Human Mitochondria and Mitochondrial Genome Function as a Single Dynamic Cellular Unit

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Abstract. $\rho^0$ HeLa cells entirely lacking mitochondrial DNA (mtDNA) and mitochondrial transfection techniques were used to examine intermitochondrial interactions between mitochondria with and without mtDNA, and also between those with wild-type (wt) and mutant-type mtDNA in living human cells. First, unambiguous evidence was obtained that the DNA-binding dyes ethidium bromide (EtBr) and 4',6-diamidino-2-phenylindole (DAPI) exclusively stained mitochondria containing mtDNA in living human cells. Then, using EtBr or DAPI fluorescence as a probe, mtDNA was shown to spread rapidly to all $\rho^0$ HeLa mitochondria when EtBr- or DAPI-stained HeLa mitochondria were introduced into $\rho^0$ HeLa cells. Moreover, coexisting wt-mtDNA and mutant mtDNA with a large deletion (Δ-mtDNA) were shown to mix homogeneously throughout mitochondria, not to remain segregated by use of electron microscopic analysis of cytochrome c oxidase activities of individual mitochondria as a probe to identify mitochondria with predominantly wt- or Δ-mtDNA in single cells. This rapid diffusion of mtDNA and the resultant homogeneous distribution of the heteroplasmic wt- and Δ-mtDNA molecules throughout mitochondria in a cell suggest that the mitochondria in living human cells have lost their individuality. Thus, the actual number of mitochondria per cell is not of crucial importance, and mitochondria in a cell should be considered as a virtually single dynamic unit.

Mammalian mitochondria are usually depicted as elongated cylindrical particles resembling bacteria, and it has been generally thought that a mammalian cell possesses hundreds of independent mitochondria, each containing several mitochondrial DNA (mtDNA) molecules (Borst and Kroon, 1969; Nass, 1969; Clayton, 1984; Attardi and Schatz, 1988; Wallace, 1992). A yeast strain was proposed to contain a single highly branched mitochondrion per cell (Hoffmann and Avers, 1973), but subsequent studies demonstrated that the number of mitochondria per yeast cell varied depending on the yeast strain, stage in the life cycle, and physiological conditions (Stevens, 1981; Miyakawa et al., 1984). Variation in the number and morphology of mitochondria in mammalian cells of different cell types (Johnson et al., 1980), and even of the same cell type (Posakony et al., 1975) has also been suggested.

The idea that yeast mitochondria interact with each other has received support from morphological (Stevens, 1981; Miyakawa et al., 1984) and genetic (Dujon et al., 1974; Clark-Walker and Miklos, 1975) findings. Although extensive mtDNA recombination was not observed in mammalian cells (Hayashi et al., 1985; King and Attardi, 1988), intermitochondrial interaction of the mitochondrial genetic system was suggested by the translational complementation of mitochondrial rRNA observed in heteroplasmic cells with chloramphenicol-sensitive and -resistant mtDNA (Oliver and Wallace, 1982; Gillespie et al., 1986). Our previous study also provided biochemical evidence for intermitochondrial interactions through translational complementation and competition of mitochondrial tRNAs (Hayashi et al., 1991b).

In this study, we examined how such intermitochondrial interactions occur using three kinds of human cells; (a) HeLa cells with HeLa mtDNA, (b) $\rho^0$ HeLa cells entirely lacking mtDNA (Hayashi et al., 1992) isolated by the procedure of King and Attardi (1989), and (c) HeLa cybrids with both wild-type mtDNA (wt-mtDNA) and deletion mutant mtDNA (Δ-mtDNA) from a patient with mitochondrial encephalomyopathy (Hayashi et al., 1991b).

