtDNA of *M. m. domesticus* by PCR analysis.

Lane 1, mixtures of DNA samples including wild-type mtDNA of *M. spretus*, ΔmtDNA, and wild-type mtDNA of *M. m. domesticus*. Lanes 2 and 3, DNA samples prepared from hearts of two F_3 mito-mice with 45.2 ± 2.5, and 65.3 ± 2.7% ΔmtDNA, respectively, in their tails.

Figure 1-2

a


1
2
3
16.3 kbp
11.6 kbp
5.1 kbp
- domesticus
- Δ
- spretus

b

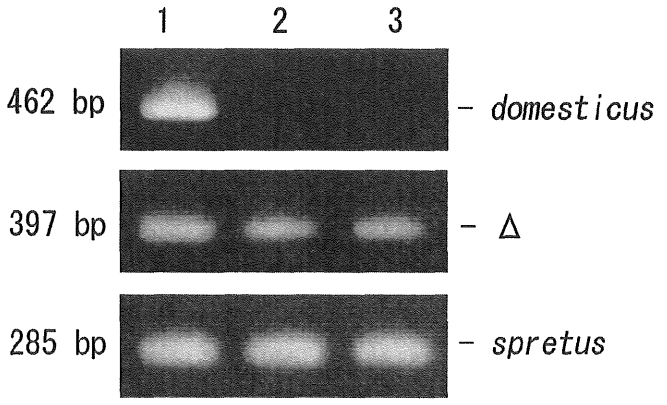

1
2
3
462 bp
397 bp
285 bp
- domesticus
- Δ
- spretus

Figure 1-3 COX histochemistry of longitudinal sections of hearts carrying 0% (left), 60.6 ± 3.4% (middle) and 85.2 ± 3.0% (right) ΔmtDNA.

Hearts consist of mononuclear cardiac muscle fibers (cardiac cells) joined end-to-end by intercalated discs (arrowheads). All cardiac cells were COX positive in heart carrying 60.6 ± 3.4% ΔmtDNA, whereas heart with 85.2 ± 3.0% ΔmtDNA consisted of COX positive and negative fibers. Scale bar, 30 μm.

Figure 1-3

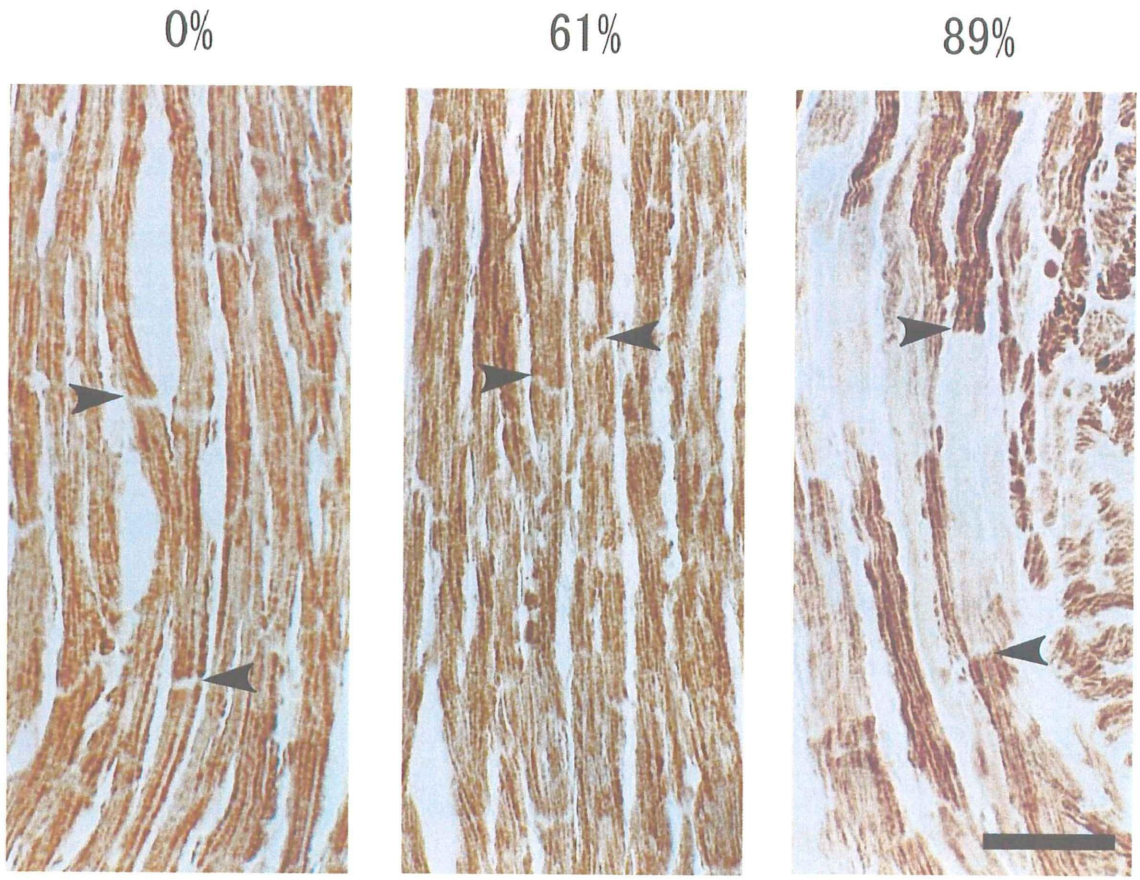


Figure 1-4 COX electronmicrographs of longitudinal sections of hearts carrying 0% (left), 60.6 ± 3.4% (middle) and 85.2 ± 3.0% ΔmtDNA (right).

Arrowheads indicate intercalated discs. Hearts carrying 60.6 ± 3.4% ΔmtDNA consist of all COX positive cells, and all individual mitochondria in each cell were COX positive. On the other hand, hearts carrying 85.2 ± 3.0% ΔmtDNA consist of cells with only COX positive mitochondria or cells with only COX negative mitochondria. No mosaic distribution of COX positive and negative mitochondria within single cardiac cells was observed, irrespective of whether hearts contained 60.6 ± 3.4% or 85.2 ± 3.0% ΔmtDNA. Scale bar, 1 μm.

Figure 1-4

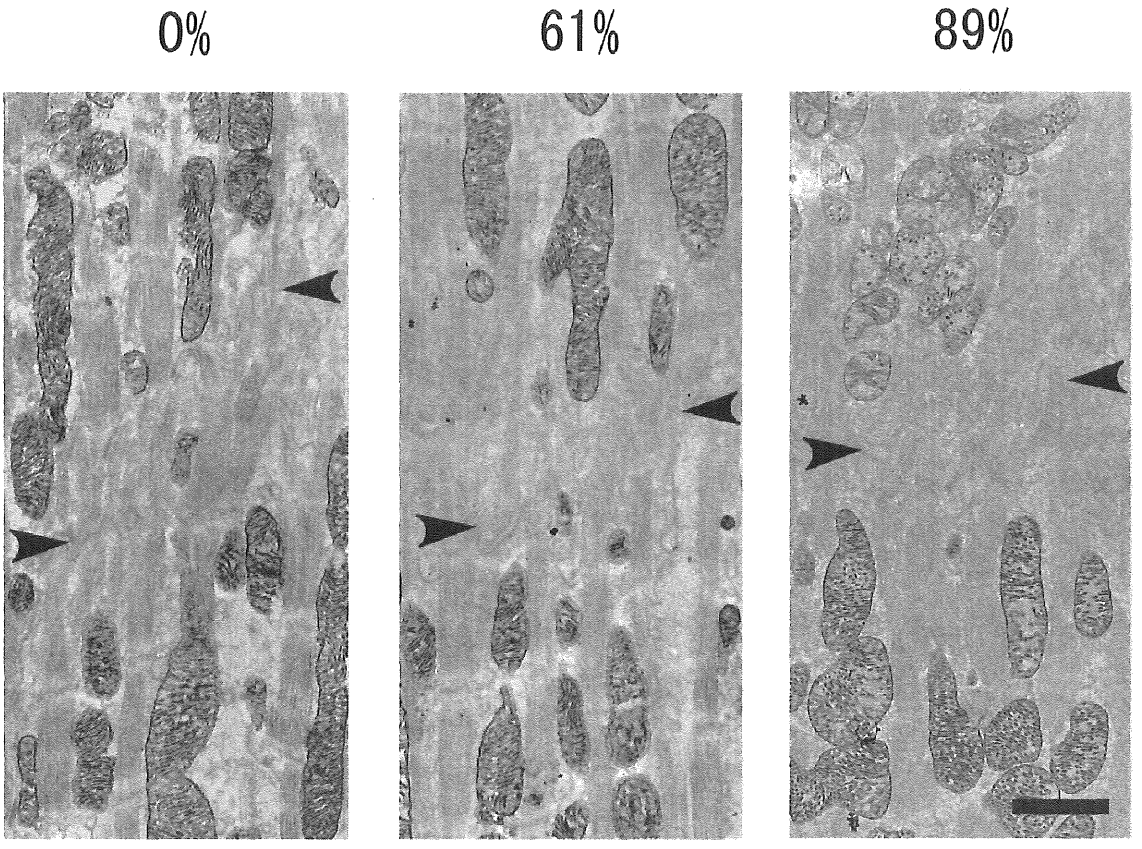
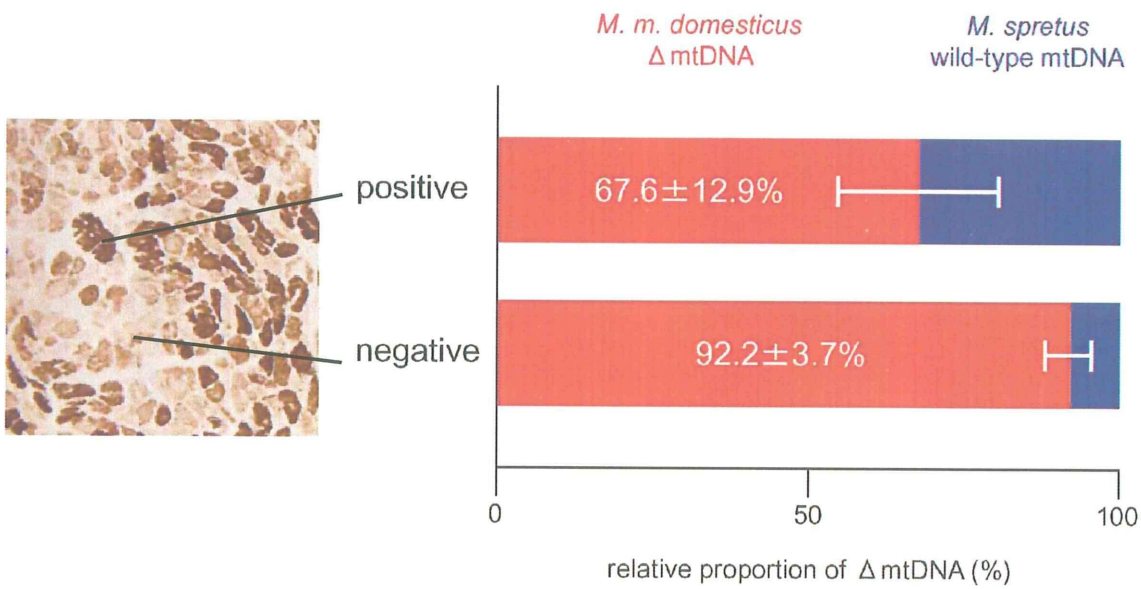


Figure 1-5 Single-cell quantitative PCR analysis of heart carrying 85.2 ± 3.0% ΔmtDNA.

COX histochemistry of heart carrying 85.2 ± 3.0% ΔmtDNA and ΔmtDNA ratios in COX positive and negative cells are shown. Three serial cryosections (10 μm) of were used for single-cell quantitative PCR. The first and third of three serial cryosections were stained with dimethylaminoazobenzene for COX activity, and COX positive and negative cells in both sections were dissected and used for quantitative PCR. COX positive cells and negative cells possessed 67.6 ± 12.9% (n=22) and 92.2 ± 3.7% (n=18) ΔmtDNA, respectively.

Figure 1-5


M. m. domesticus
Δ mtDNA
M. spretus
wild-type mtDNA
positive
negative
67.6±12.9%
92.2±3.7%
0
50
100
relative proportion of Δ mtDNA (%)

SECTION II

Rare creation of recombinant mtDNA haplotypes in mammalian somatic tissues

INTRODUCTION

Extensive recombination between mtDNAs from both parental cells has been proved in yeast (Dujon *et al.* 1974) and plant cells (Belliard *et al.* 1979; Boeshore *et al.* 1983). Moreover, the presence of recombinant mtDNA haplotypes was reported in mussel species (Ladoukakis and Zouros 2001). In mammalian species, however, there is as yet no direct evidence for mtDNA recombination, although some reports provide indirect evidence for their creation (Hayashi *et al.* 1985; Thyagarajan *et al.* 1996; Awadalla *et al.* 1999). It has been a controversial issue for many years of whether recombinant mtDNAs are created in mammalian cells (Rokas *et al.* 2003). Previous studies of my colleagues showed extensive exchanges of mtDNAs between mitochondria in human hybrid cells (Takai *et al.* 1997; Ono *et al.* 2001), and in mouse tissues (Inoue *et al.* 2000; Nakada *et al.* 2001). These findings suggested exclusion of physical barriers against recombination between heteroplasmic mtDNAs derived from different individuals or even from different species. Therefore, this issue could be resolved by precise sequence analysis of the heteroplasmic mtDNAs, which differ in many informative sequence positions. Recently, sequence analysis of PCR products of human mtDNA proved the presence of recombination between maternal and exceptionally leaked paternal mtDNAs in skeletal muscles from a patient with mitochondrial disease (Kraytsberg *et al.* 2004). However, studies using PCR for identification of recombinant mtDNA haplotypes could not completely exclude the possibility that apparent

recombinants corresponded to artifacts due to PCR jumping.

