Role of Mitochondrial DNA Mutations in Age-
Associated Mitochondrial Dysfunction in Humans

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# Role of Mitochondrial DNA Mutations in Age-Associated Mitochondrial Dysfunction in Mammals.

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## ABSTRACT

In Part 1, the role of mitochondrial DNA (mtDNA) and nuclear genome in human aging was examined by their intercellular transfer using skin fibroblasts and mtDNA-less HeLa cells ($\rho^0$-HeLa cells). I found *in vivo* age-related reductions in the activity of cytochrome *c* oxidase (COX) in human skin fibroblasts obtained from 16 donors of various ages (0-97 years old). The abnormality in mitochondria of the aged donors was not attributable to either decrease in the copy number of mtDNA molecules or increase in the copy number of deletion mutant mtDNA molecules, but to significant decrease in overall polypeptide synthesis in the mitochondria. However, intercellular mtDNA transfer experiments showed that fibroblast mtDNA from elderly donors is functionally intact. By contrast, intercellular transfer of HeLa nuclei to fibroblasts from aged donors restored COX activity, suggesting that the age-related phenotype was nuclear recessive. However, during subsequent cultivation of these hybrids, the activity gradually reduced again, associated with gradual chromosome loss. These observations support the idea that accumulation of nuclear recessive somatic mutations, but not mtDNA mutations, is responsible for the *in vivo* age-related mitochondrial dysfunction observed in human skin fibroblasts.

In Part 2, I examined age-associated changes of respiratory enzyme activities and protein synthesis in mitochondria isolated from mouse brain with high oxidative activities. COX activity increased unexpectedly with aging, while the mitochondrial translational activities showed two phases of alterations: they increased progressively up to 21 weeks after birth followed by a gradual decrease with aging. Results showed that these changes were not due to the change in

mtDNA copy number or the accumulation of deletion mutations in mtDNA. These observations suggest that the common feature of age-associated changes in both human and mouse mitochondrial functions is limited to the decrease in mitochondrial translational activity. Therefore, mouse brain can be used as a model to understand the relationships between aging and mitochondrial function by examining the cause of decrease in mitochondrial translation activity.

## ABBREVIATIONS

| | |
|---|---|
| COX | cytochrome c oxidase |
| $\Delta$mtDNA$^{4977}$ | mtDNA deleted 4977bp |
| DM170 | cell culture medium without glucose |
| FBS | fetus bovine serum |
| *g* | centrifugal force (x unit gravitational field) |
| HAT medium | selection medium including hypoxanthine, aminopterin, and thymidine |
| HeEB | cybrid cells isolated from $\rho^0$-HeLa cells introduced HeLa cell mtDNA |
| MELAS | mitochondrial encephalomyopathy, lactic acidosis, and strokelike episodes syndrome |
| mtDNA | mitochondrial DNA |
| mtTF1 | mitochondrial transcription factor |
| nts | nucleotides |
| PCR | polymerase chain reaction |
| PDL | population doubling level |
| $\rho^0$-HeLa | HeLa cells lacking mtDNA |
| RPMI1640 | cell culture medium rich in nourishment |
| RNase MRP | RNase for mitochondrial RNA processing |
| SDH | succinate dehydrogenase |
| SDS | sodium dodecyl sulfate |
| Tris | Tris(hydroxymethyl)aminomethane |
| w/v | weight per volume |

## INTRODUCTION

In broad terms theories of aging fall into two classes - those which regard aging as a programmed process akin to development, in which programmed changes in gene expression result in decreased viability, or stochastic theories which suggest that aging results from random damage to critical macromolecules. The somatic mutation theory of aging (10, 25, 56, 61, 73) belongs to the latter class and is the most popular of the stochastic theories as it provides an explanation for the progressive decline in vitality of the somaticc cells. As originally proposed, the somatic mutations of importance were considered to be nuclear mutations but a serious possibility is that mitochondrial mutations in somatic cells may be equally as important as nuclear mutations.

mtDNA is a small circular molecule which is almost exclusively maternally transmitted. It encodes 13 of the many subunits of the mitochondrial respiratory chain and oxidative phosphorylation systems. Recently, accumulation of various somatic mutations in mtDNA during lifetime and the resultant decline of mitochondrial energy production have been proposed to be involved in aging processes (47, 51, 77) and in several degenerative diseases (47, 77). In fact, there are reports that the number of cytochrome *c* oxidase (COX) negative fibers increases with age in human muscle (57, 58), and that mitochondrial respiratory function decreases with age in human muscle (75) and liver (79). This age-related decrease in energy production is proposed to be caused by the accumulation of a common mutant mtDNA with a 4977 bp-deletion ($\Delta$mtDNA$^{4977}$) in human brain (44), heart (21, 22), and liver (81) . The rates of mtDNA transcription in rat brain (27) and translation in rat skeletal muscle (4) are also reported to

decrease with age.

However, there is as yet no convincing evidence that accumulated mtDNA mutations are causal genetic factors of age-related mitochondrial dysfunction. Moreover, this age-related phenotypic change may be under control of the nuclear genome, because nuclear genes encode most mitochondrial proteins including all the factors involved in expression of the mitochondrial genome (3, 17). This problem can be solved by examining whether mtDNA in cells of aged humans and age-related mitochondrial dysfunction are co-transferred to other cells. Such intercellular mtDNA transfer is technically possible when cultured cells from aged donors are available.

Since the discovery of the intrinsic limit of cell-division, i.e. the limit of population doublings, of human diploid fibroblasts grown in culture (41), these cells have been used extensively as a model system for studying *in vitro* cellular aging (29). Goldstein *et al.* (30) explored the relationship between their limited replicative life span and energy metabolism using fibroblasts from the same donor at early and late passages, and found that there was no gross deficit in energy metabolism at increased population doubling levels (PDL), i.e. during *in vitro* aging, of cultured human fibroblasts.

On the other hand, in the Part 1 of the present study I observed *in vivo* aging related reduction of COX activity in cultured human skin fibroblasts from 16 different donors of various ages (0-97 years old). The abnormality in mitochondria of the aged humans was not attributable to either decrease in the copy number of mtDNA molecules or increase in the copy number of deletion mutant mtDNA molecules, but to significant decrease in overall polypeptide synthesis in the mitochondria. Intercellular transfer of mtDNA and nuclear

genome using $\rho^0$-HeLa cells, which completely lack mtDNA (37, 38), showed that nuclear genome, but not mtDNA of elderly donors was responsible for the *in vivo* age-related down regulation of mitochondrial energy production found in human skin fibroblasts.

Moreover, in Part 2 of present study, to investigate whether my conclusions generated from fibroblasts can be extended to other tissues that show much higher oxidative activities than fibroblasts, I used mouse tissues, since fresh human tissues with high oxidative activities were difficult to obtain from healthy subjects. An inbred mouse strain, C57BL/6, was used in view of its wide use in aging studies (19). I analyzed *in vitro* protein synthesis in isolated mitochondria, and showed that translational activity in mitochondria substantially decreased with aging, whereas COX activity increased slightly.

## MATERIALS AND METHODS

**Cells and cell culture.** Normal human skin fibroblasts lines derived from 16 different donors of various ages (0-97 years old) were obtained from the Tokyo Metropolitan Institute of Gerontology, Japanese Cancer Research Resources Bank, and Tohoku University School of Medicine (Fig. 1). $\rho^0$-HeLa cells, which are resistant to 20$\mu$M 6-thioguanine, and fibroblast lines were grown in glucose-rich medium RPMI1640 supplemented with pyruvate (0.1 mg/ml) and 10% fetal bovine serum (FBS) (37).