Using mitochondrial transfection techniques, we created an environment suitable for interaction between mitochondria with and without mtDNA and also between mitochondria with wt- and Δ-mtDNA. Then we investigated the distribution of mtDNA and its products in mitochondria of single
cells by vital mtDNA staining with fluorescence probes and by measurement of the cytochrome c oxidase (COX) activities of individual mitochondria. The results provided direct evidence for frequent fusion of host and transferred mitochondria, and subsequent rapid diffusion of mtDNA and its transcripts, ensuring their homogeneous distribution throughout the mitochondria in a cell. Thus, in living cells mitochondria have a fluid nature and function as a single dynamic cellular unit, indicating that mitochondria in a living human cell are virtually single. Our findings indicate the necessity for a reassessment of the conventional theory of mitochondrial organization, and introduce the new concept of dynamics of the mitochondrial genetic system and of organelle biogenesis in living mammalian cells.

Materials and Methods

Cell Culture

HeLa cells, ρ0 HeLa cells entirely lacking mtDNA, respiration deficient ρ− HeLa cybrids with Δ-mtDNA predominantly, and HeLa cybrids with both wt- and Δ-mtDNA were grown in either a glucose-rich medium, RPMI+pyruvate (RPMI1640 containing glucose [2 mg/ml], pyruvate [0.1 mg/ml], and 10% fetal bovine serum), or a medium without glucose (DM170 containing galactose [0.9 mg/ml], pyruvate [0.5 mg/ml], and 10% fetal bovine serum).

Fluorescence Micrographs

After weak trypsinization, HeLa cells grown in a living-cell observation chamber (OZ chamber, Elecro Science, Tokyo) were treated with DAPI (10 μg/ml) for 120 min or with EtBr (2 μg/ml) for 5 min, washed several times with cultivation medium (RPMI1640+pyruvate+10% fetal calf serum), and observed with a fluorescence microscope (TMD-EF with an objective lens NCF Fluor DL 100X, Nikon) with excitation at 365 nm (UV) and 546 nm (green), respectively. Photographs were taken using ultrasensitive film (TMZp3200, ASA3200, Kodak) to minimize phototoxicity. The same cells were then stained with R123 (10 μg/ml) for 2 min, washed several times, and photographed with excitation at 495 nm (blue).

Cytoplasmic Transfer of EtBr- or DAPI-stained Mitochondria to ρ0 HeLa Cells

EtBr- and DAPI-stained HeLa mitochondria were introduced into DAPI- and EtBr-stained ρ0 HeLa cells, respectively, as described before (Hayashi et al., 1994) with slight modifications. Briefly, HeLa cells were encultured by centrifugation in the presence of cytochalasin B (10 μg/ml), and fused to ρ0 HeLa cells. Fusion mixtures were plated in OZ chambers. Since it usually takes 4–6 h for most cells in the fusion mixture to spread sufficiently to allow observation of their mitochondria under a microscope, cybrid cells were screened under a fluorescence microscope (OZ chamber, Elecon Science, Tokyo) were treated with EtBr, their cytoplasm stained very weakly and uniformly, and no mitochondria were detected by vital staining with EtBr (Fig. 1 D), presumably due to the absence of mtDNA (Fig. 2 A). Similar results were obtained when the cells were treated with a 10 times higher concentration of EtBr. However, R123 staining (Fig. 1 E) and ultrastructural analysis (Hayashi et al., 1994) showed that the cells do have mitochondria that maintain a membrane potential. Thus, the EtBr staining of HeLa mitochondria could not be due to the interaction of EtBr with mitochondrial components other than mtDNA and its transcripts. Since EtBr is a delocalized lipophilic cation like R123, it might be taken up by mitochondria simply in accordance with their membrane potential.

Results

Identification of Mitochondria with and without mtDNA in Living Human Cells

First we studied the identification of mtDNA and its transcripts in mitochondria of living human cells by their vital staining with EtBr. When HeLa cells were treated with a low concentration of EtBr for 5 min, their mitochondria were observed by fluorescence microscopy to be stained intensely against a dark cytoplasmic background (Fig. 1 A). The brightly stained regions in the cytoplasm were confirmed to be mitochondria by the fact that they exactly corresponded to regions of the same cells stained subsequently with rhodamine 123 (R123), a membrane potential-dependent mitochondria-specific dye (Johnson et al., 1980; Chen, 1988) (Fig. 1 A and B). Moreover, the EtBr- and R123-stained mitochondria in HeLa cells were identical to the network structure detected by phase-contrast microscopy (Fig. 1 A–C). These findings indicate that all the mitochondria in living HeLa cells are stained with EtBr.