To obtain evidence for creation of recombinant mtDNA haplotypes in mammalian cells, I used two different procedures for mtDNA cloning. One was cloning of PCR products of mtDNAs, and the other was cloning of mtDNA purified by EtBr-CsCl centrifugation. For examination of *in vitro* mtDNA recombination, I used somatic hybrid cells within which two human mtDNAs possessing many different mutation sites coexist. On the other hand, for examination of *in vivo* mtDNA recombination, I carried out introduction of mitochondria into mouse zygotes to obtain mice carrying two mouse mtDNAs from different species.

MATERIALS AND METHODS

Cells and cell culture

All the cell lines and hybrid cells used in this study were grown in normal RPMI1640 medium with 0.1 mg/ml pyruvate, 50 μg/ml uridine and 10% fetal bovine serum.

Analysis of mtDNA genotypes in human hybrid cells

Total DNA extracted from 2 x 10^5 human cultured cells was used for analysis of their mtDNA genotypes. A4269G mtDNA was identified by the PCR method using a specific primer set (np 4,116-4,135 and np 4,299-4,269). The cycle times were 30 sec denaturation at 95°C, 30 sec annealing at 50°C, and 30 sec extension at 72°C for 30 cycles. The products digested with *Ssp* I were separated by 4% agarose X (Wako, Japan) gel electrophoresis in the presence of ethidium bromide (0.5 mg/ml). The A3243G mtDNA was identified by the PCR method using a specific primer set (np 3,153-3,174 and np 3,551-3,528). The cycle times were 30 sec for denaturation at 95°C, 30 sec for annealing at 45°C, and 30 sec for extension at 72°C for 30 cycles. The products digested with *Apa*I were separated by 3% agarose S (Wako, Japan) gel electrophoresis in the presence of EtBr (0.5 mg/ml).

Generation of mito-mice

Mice with exogenous ΔmtDNA, named mito-mice, were generated

as described previously (Sato *et al.* 2004). Briefly, cytoplasts containing ΔmtDNA were introduced into the perivitelline space of the B6-mt^{spr} embryos with a piezo-driven micromanipulator. The cytoplasts were fused with the embryos by applying an electric pulse. Fused embryos were transferred to the oviducts of pseudopregnant foster mothers. One F_0 female mated with C57BL/6J showed germline transmission of ΔmtDNA. An F_3 mito-mouse possessing 30.0% *M. m. domesticus* ΔmtDNA and 70.0% *M. spretus* wild-type mtDNA in the tail was used for examination of recombinant mtDNAs. For quantification of *M. m. domesticus* ΔmtDNA and *M. spretus* wild-type mtDNA, *Bgl*Ⅱ fragments of total DNA extracted from brain and skeletal muscle were transferred to a nylon membrane and hybridized with alkaline phosphate-labeled mouse probe (np 1,895-2,762). Probe labeling and signal detection were carried out as described in the protocols of the AlkPhos Direct (Amersham Pharmacia Biotech, UK).

PCR amplification of mtDNA in human hybrid cells for cloning and sequencing

Total DNA extracted from 2 x 10^5 human hybrid cells was used for amplification of mtDNA fragments including pathogenic mutation sites and the D-loop region using a primer set of human mtDNA (np 15,994-16,024 and np 4,300-4,271). The cycle times were 1 min for denaturation at 94°C, 1 min for annealing at 54.5°C, and 6 min for extension at 72°C for 30 cycles. The 4,878 bp PCR products were ligated with pUC118 (TaKaRa, Tokyo, Japan), and then introduced into DH5α (TaKaRa, Tokyo, Japan).

PCR amplification of mtDNAs in mito-mice tissues for cloning and sequencing

Total DNA was extracted from skeletal muscles of mito-mice, and mtDNA including the D-loop region was amplified with a primer set common to both *M. spretus* and *M. m. domesticus* mtDNAs (np 15,278-15,311 and np 489-468). The cycle times were 30 sec for denaturation at 95 °C, 30 sec for annealing at 55 °C and 2 min for extension at 72 °C for 30 cycles. The PCR products were ligated with pUC118 (TaKaRa, Tokyo, Japan), and then introduced into DH5α (TaKaRa, Tokyo, Japan).

Purification of mtDNA

Mitochondria and crude mtDNA were prepared from cultured human hybrid cells and mouse tissues as described (Yonekawa *et al.* 1978), with a modification of additional treating mitochondria with DNaseI to eliminate nuclear contaminants. The mtDNAs were purified by EtBr-CsCl density-gradient centrifugation at 36,000 rpm for 40 hr (SW55Ti rotor, Beckman, California, USA).

Cloning of purified human and mouse mtDNAs

Human mtDNA purified from hybrid cells was digested with *Sac*I, *EcoR*I, and *EcoR* V . After agarose gel electrophoresis, three mtDNA fragments, Fragments A (4,083 bp; np 41-4,123), Fragment B, (3,556 bp; np 3,183-6,738) and Fragment C (2,994 bp; np 9,649-12,642) possessing *Sac*I

and *EcoR*I sites, *EcoR*V sites, and *Sac*I and *EcoR*I sites, respectively, were extracted from the gels, and ligated with pBluescript II SK- and pUC118 (TaKaRa, Tokyo, Japan). The ligated vectors were introduced into DH5α and DH10B (TaKaRa, Tokyo, Japan)

Mouse mtDNA purified from skeletal muscles and brain was digested with *Mlu*I. *M. m. domesticus* mtDNA was digested at np 1,772, but *M. spretus* mtDNA was not due to the lack of *Mlu*I sites. After agarose gel electrophoresis, an 11.6 kbp fragment corresponding to whole ΔmtDNA was extracted from the gel, and ligated with pCR-XL-TOPO vector (Invitrogen, California, USA), possessing an 87 bp insertion (TAAGTTAGAGACCTTAAAATCTCCATACACCATGATGCCACAACTAGAT ACATAACATGATTTATCACA) at the TA cloning site, and then introduced into JM109 (TaKaRa, Tokyo, Japan).

Nucleotide sequence analysis

Sequence templates were prepared with a TempliPhi DNA Sequencing Template Amplification Kit (Amersham Biosciences, UK) following the manufacturer's protocol. Sequence reactions were performed by Dye Termination Methods (TaKaRa PCR Thermal Cycler GP; TaKaRa, Japan). Samples were then sequenced on MegaBACE1000 (Amersham Biosciences, UK).

Amplification of recombinant molecules using PCR

Primers specific for *M. spretus* (np 4,141-4,166, CAATTATAAAC

AATTAGGAACATGGG) and for *M. m. domesticus* (np 4,311-4,282, CATGTAAGAAGAATAAGTCCTATGTGCAGT) were used for selective amplification of recombinant molecules in mtDNA sample prepared from skeletal muscles of an F3 mito-mouse. The cycle times were 20 sec for denaturation at 95°C, 30 sec for annealing and extension at 62°C for 30 cycles.

Quantitative single cell PCR analysis

Serial cryosections (10 μm thickness) of cardiac muscle tissue carrying 87.3% ΔmtDNA excised from an F_3 mito-mouse were used for quantitative single-cell PCR. The first section was stained with dimethylaminoazobenzene for COX activity, and COX-positive and COX-negative cells were selected. Each cell in the next cryosection was dissected with PRO-300 Laser Scissors (Cell Robotics, New Mexico, USA). Quantification of *M. m. domesticus* ΔmtDNA and *M. spretus* wild-type mtDNA was carried out using a TaqMan PCR reagent kit and an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, California, USA) under the conditions recommended by the manufacturer. The primer set specific for *M. m. domesticus* ΔmtDNA was np 7,697-7,725 and 12,528-12,508. The reporter dye FAM labeled TaqMan MGB probe (Applied Biosystems, California, USA) specific for *M. m. domesticus* ΔmtDNA was np 7,750-7,758 and 12,455-12,464. The primer set specific for *M. spretus* wild-type mtDNA was np 7,442-7,469 and 7,726-7,697. The reporter dye FAM and the quencher dye TAMRA labeled probe was np 7,490-7,513.

RESULTS

First, I examined mtDNAs in human hybrid cells, which my colleagues previously isolated by fusion of two types of respiration-deficient parental cells caused by different pathogenic mutations, A3243G in the *tRNA*$^{Leu(UUR)}$ gene and A4269G in the *tRNA*^{Ile} gene of mtDNA, derived from patients with MELAS and cardiomyopathy, respectively (Ono *et al.* 2001). To allow sufficient time for recombination of parental mtDNAs in the hybrid cells, I cultivated the hybrid cells for 9 months after fusion (Fig. 2-1a). The hybrid cells I examined possessed 36% A3243G mtDNA and 64% A4269G mtDNA (Fig. 2-1a), and their coexistence restored respiratory enzyme activity to the normal level (Fig. 2-1b, c), suggesting the presence of mitochondrial complementation by extensive exchange of mtDNAs between the two parental mitochondria in the hybrid cells. This complementation indicated the coexistence of both parental A3243G and A4269G mtDNAs within the same mitochondria. Therefore, physical barriers for recombination between mtDNAs derived from different patients were excluded in the hybrid cells. Southern blot analysis of subclones isolated from the hybrid cells showed that heteroplasmy of both parental mtDNAs was maintained in all the subclones during 9 months cultivation after the fusion (data not shown).

Since many different mutation sites in parental mtDNAs were required to demonstrate the presence of their recombinants, I used 4,878bp PCR products ranging from nucleotide position (np) 15,994 to 4,300. The

products included both the pathogenic mutation sites and the D-loop region, in which many polymorphic mutations were expected to exist (Fig. 2-2a). Sequence analyses of each parental A3243G and A4269G mtDNAs and comparison of their sequences with Cambridge reference sequences (CRS) (Andrew *et al.* 1999) showed that 7 sites were specific to A3243G mtDNA, and 11 sites were specific to A4269G mtDNA (Fig. 2-2a). Then, I carried out cloning and sequence analysis of the 4,878bp PCR products of mtDNAs in the hybrid cells. Of the 100 clones I sequenced, 64 clones (clones 16-79) possessed mutations sites specific to both A3243G mtDNA and to A4269G mtDNA (Fig. 2-2b), suggesting that they apparently corresponded to recombinant mtDNA haplotypes.

For examination of *in vivo* recombination, mice with heteroplasmic mtDNAs carrying different sequences have to be generated. However, most laboratory mouse strains are derived from a single female mouse of *M. m. domesticus*, and their mtDNA sequences are almost identical (Ferris *et al.* 1982; Yonekawa *et al.* 1982). Another problem was how I could obtain clear evidence for the coexistence of both parental mtDNAs within the same mitochondria, since recombinant haplotypes could not be created unless the parental mtDNAs coexist within the same mitochondria. To overcome these problems, I used previously generated mito-mice (Sato *et al.* 2004). The mice were generated by introducing ΔmtDNA of *M. m. domesticus* (Fig. 2-3a, right) into zygotes of congenic B6-mt^{spr} strain mice carrying wild-type mtDNA of *M. spretus* (Fig. 2-3a, left), which belongs to the genus *Mus*, and so is a different species from *M. m. domesticus*.

After three generations, I obtained an F_3 mito-mouse (6 months old) carrying 60.0% ΔmtDNA of *M. m. domesticus* and 40.0% wild-type mtDNA of *M. spretus* in its tail. Since the exo- and endogenous mtDNAs were derived from different mouse species, they possessed many different mutation sites. Cytochrome *c* oxidase (COX) electronmicrographs, which can identify COX activity at the individual mitochondrial level, showed that all mitochondria from skeletal muscles with 58.5% ΔmtDNA possessed COX activity (data not shown). If exogenous COX-negative mitochondria with ΔmtDNA and endogenous COX-positive mitochondria with wild-type mtDNA do not fuse with each other, 58.5% mitochondria should be COX negative, but this was not the case. The presence of COX activity in all mitochondria suggested the coexistence of exogenous ΔmtDNA of *M. m. domesticus* and endogenous wild-type mtDNA of *M. spretus* within the same mitochondria in mito-mice tissues.

Since the exo- and endogenous mtDNAs were derived from different mouse species, they were expected to possess many different mutations. Sequence analysis of 1.5 kbp PCR products including the D-loop region showed differences due to 89 base substitutions or deletions in the mtDNAs of *M. m. domesticus* and *M. spretus*, and thus their recombinants present in tissues of the mito-mice could easily be identified. Cloning and sequence analyses were carried out using 1.5 kbp PCR products of the skeletal muscles carrying 58.5% ΔmtDNA from an F_3 mito-mouse (Fig. 2-3). Of 21 clones sequenced, 11 clones (clone 1-11) and six clones (clone 16-21) possessed exactly the same sequences as those of parental mtDNAs of *M. m.*

domesticus and *M. spretus*, respectively (Fig. 2-3b). On the other hand, the other four clones (clone 12-15) all possessed mutations specific to both *M. m. domesticus* and *M. spretus* mtDNAs.

For confirmation of such an extensive creation of mtDNA recombinants, I carried out cloning of purified mtDNA without using PCR amplification in the hybrid cells. The mtDNA prepared from the hybrid cells was purified by EtBr-CsCl centrifugation, and the purified mtDNA was digested with restriction enzymes (Fig. 2-4a). After agarose gel electrophoresis, three fragments, Fragments A, B, and C, consisting of 4,083 bp, 3,556 bp, and 2,994 bp fragments, respectively, were extracted from the gels for their cloning.