**Intercellular transfer of fibroblast mtDNA.** Intercellular transfer of mtDNA was carried out as described previously (38) by fusion of enucleated fibroblasts with $\rho^0$-HeLa cells and cybrid clones were isolated in selective medium. Briefly, skin fibroblasts grown on round glass discs were enucleated by centrifugation (23,000 x*g*, at 34°C for 10 min) in the presence of cytochalasin B (Sigma; 10 $\mu$g/ml). The resulting cytoplasts were mixed with $\rho^0$-HeLa cells, and fusion was carried out in the presence of 50% (w/v) polyethylene glycol 1500 (Boehringer Mannheim). The fusion mixture was cultivated in selective medium without glucose (DM170, Kanto Kagaku, Tokyo) supplemented with 20 $\mu$M 6-thioguanine and 10% FBS (38). The residual nonenucleated parental fibroblasts, and hybrids between nonenucleated fibroblasts and $\rho^0$-HeLa cells were completely eliminated with 20 $\mu$M 6-thioguanine. Unfused parental $\rho^0$-HeLa cells were removed by culture in DM170 medium, since they could not grow in the medium without glucose due to the complete absence of mtDNA (38). On day 14 after fusion, cybrid colonies grown in selective medium were picked up and cloned by the cylinder method (35). Cybrids were cultivated in normal medium (RPMI1640 +

pyruvate + 10% FBS). As a control, HeLa mtDNA was introduced into $\rho^0$-HeLa cells by the fusion of $\rho^0$-HeLa cells with enucleated wild-type HeLa cells, which are sensitive to 6-thioguanine. The cybrids named HeEB cells, i.e., $\rho^0$-HeLa cells containing mtDNA from wild-type HeLa cells, were isolated in selective medium DM170 with 20 $\mu$M 6-thioguanine, in which unfused $\rho^0$-HeLa cells, wild-type HeLa cells, and their hybrids could not survive.

**Intercellular transfer of HeLa nuclear genome.** Intercellular transfer of HeLa nuclei to TIG102 fibroblasts from a 97-year-old woman was attained by fusion of TIG102 fibroblasts with $\rho^0$-HeLa cells using polyethylene glycol 1500 (35). The fusion mixture was selected with HAT medium (Sigma), and colonies grown in the selection medium were cloned as described above.

**Chromosome analysis.** The chromosome compositions of cybrid and the hybrid clones of log-phase cells were analyzed immediately after cloning using air-dried chromosome preparations as described previously (34). At least 20 metaphase spreads of each clone isolated were counted.

**Fractionation of mitochondria.** Mice of the C57BL/6 strain were sacrificed by cervical dislocation. The brain and thigh muscles were removed immediately and washed in isolation buffer (0.2 M sucrose, 0.13 M NaCl and 1 mM Tris-HCl pH7.4). Tissues were cut into small pieces and homogenized in the buffer with 10 strokes using a Teflon-glass Potter homogenizer. The homogenate was centrifuged at 500x*g* for 10 min. Supernatant (post nuclear fraction) was centrifuged at 8,000x*g* for 15 min. The pellet was used as the mitochondrial fraction. All the procedures were performed at 4°C.

**Southern blot analysis.** Total DNA extracted from 2

x $10^5$ cells or 0.1 g brain tissues was digested with the single-cut restriction enzyme, *Pvu*II for human mtDNA or *Xho*I for mouse mtDNA, to estimate the contents of mtDNA per cell. The fragments separated by agarose gel electrophoresis were then transferred to nitrocellulose membranes and hybridized with [$\alpha$-$^{32}$P]dCTP-labeled HeLa mtDNA or [$\alpha$-$^{32}$P]dATP-labeled mouse mtDNA. The membranes were exposed to imaging plates (Fuji Film, Tokyo) for 5 min, and radioactivity was measured with a bioimage analyzer, Fujix BAS 2000 (Fuji Film). To estimate the contents of mtDNA per mitochondrion, the equivalent amounts of mitochondrial fraction were blotted on a membrane and hybridized with [$\alpha$-$^{32}$P]dATP-labeled mouse mtDNA.

**PCR analysis for human mtDNA.** Total DNA (100 ng/ml) extracted from 2 x $10^5$ cells was used for amplification. The nucleotide sequence from position 7901 to 7920 on the light strand and from 13650 to 13631 on the heavy strand, which have frequently been used (44, 80) for detection of a common $\Delta$mtDNA$^{4977}$, were used as oligonucleotide primers. The cycle times were 15 sec denaturation at 95°C, 15 sec annealing at 50°C, and 120 sec extension at 72°C for 30 cycles. The products were separated on agarose gels containing ethidium bromide (0.5 $\mu$g/ml).

**PCR analysis for mouse mtDNA.** PCR amplifications were performed as described in (16). Primers for the initial PCR amplifications corresponded to mouse mtDNA nucleotides (nts) 7506-7530. Nested PCR primers corresponded to nts 7539-7563. To ensure that the products were true deletions and not the result of primer misannealing, primer shift analyses were performed. Primers for primer shift analyses corresponded to nts 7590-7614 and nts 14914-14917.

**Analysis of mitochondrial translation products.** Mitochondrial translation products were labeled with [$^{35}$S] methionine as described in (7, 50). Proteins were separated by SDS/urea/polyacrylamide gel electrophoresis. The dried gel was exposed to an imaging plate for 6 hr, and the labeled polypeptides were located with a bioimaging analyzer.

**Analysis of COX activity.** For biochemical analysis, log-phase cells were harvested, and COX activity was measured as the rate of cyanide-sensitive oxidation of reduced cytochrome c as described before (53). For cytochemical analysis, cells grown on coverslips were fixed with freshly prepared 4% glutaraldehyde/ 0.1 M phosphate buffer, pH 7.4, for 10 min, and stained with COX (63) for 2 hrs at 37°C.

# Part 1. Analyses of human skin fibroblasts using enucleation and cell fusion technics.

## RESULTS

In this study, 16 lines of cultured norman human skin fibroblasts isolated from 16 persons of various ages (0-97 years old) were used for studying "*in vivo* aging". First, I compared the COX activities of these fibroblasts, and found that the activities decreased gradually as the fibroblast donors' age increased (Fig. 1a). Although the population doubling levels (PDL), i.e. *in vitro* aging levels, of cultured human fibroblasts lines examined were not constant, they did not affect the COX activities (Fig. 1b), consistent with reports by Goldstein *et al.* (30) and Sun *et al.* (72). These results suggest that fibroblast lines from donors of various ages provide a good model system for studying *in vivo* aging.

The above data represent overall COX activities of the cells not the activities of single cells. To determine whether a small number of fibroblasts from elderly donors show normal COX activities, like muscle fibers from aged donors (57), I carried out a cytochemical analysis of COX activities at the individual cell level using TIG102 fibroblasts from a 97-year-old woman and TIG3S fibroblasts from a fetus. As shown in Fig. 2, the COX activities in TIG102 fibroblasts were much lower than those in TIG3S fibroblasts, and the decrease was uniform in all the individual cells (Fig. 2b).

Since three subunits of COX are encoded by mtDNA, the observed age-related reduction of COX activity could be due to reduction of the amount of normal mtDNA or to down regulation of post-transcriptional activity in the mitochondria. To examine these possibilities, I first compared the total amounts of mtDNA per cell in TIG102 and TIG3S fibroblasts by Southern blot analysis after digestion of total DNA samples with a single-cut restriction enzyme, *Pvu*II (Fig. 3a). The

results showed that there were no large mtDNA deletions in the mtDNA of either the aged subject or the fetus, and that the amount of normal sized mtDNA was slightly increased in TIG102 fibroblasts from the aged donor, consistent with previous observations (28, 69). Thus the age-related decrease of COX activity in fibroblasts (Figs. 1 and 2) is not due to decrease in the copy number of mtDNA or to accumulation of large-scale deletion mutations in the mtDNA.

