Inter-mitochondrial complementation: Mitochondria-specific system preventing mice from expression of disease phenotypes by mutant mtDNA

KAZUTO NAKADA[1,2,3], KIMIKO INOUE[1,5], TOMOKO ONO[1], KOTOYO ISOBE[1], ATSUO OGURA[5], YU-ICHI GOTO[4], IKUYA NONAKA[3] & JUN-ICHI HAYASHI[1,2]

[1]*Institute of Biological Sciences,* [2]*Center for Tsukuba Advanced Research Alliance, University of Tsukuba, Ibaraki, Japan*
[3]*Department of Ultrastructural Research,* [4]*Department of Mental Retardation and Birth Defect Research, National Institte of Neuroscience, National Center of Neurology and Psychiatry, Tokyo, Japan*
[5]*Department of Veterinary Science, National Institute of Infectious Diseases, Tokyo, Japan*
K.N. and K.I. contributed equally to this study.
Correspondence should be addressed to J.-I.H.; email: jih45@sakura.cc.tsukuba.ac.jp

Here we investigated the pathogenesis of deletion mutant mitochondrial (mt)DNA by generating mice with mutant mtDNA carrying a 4696-basepair deletion (ΔmtDNA4696), and by using cytochrome *c* oxidase (COX) electron micrographs to identify COX activity at the individual mitochondrial level. All mitochondria in tissues with ΔmtDNA4696 showed normal COX activity until ΔmtDNA4696 accumulated predominantly; this prevented mice from expressing disease phenotypes. Moreover, we did not observe coexistence of COX-positive and -negative mitochondria within single cells. These results indicate the occurrence of inter-mitochondrial complementation through exchange of genetic contents between exogenously introduced mitochondria with ΔmtDNA4696 and host mitochondria with normal mtDNA. This complementation shows a mitochondria-specific mechanism for avoiding expression of deletion-mutant mtDNA, and opens the possibility of a gene therapy in which mitochondria possessing full-length DNA are introduced.

Mice with a pathogenic mutant mitochondrial (mt)DNA could provide an ideal system for investigating how mutant mtDNA is transmitted to subsequent generations and distributed in various tissues, a process resulting in mitochondrial diseases of various clinical phenotypes[1]. Many lines of mice have been generated with mitochondrial disorders that result from disruption of nuclear DNA-coded factors that are related to mitochondrial function[2,3]. However, no procedures are available to generate disease models by direct introduction of artificially mutated mouse mtDNA into mouse cells or embryos. The only procedures to introduce exogenous mtDNA are microinjection of mitochondria or cell-fusion techniques. Therefore, mice with mitochondrial dysfunction might be generated either by introducing mitochondria from different species[5] or mouse mitochondria with mutant mtDNAs that had accumulated in somatic tissues[6]. We recently generated mice with mitochondrial dysfunction by introducing mitochondria with somatic mutant mtDNA into mouse zygotes[6]. We introduced mouse mutant mtDNA with a 4696-bp deletion (ΔmtDNA4696) accumulated in somatic cells into mouse cells without mtDNA (ρ^0 mouse cells) by isolating Cy4696 cybrids with various proportions of ΔmtDNA4696. We then introduced respiration-deficient mitochondria with only ΔmtDNA4696 into mouse zygotes using electrofusion with the enucleated Cy4696 cybrids. In contrast with mtDNA in mitochondria from sperm[8] or spermatid[9], ΔmtDNA4696 in mitochondria from somatic cells was not rejected by the embryos, and the ΔmtDNA4696 and/or a partially duplicated form was transmitted maternally from F0 mice to produce F2–F3 mice with 60–85% ΔmtDNA4696.

To delineate the precise mechanisms of the early pathogenesis of ΔmtDNA4696 (Fig. 1*a*), we used cytochrome *c* oxidase (COX) electron micrographs, which can identify respiratory enzyme COX activity at the individual mitochondrial level. In six-month-old F3 male mice containing various proportions of ΔmtDNA4696 in their tissues, we found extensive *in vivo* genetic complementation between exogenously introduced COX^- mitochondria with ΔmtDNA4696, and recipient COX^+ mitochondria with wild-type mtDNA. This is the first report to show the occurrence of extensive *in vivo* inter-mitochondrial complementation, which prevented mice from expressing disease phenotypes by accumulated ΔmtDNA4696. Our findings extend the conventional concept of mitochondrial complementation from *in vitro* to *in vivo*, and from intra- to inter-mitochondria, which corresponds to a mitochondria-specific system for preventing mice from expressing various mtDNA lesions.

COX activity of mitochondria in cybrids with ΔmtDNA4696

We examined the precise distribution and expression profiles of ΔmtDNA4696 at the levels of tissues, cells and mitochondria using Southern-blot and PCR analyses, COX histochemistry and COX electron micrographs. As the COX activity of individual mitochondria can be identified on COX electron micrographs, we could determine whether exogenously introduced ΔmtDNA4696 and recipient wild-type mtDNA are dis-