Fragment A included 11 CRS sites that are specific to either A4269G or A3243G mtDNA (Fig. 2-4b). Of 85 clones of Fragment A, 30 clones (clones 1-30) and 55 clones (clones 31-85) possessed sequences identical to those of parental A3243G and A4269G mtDNAs, respectively, although single somatic mutations were observed in 9 clones (clones 25-33) (Fig. 2-4b). Fragment B included 10 CRS sites that are specific to either A4269G or A3243G mtDNA. Of 101 clones of Fragment B, 44 clones (clones 1-44) and 57 clones (clones 45-101) possessed sequences identical to those of parental A3243G and A4269G mtDNA, respectively, although single somatic mutations were observed in 16 clones (clones 41-56) (Fig. 2-4b). Fragment C included 7 CRS sites that are specific to either parental A4269G or A3243G. Of 85 clones of Fragment C, 35 clones (clones 1-35) and 50 clones (clones 36-85) possessed sequences identical to those of parental A3243G and

A4269G mtDNAs, respectively (Fig. 2-4b). These observations suggested that there were no recombinant mtDNAs in any of the clones I examined. I sequenced more than 85 clones, which covered 60% of the whole mitochondrial genome (Fig. 2-4). Thus, even if recombinant mtDNA haplotypes were present in the hybrid cells, their frequency would be less than 2%. These observations suggested that most apparent recombinants observed in PCR products (Fig. 2-2b) corresponded to jumping artifacts rather than to real recombinants.

In these experiments, I used human hybrid cells isolated by the fusion of somatic cells. Therefore, it is still possible that mtDNA recombination is an event that frequently occurs in tissues or in female germ line cells, but not in cultivated somatic cells.

This time, to avoid PCR jumping artifacts, I used mouse mtDNA purified by EtBr-CsCl centrifugation for cloning and sequencing. From an F_3 mito-mice carrying 30.0% ΔmtDNA in its tail, skeletal muscles and brain were excised. Southern blot analysis of *Bgl*Ⅱ digests showed that mtDNA samples purified from mito-mouse skeletal muscles and brain contained 30.7% and 34.2% ΔmtDNA of *M. m. domesticus*, respectively (Fig. 2-5b). To exclude the possibility of recombination between two plasmids carrying ΔmtDNA and *M. spretus* mtDNA after cloning in *E. coli* cytoplasm, I carried out exclusive cloning of ΔmtDNA by digestion of purified mouse mtDNA with restriction enzyme *Mlu* I , so that *M. spretus* mtDNA could not be cloned due to the absence of the *Mlu* I site (Fig. 2-5a).

After agarose gel electrophoresis, an 11.6 kbp fragment

corresponding to whole linear ΔmtDNA was extracted from the gels for its cloning. Sequencing of 107 and 56 clones of whole ΔmtDNA purified from skeletal muscles and brain, respectively, showed that only one clone (clone 10) of muscle ΔmtDNA contained 37-65 bp and 121-135 bp regions in which the sequences were identical to those of *M. spretus* mtDNA (Fig. 2-5c, d). The remaining 162 ΔmtDNA clones did not contain sequences identical to that in *M. spretus* mtDNA. These observations suggested that the frequency of recombinant mtDNA haplotypes in mito-mice tissues was less than 1%. On the other hand, while 11 clones (9 in skeletal muscles and 2 in brain) of 162 clones possessed somatic mutations (Fig. 2-5c), no clones shared the same somatic mutations, suggesting that clonal expansion of ΔmtDNA haplotypes carrying specific somatic mutations did not occur in mouse tissues.

Then, for obtaining the evidence for the presence of the recombinant molecules in the mtDNA samples before cloning, I carried out their selective amplification using a mismatch primer set containing sequences specific to *M. spretus* and *M. m. domesticus*, respectively (Fig. 2-6a). In 30 cycles of the PCR, an expected 171bp fragment was exclusively amplified from the mito-mouse mtDNA sample, but not from both parental mtDNAs and from their mixture (Fig. 2-6b). However, 50 cycles of the PCR produced the 171 bp fragment from the mixture (Fig. 2-6b), suggesting that all the 171bp fragments corresponded to PCR jumping products. For excluding this possibility, I carried out cloning and sequencing of the 171 bp fragments amplified from the mito-mouse mtDNA and from mixture of

parental mtDNAs, respectively. The results showed that all clones from the mito-mouse mtDNA showed identical sequence to that of the recombinant clone 10, whereas all clones from the mixture mtDNAs possessed different sequences from that of the recombinant clone 10 (Fig. 2-6c). Therefore, former clones corresponded to real recombinants, whereas latter clones corresponded to PCR jumping artifacts. These observations provided unambiguous evidence for rare creation of the recombinant molecules identical to clone 10 in skeletal muscles of the mito-mouse (Fig. 2-5d).

DISCUSSION

I observed very rare mtDNA recombination only in skeletal muscles from mito-mice. Then, a question is what is the biological significance of sequence changes in mammalian mtDNA. One explanation for creating such products is complementation of mitochondrial dysfunction caused by pathogenic mutations in mtDNA. In this case, the restoration of respiratory function in the hybrid cells (Fig. 2-1c) could be attained by production of new mtDNA haplotypes with neither A3243G nor A4269G mutations by recombination, because the accumulation of more than 5% recombinant haplotypes without both mutations may restore respiratory function in the hybrid cells (Chomyn *et al.* 1992). However, this study unambiguously showed that no such forms were present in 101 clones of Fragment B (Fig. 2-4b), although the hybrid cells showed restoration from respiration deficiency (Fig. 2-1c). Such a low frequency of recombination, even if it occurred, between heteroplasmic mtDNA would not complement respiration defects caused by various mutations in mtDNA. Thus, the observed restoration in human hybrid cells was entirely due to exchange of mtDNAs or their products between mitochondria, not to recombination between mtDNAs.

Another explanation for creating such products is generation of sequence divergence in the mammalian mtDNA population. Creation of recombinant mtDNA haplotypes was reported in species that show biparental mtDNA inheritance (Dujon *et al.* 1974) or leakage of paternal

mtDNA (Ladoukakis *et al.* 2001). In these cases, recombination occurred between maternal and paternal mtDNAs, and its biological meaning should be to provide extensive variations of mtDNA haplotypes in the species. In mammalian species, however, rapid and complete elimination of paternal mtDNA from zygotes (Kaneda *et al.* 1995; Shitara *et al.* 1998) prevents the coexistence of mtDNAs from both parents within individuals, resulting in the homoplasmic nature of the maternal mtDNA population in each individual. Thus, recombination of homoplasmic mtDNAs to create homoplasmic mtDNAs does not seem to have any biological significance in such an asexual genetic system.

While the transmission of paternal mtDNA was reported in a patient with mitochondrial disease (Shwartz and Vissing 2002), and in mouse interspecies F_1 hybrids (Shitara *et al.* 1998), subsequent studies showed no paternal mtDNA leakage in patients with mitochondrial diseases (Taylor *et al.* 2003; Filosto *et al.* 2003), and no transmission of the leaked paternal mtDNA to following generations (Shitara *et al.* 1998). Therefore, paternal mtDNA leakage is rare and its transmission is even rarer in mammalian species. Moreover, this study shows that the frequency of creation of new haplotypes with sequence changes between heteroplasmic mtDNAs is very low in mammalian species (Figs. 2-4b and 2-5c). All these observations suggest that transmission of recombinant mtDNA haplotypes through female germ lines to the following generation is an extremely rare event, even when paternal mtDNA leaks by some defect of the machinery required to ensure complete elimination of sperm mtDNA from zygotes.

Therefore, the biological significance of sequence changes in mammalian mtDNA seems to be neither the generation of sequence variations in the mammalian mtDNA population, nor complementing respiration defects caused by deleterious somatic mutations in mtDNA (Fig. 2-1c).

On the other hand, it is highly likely that the clone with sequence changes simply reflect a product resulting from the mtDNA repair reaction upon strand breakage. Due to the high concentration of reactive oxygen species (ROS) in mitochondria, there should be frequent creation of single strand breakage in mtDNA, resulting in some strand invasion and displacement between homologous or heterologous mtDNA molecules in heteroplasmic cells. In the case of strand invasion between heterologous mtDNA molecules, nucleotide mismatches in the heteroduplex should be subsequently repaired by the mismatch repair activity in mitochondria (Mason *et al.* 2003). If invaded strands are repaired, there should be no sequence changes, but in the case of repair of recipient strands, the repair process should create sequences identical to those of the invaded mtDNA molecules. In fact, the sequence changes I observed were restricted to very small regions (Fig. 2-5c). Thus, the observed recombinant mtDNA haplotype would simply reflect the gene conversion products in the repair of damaged mtDNA molecules, rather than the classic recombination for creating sequence divergence in the mtDNA population.

This study also resolves a controversial issue of whether clonal expansion of mtDNA and resultant expression of respiration defects occurs

in mouse tissues. There have been many reports suggesting age-associated clonal expansion of some mtDNA haplotypes carrying somatic mutations in human tissues (Michikawa *et al.* 1999; Chinnery *et al.* 2002; Trifunovie *et al.* 2004). Recently, Trifunovic *et al.* (2004) reported generation of ageing-model mice expressing proof-reading-deficient mtDNA polymerase, in which increased levels of somatic mutations in mtDNA appear to be associated with respiration defects, reduced life span, and premature onset of ageing-related phenotypes. They assumed that clonal expansion of mtDNA haplotypes with various somatic mutations should be one of factors responsible for respiration defects and resultant expression of ageing phenotypes. However, sequence studies of ΔmtDNA in mito-mice showed that 11 of 163 clones possessed somatic mutations, but no clones shared the same somatic mutations (Fig. 2-5c), suggesting no clonal expansion of the specific mtDNA haplotypes carrying somatic mutations in mouse tissues. For precise examination of clonal expansion, COX histochemistry (Fig. 2-7a) and quantitative analysis of ΔmtDNA were carried out using serial sections of the same cardiac cells (Fig. 2-7b), since they could provide ideal system for showing both respiratory function and the proportion of mutated mtDNA within the same cells. I used the heart of an F_3 mito-mouse containing 87.3% ΔmtDNA, and the results showed that COX-positive and -negative fibers (cardiac cells) possessed 73.0±5.0% and 92.5±3.2% ΔmtDNA, respectively, suggesting the absence of clonal expansion of ΔmtDNA in cardiac cells. The results in Fig. 2-5c and Fig. 2-7 provided direct evidence that no clonal expansion of specific mtDNA haplotypes carrying some

mutations occurred in cells of the 6 months old mito-mice. Thus, clonal expansion of mtDNA may occur in aged human tissues, but not in aged mouse tissues. Probably, longer time is required to attain clonal expansion of mtDNA in mouse tissues as well as in human tissues.

This study resolves another controversial issue of whether mammalian mitochondria interact with each other and exchange their genetic products (Attardi *et al.* 2002). Previous reports of my colleagues provided evidence for interactions between mitochondria based on the observation that expression of respiration defects induced by exogenous ΔmtDNA was prevented by endogenous wild-type mtDNA of the same mouse species in mito-mice (Nakada *et al.* 2001). Although this could be explained by assuming clonal expansion of wild-type mtDNA preexisting in exogenous mitochondria (Attardi *et al.* 2002), this possibility was completely excluded by the findings that the exo- and endogenous mtDNA genotypes come into close proximity to convert their sequences (Fig. 2-5c), and that clonal expansion of specific mtDNA haplotypes did not occur in mouse tissues (Figs 2-5c and 2-7). Therefore, mammalian mitochondria have the abilities not only to exchange genetic contents, but also to interact mtDNA molecules and create gene conversion products (Fig. 2-5c). These observations support "interaction theory of mitochondria" (Hayashi *et al.* 1994; Nakada *et al.* 2001a, b; Ono *et al.* 2001), which proposes that free mixing of mitochondrial matrix components throughout the mitochondrial network would protect mammalian mitochondria from direct expression of respiration defects caused by accumulated somatic mutations in mtDNAs, or by ROS-induced

mtDNA strand breakage.

Figure 2-1 Coexistence of parental pathogenic mutated mtDNAs within hybrid cells and its effect on phenotypic expression of mitochondrial respiratory function.

ρ^+ 143B, 143BTK- cells; ρ^+ HeLa, HeLa cells; ρ^0 143B, mtDNA-less 143BTK- cells; ρ^0 HeLa, mtDNA-less HeLa cells; *syn*- HeLa and *syn*-143B are respiration-deficient HeLa cells and 143BTK- cells due to A4269G and A3243G pathogenic mutations in the $tRNA^{Ile}$ gene and $tRNA^{Leu(UUR)}$gene, respectively. The *syn*- cells were used as parental cells for isolation of their hybrid cells (*syn*- 143B x *syn*- HeLa). 9m represents the cultivation time (months) of the hybrid cells after fusion.

a, Analyses of mtDNA genotypes of parental cells and their hybrid cells. For identification of the mutated A3243G mtDNA (3243^+) and A4269G mtDNA (4269^+), I carried out restriction enzyme analyses of the PCR products. As the result of gain of an *Apa* I site by an A3243G substitution, the PCR products of A3243G mtDNA (3243^+) produce a 309 bp fragment, whereas those of A4269G and wild-type mtDNAs without the mutation (3243^-) produce a 399 bp fragment. On the other hand, A4269G mtDNA (4269^+) produces a 184 bp fragment because of the loss of an *Ssp* I site by an A4269G substitution, whereas A3243G and wild-type mtDNAs without the mutation (4269^-) produce a 153 bp fragment. Hybrid cells show the coexistence of parental A3243G mtDNA (3243^+) and A4269G mtDNA (4269^+).

b, Analysis of phenotype of mitochondrial translation activity. After specific [^{35}S]-methionine labeling of mitochondrial translation products,

mitochondrial proteins were separated by SDS-PAGE. ND5, COI, ND4, Cytb, ND2, ND1, COII, COIII, ATP6, ND6, ND3, ND3', ATP8, and ND4L are polypeptides encoded by mtDNA genes (cf. Fig. 2a). No activities are observed in ρ^0 HeLa and ρ^0 143B due to their mtDNA depletion, and in *syn*$^-$ HeLa and *syn*$^-$ 143B due to pathogenic mutations in tRNA genes. Hybrid cells show restored mitochondrial translation activities.

c, Analysis of phenotype of respiratory enzyme activity. Examination of enzyme activity of complex Ⅳ (cytochrome *c* oxidase; COX), one of the respiratory enzyme complexes. No cells without mitochondrial translation activities show COX activity, while hybrid cells show restoration of the reduced COX activity in their parental syn$^-$ cells.