Recently, mtDNA molecules with large-scale deletion mutations that were not detectable by Southern blot analysis were observed in human brain, muscle, and liver by the polymerase chain reaction (PCR) technique. Of the large-scale deletions, 4977 bp deletion of mtDNA was found to accumulate in these organs with increase in age (77). Using the PCR amplification conditions described by Ikebe *et al.* (44), I examined whether the 4977 bp-deletion mutant mtDNA ($\Delta$mtDNA$^{4977}$) was present in aged or fetal fibroblasts. However, no $\Delta$mtDNA$^{4977}$ was detected in DNA samples of TIG102 fibroblasts from the aged donor or fetal TIG3S fibroblast, even under the conditions of PCR amplification that could detect 0.002% of $\Delta$mtDNA$^{4977}$ molecules in control samples (Fig. 3b).

Second, to examine whether mitochondria of aged subjects show down regulation of post-transcription, I compared the activities for protein synthesis of mitochondria in fibroblasts of the aged subject and the fetus. Mitochondrial translation products were analyzed by SDS-polyacrylamide gel electrophoresis after pulse-labeling the fibroblasts with [$^{35}$S]methionine in the presence of emetine, a specific inhibitor of protein synthesis in the cytoplasm. In TIG102 fibroblasts, significant decreases of radioactivity were observed not only in three COX subunits (COI, COII, and COIII) but also in all

other polypeptides encoded by mtDNA (Fig. 4). These results suggest that the reduction in [$^{35}$S]methionine label incorporated into fibroblasts from aged subjects is the result of a decrease in overall mitochondrial protein synthesis, or the result of an increase in the turnover of mitochondrial translation products due to the lack of nuclear-encoded gene products with which to assemble into the inner membrane protein complexes.

Since subunits of COX are encoded by nuclear and mitochondrial genomes, and since mitochondrial protein synthesis is under the control of both genomes, it was necessary to determine which genome was responsible for the age-related down regulation of mitochondrial protein synthesis and COX activity. For this purpose I used the fetal fibroblast line TIG3S and fibroblast line TIG102 from an old subject as mtDNA donors. $\rho^0$-HeLa cells, which have no mtDNA and no COX activity (38), were used as mtDNA recipient cells. On transfer of mtDNA from these two types of fibroblasts to $\rho^0$-HeLa cells, several cybrid clones were obtained, respectively. As a control experiment, I introduced HeLa cell mtDNA into $\rho^0$-HeLa cells, and isolated cybrids (named HeEB) with HeLa mtDNA. Karyotype analysis showed that all the cybrid clones had a modal chromosome number of 50 (range, 47-54), which was the same as that of $\rho^0$-HeLa cells (50; range 47-52). On the other hand, the restriction patterns with *HhaI*, which can distinguish HeLa mtDNA from other human mtDNA (28, 67), showed that the mtDNA in all the cybrid clones were derived exclusively from those of the mtDNA donor cells.

The COX activities and mitochondrial protein syntheses of cybrid clones with mtDNA from TIG3S and TIG102 fibroblasts were compared. As shown in Figs. 4 and 5, irrespective of whether mtDNA population was imported from fibroblasts of aged or fetal donors, all the cybrid clones showed

similar activities of COX and mitochondrial protein synthesis to those of cybrid clone HeEB with HeLa mtDNA. Since COX activity was reduced similarly in individual TIG102 fibroblasts (Fig. 2b), these cybrid clones were unlikely to be restricted to those with mtDNA imported from TIG102 fibroblasts with normal COX activity. Accordingly, the phenotype of reduced mitochondrial function observed in fibroblasts of the aged subject was not co-transferred to $\rho^0$-HeLa cells with the mtDNA. Similar results were obtained when mtDNA of fibroblast line TIG106 from another aged subject was introduced into $\rho^0$-HeLa cells. Moreover, on PCR analysis, no $\Delta$mtDNA$^{4977}$ molecules were found in any cybrid clone (Fig. 3b). As all the cybrid clones share the same nuclear background as HeLa cells, these observations clearly suggest that mtDNA molecules in fibroblasts from the aged subject are functionally intact. Therefore, the age-related mitochondrial dysfunction observed in fibroblasts of old subjects is not due at least to the accumulation of various kinds of somatic mutations in mtDNA.

Then, to examine whether the reduced COX activity in cultured fibroblasts is inherited in a nuclear-dominant way or a nuclear-recessive way, HeLa nuclei were introduced to TIG102 fibroblasts. Since $\rho^0$-HeLa cells were completely without mtDNA, introduction of HeLa nuclei to the aged fibroblasts was simply attained by the fusion of TIG102 fibroblasts with $\rho^0$-HeLa cells. I subsequently isolated their hybrid clones in the selective medium (cf. Materials and Methods), and examined their COX activity. The results showed that the reduced COX activity in the aged fibroblasts recovered completely by the introduction of HeLa nuclei (Fig. 6), suggesting that human age-related mitochondrial dysfunction was nuclear recessive. Since HeLa nuclei-donor cells, i.e. $\rho^0$-HeLa cells, showed no

COX activity (Fig. 6), restoration of COX activity in the hybrids must be the results of cooperation between the imported HeLa nuclear genome and mtDNA of host fibroblasts of the aged subject.

During their subsequent cultivation, however, the hybrids gradually lost COX activity (Fig. 6) in a similar way to the gradual age-related loss of COX activity observed in human skin fibroblasts (Fig. 1). Moreover, the gradual loss of COX activity in the hybrids was paralleled by gradual chromosome loss (Fig. 6). Therefore, these observations suggest that nuclear genes of HeLa cells responsible for the restoration of COX activity are located separately on several chromosomes.

## DISCUSSION

Similar decrease of mitochondrial respiratory activity related to donor age has been observed in human muscle (57, 58, 75) and liver (79). The amount of the common large-scale deletion mutant $\Delta$mtDNA$^{4977}$, which was first found to accumulate predominantly in skeletal muscles of patients of mitochondrial myopathy (43, 66), was shown to increase with age in human skeletal muscle, liver, and brain (21, 22, 44, 81). However, its contents in old subjects were very small (0.005-0.1%) compared with those in patients with mitochondrial myopathy (20-90%). Since reduction of COX activity was heterogeneous in muscle fibers of the aged subjects (57, 58), and accumulation of $\Delta$mtDNA$^{4977}$ was also heterogeneous in brain tissues (20, 70), focal accumulation of a small amount of the mutant mtDNA possibly induced age-related mitochondrial dysfunction in limited regions of these organs. It is also possible that in addition to this common deletion, the accumulations of various other unidentified somatic mutations in the mtDNA population cause the age-related mitochondrial dysfunction (23, 48).

On *in vivo* aging of normal human skin fibroblasts, however, donor age-related reduction of COX activity was not heterogeneous in individual fibroblasts from the same donors, and transfer of their mtDNA to $\rho^0$-HeLa cells showed that the fibroblast mtDNA in aged donors was functionally intact. Moreover, $\Delta$mtDNA$^{4977}$ was not detected in any cybrid clones or in mtDNA donor fibroblasts by PCR, which can detect 0.002% of $\Delta$mtDNA$^{4977}$. Large-scale deletion mutant mtDNA molecules presumably do not accumulate in dividing tissues due to selection against the survival of cells containing these deletion mutants (42), but they could be propagated predominantly in

blood cells of cases of Pearson syndrome (65) and in cybrid clones isolated by fusion of $\rho^0$-HeLa cells with enucleated fibroblasts from a patient with Kearns-Sayre syndrome (38). Accordingly, it is also unlikely that deletion mutations in mtDNA of fibroblasts from aged subjects were selectively eliminated during the enucleation, cell fusion and cloning processes. These considerations indicate that accumulation of various kinds of mtDNA somatic mutations, even if it occurs as supposed in aged brain and muscle, is not involved in the donor age-related decrease in mitochondrial respiratory function observed in human skin fibroblasts (Fig. 1a).