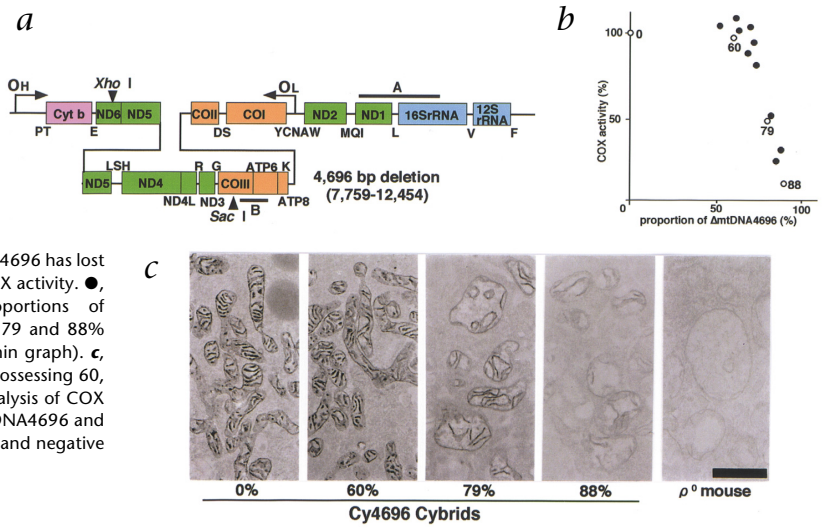

Fig. 1 Characterization of ΔmtDNA4696 in somatic cybrids. ***a***, Gene map of ΔmtDNA4696. The deleted region extended from nucleotide position 7,759 in the $tRNA^{Lys}$ gene to 12,454 in the *ND5* gene. Wild-type mtDNA possesses one *Xho*I and one *Sac*I site, whereas ΔmtDNA4696 possesses one *Xho*I site but no *Sac*I site, which is included in the deleted region. Regions A (1,751–3,803) and B (8,410–8,964) indicate probes used for Southern-blot analysis. Note that ΔmtDNA4696 has lost the B region. ***b***, Biochemical estimation of COX activity. ●, Cy4696 cybrids possessing various proportions of ΔmtDNA4696; ○, Cy4696 cybrids with 60, 79 and 88% ΔmtDNA4696 (as indicated by numbers within graph). ***c***, COX electron micrographs. Cy4696 cybrids possessing 60, 79 and 88% ΔmtDNA4696 were used for analysis of COX activity. Cy4696 cybrids possessing 0% ΔmtDNA4696 and parental ρ^0 mouse cells were used as positive and negative controls, respectively. Scale bar, 1 μm.

tributed uniformly throughout mitochondria or segregated and confined into different organelles within single cells. This information would help in understanding how exogenously introduced ΔmtDNA4696 expresses disease phenotypes in various tissues.

First, we confirmed the reliability of COX electron micrographs by the use of Cy4696 cybrids with only wild-type mtDNA and respiration-deficient ρ^0 mouse cells as positive and negative controls, respectively (Fig. 1*b*). All mitochondria in Cy4696 cybrids with only wild-type mtDNA were COX^+, whereas we observed no COX^+ mitochondria in ρ^0 mouse cells (Fig. 1*c*). Then, we examined Cy4696 cybrids with 60% ΔmtDNA4696, which showed normal COX activity on biochemical assay, and Cy4696 cybrids with 88% ΔmtDNA4696, which showed extremely low COX activity (Fig. 1*b*), and have been used as ΔmtDNA4696 donors for its introduction into mouse embryos[7]. We also examined Cy4696 cybrids with 79% ΔmtDNA4696, which showed 50% COX activity (Fig. 1*b*). COX electron micrographs showed that all mitochondria in Cy4696 cybrids with 88% ΔmtDNA4696 had entirely lost COX activity and were considerably swollen with a disorganized inner membrane like mitochondria in ρ^0 mouse cells (Fig. 1*c*), whereas all mitochondria in Cy4696 cybrids with 60% ΔmtDNA4696 were COX^+ (Fig. 1*c*), which is due to coexistence of ΔmtDNA4696 and wild-type mtDNA, and presumed complementation within mitochondria (intra-mitochondrial complementation). Cy4696 with 79% ΔmtDNA4696, however, possessed mitochondria with slightly swollen morphology and insufficient COX staining ($COX^{+/-}$, Fig. 1*c*).

COX activity of mitochondria in tissues with ΔmtDNA4696

The absence of complementation between mitochondria (that is, the absence of inter-mitochondrial complementation) was seemingly indicated by coexistence of different types of respiration-deficient mitochondria within single cells[10]. If this is so, in mice with mtDNA-based disease, inter-mitochondrial complementation could not occur between COX^+ mitochondria from host embryos and COX^- mitochondria from Cy4696 cybrids carrying predominantly ΔmtDNA4696. Therefore, cells of diseased mice with 60% ΔmtDNA4696 would show a mosaic distribution of 60% COX^- mitochondria with exogenous ΔmtDNA4696 and 40% COX^+ mitochondria with normal host mtDNA on COX electron micrographs.

An ideal system to test this possibility, would be skeletal muscles with various proportions of ΔmtDNA4696, given that the proportion of ΔmtDNA4696 in each muscle fiber could be positively identified by single-fiber PCR, and directly compared with COX activities within the same single fibers using