Figure 2-1

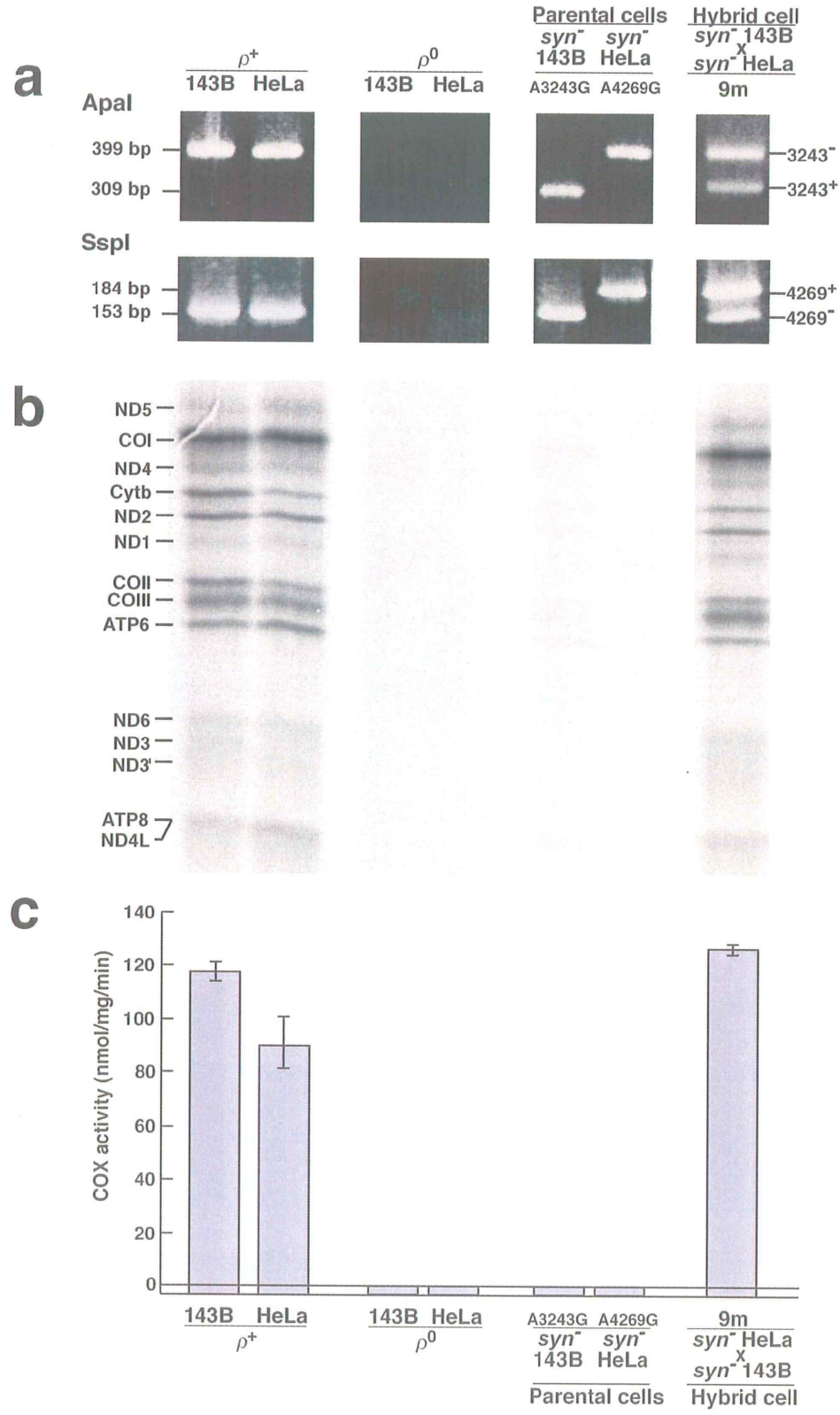

a
ρ+
143B HeLa
ρ0
143B HeLa
Parental cells
syn- 143B
syn- HeLa
A3243G A4269G
Hybrid cell
syn- 143B x syn- HeLa
9m
ApaI
399 bp
309 bp
3243-
3243+
SspI
184 bp
153 bp
4269+
4269-
b
ND5
COI
ND4
Cytb
ND2
ND1
COII
COIII
ATP6
ND6
ND3
ND3'
ATP8
ND4L
c
COX activity (nmol/mg/min)
140
120
100
80
60
40
20
0
143B HeLa
ρ+
143B HeLa
ρ0
A3243G A4269G
syn- 143B
syn- HeLa
Parental cells
9m
syn- HeLa x syn- 143B
Hybrid cell

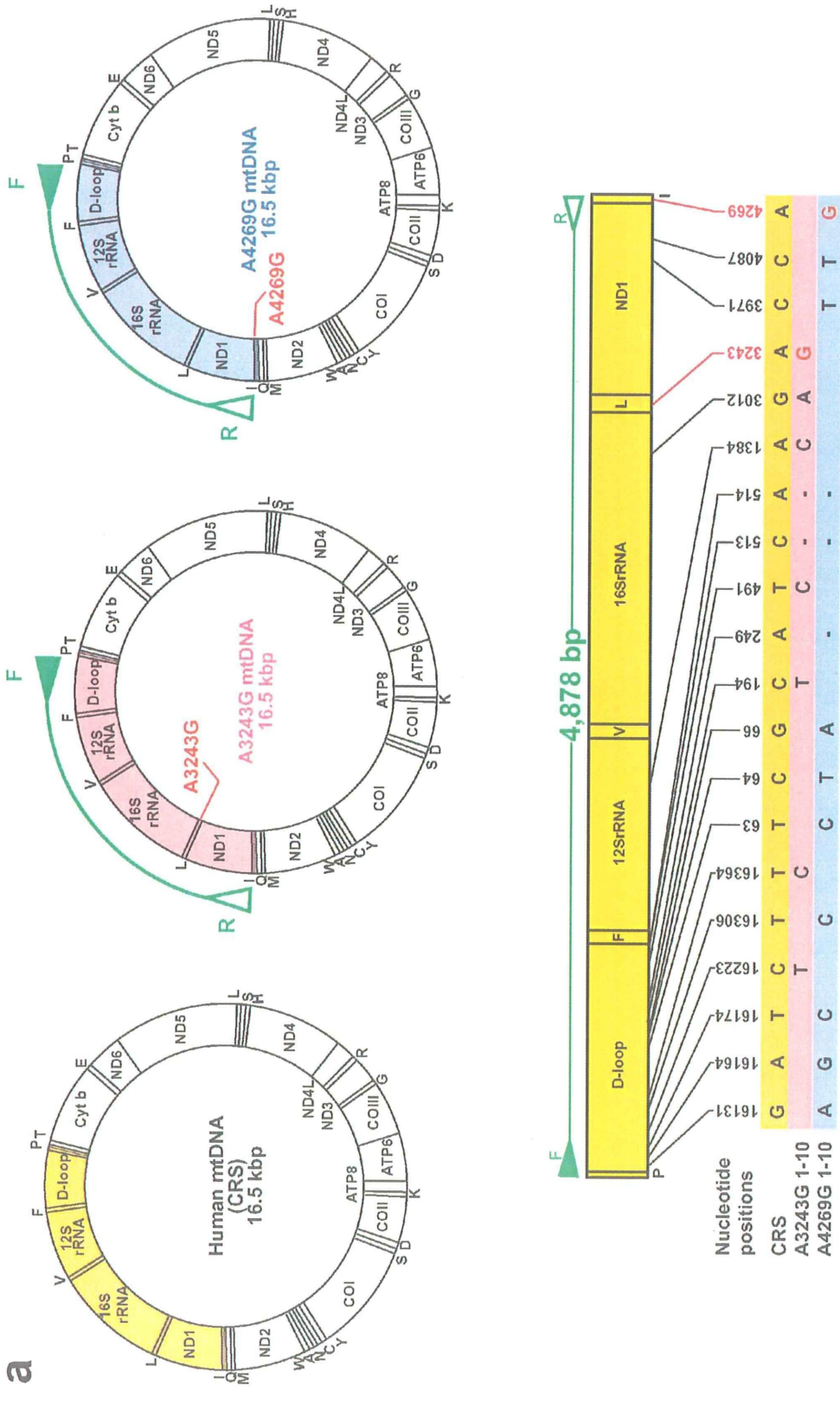
Figure 2-2 Sequence analysis of cloned PCR fragments from hybrid cells carrying parental A3243G and A4269G mtDNAs.

a, Gene maps of human mtDNAs. Closed and open green arrowheads on the maps represent forward primer (F) and reverse primer (R), respectively, used for amplification of 4,878 bp products (arcs). The cloned regions of 4,878bp PCR products include the D-loop and the pathogenic mutation sites of both parental A3243G and A4269G mtDNAs, and are shown in pink and blue regions, respectively, on the gene maps. The corresponding region of the Cambridge reference sequence (CRS) (Bibb *et al.* 1981) is shown in yellow. CRS shown in the yellow lane was used as a human mtDNA standard for comparison with the two parental mtDNA sequences. A3243G 1-10, sequence of 10 clones of the PCR products amplified from A3243G mtDNA is shown in the pink lane. A4269G 1-10, sequence of 10 clones of the PCR products amplified from A4269G mtDNA is shown in the blue lane. The results on the sequences of the cloned 4,878 bp PCR products show that 20 sites in 4,878 bp sequences are different from that of CRS. The nucleotide positions of these 20 sites in CRS are shown in the yellow lane. Of 20 sites, 2 are common to A4269G and A3243G mtDNAs, and the other 18 sites are specific to either A4269G or A3243G mtDNA. Of 18 sites, six polymorphic mutations and one pathogenic mutation are specific to A3243G mtDNA, while 10 polymorphic and one pathogenic mutation are specific to A4269G mtDNA. Polymorphic mutation sites common to both A4269G and A3243G are shown by black letters. Polymorphic mutation sites specific to A3243G

mtDNA or A4269G mtDNA are shown by black lines and letters. Pathogenic mutation sites, A4269G and A3243G, are shown by red lines and letters.

b, Sequences of the cloned 4,878 bp PCR products of mtDNAs from the hybrid cells carrying parental A3243G and A4269G mtDNAs. As a reference, 18 sites of CRS that are specific to either A4269G or A3243G mtDNA are shown in the yellow lane. Mutation sites specific to A3243G mtDNA are shown in pink, and those specific to A4269G mtDNA are shown in blue. Of 100 clones I sequenced, 15 clones (clones 1-15) and 21 clones (clones 80-100) possessed sequences completely identical to those of parental A3243G and A4269G mtDNAs, respectively. However, the other 64 clones (clones 16-79) were apparent recombinants due to mutations derived from different parental mtDNAs. For example, clones 16-43 possess the A3243G mutation, but possess some polymorphic mutations derived from A4269G mtDNA. Clones 44-51 possess both A3243G and A4269G mutations. Clones 52-71 possess the A4269G mutation, but also some polymorphic mutations derived from A3243G mtDNA. Clones 72-79 do not possess either pathogenic mutation. I observed 35 somatic mutations specific to each clone (data not shown).