The accumulation of somatic mutations in mtDNA is supposed to play a significant role in carcinogenesis (1, 5, 60, 68) and aging (47, 51, 77) for the following reasons: mitochondria are highly oxygenic organelles due to their energy production function; mtDNA lacks histones, which protect DNA from mutagenic damage; mtDNA repair systems are limited. Moreover, this possibility is supported by recent findings that the hydroxyl-radical adduct, 8-OH-dG (32) and very small amounts of deletion mutations (20-22, 44, 70, 71, 81, 82) and a point mutation (59) accumulate in mtDNA during aging. In fact, mammalian mtDNA evolved 10 times faster than single-copy nuclear DNA (8). On the other hand, many studies have failed to demonstrate accumulation of somatic mutations in mtDNA populations of human individuals. For example, the mtDNA mutation levels within single individuals have been shown to be extremely limited (9, 54), and thus about $10^{16}$ mtDNA molecules within an individual are considered to be almost identical to one another (11). Even in the case of the heteroplasmic mtDNA molecules in a MELAS patient, the mutation was not somatic, and no other mutations were observed between them (46). Bodenteich *et al.* (6) detected no

age-related accumulation of somatic mutations in mtDNA of human retina, even though this tissue is postmitotic, and is constantly exposed to UV-light in life. Moreover, Monnat and Realy (55) observed no accumulation of somatic mutations in the mtDNA of different tissues within one individual. Furthermore, no somatic mutations were induced in the mtDNA of HeLa cells by treatment with chemical carcinogens (52). However, approaches using mtDNA sequence analysis could not provide direct evidence of whether accumulation of somatic mutations in mtDNA plays a causal role in aging and/or carcinogenesis.

Cytoplasmic mtDNA transfer techniques should resolve this issue by testing whether mtDNA genotypes and the phenotypes related to carcinogenesis or aging can be co-transferred to other cells. These techniques have been used successfully for demonstrating that accumulation of mtDNA mutations is responsible for mitochondrial dysfunction observed in mitochondrial diseases (14, 15, 38, 45). These techniques have also shown that mtDNA mutations are not involved in the expression of tumorigenicity in HeLa (39, 69) or rat glioma (33) cells, and that the phenotype of carcinogen-induced tumorigenicity expressed in mouse skin fibroblasts is transmitted through the nuclear genome only, not through the mitochondrial genome (36). In this work, intercellular transfer of the mitochondrial genome from fibroblasts of aged subjects to $\rho^0$-HeLa cells provided convincing evidence that the *in vivo* age-related mitochondrial dysfunction found in human skin fibroblasts is not controlled by the mitochondrial genome.

By contrast, intercellular transfer of HeLa nuclear genome to fibroblasts from aged donor clearly showed that the reduced COX activity in the aged fibroblasts can be restored by introduction of HeLa nuclear genome, suggesting that human

age-related mitochondrial dysfunction was inherited in a nuclear-recessive way. Moreover, these cells subsequently began to lose the restored COX activity during cultivation in association with gradual chromosome loss. Accordingly, nuclear genes of HeLa cells, which were responsible for the restoration of COX activity, appeared to be localized separately on several chromosomes. These observations support the idea that accumulation of nuclear-recessive somatic mutations in genes located on various chromosomes is responsible for the age-related mitochondrial dysfunction observed in human skin fibroblasts (cf. Fig. 1). This age-related phenotype appears to be quite different from the age-related mortality phenotype observed in human diploid fibroblasts, where mortality is inherited in a nuclear-dominant way, because hybrids obtained from fusion of normal human fibroblasts with various transformed human cell lines exhibited limited potential for cell division (29, 62).

## Part 2. Analyses of mouse brain using *in vitro* mitochondrial translation system.

## RESULTS

Both COX and SDH activities were analyzed using mitochondria isolated from mice of various ages (1-98 weeks). The subunits of COX are encoded by both mtDNA and nuclear DNA, while those of SDH are encoded only by nuclear DNA. The SDH activity in mitochondria isolated from the brain did not change substantially with aging (Fig. 7), indicating that I had isolated intact mitochondria. On the other hand, COX activities constantly increased with aging (Fig. 7). This seems to be inconsistent with the previous observations that COX activity in human muscle decreased with aging (75).

To understand why COX activity increased slightly with aging, I analyzed mitochondrial translation activity in isolated mitochondria from mouse brains. Figure 2 shows that the amounts of [$^{35}$S] methionine incorporation into newly synthesized polypeptides in the mitochondria increased progressively up to 21 weeks after birth, then decreased gradually with aging. Therefore, an apparent increase in COX activity with aging was not due at least to an increase in mitochondrial protein synthesis activity. A similar shift was also observed in the mitochondria of mouse skeletal muscle (Fig. 8), suggesting that the changes in mitochondrial translation activity observed in brain mitochondria during a life span were not a brain-specific phenomenon, but can be generalized to other oxidative organs in mice.

Next, using dot blot and Southern blot analyses I studied the change in mtDNA copy numbers and the accumulation of deletion mutations with aging (Fig. 9). Southern blot analysis was carried out after digestion of total cellular DNA samples with a single-cut restriction enzyme, *XhoI*. The results showed that the total amounts of mtDNA per mitochondrion did not

change substantially with aging (Fig. 9A), and that there were no detectable amounts of mtDNA deletion mutations in the DNA samples of both the aged and young mice brains (Fig. 9B), consistent with our previous observations (40). Moreover, the amounts of both mtDNA and mitochondria in a single cell increased progressively during postnatal development (Fig. 9B), while the amounts of mtDNA per mitochondrion remained constant throughout a life span (Fig. 9A).

On the other hand, PCR analysis of mtDNA in the brain showed that at least 4-5 kinds of deletion mutations of mtDNA were present, even in the fetus as well as in the aged mice (Fig. 10). However, the number of deletion mutations did not increase with age (Fig. 10), and their amounts were not sufficient for detection by Southern blot analysis (Fig. 9B). These observations suggest that accumulation of deletion mutations in mtDNA and/or decreased copy number of mtDNA are not responsible for the observed age-associated decrease in mitochondrial translational activity.

## DISCUSSION

The decrease in mitochondrial translation activity with aging may be one of the common phenomena that are shared by both mouse and human tissues. However, the age-associated increase in COX activity found in mitochondria of mouse brain (Fig. 7) seems to be inconsistent with the observations that translation activity in human (40) and mouse mitochondria (Fig. 8) decreased with aging. This discrepancy may be explained by assuming that mice have some specific mechanisms to compensate for the age-related decrease in mitochondrial translation activity, such as a mean of decreasing the turn-over rate of mitochondrial respiratory enzyme complexes.

The most important findings in this study were that the translation activity of mitochondria isolated from both mouse brain and skeletal muscle showed two phases of alterations after birth; it increased progressively up to 21 weeks, then turned to a gradual decrease with aging (Fig. 8). This shift of mitochondrial translation activity was not due at least to the change in mtDNA copy numbers, since dot blot analysis showed that the amount of mtDNA per mitochondrion did not change substantially during postnatal development and senescence (Fig. 9A), although the amounts of both mtDNA and mitochondria in a single cell increased during postnatal development (Fig. 9B).

The mechanisms that can explain the initial increase and subsequent decrease of mitochondrial translational activities during postnatal development and senescence, respectively, might be totally different. The former phase probably belongs to one of the programmed processes of postnatal developmental stages of the mouse to attain formation of fully functional and mature organs. On the other hand, the latter may include much more random processes, such as random accumulation of

somatic mutations in mtDNA and/or nuclear DNA due to the oxidative stresses during a life span. In this study, however, deletion mutants of mtDNA were not detectable by Southern blot analysis any of all the brain mtDNA samples tested (Fig. 9B). As I showed previously (38), the accumulation of deletion mutant mtDNA to the level of more than 60% of total mtDNA was necessary to have an effect on mitochondrial translation activity. Therefore, even though various kinds of mtDNA deletions were observed in mouse brains with the use of PCR analysis (Fig. 10), they did not accumulate to a level that could account for the observed decrease of mitochondrial translation activities (Fig. 9B).