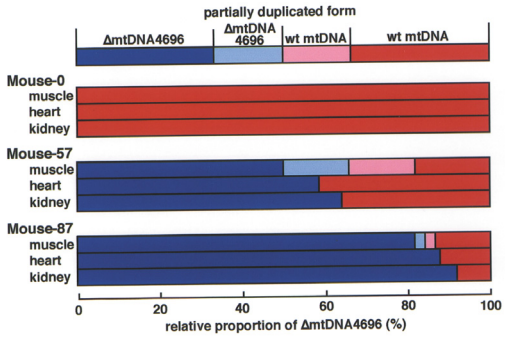

Fig. 2 Distribution of ΔmtDNA4696, a partially duplicated mtDNA and wild-type mtDNA in skeletal muscle, heart and kidney of mouse-0, -57 and -87, which respectively possessed 0%, 57% and 87% ΔmtDNA4696 in their tails. The solid bars represent amounts of ΔmtDNA4696 including those of ΔmtDNA4696 and of the ΔmtDNA4696 region of the partially duplicated mtDNA. The partially duplicated form consisting of one ΔmtDNA4696 and one full-length mtDNA was detectable in skeletal muscle, but not in heart or kidney. Considering the presence of intermitochondrial interaction, one molecule of the duplicated form corresponds to one ΔmtDNA4696 and one wild-type mtDNA. Therefore, the amount of ΔmtDNA4696 required for inducing mitochondrial dysfunction should be equivalent to the sum of the amounts of ΔmtDNA4696 and ΔmtDNA4696 in the duplicated form. Note that the proportion of ΔmtDNA4696 did not vary significantly in different tissues of the same individual. wt, wild-type.

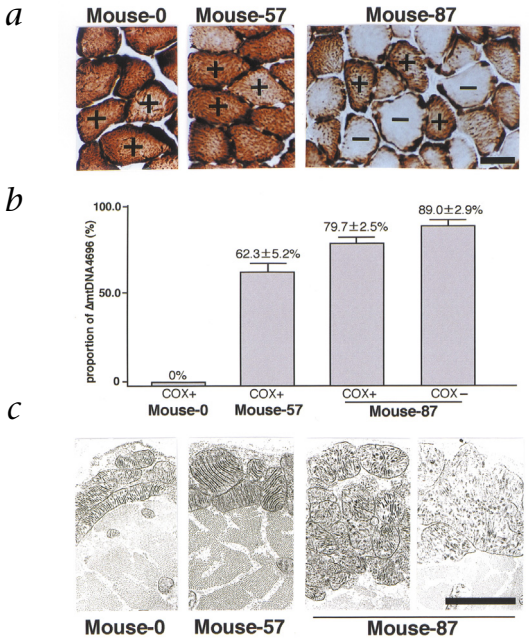

Fig. 3 Pathogenesis of ΔmtDNA4696 in skeletal muscles from mouse-0, -57 and -87. ***a*** and ***b***, COX histochemistry (*a*) and single-fiber PCR analysis (*b*) of serial cross sections of skeletal muscles. Southern-blot analysis showed that skeletal muscles of mouse-0, -57 and -87 possessed 0%, 66% and 85% ΔmtDNA4696, respectively. All muscle fibers in mouse-57 were COX^+, while muscle fibers in mouse-87 consisted of a mixture of COX^+ and COX^- fibers. The subsarcolemmal regions in the COX^- fibers were stained, because these regions in fibers with predominant ΔmtDNA4696 usually accumulate a significant amount of mitochondria to compensate for low respiratory function, which would account for COX histochemical staining, even when each mitochondrion showed low COX activity. Scale bar in *a*, 30 μm. ***c***, COX electron micrographs. Note that mitochondrial outer membranes were not stained with COX. Scale bar, 1 μm.

serial cross sections. We performed Southern-blot analysis of tail DNA, and selected DNA of three mice, mouse-0, -57 and -87 containing 0%, 57% and 87% ΔmtDNA4696, respectively, for further analyses. The ΔmtDNA4696 proportions did not vary significantly from other tissues of the same mice[7]. In fact, skeletal muscle of mouse-0, -57 and -87 contained 0%, 66% and 85% ΔmtDNA4696, respectively (Fig. 2), and we used these samples for COX histochemistry, single-fiber PCR and COX electron micrographs. When we used mouse-57 skeletal muscle with 66% ΔmtDNA4696, including 16% ΔmtDNA4696 as the duplicated form (Fig. 2), COX histochemistry and single-fiber PCR analysis of their serial cross sections showed that all fibers possessed ΔmtDNA4696 (62.3 ± 5.2%), but possessed normal COX activities, comparable with those of fibers without ΔmtDNA4696 (Fig. 3*a* and *b*). Moreover, all individual mitochondria in muscle fibers of mouse-57 showed COX activity on the COX electron micrographs (Fig. 3*c*). On the other hand, mouse-87 skeletal muscle with 85% ΔmtDNA4696 gave totally different features of COX histochemistry: 41.3% of fibers were insufficiently stained with COX and possessed 89.0 ± 2.9% ΔmtDNA4696, whereas 58.7% were stained with COX but possessed 79.7 ± 2.5% ΔmtDNA4696 (Fig. 3*a* and *b*). Moreover, COX electron micrographs showed that mitochondria were accumulated in subsacrolemmal regions of all fibers in compensation for respiration deficiency irrespective of whether mitochondria were COX^- or COX^+, but again coexistence of COX^- and COX^+ mitochondria did not occur within any single fiber (Fig. 3*c*). This region of COX^- fibers usually accumulates a significant number of proliferated abnormal mitochondria, which would provide signals of COX staining in histochemical analysis (Fig. 3*a* and *b*), even when each mitochondrion showed very low COX activity (Fig. 3*c*). These observations could not be obtained if there were not continuous inter-mitochondrial complementation between COX^+ mitochondria derived from host embryos and COX^- mitochondria from ΔmtDNA4696 donor cybrids.