In contrast, when living ρ0 HeLa cells lacking mtDNA were treated with EtBr, their cytoplasm stained very weakly and uniformly, and no mitochondria were detected by vital staining with EtBr (Fig. 1 D), presumably due to the absence of mtDNA (Fig. 2 A). Similar results were obtained when the cells were treated with a 10 times higher concentration of EtBr. However, R123 staining (Fig. 1 E) and ultrastructural analysis (Hayashi et al., 1994) showed that the cells do have mitochondria that maintain a membrane potential. Thus, the EtBr staining of HeLa mitochondria could not be due to the interaction of EtBr with mitochondrial components other than mtDNA and its transcripts. Since EtBr is a delocalized lipophilic cation like R123, it might be taken up by mitochondria simply in accordance with their membrane potential.
Identification of mtDNA and/or its transcripts in living cells by their vital staining with EtBr. A-C, HeLa cells; D, E, ρ₀ HeLa cells; F, G, ρ⁻ HeLa cybrids with 93% Δ-mtDNA, established previously (Hayashi et al., 1991b) by the fusion of ρ₀ HeLa cells with enucleated skin fibroblasts derived from a patient with mitochondrial encephalomyopathy (Kearns-Sayre syndrome); H, human skin fibroblasts. Fluorescence micrographs of EtBr staining (A, D, F) and R123 staining (B, E, G, H). Phase-contrast micrographs (C), (A-C), (D and E), and (F and G), respectively, are the same cells. The shape of mitochondria in the three images (A, B, and C) were slightly different because of the rapid and three-dimensional movement of the mitochondria in the living cells during the time required for R123 staining (2 min) and the interval between the times of fluorescence and phase-contrast photographs (0.5 min), and because of the small focal depth of the 100X lens. Note that EtBr did not stain nuclei when cells were treated with a low concentration of EtBr (2 μg/ml) for a short time (5 min), although nucleolar regions of the nucleus were preferentially stained (A, D, F). Bar, 40 μm.

Figure 2. Analyses of mtDNA and its translation products in HeLa cells, ρ₀ HeLa cells, and ρ⁻ HeLa cybrids. (A) Southern blot analysis of PvuII-cut mtDNA. 16.5 kbp, wt-mtDNA; 11.5 kbp, Δ-mtDNA with a 5196-bp deletion. Total DNA (5 μg/lane) extracted from cells was analyzed using [α²⁵P]dCTP-labeled HeLa mtDNA as a probe. (B) Protein synthesis in mitochondria. After [³⁵S]methionine-labeling of mitochondrial translation products in the presence of emetine (0.2 mg/ml), proteins of the mitochondrial fraction (50 μg/lane) were separated by SDS/urea/polyacrylamide gel electrophoresis. ND5, COI, ND4, Cyb, ND2, ND1, COII, COIII, ATP6, ND6, ND3, ATP8, and ND4L are polypeptides assigned to mtDNA genes.

Diffusion of mtDNA from Imported HeLa Mitochondria to Host ρ₀ HeLa Mitochondria

We next transferred EtBr-stained HeLa mitochondria to un stained ρ₀ HeLa cells by fusion of enucleated HeLa cells with ρ₀ HeLa cells, and 6 h later examined whether the mtDNA and/or its transcripts from HeLa cells had penetrated into the mitochondria of the host ρ₀ HeLa cells by monitoring EtBr fluorescence. Then we stained the same cells, i.e., cybrids (Fig. 3 legend), with R123 to identify host ρ₀ HeLa mitochondria as well as the imported HeLa mitochondria. Fig. 3 shows that the cybrid mitochondria stained with EtBr are completely identical to those subsequently stained with R123, i.e., that ρ₀ HeLa mitochondria that were not stained with EtBr but stained with R123 appear to be lost in the cybrid. To obtain the reproducibility of our observations, we tried the mtDNA transfer experiment three times, and the same results as those in Fig. 3 were observed in all three independent cybrid cells.