In patients with mitochondrial encephalomyopathies, accumulation of mutations, particularly of $\rho^-$ and *syn*$^-$ mutations, mtDNA were demonstrated to cause the decreases in mitochondrial translation activities (14, 31, 38, 43, 45, 77), where the mitochondrial transcription level corresponded to the increase probably by the compensatory reactions (42). In the case of aging, however, both the mitochondrial transcription (26) and translation levels (Fig. 8) decreased simultaneously with aging. Because of such different reactions of the mitochondrial transcriptional activities between mitochondrial encephalomyopathies and aging, accumulation of mutations in mtDNA can not be defined as a cause of the decrease of mitochondrial translation activity observed in the aged subjects.

In Part 2, any explanation for why the mitochondrial translation activity showed two phases during the postnatal development and subsequent senescence of mice must remain speculative. This problem can be approached by the investigation of nuclear DNA-encoded factors that regulate mitochondrial transcription and translation, such as mtTF1 (74), RNase MRP (13), and endonuclease G (24).

## CONCLUSION

In the Part 1 of this work, I found an *in vivo* age-related decrease of mitochondrial energy production in cultured human skin fibroblasts obtained from 16 normal donors of various ages (0-97 years old), and showed that this phenotype was due to accumulation of nuclear recessive somatic mutations, but not due to decrease in the copy number of mtDNA molecules or the accumulation of various kinds of somatic mutations in mtDNA in fibroblasts of aged persons. And in the Part 2 of this work, I studied activities of respiratory enzymes and protein synthesis in mitochondria isolated from brains using an inbred mouse strain C57BL/6, and found that translation of proteins encoded by mtDNA substantially decreased with aging, whereas COX activity increased slightly. From this finding, it can be proposed that even when somatic mtDNA mutations accumulate with age in highly oxidative organs of mice, their amounts should not be large enough to induce a decrease in COX activity.

These findings can be summarized as follows:

1. Mitochondrial respiratory functions in human skin fibroblasts decrease with aging.
2. Nuclear DNA, but not mtDNA, is responsible for age-associated mitochondrial dysfunction observed in human skin fibroblasts.
3. Mitochondrial translation activity in mouse brain decrease with aging.
4. The decrease in mouse mitochondrial translation activity is not caused by accumulation of mtDNA mutations.

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# FIGURES

Fig. 1. Biochemical analysis of COX activities of 16 lines of cultured human skin fibroblasts isolated from donors of various ages.

a, influence of *in vivo* age on COX activity. 1, TIG3S (fetus, M, 19 PDL); 2, TUMP403 (1 yr, M, 8 PDL); 3, TUMP304 (10 yr, F, 9 PDL); 4, TUMP415 (20 yr, F, 8 PDL); 5, TUMP300 (27 yr, M, 8 PDL); 6, TUMP372 (30 yr, M, 7 PDL); 7, TIG114 (36 yr, M, 18 PDL); 8, TUMP391 (37 yr, M, 7 PDL); 9, TUMP474 (40 yr, F, 6 PDL); 10, TIG112 (40 yr, F, 14 PDL); 11, TIG108 (40 yr, F, 15 PDL); 12, TUMP386 (57 yr, M, 8 PDL); 13, TUMP363 (65 yr, F, 7 PDL); 14, TIG103 (69 yr, F, 14 PDL); 15, TIG106 (80 yr, F, 13 PDL); 16, TIG102 (97 yr, F, 16 PDL). Donor ages, sexes, and population doubling levels (PDL), i.e.*in vitro* aging levels, of fibroblasts are indicated in parentheses. All TIG fibroblast lines except TIG103, TIG106, and TIG102 were obtained from the Tokyo Metropolitan Institute of Gerontology. TIG103, TIG106, and TIG102 were obtained from the Japanese Cancer Research Resources Bank. All TUMP fibroblast lines were obtained from the Department of Pediatrics, Tohoku University School of Medicine. b, influence of *in vitro* age on COX activity. 6, TUMP372 fibroblasts; 12, TUMP386 fibroblasts.



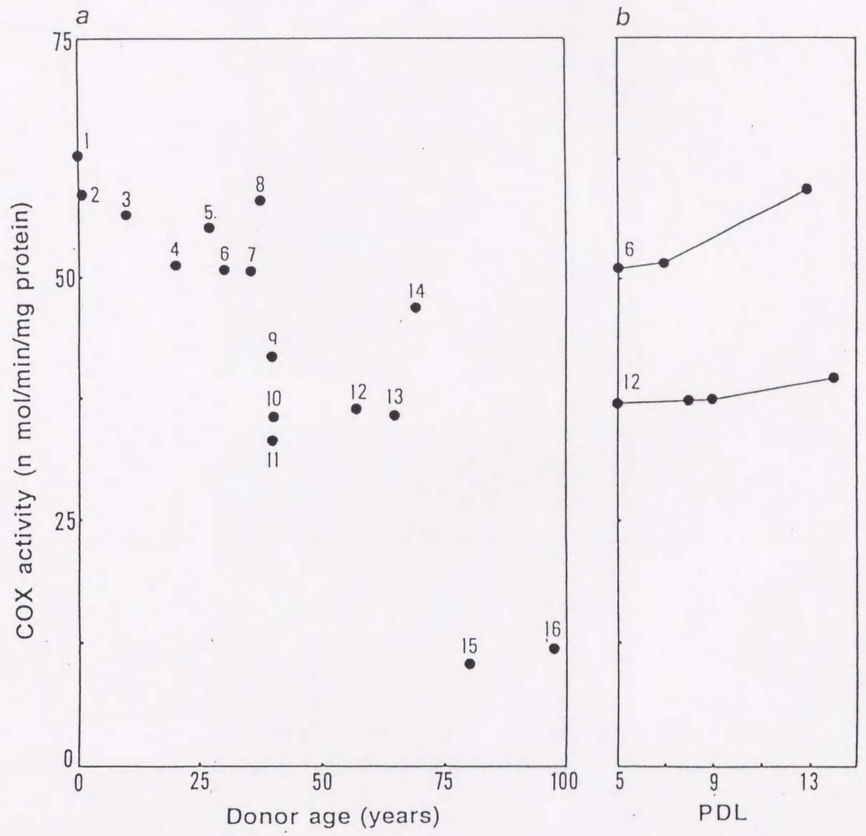


Fig. 1

Fig. 2. Cytochemical analysis of COX activities of skin fibroblasts of a fetus and old person.
Cells were stained cytochemically for COX activity. a, TIG3S fibroblasts isolated from a fetus; b, TIG102 fibroblasts isolated from a 97-year-old woman. Bar, 80$\mu$m.