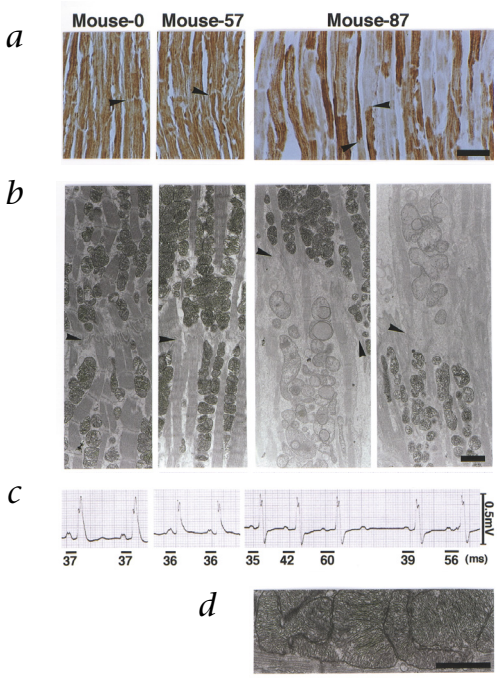

Fig. 4 Pathogenesis of ΔmtDNA4696 in hearts from mouse-0, -57 and -87. ***a*** and ***b***, COX histochemistry (*a*) and COX electron micrographs (*b*) of longitudinal sections of cardiac muscles. Hearts of mouse-57 and -87 possessed 58% and 88% ΔmtDNA4696, respectively. Cardiac muscle fibers consist of mononuclear cells joined end-to-end by intercalated discs (arrowheads). All cardiac muscle fibers (with 58% ΔmtDNA4696) of mouse-57 showed normal COX staining, while those (with 88% ΔmtDNA4696) of mouse-87 consisted of COX^+ and COX^- cells. Moreover, all mitochondria in single fibers are either COX^+ or COX^-, and no coexistence of COX^+ and COX^- mitochondria within single cells was detectable on COX electron micrographs. Scale bars in *a* and *b*, 30 μm and 1 μm, respectively. ***c***, Electrocardiogram measurement of PQ intervals. Note that only mouse-87 showed elongated PQ intervals and auriculoventricular block with Wenckebach periodicity. ***d***, Electron micrograph of mitochondria in cardiac muscle fibers of mouse-87. The ultrastructure of mitochondria in cardiac muscle fibers of mouse-87 was examined using ultra-thin sections stained with both uranyl acetate and lead nitrate. They show ultrastructural features indicating mitochondrial fusion and/or fission. Unlike in COX electron micrographs, the mitochondrial outer membrane could be identified. Scale bar, 1 μm.

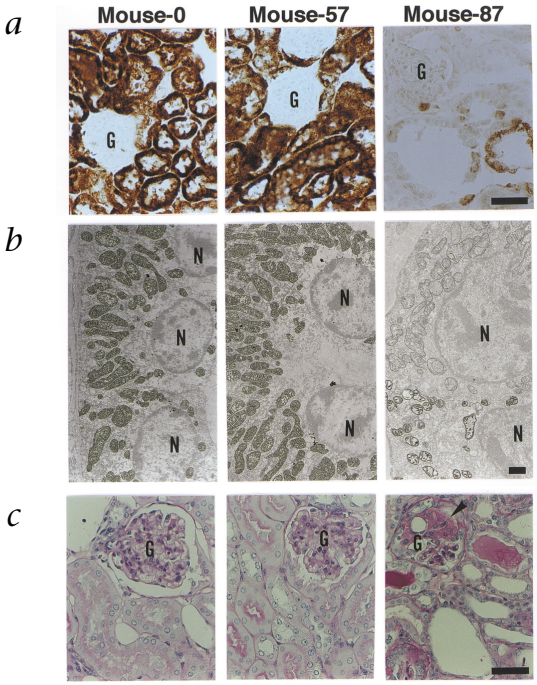

Fig. 5 Pathogenesis of ΔmtDNA4696 in kidneys from mouse-0, -57 and -87. ***a*** and ***b***, COX histochemistry (*a*) and COX electron micrographs (*b*) of kidneys. Kidneys of mouse-57 and -87 possessed 64% and 92% ΔmtDNA4696, respectively. Note that cells of glomeruli ('G') of all three mice were not sufficiently stained with COX due to scarcity of mitochondria. All cells in renal tubules of mouse-57 showed normal COX staining, while those of mouse-87 consisted of COX^+ and COX^- cells. Moreover, all mitochondria in single cells were either COX^+ or COX^-, and the coexistence of COX^+ or COX^- mitochondria within single cells was not observed in COX electron micrographs. N, nucleus. Scale bars in *a* and *b*, 30 μm and 1μm, respectively. ***c***, Histopathology of the renal cortex of the kidneys. 1-μm sections of kidney were stained with periodic acid Schiff reaction. Only mouse-87 with 92% ΔmtDNA4696 in the kidney showed a dilated lumen of renal tubules with casts, and segmental glomerular sclerosis (arrowhead). Scale bar, 30 μm.

We obtained similar results in hearts (Fig. 4) and kidneys (Fig. 5). In COX histochemistry and COX electron micrographs, we observed no COX^- cells in mouse-57 heart with 58% ΔmtDNA4696 (Fig. 4*a*) and in mouse-57 kidney with 64% ΔmtDNA4696 (Fig. 5*a*). However, mouse-87 heart with 88% ΔmtDNA4696 (Fig. 4*a*) and mouse-87 kidney with 92% ΔmtDNA4696 (Fig. 5*a*) consisted of both COX^+ and COX^- cells. COX electron micrographs clearly showed that each cell had either COX^+ or COX^-, and sometimes $COX^{+/-}$ mitochondria, and we observed no mosaic distribution of different types of mitochondria within any single cell (Figs. 4*b* and 5*b*). All mitochondria in one kidney cell of mouse-87 (Fig. 5*b*) showed intermediate COX activity, which is comparable to that of mitochondria in a cybrid with 79% ΔmtDNA4696 expressing 50% COX activity (Fig. 1*c*) suggesting that the kidney cell probably possessed about 79% ΔmtDNA4696 and expressed 50% COX activity.