Figure 2-2


a
Human mtDNA (CRS) 16.5 kbp
A3243G mtDNA 16.5 kbp
A3243G
A4269G mtDNA 16.5 kbp
A4269G
ND1
ND2
COI
COII
ATP8
ATP6
COIII
ND3
ND4L
ND4
ND5
ND6
Cyt b
D-loop
12S rRNA
16S rRNA
F
R
4,878 bp
D-loop
12SrRNA
16SrRNA
ND1
4269
4087
3971
3243
3012
1384
514
513
491
249
194
66
64
63
16364
16306
16223
16174
16164
16131
Nucleotide positions
CRS
A3243G 1-10
A4269G 1-10

b

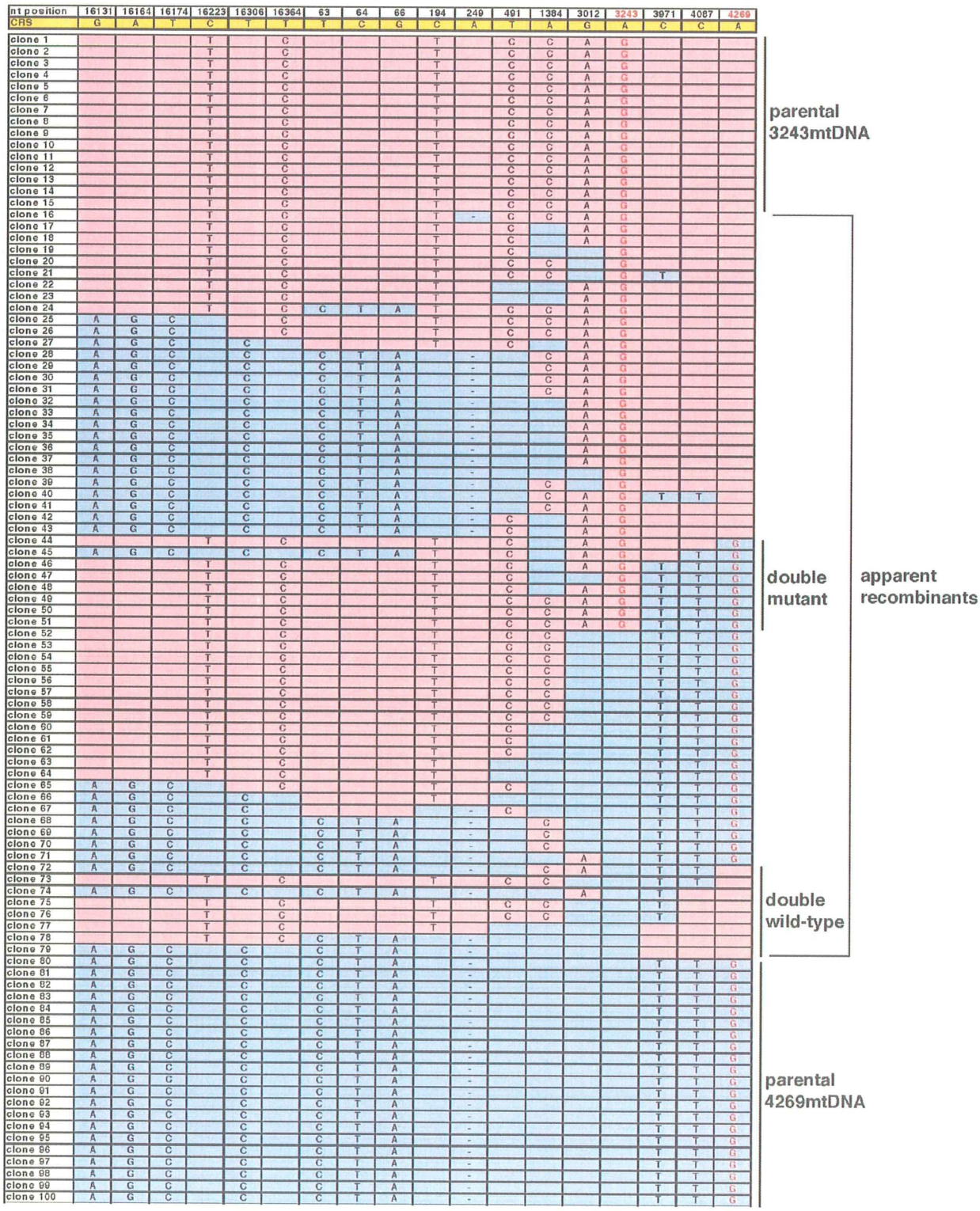

parental 3243mtDNA
apparent recombinants
double mutant
double wild-type
parental 4269mtDNA

Figure 2-3 Sequence analysis of cloned PCR fragments from mito-mice.

a, Gene maps of wild-type mtDNA of *M. spretus* (green circle) and ΔmtDNA of *M. m. domesticus* (smaller red circle). The open arc in the ΔmtDNA map corresponds to the deleted region expanded from $tRNA^{Lys}$ to *ND5* genes (np 7,759-12,454). Closed and open arrowheads on the maps represent forward primer and reverse primer, respectively, used for amplification of PCR products (arcs).

b, Sequences of the cloned PCR products of mito-mice carrying 58.5% ΔmtDNA in its skeletal muscles. Polymorphic sites specific to *M. m. domesticus* are shown in red lanes, and those specific to *M. spretus* are shown in green lanes. Of 85 polymorphic sites between *M. m. domesticus* and *M. spretus*, 25 are shown. Of 21 clones I sequenced, 11 clones (clones 1-11) and 5 clones (clones 16-21) possessed sequences completely identical to those of parental *M. m. domesticus* and *M. spretus* mtDNAs, respectively. However, the other 4 clones (clones 11-15) were apparent recombinants due to mutations derived from different parental mtDNAs.

Figure 2-3

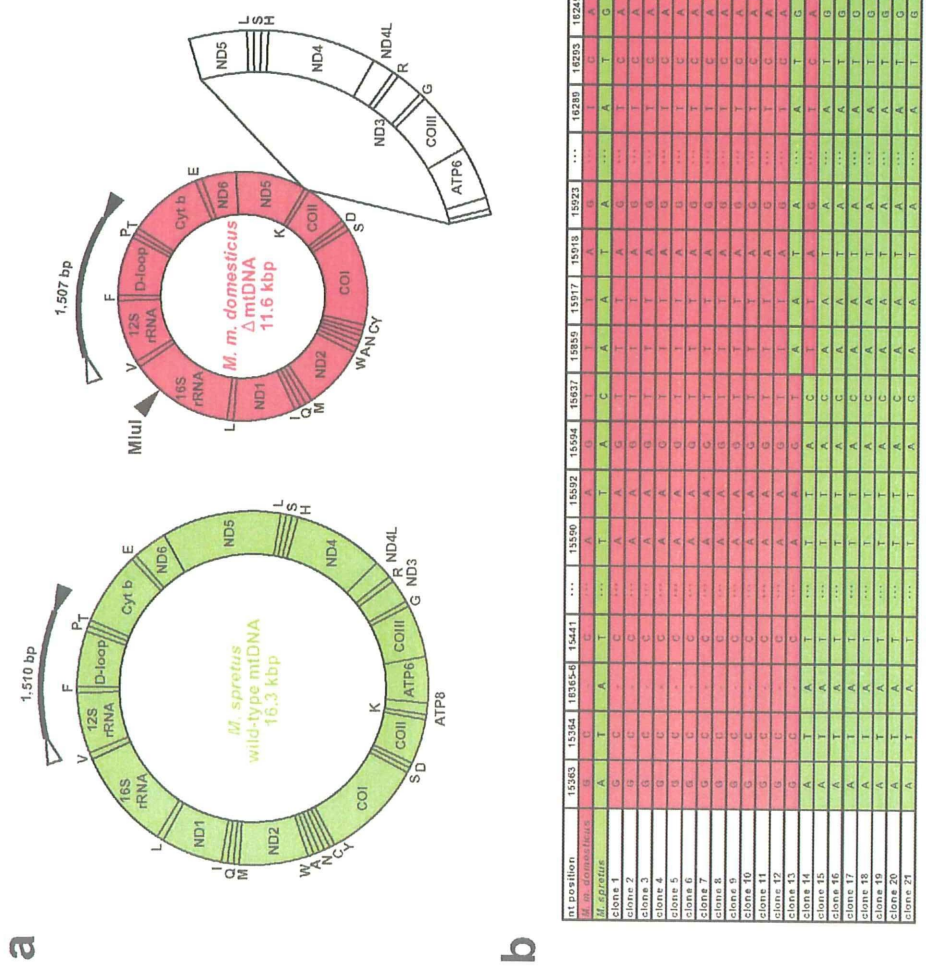
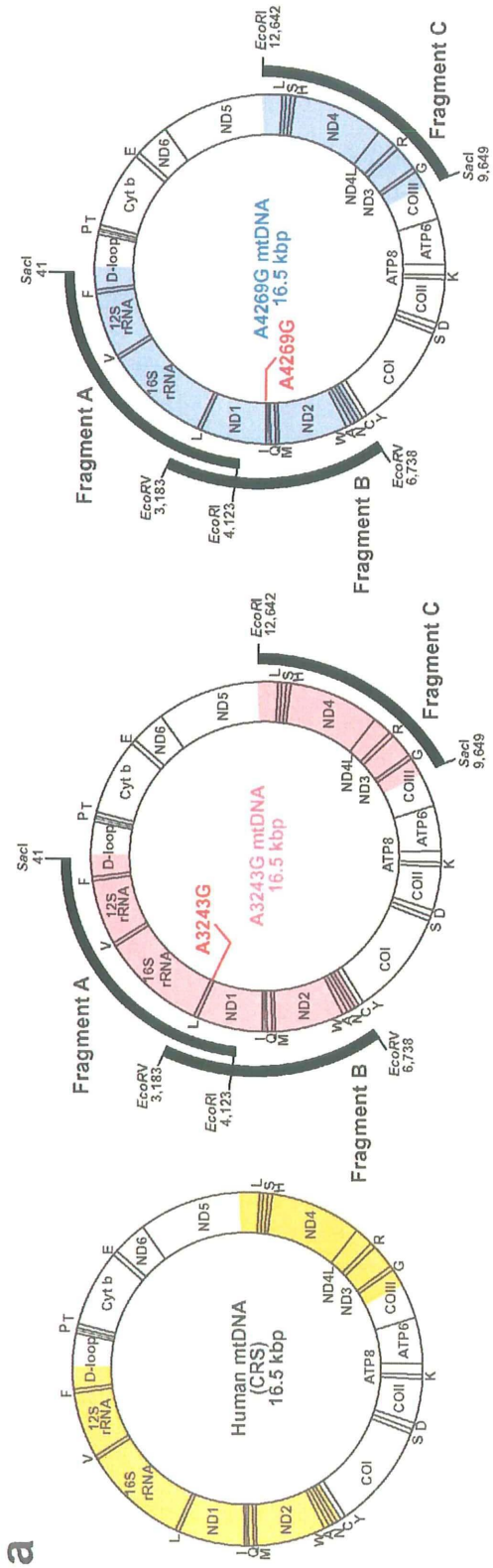

a
M. spretus
wild-type mtDNA
16.3 kbp
M. m. domesticus
ΔmtDNA
11.6 kbp
1.510 bp
1.507 bp
MluI
16S rRNA
12S rRNA
D-loop
Cyt b
ND1
ND2
COI
COII
ATP6
ATP8
COIII
ND3
ND4L
ND4
ND5
ND6
b
parental M. m. domesticus mtDNA
aparent recombinants
parental M. spretus mtDNA

Figure 2-4 Sequence analysis of purified mtDNA from hybrid cells carrying parental A3243G and A4269G mtDNAs.

a, Gene maps of human mtDNAs indicating sequenced regions. Cloning and sequencing were carried out using Fragments A (4,083 bp), B (3,556 bp), and C (2,994 bp) purified from the hybrid cells carrying parental A3243G and A4269G mtDNAs (arcs). Sequenced regions corresponding to CRS, parental A3243G and A4269G mtDNAs are shown in yellow, pink and blue, respectively.

b, Sequences of the cloned mtDNA fragments, Fragments A, B, and C. CRS shown in the yellow lane is used as a human mtDNA standard for comparison with both parental mtDNA sequences. Pink and blue lanes represent the sequences of A3243G mtDNA and A4269G mtDNAs, respectively, purified from parental cells.

Figure 2-4


a
Human mtDNA
(CRS)
16.5 kbp
A3243G mtDNA
16.5 kbp
A3243G
A4269G mtDNA
16.5 kbp
A4269G
Fragment A
Fragment B
Fragment C
EcoRI
12,642
SacI
9,649
EcoRV
6,738
EcoRI
4,123
EcoRV
3,183
SacI
41
ND1
ND2
COI
COII
ATP8
ATP6
COIII
ND3
ND4L
ND4
ND5
ND6
Cyt b
D-loop
12S rRNA
16S rRNA