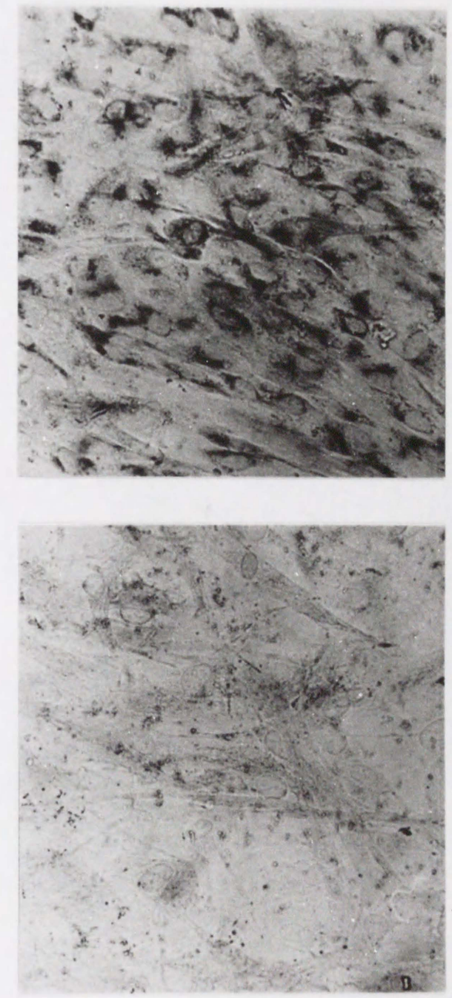


Fig. 2

Fig. 3. Southern blot and PCR analyses of mtDNA.
a, Comparison of amounts of mtDNA in TIG3S and TIG102 fibroblasts by Southern blot analysis of *Pvu*II restriction patterns of mtDNA. $\rho^0$, $\rho^0$-HeLa cells; Ft, TIG3S fibroblasts from a fetus; Ag, TIG102 fibroblasts from a 97-year-old woman. Total DNA (5 $\mu$g/lane) extracted from the cells was analyzed. b, Screening of $\Delta$-mtDNA$^{4977}$ in fibroblasts and cybrid clones by PCR amplification. The control DNA sample containing 20% $\Delta$-mtDNA$^{4977}$ prepared from a patient with Kearns-Sayre syndrome (kindly provided from Drs. R. Sakuta and I. Nonaka, National Center of Neurology and Psychiatry, Japan) was serially diluted with total DNA prepared from TIG3S fibroblasts to determine the minimal detectable content of $\Delta$-mtDNA$^{4977}$ in my PCR amplification conditions (see, Methodology section). $\rho^0$, $\rho^0$-HeLa cells; Control 1, 2, 3, 4, and 5 are DNA samples containing 2%, 0.2%, 0.02%, 0.002%, and 0.0002% $\Delta$-mtDNA$^{4977}$, respectively; Experimental 1, 2, 3, 4, 5, 6, and 7 are DNA samples of TIG3S, TIG102, cybrid clones Ft2, Ag1, Ag2, Ag3, and HeEB, respectively. Ft2 is a cybrid clone with TIG3S mtDNA; Ag1, Ag2, and Ag3 are cybrid clones with TIG102 mtDNA; HeEB is a cybrid clone with HeLa mtDNA. M, molecular weight standards ($\phi$X174/*Hinc*II digests; Toyobo, Osaka). Fragments of 5750 bp and 773 bp are PCR products amplified from wild-type mtDNA and from $\Delta$-mtDNA$^{4977}$, respectively.



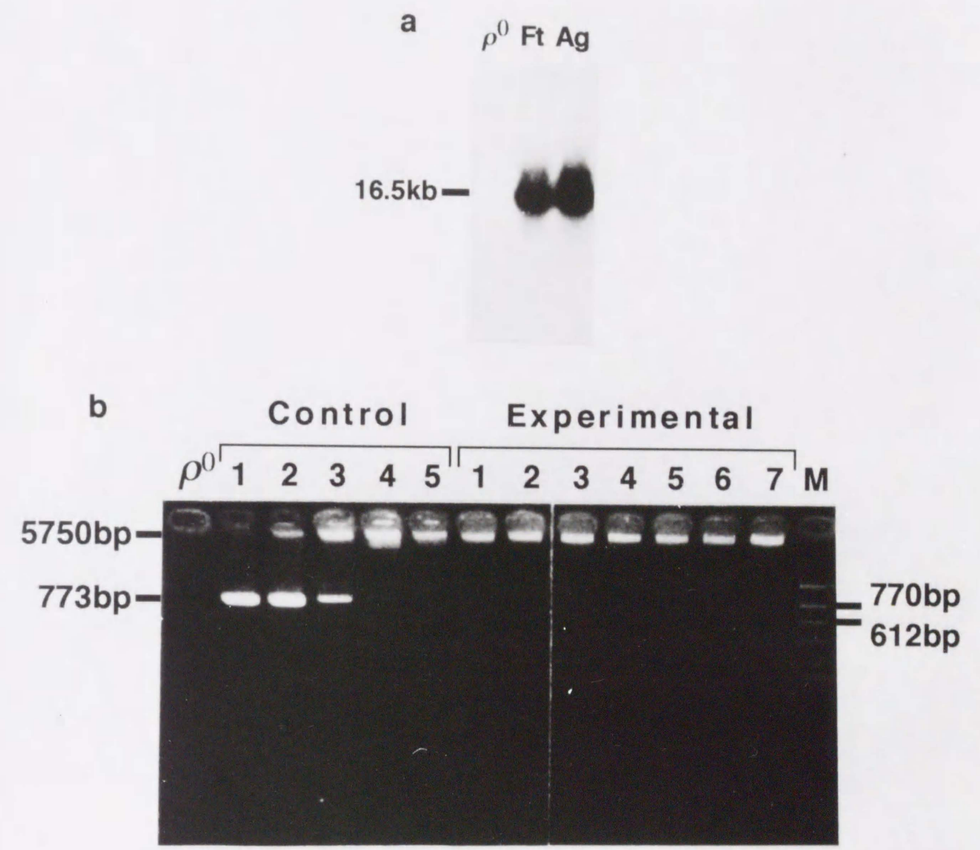


Fig. 3

Fig. 4. Analysis of mitochondrial protein synthesis in skin fibroblasts from a fetus and aged human subject and cybrids. 1, TIG3S fibroblasts; 2, TIG102 fibroblasts; 3, $\rho^0$-HeLa cells; 4, cybrid clone Ft2 ($\rho^0$-HeLa cells imported mtDNA from TIG3S fibroblasts); 5, 6 and 7 are cybrid clones Ag1, Ag2 and Ag3 ($\rho^0$-HeLa cells imported mtDNA from TIG102 fibroblasts), respectively; 8, cybrid clone HeEB ($\rho^0$-HeLa cells imported mtDNA from HeLa cells); 9, HeLa cells. After specific [$^{35}$S]methionine labeling of mitochondrial translation products in the presence of emetine, proteins of the mitochondrial fraction (50 $\mu$g/lane) were separated by SDS/urea/polyacrylamide gel electrophoresis. ND5, COI, ND4, Cytb, ND2, ND1, COII, COIII, ATP6, ND3, ATP8, and ND4L are polypeptides assigned to mtDNA genes.



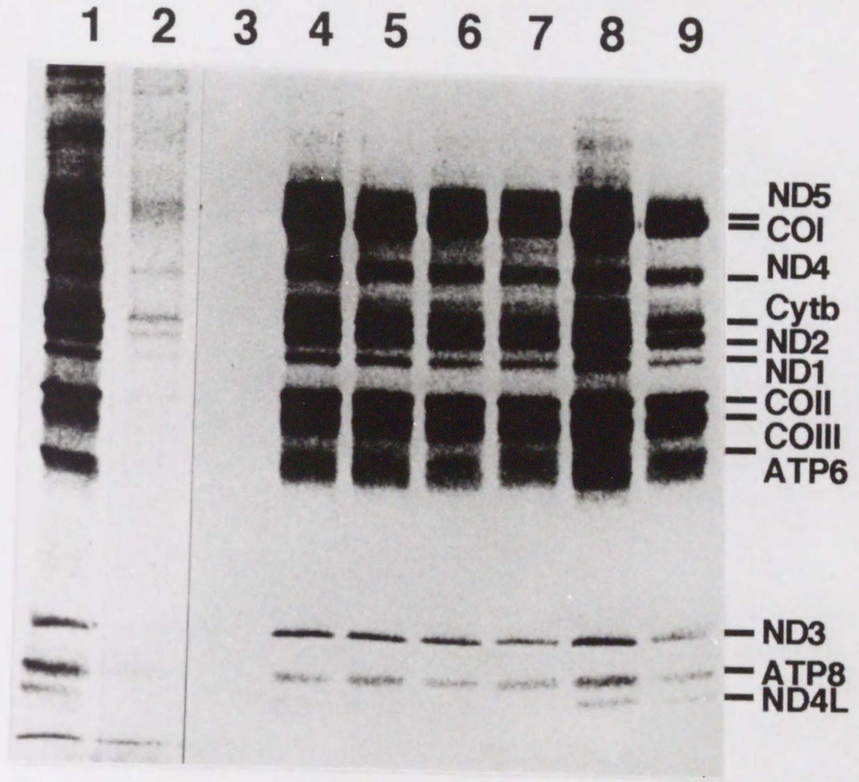


Fig. 4

Fig. 5. Comparison of activities in COX (a) and mitochondrial translation (b) of cells with mtDNA derived from fetal and aged human skin fibroblasts.
1, TIG3S fibroblasts; 2,TIG102 fibroblasts; 3 and 4, cybrid clones Ft1 and Ft2 ($\rho^0$-HeLa cells imported mtDNA from TIG3S fibroblasts); 5, 6 and 7, cybrid clones Ag1, Ag2, and Ag3 ($\rho^0$-HeLa cells imported mtDNA from TIG102 fibroblasts); 8, cybrid clone HeEB ($\rho^0$-HeLa cells imported mtDNA from HeLa cells).