The homogeneous distribution of COX activity throughout mitochondria within single cells of tissues with ΔmtDNA4696 (Figs. 3*c*, 4*b* and 5*b*) indicates the presence of extensive *in vivo* inter- as well as intra-mitochondrial complementation in all the tissues we examined. This complementation would be attained by frequent mitochondrial fusion followed by rapid exchange of their contents. Moreover, COX electron micrographs (Figs. 3*c*, 4*b*, and 5*b*) and conventional electron micrographs (Fig. 4*d*) also provided morphological features supporting the idea of frequent interorganellar interactions, although we could not distinguish whether these represented organellar fusion or fission.

Pathogenesis of ΔmtDNA4696 in tissues

If ΔmtDNA4696-induced decline of respiratory function is responsible for the pathogenesis, these observations predict that disease phenotypes would not manifest even when tissues had accumulated around 60% ΔmtDNA4696. Hearts and kidneys of mouse-0, -57 and -87 were used to test this hypothesis. We recorded electrocardiograms, and found that elongated PQ intervals and auriculoventricular block with Wenckebach periodicity were preferentially observed in mouse-87, but not in mouse-0 or -57 (Fig. 4*c*). Similar coordination between of abnormal electrocardiograms and decreased COX activity was reported in mouse heart with a disrupted nuclear gene for mitochondrial transcription factor A (ref. 3). In mouse-87, kidney possessing 92% ΔmtDNA4696 showed dilatation of the cortical proximal and distal tubules

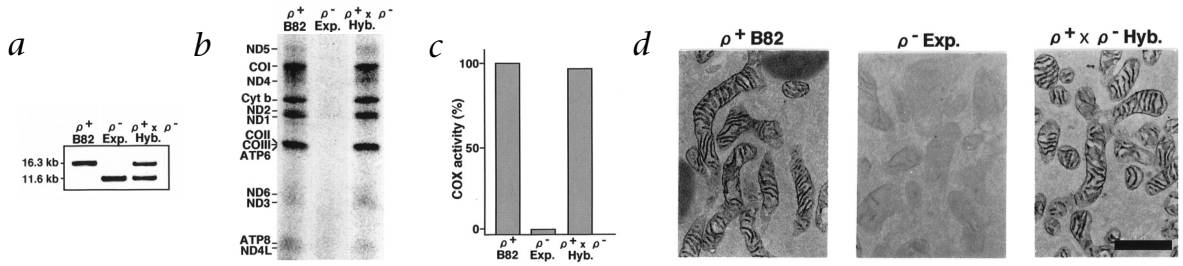

Fig. 6 Effect of coexistence of similiar amounts of COX^- and COX^+ mitochondria within the same cells. ρ^+ B82, ρ^+ B82TK$^-$ cells with only wild-type mtDNA; ρ^- Exp., ρ^- fibroblast clone with only ΔmtDNA4696 isolated from explants of embryos with a predominant amount of ΔmtDNA4696; $\rho^- \times \rho^+$, hybrids with 63% ΔmtDNA4696 isolated by the fusion of the ρ^- fibroblasts and ρ^+ B82TK$^-$ cells. ***a***, Southern-blot analysis of *Xho*I fragments hybridized with [α-^{32}P]dATP-labeled mouse mtDNA probes. ***b***, analysis of mitochondrial translation products labeled with [^{35}S]methionine. ND5, COI, ND4, Cytb, ND2, ND1, COII, COIII, ATP6, ND6, ND3, ATP8 and ND4L are polypeptides assigned to mtDNA genes. ***c***, biochemical analysis of COX activity. ***d***, COX electron micrographs. Photographs are representative of those in the entire experiments. Scale bar, 1 μm.

and segmental glomerular sclerosis (Fig. 5*c*). These abnormalities were not observed in mouse-0 or -57 (Fig. 5*c*).

In summary, all tissues with about 60% ΔmtDNA4696 showed normal mitochondrial respiratory function without expressing disease phenotypes, whereas tissues with more than 85% ΔmtDNA4696 simultaneously expressed mitochondrial dysfunction and disease phenotypes, such as elongated PQ intervals, auriculoventricular block and renal failure. Thus, mitochondrial dysfunction caused by ΔmtDNA4696 resulted in expression of disease phenotypes which corresponded to those in human subjects with predominantly ΔmtDNA (refs. 11-15).