b

Fragment A

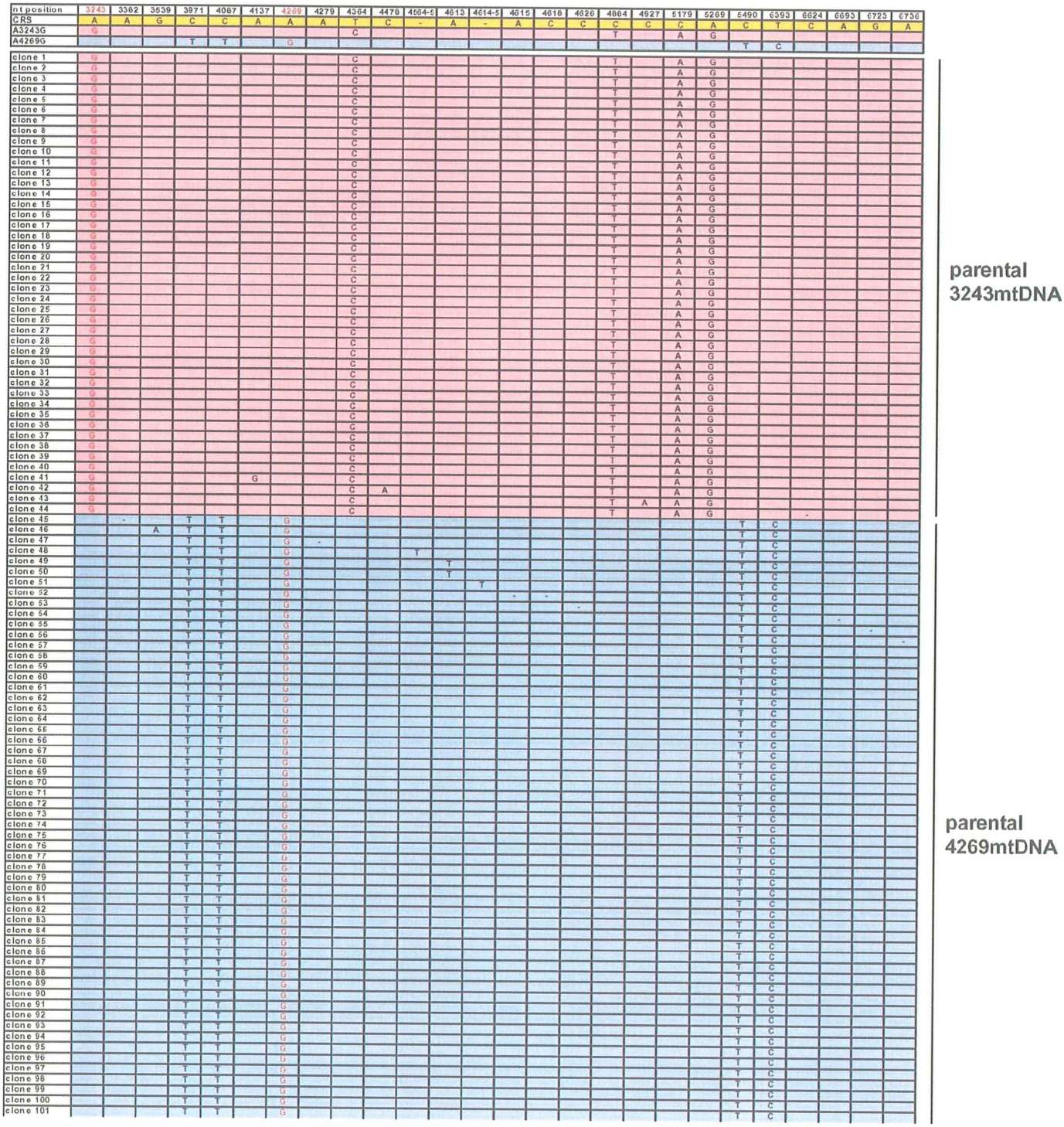
nt position	63	64	66	194	248	303-4	491	879	1384	2175	2455	2779	2830	3012	3243	3971	4087
CRS	T	C	G	C	A	-	T	G	A	G	G	G	G	G	A	C	C
A3243G				T			C		C					A	G		
A4269G	C	T	A		-											T	T
clone 1				T			C		C					A	G		
clone 2				T			C		C					A	G		
clone 3				T			C		C					A	G		
clone 4				T			C		C					A	G		
clone 5				T			C		C					A	G		
clone 6				T			C		C					A	G		
clone 7				T			C		C					A	G		
clone 8				T			C		C					A	G		
clone 9				T			C		C					A	G		
clone 10				T			C		C					A	G		
clone 11				T			C		C					A	G		
clone 12				T			C		C					A	G		
clone 13				T			C		C					A	G		
clone 14				T			C		C					A	G		
clone 15				T			C		C					A	G		
clone 16				T			C		C					A	G		
clone 17				T			C		C					A	G		
clone 18				T			C		C					A	G		
clone 19				T			C		C					A	G		
clone 20				T			C		C					A	G		
clone 21				T			C		C					A	G		
clone 22				T			C		C					A	G		
clone 23				T			C		C					A	G		
clone 24				T			C		C					A	G		
clone 25				T			C	A	C					A	G		
clone 26				T			C		C			T		A	G		
clone 27				T			C		C	A				A	G		
clone 28				T			C		C	A				A	G		
clone 29				T			C		C				A	A	G		
clone 30				T			C		C				A	A	G		
clone 31	C	T	A		-	C										T	T
clone 32	C	T	A		-	C										T	T
clone 33	C	T	A		-						A					T	T
clone 34	C	T	A		-											T	T
clone 35	C	T	A		-											T	T
clone 36	C	T	A		-											T	T
clone 37	C	T	A		-											T	T
clone 38	C	T	A		-											T	T
clone 39	C	T	A		-											T	T
clone 40	C	T	A		-											T	T
clone 41	C	T	A		-											T	T
clone 42	C	T	A		-											T	T
clone 43	C	T	A		-											T	T
clone 44	C	T	A		-											T	T
clone 45	C	T	A		-											T	T
clone 46	C	T	A		-											T	T
clone 47	C	T	A		-											T	T
clone 48	C	T	A		-											T	T
clone 49	C	T	A		-											T	T
clone 50	C	T	A		-											T	T
clone 51	C	T	A		-											T	T
clone 52	C	T	A		-											T	T
clone 53	C	T	A		-											T	T
clone 54	C	T	A		-											T	T
clone 55	C	T	A		-											T	T
clone 56	C	T	A		-											T	T
clone 57	C	T	A		-											T	T
clone 58	C	T	A		-											T	T
clone 59	C	T	A		-											T	T
clone 60	C	T	A		-											T	T
clone 61	C	T	A		-											T	T
clone 62	C	T	A		-											T	T
clone 63	C	T	A		-											T	T
clone 64	C	T	A		-											T	T
clone 65	C	T	A		-											T	T
clone 66	C	T	A		-											T	T
clone 67	C	T	A		-											T	T
clone 68	C	T	A		-											T	T
clone 69	C	T	A		-											T	T
clone 70	C	T	A		-											T	T
clone 71	C	T	A		-											T	T
clone 72	C	T	A		-											T	T
clone 73	C	T	A		-											T	T
clone 74	C	T	A		-											T	T
clone 75	C	T	A		-											T	T
clone 76	C	T	A		-											T	T
clone 77	C	T	A		-											T	T
clone 78	C	T	A		-											T	T
clone 79	C	T	A		-											T	T
clone 80	C	T	A		-											T	T
clone 81	C	T	A		-											T	T
clone 82	C	T	A		-											T	T
clone 83	C	T	A		-											T	T
clone 84	C	T	A		-											T	T
clone 85	C	T	A		-											T	T

parental 3243mtDNA

parental 4269mtDNA

b

Fragment B



b

Fragment C

nt position	9825	10311	10398	10400	10610	10876	12407	
CRS	T	G	A	C	T	T	G	
A3243G	A		G	T		C		
A4269G		A			C		A	
clone 1	A		G	T		C		parental 3243mtDNA
clone 2	A		G	T		C		
clone 3	A		G	T		C		
clone 4	A		G	T		C		
clone 5	A		G	T		C		
clone 6	A		G	T		C		
clone 7	A		G	T		C		
clone 8	A		G	T		C		
clone 9	A		G	T		C		
clone 10	A		G	T		C		
clone 11	A		G	T		C		
clone 12	A		G	T		C		
clone 13	A		G	T		C		
clone 14	A		G	T		C		
clone 15	A		G	T		C		
clone 16	A		G	T		C		
clone 17	A		G	T		C		
clone 18	A		G	T		C		
clone 19	A		G	T		C		
clone 20	A		G	T		C		
clone 21	A		G	T		C		
clone 22	A		G	T		C		
clone 23	A		G	T		C		
clone 24	A		G	T		C		
clone 25	A		G	T		C		
clone 26	A		G	T		C		
clone 27	A		G	T		C		
clone 28	A		G	T		C		
clone 29	A		G	T		C		
clone 30	A		G	T		C		
clone 31	A		G	T		C		
clone 32	A		G	T		C		
clone 33	A		G	T		C		
clone 34	A		G	T		C		
clone 35	A		G	T		C		
clone 36		A			C		A	parental 4269mtDNA
clone 37		A			C		A	
clone 38		A			C		A	
clone 39		A			C		A	
clone 40		A			C		A	
clone 41		A			C		A	
clone 42		A			C		A	
clone 43		A			C		A	
clone 44		A			C		A	
clone 45		A			C		A	
clone 46		A			C		A	
clone 47		A			C		A	
clone 48		A			C		A	
clone 49		A			C		A	
clone 50		A			C		A	
clone 51		A			C		A	
clone 52		A			C		A	
clone 53		A			C		A	
clone 54		A			C		A	
clone 55		A			C		A	
clone 56		A			C		A	
clone 57		A			C		A	
clone 58		A			C		A	
clone 59		A			C		A	
clone 60		A			C		A	
clone 61		A			C		A	
clone 62		A			C		A	
clone 63		A			C		A	
clone 64		A			C		A	
clone 65		A			C		A	
clone 66		A			C		A	
clone 67		A			C		A	
clone 68		A			C		A	
clone 69		A			C		A	
clone 70		A			C		A	
clone 71		A			C		A	
clone 72		A			C		A	
clone 73		A			C		A	
clone 74		A			C		A	
clone 75		A			C		A	
clone 76		A			C		A	
clone 77		A			C		A	
clone 78		A			C		A	
clone 79		A			C		A	
clone 80		A			C		A	
clone 81		A			C		A	
clone 82		A			C		A	
clone 83		A			C		A	
clone 84		A			C		A	
clone 85		A			C		A	

Figure 2-5 Sequence analysis of ΔmtDNA purified from tissues of an F_3 mito-mouse.

a, Gene maps of wild-type mtDNA of *M. spretus* (green circle) and ΔmtDNA of *M. m. domesticus* (smaller red circle). The open arc in the ΔmtDNA map corresponds to the deleted region expanded from $tRNA^{Lys}$ to *ND5* genes (np 7,759-12,454). No *Mlu* I site is present in wild-type mtDNA of *M. spretus*, whereas ΔmtDNA of *M. m. domesticus* possesses one *Mlu* I site.

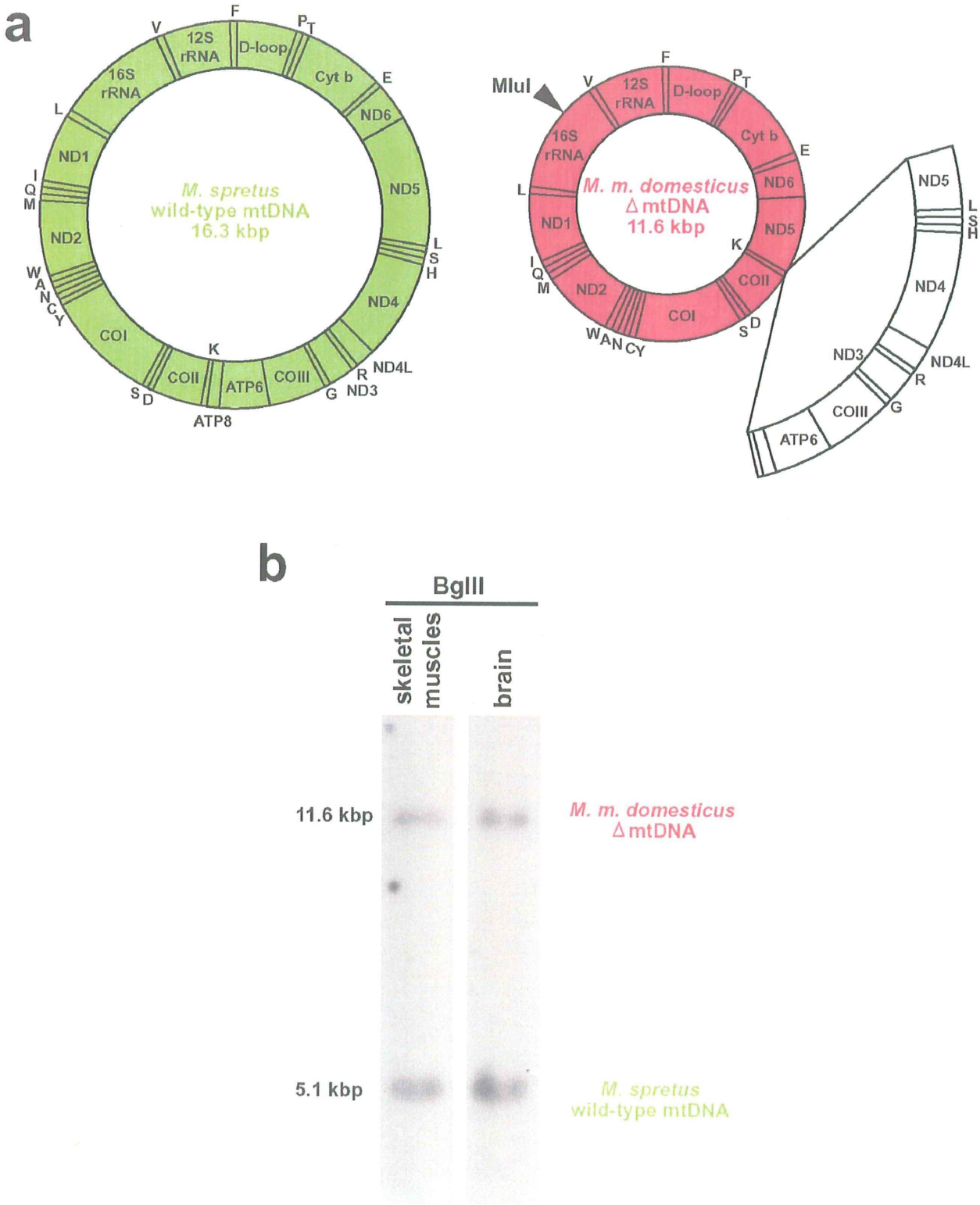
b, Analysis of mtDNA composition in skeletal muscles and brain from an F_3 mito-mouse by Southern blot analysis. Wild-type mtDNA of *M. spretus* gives three *Bgl* II fragments (8.3, 5.1, and 2.9 kbp), and one 5.1 kbp fragment is detectable by the probe (nt. 1,895-2,762). ΔmtDNA of *M. m. domesticus* gives one linear 11.6 kbp *Bgl* II fragment. Skeletal muscles and brain from an F_3 mito-mouse possessing 30.7% and 34.2% ΔmtDNA, respectively, were used for mtDNA purification and subsequent exclusive cloning and sequencing of ΔmtDNA.

c, Sequences of the cloned ΔmtDNA purified from skeletal muscles and brain of an F_3 mito-mouse possessing 30.7% and 34.2% ΔmtDNA, respectively. Green and red lanes correspond to mtDNA sequences of *M. spretus* mtDNA and *M. m. domesticus* (ref. 32), respectively. Of 107 clones of ΔmtDNA from skeletal muscles, only one clone (clone 10) possesses two regions carrying sequences specific to *M. spretus* mtDNA in np 3,350-3,386 (*ND1* gene) and np 4,093-4,213 (*ND2* gene), indicating the occurrence of sequence changes in mouse mtDNA in skeletal muscles. Of the remaining 106 clones, 97 possess

completely identical sequences to those of *M. m. domesticus* mtDNA, while 9 clones (clones 1-9) possess somatic mutations, since all of them were not found in sequences of parental *M. spretus* and *M. m. domesticus* mtDNAs. On the other hand, none of 56 clones of ΔmtDNA from brain show sequence changes, while 2 clones (clones 1 and 2) possess somatic mutations.