These observations suggest that all mitochondria of the host ρ₀ HeLa cells also possessed mtDNA after the import of HeLa mitochondria. If the imported HeLa mitochondria
Figure 3. Distribution of cytoplasmically transferred HeLa mtDNA and/or its transcripts in mitochondria of cybrids. (A-C) fluorescence micrographs; (D) phase-contrast micrograph of the same cybrid cell. The cybrid cell with both DAPI-stained nuclei (A) and EtBr-stained mitochondria (B) was subsequently stained with R123 (C). HeLa cells were treated with EtBr (2 μg/ml) for 5 min, enucleated by centrifugation and fused to ρ0 HeLa cells. Fusion mixtures were plated in OZ chambers. Before fusion, the host ρ0 HeLa cells were prestained with DAPI (2 μg/ml) for 15 min to identify ρ0 HeLa nuclei. It usually takes 4–6 h for most cells in the fusion mixture to spread sufficiently to allow observation of their mitochondria under a microscope. So 6 h after plating, cells with DAPI-stained (A) but not EtBr-stained nuclei and with EtBr-stained mitochondria (B), i.e., cybrid cells, were screened under a fluorescence microscope using UV (365 nm) (A) and blue (495 nm) excitation (B), respectively. The same cybrid cell was then stained with R123 for 2 min to identify all mitochondria in the cell (C). Bar, 40 μm.

did not fuse with swollen ρ0 HeLa mitochondria but simply took on a normal network appearance within 6 h, or if they did fuse but the EtBr-stained HeLa mtDNA and its transcripts did not spread rapidly to all the mitochondria of the host ρ0 HeLa cells, swollen ρ0 HeLa mitochondria that were stained with R123 but not with EtBr (Fig. 1 D and E) should be found in the cybrid, contrary to our observations (Fig. 3 B and C). The possibility that free EtBr in the enucleated HeLa cytoplasm stained mitochondria of the host cells was excluded by the use of ρ0 HeLa cells that have no mtDNA as host cells. The possibility that the apparent loss of R123 staining of ρ0 HeLa mitochondria in the cybrid was due simply to artificial, cell fusion-induced loss of the membrane potential was unlikely, because swollen ρ0 mitochondria were detected by R123 staining of multinucleated homokaryons obtained by fusion of ρ0 HeLa cells. The possibility that swollen ρ0 HeLa mitochondria degraded preferentially by introduction of HeLa mitochondria was also unlikely, because the swollen ρ0 mitochondria containing Δ-mtDNA predominantly also disappeared by introduction of HeLa mitochondria without degradation of Δ-mtDNA (data not shown). Accordingly, the observations in Fig. 3 show that the transferred HeLa mitochondria fused with the host ρ0 HeLa mitochondria and that EtBr-stained HeLa mtDNA and/or its transcripts spread rapidly to all the ρ0 HeLa mitochondria within 6 h.