Inter-mitochondrial complementation in $\rho^- \times \rho^+$ hybrids

To directly prove the presence of interaction between distinct mitochondria, we isolated completely respiration-deficient ρ^- fibroblasts with only ΔmtDNA4696 from explants of embryos with a predominant amount of ΔmtDNA4696 (Fig. 6*a*). The explanted ρ^- cells entirely lost mitochondrial translation activity (Fig. 6*b*) due to the loss of six tRNA genes in ΔmtDNA4696 (Fig. 1*a*), resulting in formation of overall COX^- mitochondria (Fig. 6*c* and *d*). Then, we induced coexistence of COX^- and COX^+ mitochondria within the same cells by isolating hybrids between the explanted ρ^- cells and ρ^+ $B82TK^-$ cells with only COX^+ mitochondria, using nutritional and hypoxanthine/aminopterin/thymidine (HAT) selection medium for excluding both parental cells, respectively. We selected one $\rho^- \times \rho^+$ hybrid colony possessing 63% ΔmtDNA4696 (Fig. 6*a*) and examined its respiration properties. If there were no interaction between COX^- and COX^+ mitochondria from both parents in the hybrids, 63% mitochondria should be COX^- and the remaining 37% should be COX^+. However, the results showed that all mitochondria were COX^+ (Fig. 6*d*). Moreover, translation and COX activities in mitochondria of the hybrids returned to normal levels (Fig. 6*b* and *c*). These features could not be obtained if there were no fusion of COX^- and COX^+ mitochondria followed by exchange of their genetic contents—the six tRNAs from COX^+ mitochondria to COX^- mitochondria in particular.

Discussion

Here, COX electron micrographs clearly showed that all mitochondria in tissues with ΔmtDNA4696 were COX^+ until the deleted mtDNA became predominant. The appearance of COX^- mitochondria was limited to tissues with more than 85% ΔmtDNA4696, and correlated with the onset of disease phenotypes. Moreover, we did not observe the coexistence of COX^+ and COX^- mitochondria within single cells, irrespective of whether the tissues contained low or high concentrations of ΔmtDNA4696 (Figs. 3–5). If exchange of mtDNA did not occur between exogenous COX^- mitochondria carrying ΔmtDNA4696 from cybrids and host COX^+ mitochondria from recipient zygotes, or even if it occurred, but did not occur frequently and continuously, mitochondria in the mice would continue to possess either ΔmtDNA4696 or wild-type mtDNA. Under these circumstances, cells in tissues with 60% and 85% ΔmtDNA4696 should have 60% and 85% of COX^- mitochondria, respectively, but this was not the case (Figs. 3–5). These observations could be explained by assuming *in vivo* inter-mitochondrial complementation between COX^- mitochondria carrying ΔmtDNA4696 and COX^+ mitochondria carrying wild-type mtDNA.

Moreover, using *in vitro* explantation experiments we provided definitive evidence for the presence of inter-mitochondrial complementation (Fig. 6). Coexistence of COX^+ and COX^- mitochondria from ρ^+ cells and explanted ρ^- cells, respectively, within the same hybrids carrying 63% ΔmtDNA4696 resulted in complete recovery of translation and COX activities in their total mitochondria (Fig. 6). A possible mechanism for such complementation is as follows: even though COX^- mitochondria from the explanted ρ^- cells possessed only ΔmtDNA4696 missing six tRNA genes (Fig. 1*a*), proteins encoded by ΔmtDNA4696 could be translated with the help of the tRNAs transcribed from wild-type mtDNA in COX^+ mitochondria from ρ^+ cells, by fusion of COX^+ and COX^- mitochondria and subsequent exchange of their genetic contents. On the contrary, progressive reduction of respiratory function and resultant onset of disease phenotypes appeared only when cells contained more than 85% ΔmtDNA4696. In cells where the proportion of ΔmtDNA4696 is more than 85%, mitochondrial translation might become limiting due to insufficient amounts of the six tRNAs transcribed solely from remaining wild-type mtDNA. Thus, the dynamics of translation might be shifted from complementation to competition of the tRNAs in cells with 70–85% ΔmtDNA4696 (Fig. 1*b*), resulting in progressive inhibition of overall mitochondrial translation and subsequent reduction of the activities of mitochondrial respiratory complexes, followed by the onset of the disease phenotypes in tissues with more than 85% ΔmtDNA4696.

A lack of *in vitro* inter-mitochondrial complementation was proposed based on observations that the coexistence of human respiration-deficient mitochondria containing different pathogenic mutant mtDNAs derived from different patients within single cells did not restore reduced mitochondrial respiratory function[10]. However, we recently showed that the failure of frequent isolation of respiration-competent cells by coexistance of differrent types of respiration-deficient mitochondria[10] is not caused by the absence of intermitochondrial complementation, but by the slow recovery of respiratory function, which required more than 10 days to be respiration competent[15].

The occurrence of frequent and continuous complementation throughout mitochondria in all tissues shown here provided two new concepts regarding the mitochondrial genetic system in living animals. One was the loss of the individuality of each mitochondrion within single cells. Mammalian cultured cells have been thought to contain hundreds of independent mitochondria[17], but our previous observations gave the wholly different view that mitochondria function as a single unit, indicating that they lose individuality at least *in vitro*[18]. Here we provide convincing *in vivo* evidence to extend this idea to all mitochondria in living individuals by showing uniform distribution of COX activity throughout mitochondria in single cells possessing various proportions of ΔmtDNA4696. However, this does not imply that ΔmtDNA4696 and wild-type mtDNA distributed completely uniformly throughout mitochondria, because there is a slight variation in the proportion of ΔmtDNA4696 between mother and offspring[7], between different tissues (Fig. 2), and even between different cells of the same tissues (Fig. 3*b*).