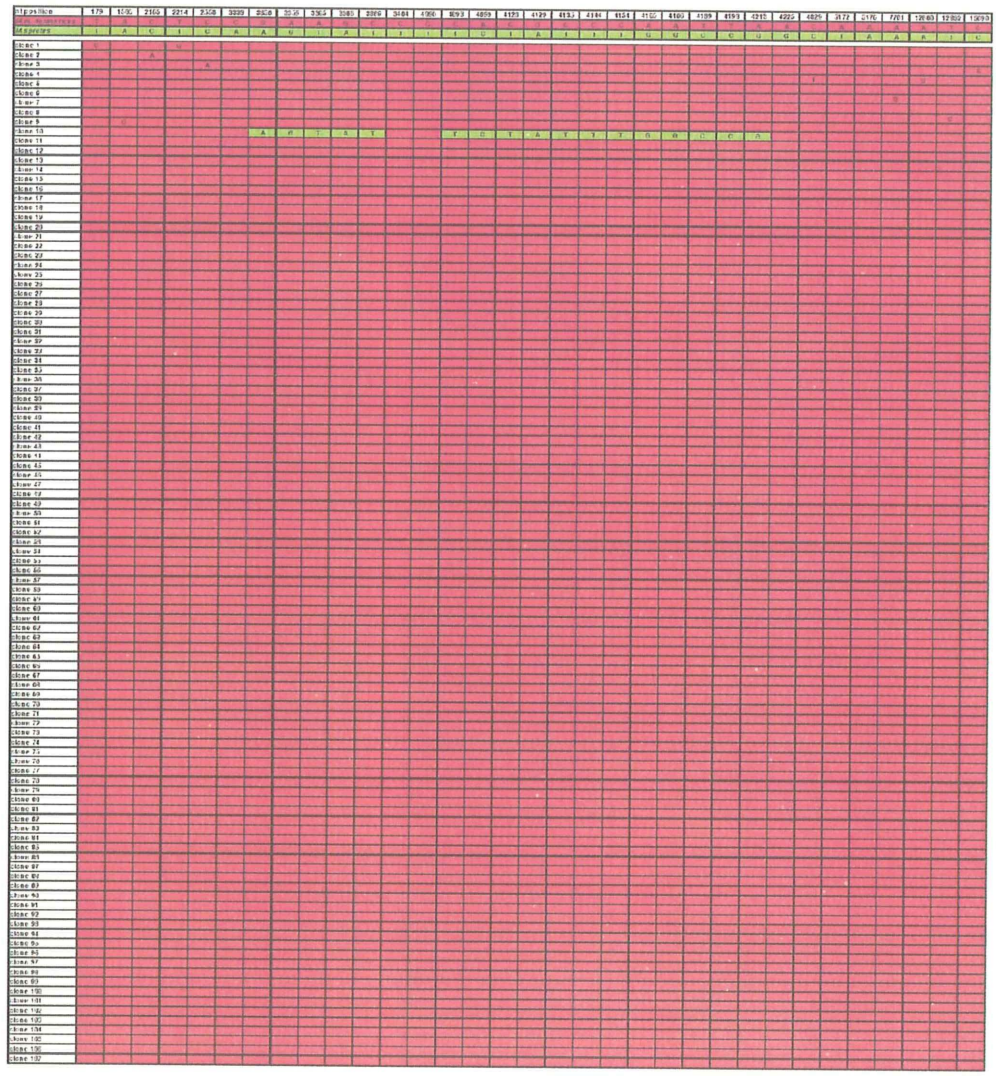
d, gene maps of ΔmtDNA (left) and clone 10 carrying two regions with sequences of *M. spretus* mtDNA (right). Green and red regions correspond to sequences of *M. spretus* and *M. m. domesticus* mtDNAs, respectively.

Figure 2-5

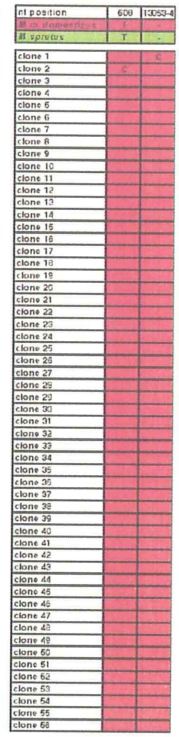

a
M. spretus
wild-type mtDNA
16.3 kbp
12S rRNA
D-loop
Cyt b
ND6
ND5
ND4
ND4L
ND3
ATP6
COIII
COII
COI
ND2
ND1
16S rRNA
MluI
M. m. domesticus
ΔmtDNA
11.6 kbp
b
BglII
skeletal muscles
brain
11.6 kbp
M. m. domesticus
ΔmtDNA
5.1 kbp
M. spretus
wild-type mtDNA

c

skeletal muscles



brain



d

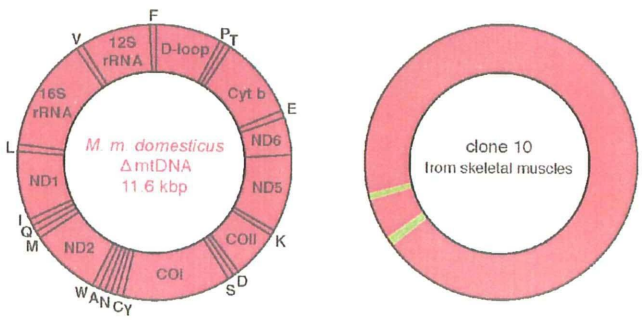


Figure 2-6 Identification of the specific recombinant mtDNA haplotype carrying identical sequence to clone 10.

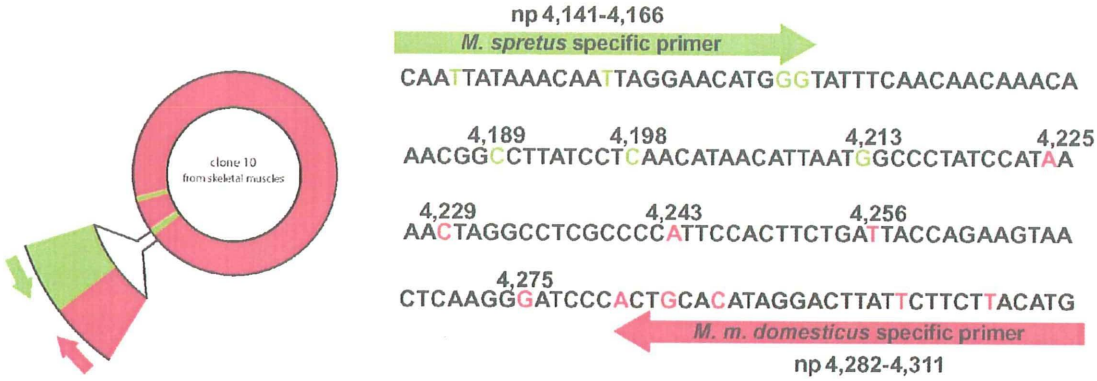
a, Gene map of clone 10 and nucleotide sequence (np 4,141-np 4,311) around a recombination break point. Polymorphic mutations specific to *M. spretus* and *M. m. domesticus* mtDNAs were shown by green and red letters, respectively. Green and red arrows indicate primers specific to *M. spretus* and *M. m. domesticus* mtDNAs, respectively, for selective amplification of the recombinants.

b, Selective amplification of the recombinants. *M. m. domesticus* mtDNA, purified mtDNA from liver of B6 strain mouse; *M. spretus* mtDNA, purified mtDNA from liver of B6mtspr strain mouse; mixture, mixture of *M. spretus* and *M. m. domesticus* mtDNAs; mito-mouse, purified mtDNA from skeletal muscles of F3 mito-mouse. Fragments with 171 bp correspond to the recombinants.

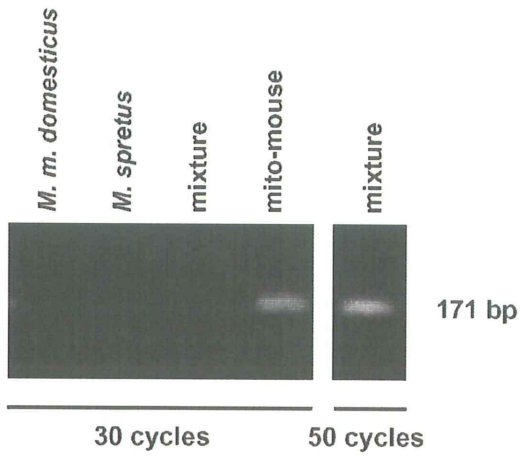
c, Sequences of cloned 171 bp fragments. Red and green regions correspond to sequences of *M. m. domesticus* and *M. spretus* mtDNAs, respectively.

Figure 2-6

a



b



c

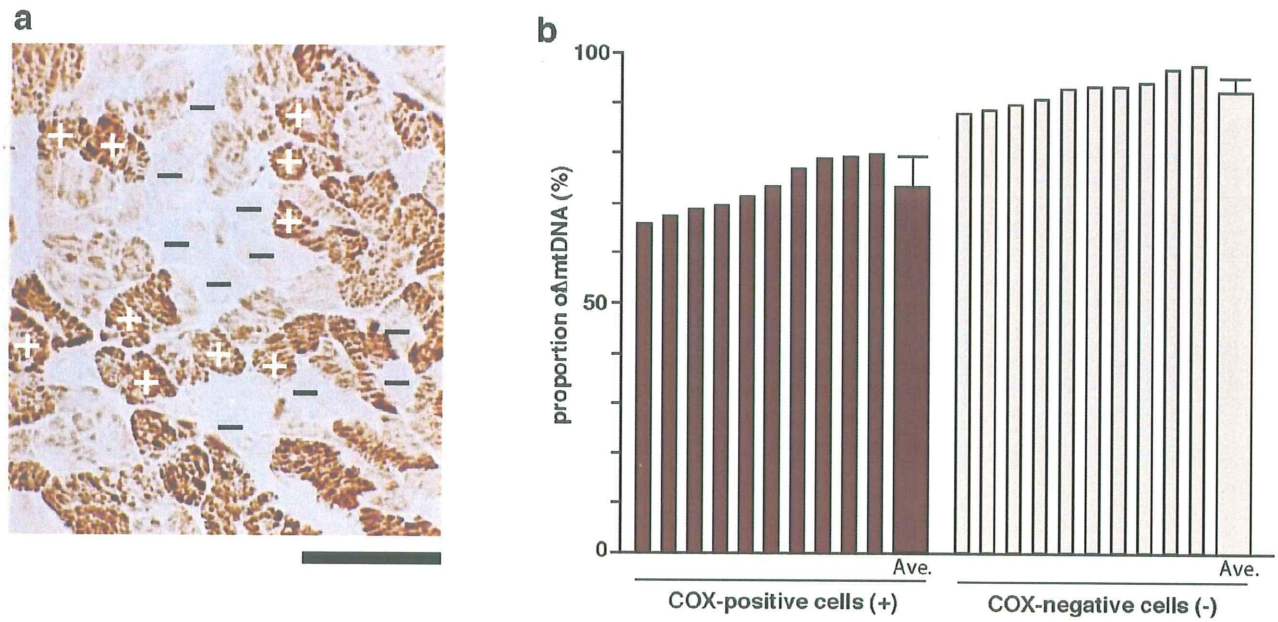
nt position		4189	4198	4213	4225	4229	4243	4256	4275
M. m. domesticus mtDNA		T	T	A	A	C	A	T	G
M. spretus mtDNA		C	C	G	G	T	C	C	A
recombinant mtDNA		C	C	G	A	C	A	T	G
mito-mouse mtDNA	cl. 1	C	C	G	A	C	A	T	G
	cl. 2	C	C	G	A	C	A	T	G
	cl. 3	C	C	G	A	C	A	T	G
	cl. 4	C	C	G	A	C	A	T	G
	cl. 5	C	C	G	A	C	A	T	G
	cl. 6	C	C	G	A	C	A	T	G
	cl. 7	C	C	G	A	C	A	T	G
	cl. 8	C	C	G	A	C	A	T	G
mixture of parental mtDNAs	cl. 1	C	C	G	G	T	C	C	G
	cl. 2	C	C	G	G	T	C	C	G
	cl. 3	C	C	G	G	T	C	T	G
	cl. 4	C	C	G	G	T	C	T	G
	cl. 5	C	C	G	G	T	A	T	G
	cl. 6	C	C	A	A	C	A	T	G
	cl. 7	C	C	A	A	C	A	T	G
	cl. 8	C	T	A	A	C	A	T	G

Figure 2-7 Examination of clonal expansion of ΔmtDNA in individual cardiac muscle cells of an F_3 mito-mice.

a, COX histochemistry of a heart carrying 87.3% ΔmtDNA. COX-positive (+) and COX–negative cells (-) are observed. Scale bar, 5 μm.

b, Brown and gray bars represent the proportion of ΔmtDNA in serial sections of COX-positive (n=10) and COX–negative cells (n=10), respectively. The average proportions of COX-positive and COX-negative cells are 73.0±5.0% and 92.5±3.2%, respectively.