Since EtBr stains both mtDNA and its transcripts (mtRNA) in mitochondria, we repeated the same experiments using a DNA-specific binding dye to exclude the possibility that mtRNA, but not mtDNA, spread rapidly to all the ρ0 HeLa mitochondria. The DNA-specific binding dye, 4',6-diamidino-2-phenylindole (DAPI), which is not a delocalized lipophilic cation like R123 and thus can bypass membrane potential-dependent staining of mitochondria, is usually used as a fluorescent probe for vital mtDNA staining in yeast (Williamson and Fennell, 1974) and trypanosomes (Hajduk, 1976; Robinson and Gull, 1991), but it does not stain mtDNA in living human cells under conventional conditions (Russell et al., 1975; Satoh and Kuroiwa, 1991). In this study, we examined various conditions for the vital staining of HeLa cell mtDNA with DAPI, and found that mitochondria-like structures in HeLa cell cytoplasm were weakly stained only when the cells were subjected to mild pretreatment with trypsin and then treated with a high concentration of DAPI. Fig. 4 shows that the DAPI-stained regions in the HeLa cytoplasm were confirmed to be mitochondria by the fact that they exactly corresponded to regions of the same cells stained subsequently with R123. Moreover, ρ0 HeLa mitochondria were not stained with DAPI, suggesting that DAPI stained only mtDNA in living HeLa cells. As shown in Fig. 5, similar results to those in Fig. 3 were obtained when DAPI-stained HeLa mitochondria were introduced into ρ0 HeLa cells. These observations suggest that mtDNA spread rapidly from HeLa mitochondria to ρ0 HeLa mitochondria within 6 h after import of the HeLa mitochondria.

Identification of Heteroplasmic wt- and Δ-mtDNA in Mitochondria of Single Cybrids

The above interpretation predicts that coexisting wt-mtDNA and mutant-type mtDNA within a cell should mix homogeneously throughout the mitochondria, and not remain segregated. We examined this prediction using HeLa cybrids containing wt-mtDNA and deletion-mutant mtDNA, Δ-mtDNA, as individual mitochondria with predominantly wt- or Δ-mtDNA can be distinguished unambiguously by electron microscopic analysis of their COX activity (Fig. 6, A and B): ρ− HeLa mitochondria in cells containing predominantly Δ-mtDNA showed no COX activity (Fig. 6 B), presumably...
Figure 4. Identification of mtDNA in living HeLa cells by their vital staining with DAPI. (A and B) HeLa cells; (C and D) \( \rho^0 \) HeLa cells. Fluorescence micrographs of DAPI staining (A and C) and subsequent R123 staining (B and D). (A and B) and (C and D), respectively, are the same cells. It should be noticed that DAPI stained nuclei much more intensely than mitochondria (A). Bar, 20 \( \mu m \).

Figure 5. Distribution of cytoplasmically transferred HeLa mtDNA in mitochondria of cybrids. A cybrid cell with both DAPI-stained mitochondria (A) and EtBr-stained nuclei (B) was subsequently stained with R123 (C). Mitochondria of HeLa cells stained with DAPI were introduced into EtBr-stained \( \rho^0 \) HeLa cells. 6 h after fusion, cybrid cells which had DAPI-stained mitochondria but not DAPI-stained nuclei (A) and had EtBr-stained nuclei (B) were screened under a fluorescence microscope using UV (365 nm) (A) and blue (495) excitation (B), respectively. The same cybrid cell was then stained with R123 for 2 min to identify all mitochondria in the cybrid cell (C). Note that on EtBr staining only nucleolar regions of the nucleus were preferentially stained with EtBr (B). Bar, 10 \( \mu m \).

Figure 6. Identification of COX activities in individual mitochondria by electron microscopy. (A) COX+ mitochondria in HeLa cells; (B), COX- mitochondria in \( \rho^0 \) HeLa cybrids containing 93\% \( \Delta \)-mtDNA, observed in thin sections (0.07 \( \mu m \)) with a standard electron microscope (at 100 kV); (C) COX+ mitochondria in HeLa cells observed in a thick section (2 \( \mu m \)) with a high-voltage electron microscope (at 1000 kV). COX+ mitochondria (A) were darkly stained compared to COX- mitochondria (B). Thick sections (2 \( \mu m \)) of HeLa cells were used to confirm the continuous structure of mitochondria visualized by fluorescence microscopy (Fig. 2 A) at the ultrastructural level. These thick sections were examined using a high-voltage electron microscope. The long branched filamentous structures (C) are mitochondria as they showed COX activity. Bar, 1 \( \mu m \).