The other important concept was that extensive *in vivo* inter-mitochondrial complementation could prevent human subjects from phenotypic expression of respiration defects

caused by various pathogenic mutant mtDNAs created in somatic tissues with age. It has been generally thought that age-associated accumulation of various somatic mutations in mtDNAs is responsible for age-associated mitochondrial dysfunction. However, no reports directly proved that mtDNA lesions in aged subjects could induce mitochondrial defects. We reported recently that introduction of mtDNA in autopsied brain tissues from aged human subjects into ρ^0 HeLa cells completely restored respiratory function, even though the brain tissues and their cybrids possessed mtDNAs with various pathogenic mutations[19]. These observations could be explained by the presence of the extensive *in vivo* inter-mitochondrial complementation shown here. Therefore, mitochondria have an effective system for preventing individuals from expressing mtDNA lesions, irrespective of whether they are transmitted maternally or newly acquired with age, although this system does not completely exclude the possibility that mtDNA defects can lead to age-associated changes.

Finally, *in vivo* inter-mitochondrial complementation could open the possibility of gene therapy by introduction of mitochondria possessing DNA with sequences missing in ΔmtDNA4696; however, no effective procedures are yet available for *in vivo* introduction of mitochondria into mammalian cells.

Methods

Cells and mice. ρ^0 mouse cells[6] and Cy4696 cybrids with 0%, 60% and 88% ΔmtDNA4696 (ref. 7) were grown in normal medium: RPMI1640 (Nissui Seiyaku, Tokyo) containing 10% FCS, 50 μg/ml uridine and 0.1 mg/ml pyruvate. For examination of pathogenesis, we selected three 6-month-old male mice of the F3 generation[7], called mouse-0, -57 and -87, with 0%, 57% and 87% ΔmtDNA4696, respectively, in their tails.

Isolation of ρ^- fibroblasts from explants and isolation of $\rho^- \times \rho^+$ hybrids. Completely respiration-deficient ρ^- fibroblasts due to a predominant amount of ΔmtDNA4696 were isolated from skin explants of embryos with only ΔmtDNA4696 in their tails. $\rho^- \times \rho^+$ hybrids were isolated by the fusion of the ρ^- cells with ρ^+ B82TK$^-$ cells, which possess only wild-type mtDNA but lack thymidine kinase (TK) activity. HAT and nutritional selection medium without pyruvate and uridine were used for excluding parental ρ^+ B82TK$^-$ cells and respiration-deficient ρ^- cells, respectively. One $\rho^- \times \rho^+$ hybrid colony possessing 63% ΔmtDNA4696 was used for precise examination of inter-mitochondrial complementation.

Analysis of mitochondrial translation products. Mitochondrial translation products were labeled with [^{35}S]methionine as described[11] with slight modifications. Briefly, 2×10^6 cells in a culture dish were incubated in methionine-free medium containing 2% FCS for 45 min at 37 °C. Then the cells were labeled with [^{35}S]methionine for 2 h in the presence of emetine (0.2 mg/ml). Proteins in the mitochondrial fraction were separated by 0.85% SDS, 12% PAGE.

Southern-blot analysis. Total DNA (2–3 μg) extracted from cells and tissues was digested with the restriction enzyme *Xho*I or *Sac*I. Restriction fragments were separated in 1.0% agarose gel, transferred to a nylon membrane and hybridized with [α-^{32}P]dATP-labeled mouse mtDNA probes. The membrane was washed and exposed to an imaging plate for 2 h and radioactivities of fragments were measured with a bioimaging analyzer, Fujix BAS 2000 (Fuji Photo Film, Tokyo, Japan). Southern-blot analysis was carried out as described[6] for identification and quantification of ΔmtDNA4696 as well as partially duplicated and wild-type mtDNA. Briefly, total DNA was digested with either *Xho*I or *Sac*I, and probes A and B (Fig. 1*a*) were used for hybridization. *Sac*I site and probe B were included in the deleted region of ΔmtDNA4696. On *Xho*I digestion, the partially duplicated mtDNA gave one 16.3-kb and one 11.6-kb fragment, and thus could not be distinguished from a mixture of wild-type mtDNA and ΔmtDNA4696. On the other hand, since the *Sac*I site was lost in ΔmtDNA4696, *Sac*I digestion of the partially duplicated mtDNA produced one 27.9-kb linear fragment, whereas wild-type mtDNA gave one 16.3-kb linear fragment, and ΔmtDNA4696 gave one uncut band, the mobility of which was much slower than that of the 16.3-kb linear fragment due to its circular form. These uncut bands did not correspond to the partially duplicated mtDNA or wild-type mtDNA, since they did not show any signals when the B region, which was lost in ΔmtDNA4696, was used as a probe.

Analyses of COX activity. Estimation of COX activity was carried out by examining the rate of cyanide-sensitive oxidation of reduced cytochrome *c* (ref. 20) with modifications. Biochemical analysis was based on the procedure described before[21]. In histochemical analysis, skeletal muscle (*Musculus soleus*), heart and kidney were excised from the animals, and 10-μm cryosections from the tissues were stained for COX activity. COX electron micrographs were carried out as described[22] with slight modifications. Briefly, 25-μm cryosections and cultured cells attached to the slide glass or dish, respectively, 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-fiber PCR analysis. Two serial cryosections (10- and 20-μm) skeletal muscle (*Musculus soleus*) were used for single-fiber PCR. The former sections were stained with dimethylaminoazobenzene for COX activity, and COX$^-$ and COX$^+$ fibers were selected for PCR analysis. The latter thicker sections were used for dissection of cytoplasm corresponding to the COX$^-$ and COX$^+$ fibers with a sharp microcapillary. PCR amplification was carried out with three primers, nucleotide positions 7576–7595, 12,260–12,280 and 12,479–12,459 of mouse mtDNA (ref. 7). Mutant ΔmtDNA4696 and wild-type mtDNA gave 210-bp and 220-bp fragments, respectively, and the proportion of ΔmtDNA4696 in each fiber was analyzed with an FM-BIO image analyzer (Hitachi). Quantification was confirmed by the observation that the proportion of ΔmtDNA4696 calculated by this PCR analysis was almost equivalent to that calculated by Southern-blot analysis of the same muscle samples.