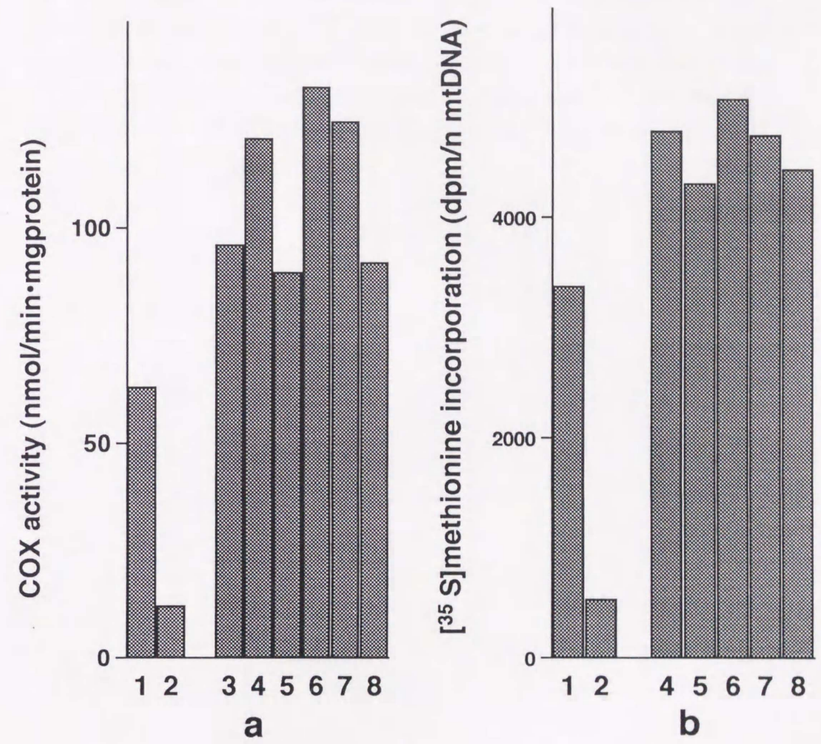


Fig. 5

Fig. 6. Recovery of the reduced COX activity in the aged fibroblasts by introduction of HeLa nuclei.
$\rho^0$, $\rho^0$-HeLa cells; TIG102, TIG102 fibroblasts from a 97-year-old woman; Hybrids, TIG102 fibroblasts with imported HeLa nuclei. Hybrids-1, -2, and -3 are COX activities of hybrids examined 2 weeks, 5 weeks, and 15 weeks after the cloning, respectively. Modal chromosome numbers of $\rho^0$-HeLa cells, TIG102, Hybrids-1, -2, and -3 were 50, 42, 89, 80, and 77, respectively.



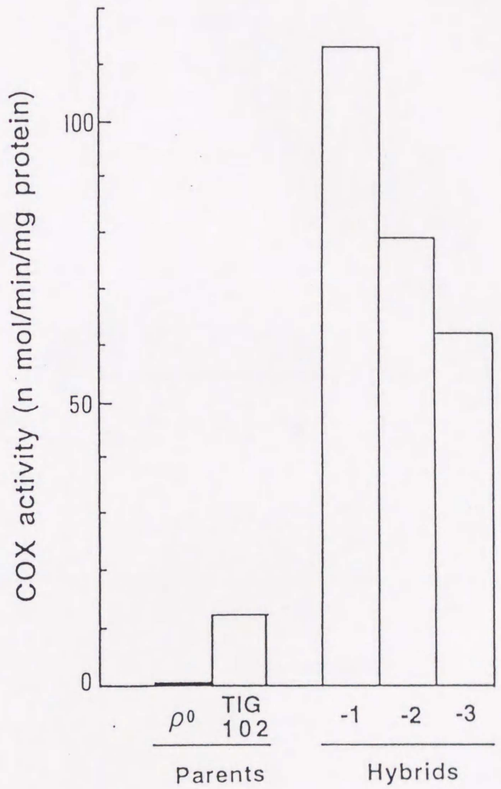


**Fig. 6**

Fig. 7. Respiration enzyme activities in mitochondria isolated from mice of various ages were analyzed as described in "Materials and Methods".
Open squares: COX activity; filled squares: SDH activity.



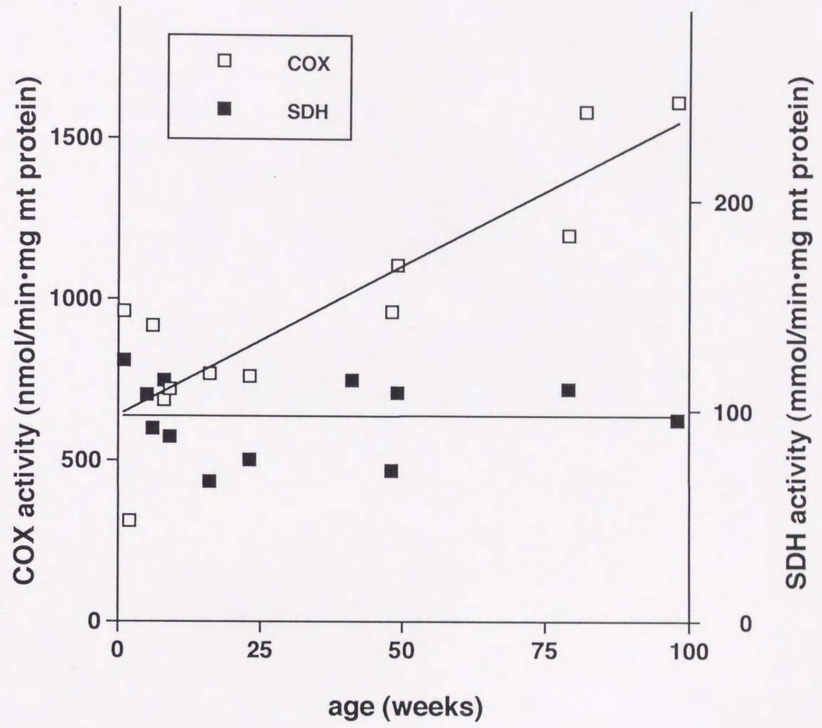


Fig. 7

COX
SDH
1500
1000
500
0
200
100
0
0
25
50
75
100
age (weeks)

Fig. 8. a. Analyses of polypeptides translated in mitochondria isolated from mice of various ages. Isolated mitochondria were radiolabeled for 2 hours with [$^{35}$S]methionine in the presence of 250 $\mu$g/ml emetine. Next, mitochondrial proteins (100 $\mu$g) were analyzed by SDS/polyacrylamide gel electrophoresis. Lanes 1-7 are mitochondrial proteins of mice aged 2, 7, 9, 20, 47, 56, and 96 weeks, respectively. b. Quantitative analysis of translation activities in brain mitochondria. c. Quantitative analysis of translation activities in muscle mitochondria.