Figure 2-7


a
b
100
50
0
proportion of mtDNA (%)
Ave.
Ave.
COX-positive cells (+)
COX-negative cells (-)

GENERAL DISCUSSION

The occurrence of mitochondrial interaction and mtDNA recombination in mammals have been a controversial issue for a long time. In this study, I confirmed the presence of mitochondrial interaction and mtDNA recombination, using mito-mice heteroplasmic between *M. m. domesticus* ΔmtDNA and *M. spretus* wild-type (normal) mtDNA. Pathogenicity of ΔmtDNA and sequence divergence between *M. m. domesticus* and *M. spretus* mtDNA (7.1%) enabled these confirmation.

In mito-mice, uniform distribution of COX-positive and -negative mitochondria in any single cells confirmed the presence of *in vivo* mitochondrial interaction and its biological significance; preventing individuals from expression of respiratory defects by pathogenic mutant mtDNA. Moreover, the finding of recombinant mtDNA between *M. m. domesticus* and *M. spretus* mtDNA also supports the presence of interaction between mitochondria. An occurrence of recombination requires the physical interaction of exogenously introduced mtDNA and recipient zygote derived mtDNA.

There have been many reports that showed relationships between mutations accumulated in mtDNA and aging. Point mutations and deletions of mtDNA accumulate in a variety of tissues with aging (Corral-Debrinski *et al.* 1992; Melov *et al.* 1995; Michikawa *et al.* 1999). Based on these observations, "mitochondrial theory of aging", which postulated mtDNA mutations were the cause of aging, was established. However, the presence of mitochondrial interaction showed in this study casts a question to the theory. The mtDNA mutations accumulate with

aging, but such mutations can occur randomly at any positions on the mtDNA. Therefore, randomly mutated mtDNA or its products in each mitochondrion were mixed by mitochondrial interaction, and defective products are complemented by each other. Although it is known that deletions and some point mutations have replication advantage over wild-type mtDNA in mitochondrial disease patients (Corral-Debrinski *et al.* 1992; Cortopassi *et al.* 1992; Yoneda *et al.* 1992), accumulation of specific mutant mtDNA was not observed in normal aging individuals. Recently, Trifunovic *et al.* (2004) reported generation of ageing-model mice expressing proof-reading-deficient mtDNA polymerase, in which increased levels of somatic mutations in mtDNA appear to be associated with respiratory defects, reduced life span, and premature onset of ageing-related phenotypes. However, they showed no evidence of identical mutant mtDNA accumulation in single cell that is needed to cause respiratory defects. Thus, more precise examination should be needed to conclude that mtDNA mutations are the cause of aging.

It is considered that efficiency of mitochondrial interaction depends on mitochondrial fusion and fission. Mitochondrial fusion and fission proteins are highly conserved from yeast to human. In human, mitochondrial fusion and fission are regulated by GTPase protein Mitofusin (Santel *et al.* 2001; Rojo *et al.* 2002) and dynamin-related protein (Smirnova *et al.* 1998), respectively. Moreover, efficiency of mitochondrial fusion and fission is supposed to depend on intracellular mitochondrial movement by motor proteins. In mammalian and Drosophila, kinesin family members

have been shown to be involved in microtubule-dependent mitochondrial transportation (Nangaku *et al.* 1994; Pereira *et al.* 1997; Tanaka *et al.* 1998). In this study, I examined only cardiac muscles, but other tissues might have different expression of fusion, fission and motor proteins and different efficiency of mitochondrial interaction. Such differences of mitochondrial interaction efficiency might be related to different threshold of respiratory defects between tissues.

So far, mtDNA sequences have been used for construction of phylogenetic trees and evolutionary studies, assuming maternal inheritance and no recombination. However, the presence of mtDNA recombination is shown in this study, and it is essential to understand the effects of mtDNA recombination on such studies.

As shown in Section II, recombinant mtDNA was found in artificially generated heteroplasmic mice carrying two types of mtDNAs. However, in natural population, inheritance of mtDNA is strictly maternal and paternal inheritence, that could generate extensive heteroplasmic conditions, does not occur, except for interspecies crossing. In interspecies F_1 hybrid of fruitflies (Kondo *et al.* 1990), mice (Gyllensten *et al.* 1991; Kaneda *et al.* 1995) and cows (Sutovsky *et al.* 1999), paternal mtDNA leakage was found, but interspecies crossings hardly occur in natural population. Moreover, such leaked paternal mtDNA was not transmitted to following generations (Shitara *et al.* 1998). During oocyte differentiation, leaked paternal mtDNA must have disappeared by stochastic segregation. These lines of evidence mean that a period of heteroplasmic state that

enables mtDNA recombination between paternal and maternal mtDNA is extremely short; before primordial germ cells stop proliferation in embryo (13.5 dpc). Although F_3 mito-mice examined in this study maintained heteroplasmic state for three generations, observed frequency of mtDNA recombination is extremely low. It is unlikely that recombinant mtDNA is created during 13.5 days and transmitted to the following generations. The transmission of paternal mtDNA and mtDNA recombination between paternal and maternal mtDNA was reported in a patient with mitochondrial disease (Shwartz and Vissing 2002; Kraytsberg *et al.* 2004). However, paternal transmission manner in this patient is curious; paternal mtDNA, which is less than 0.1% of total mtDNA in zygotes, overwhelmed maternal mtDNA in skeletal muscles of this patient (90%). I suppose that all observations in this patient are very rare exceptional case, caused by nuclear genome defects of paternal mtDNA exclusion or mtDNA maintenance. Thus, I conclude that the possibility of mtDNA recombination by paternal mtDNA is very rare, and there is no need to reassess the way of phylogenetic reconstruction and the previously constructed phylogenetic trees.

What is the biological significance of the rare mtDNA recombination? One explanation is the generation of sequence divergence in mtDNA population. Recombination in nuclear genome, such as cross-over during meiosis, generates sequence divergence. In yeast, during 20 generations, about 48hrs, newly generated recombinant mtDNA segregated completely and homoplasmic recombinant mtDNA cells were established (Dujon *et al.* 1974). In sea mussels (shellfish), recombinant

mtDNAs between paternal and maternal mtDNA were found (Ladoukakis *et al.* 2001), and such genotypes were fixed in mussel population (Burzynski *et al.* 2003). In both cases, recombinant mtDNAs were fixed in population, and its biological meaning should be to provide a variety of mtDNA haplotypes. Although both yeast and mussels show biparental mtDNA inheritance, paternal mtDNA is completely eliminated from zygotes in mammalian species (Kaneda *et al.* 1995; Shitara *et al.* 1998). Recombination between identical maternal molecules would not make any sequence divergence.

Then, I conclude that the mtDNA recombination has no biological significance to generate sequence divergence, but it is by-products of mtDNA repair. Synthesis-dependent strand annealing (SDSA), which is well examined as a repair process of DNA double-strand breaks (DSB) in Drosophila (Nassif *et al.* 1994; Adams *et al.* 2003), is one candidate for generation of recombinant mtDNA. SDSA repair of DSB is accomplished in the following way (Figure 3). In the SDSA model, processing of a DSB generates 3' single-stranded tails, which invade homologous templates and prime DNA synthesis. Nascent DNA strands are displaced from the template and can then anneal with complementary DNA from the recipient chromosome. The SDSA, therefore, results in gene conversion. Recently, mtDNA repair machineries, such as base excision repair, have been reported in mammalian mitochondria. It would be possible that SDSA-like repair machinery is found in mitochondria.

If mtDNA repair generates recombinant mtDNA, more

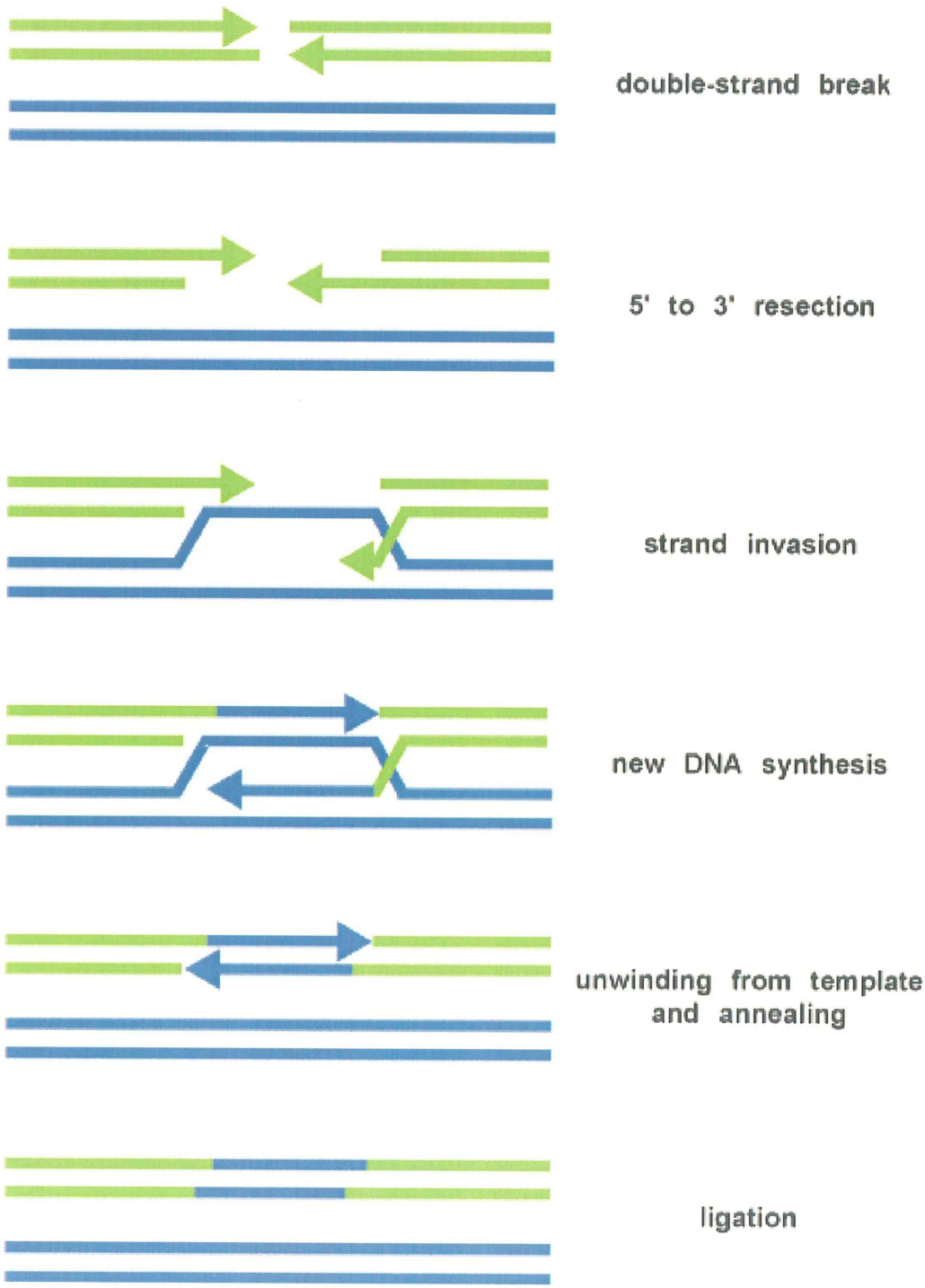
recombinant mtDNA would be observed in cells with more mtDNA lesions. In mito-mice, ΔmtDNA accumulation causes respiratory defects and more ROS generation. In such cells, it is possible that mtDNA repair machinery is accelerated and more recombinants are observed. It is necessary to assess the mtDNA recombination in mito-mice tissues carrying more ΔmtDNA and showing respiratory defects.

Moreover, recombination observed in this study was between *M. m. domesticus* ΔmtDNA and *M. spretus* wild-type mtDNA. It is possible that large sequence divergence and difference in size between these mtDNAs suppressed the occurrence of recombination. To clarify this possibility, it is needed to examine the frequency of mtDNA recombination in mice carrying less divergent and same size mtDNAs, such as NZB and B6 mtDNAs. NZB and B6 mtDNAs are same in size (16.3 kbp) and showing a high sequence homology (99.4%).

Figure 3 DSB repair process by SDSA.

A DSB is introduced into one of two homologous chromosomes (green). The 5' ends are then resected by an exonuclease to expose the 3' ends (shown with arrowheads) in single-stranded form. With the help of RecA-like proteins, the 3' ends invade into complementary homologous regions (blue). Then the 3' ends are used as primers for new DNA synthesis, using the donor chromosome strands (blue) as template. After sufficient synthesis to permit the new strands to anneal with each other, the new strands are unwound from the blue template and allowed to anneal with each other. Gaps are filled in by a polymerase and nicks are sealed by a ligase.

Figure 3


double-strand break
5' to 3' resection
strand invasion
new DNA synthesis
unwinding from template and annealing
ligation

CONCLUSION

Conclusions of this study are as follows.

The results in Section I showed unambiguous evidence for the presence of *in vivo* mitochondrial interaction.

- The occurrence of mitochondrial interaction n is accomplished by inter-exchange of genetic contents between mitochondria.
- The occurrence of mitochondrial interaction shows that mitochondria function as a single unit in each cells.

The results in Section II showed the presence of mtDNA recombination in mammals.

- The frequency of mtDNA recombination is very low.
- The occurrence of mammalian mtDNA recombination could not confuse phylogenetic reconstruction.
- mtDNA recombinant is considered to be side-products of mtDNA repairing.

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