Acknowledgments

This work was supported by a grant for a Research Fellowship from the Japan Society for Promotion of Science for Young Scientists (to K.N., K.I. and K.I.); by a grant for the Hayashi project of TARA, University of Tsukuba; by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to J.-I.H.); and by Health Sciences Research Grants for Research on Brain Science from the Ministry of Health and Welfare of Japan (to J.-I.H, I.N. and Y.-i.G.).

RECEIVED 13 MARCH; ACCEPTED 31 MAY 2001

1. Larsson, N.-G. & Clayton, D.A. Molecular genetic aspects of human mitochondrial disorders. *Annu. Rev. Genet.* **29**, 151–178 (1995).
2. Wallace, D.C. Mitochondrial diseases in man and mouse. *Science* **283**, 1482–1488 (1999).
3. Wang, J. *et al.* Dilated cardiomyopathy and atrioventricular conduction blocks induced by heart-specific inactivation of mitochondrial DNA gene expression. *Nature Genet.* **21**, 133–137 (1999).
4. Yamaoka, M. *et al.* Complete repopulation of mouse mitochondrial DNA-less cells with rat mitochondrial DNA restores mitochondrial translation but not mitochondrial respiratory functions. *Genetics* **155**, 301–307 (2000).
5. Yamaoka, M., *et al.* Mice with only rat mtDNA are required as models of mitochondrial diseases. *Biochem. Biophys. Res. Commun.* **282**, 707–711 (2001).
6. Inoue, K. *et al.* Isolation and characterization of mitochondrial DNA-less mouse cell lines and their application for trapping mouse synaptosomal mitochondrial DNA with deletion mutations. *J. Biol. Chem.* **272**, 15510–15515 (1997).
7. Inoue, K. *et al.* Generation of mice with mitochondrial dysfunction by introducing mouse deletion mutant mtDNA into zygotes. *Nature Genet.* **26**, 176–181 (2000).
8. Kaneda, H. *et al.* Elimination of paternal mitochondrial DNA in intraspecific crosses during early mouse embryogenesis. *Proc. Natl. Acad. Sci. USA* **92**, 4542–4546 (1995).
9. Shitara, H. *et al.* Selective and continuous elimination of mitochondria microinjected into mouse eggs from spermatids, but not from liver cells, occurs through-

out embryogenesis. *Genetics* **156**, 1277–1284 (2000).
10. Yoneda, M., Miyatake, T. & Attardi, G. Complementation of mutant and wild-type human mitochondrial DNAs coexisting since the mutation event and lack of complementation of DNAs introduced separately into a cell with distinct organelles. *Mol. Cell. Biol.* **14**, 2699–2712 (1994).
11. Lombes, A., Bonilla, E. & DiMauro, S. Mitochondrial encephalomyopathies. *Rev. Neurol.* **145**, 671–689 (1989).
12. Muller-Hocker, J. Mitochondria and aging. *Brain Pathol.* **2**, 149–158 (1992).
13. Szabolcs, M.J. *et al.* Mitochondrial DNA deletion: a cause of chronic tubulointerstitial nephropathy. *Kidney Int.* **45**, 1388–1396 (1994).
14. Sciacco, M. *et al.* Distribution of wild-type and common deletion forms of mtDNA in normal and respiration-deficient muscle fibers from patients with mitochondrial myopathy. *Hum. Mol. Genet.* **3**, 13–19 (1994).
15. Hayashi, J.-I. *et al.* Introduction of disease-related mitochondrial DNA deletions into HeLa cells lacking mitochondrial DNA results in mitochondrial dysfunction. *Proc. Natl. Acad. Sci. USA* **88**, 10614–10618 (1991).
16. Clayton, D.A. Transcription of the mammalian mitochondrial genome. *Ann. Rev. Biochem.* **53**, 573–594 (1984).
17. Ono, T., Isobe, K., Nakada, K. & Hayashi, J.-I. Human cells are protected from mitochondrial dysfunction by complementation of DNA products in fused mitochondria, *Nature Genet.* **28**, 272–275 (2001).
18. Hayashi, J.-I., Takemitsu, M., Goto, Y.-i. & Nonaka, I. Human mitochondria and mitochondrial genome function as a single dynamic cellular unit. *J. Cell Biol.* **125**, 43–50 (1994).
19. Ito, S. *et al.* Functional integrity of mitochondrial genomes in human platelets and autopsied brain tissues from elderly patients with Alzheimer's disease. *Proc. Natl. Acad. Sci. USA* **96**, 2099–2103 (1999).
20. Seligman, A.M., Karnovsky, M.J., Wasserkrug, H.L. & Hanker, J.S. Nondroplet ultrastructural demonstration of cytochrome oxidase activity with a polymerizing osmophilic reagent, diaminobenzidine (DAB). *J. Cell Biol.* **38**, 1–14 (1968).
21. Miyabayashi, S. *et al.* Cytochrome *c* oxidase deficiency in two siblings with Leigh encephalomyelopathy. *Brain Dev.* **6**, 362–372 (1984).
22. Nonaka, I., Koga, Y., Ohtaki, E. & Yamamoto, M. Tissue specificity in cytochrome *c* oxidase deficient myopathy. *J. Neurol. Sci.* **92**, 193–203 (1989).