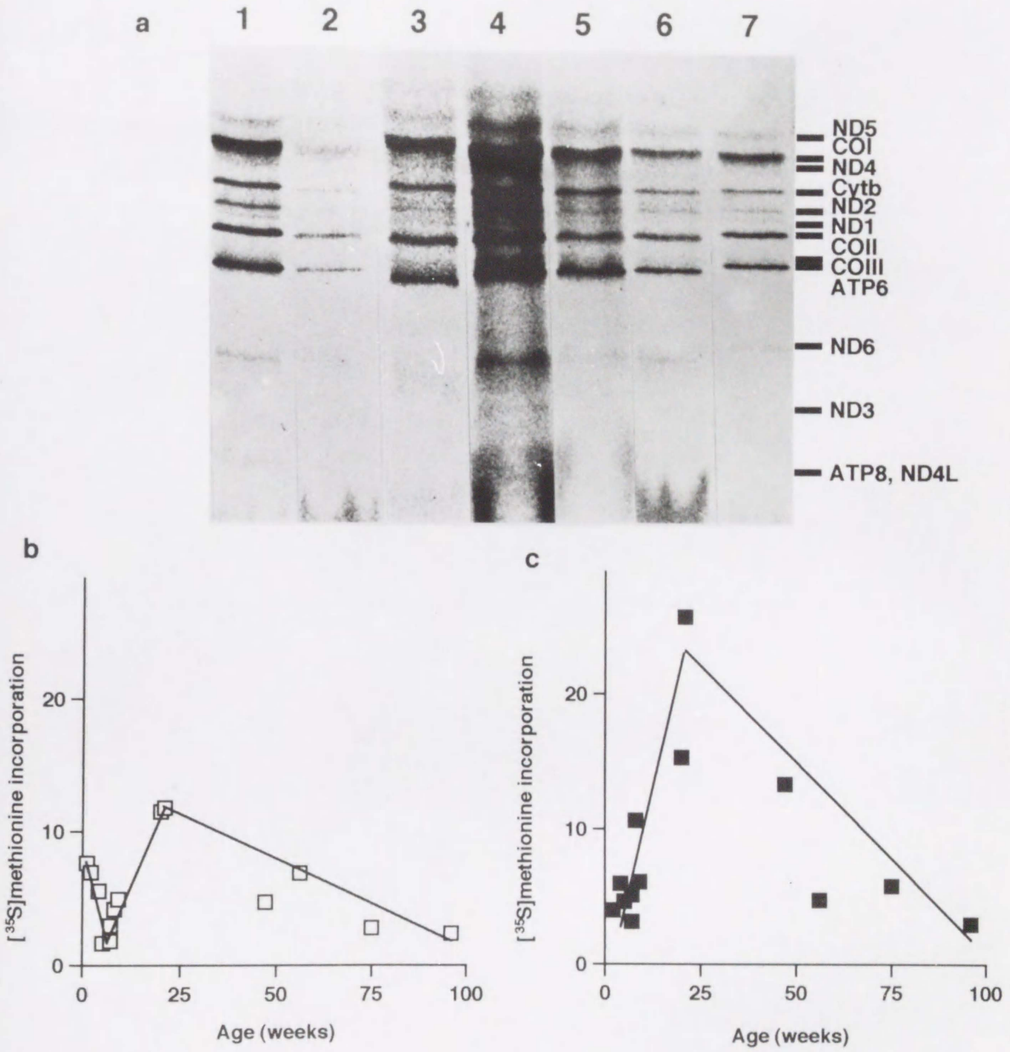


Fig. 8

Fig. 9. Relative contents of mtDNA in brain of 4-, 22-, and 67-week-old mice.
A. Quantitative analyses of mtDNA contents per mitochondrial protein. Dot blot analysis was carried out on each blotting mitochondrial fraction (10 $\mu$g protein) on a NYTRAN membrane followed by hybridization with [$\alpha$-$^{32}$P]dATP-labeled mouse mtDNA. B. Southern blot analysis of mtDNA. Total DNA was extracted from brain, and equivalent amounts of DNA (2 $\mu$g) were digested by *Xho*I, electrophoresed on a 0.6% agarose gel and hybridized with [$\alpha$-$^{32}$P]dATP-labeled mouse mtDNA. Lanes 1, 2, and 3 correspond to 4-, 22-, and 67-week-old mice, respectively.



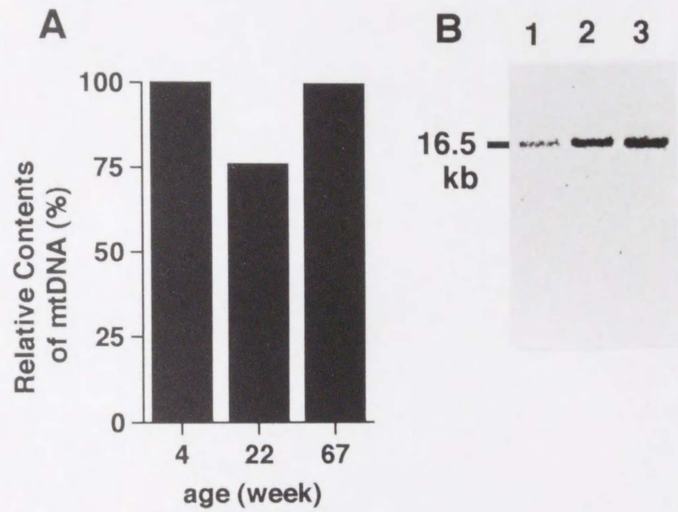


Fig. 9

Fig. 10. PCR amplification products of deletion mutants of mtDNA.
Total cellular DNA from brains of mouse aged 0 (18-day fetus, lanes 1 and 2), 4 (lanes 3 and 4), 22 (lanes 5 and 6), and 98 (lanes 7 and 8) weeks were amplified as described in "MATERIALS AND METHODS". M: size marker; lanes 1, 3, 5, and 7 are no shift analysis; lanes 2, 4, 6, and 8 are primer shift analysis producing 50 bps shorter products than no shift analysis.



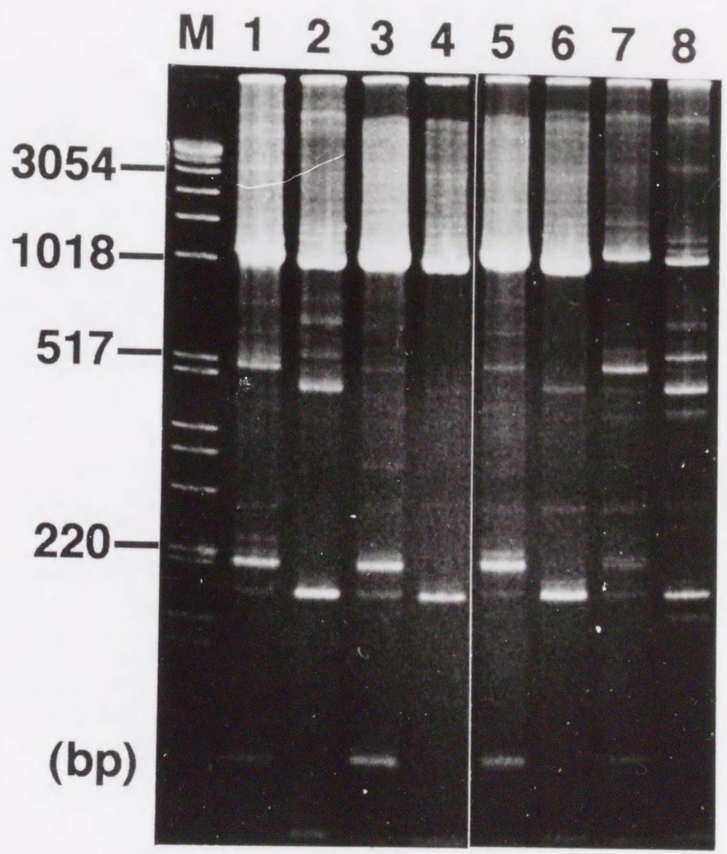


Fig. 10

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