as the result of the absence of mitochondrial protein synthesis due to deletion of five tRNA genes in \( \Delta \)-mtDNA (Hayashi et al., 1991b). Accordingly, COX activity of individual mitochondria can be used as a probe to identify mitochondria with wt- or \( \Delta \)-mtDNA in a cell. Thus, if, contrary to our prediction, there is little or no exchange of wt- and \( \Delta \)-mtDNA molecules between mitochondria, their inter-mitochondrial segregation should occur very fast because each mitochondrion is thought to have only a few mtDNA molecules. In this case, mitochondria possessing either wt- or \( \Delta \)-mtDNA predominantly, and the ratio of COX+ to COX- mitochondria in a cell should be proportional to the ratio of wt- to \( \Delta \)-mtDNA.
Living human cells are treated with a low concentration of A-mtDNA. Without mtDNA, and also between those with wt- and mutant mtDNA, mitochondrial interactions occur in human cells by creating an environment suitable for interaction between mitochondria with and without mtDNA, and also between those with wt- and Δ-mtDNA.

In this study we obtained unambiguous evidence that when living human cells are treated with a low concentration of EtBr for a short time the EtBr exclusively stains mtDNA and its transcripts. EtBr is known to be able to interact with proteins and other compounds besides DNA. Moreover, it can be taken up by mitochondria in accordance with the membrane potential, since it is a delocalized lipophilic cation like R123. However, the following evidence supports the idea that EtBr exclusively stains mitochondria with mtDNA in living human cells. We used 18HeLa cells and pHeLa hybrids: both totally lack mitochondrial protein synthesis (Fig. 2 B), but the latter cells do have mtDNA (Fig. 2 A). Both pHeLa and pHeLa mitochondria were stained with R123 (Fig. 1 E and G), whereas the pHeLa mitochondria were stained with EtBr (Fig. 1 F), but the pHeLa mitochondria were not (Fig. 1 D). Since mitochondria without mtDNA were not stained with EtBr, the EtBr staining of mitochondria was not due to the interaction of EtBr with mitochondrial compounds other than mtDNA and its transcripts. If EtBr was taken up by mitochondria simply in accordance with the membrane potential, and if the membrane potential in pHeLa mitochondria was not high enough to allow accumulation of enough EtBr for its detection due to the absence of mitochondrial protein synthesis, pHeLa mitochondria should also not be stained with EtBr, contrary to the results in Fig. 1 F. Therefore, the possibility that EtBr was simply taken up by mitochondria in accordance with the membrane potential can also be ruled out by the fact that pHeLa mitochondria were stained with EtBr although they completely lack mitochondrial protein synthesis. There was a possibility that mtRNA but not mtDNA spreads rapidly to all the pHeLa mitochondria in the experiments shown in Fig. 3, although EtBr preferentially binds to DNA rather than to RNA, and the contents of mtRNA in mitochondria are comparable to those of mtDNA (Attardi and Schatz, 1988; Cantatore and Saccone, 1987). However, this possibility was excluded by the results of mtDNA transfer experiments using the DNA-specific dye DAPI (Fig. 5). Moreover, since DAPI is not a delocalized lipophilic cation like R123 and EtBr, it can bypass membrane potential-dependent binding to mitochondria. Therefore, we conclude that mtDNA spread rapidly from HeLa mitochondria to all pHeLa mitochondria when HeLa mitochondria are introduced into pHeLa cells.

Using EtBr and DAPI fluorescence, we found that the cytoplasmically transferred HeLa mitochondria fused with the host pHeLa mitochondria, and HeLa mtDNA and its transcripts spread rapidly to all the pHeLa mitochondria within 6 h (Figs. 3 and 5). This finding suggested that coexisting wt- and Δ-mtDNA within a cell mix homogeneously throughout the mitochondrial population in a cell and do not remain segregated. However, it is well known that coexisting wt- and mutant mtDNA (Birky, 1983; Wallace, 1986) and coexisting mtDNA of different species (Hayashi et al., 1983) in a cell segregate in a stochastic way, and that during one year of cultivation each cell of the progeny cell population of single clones possesses either species of mtDNA predominantly (Hayashi et al., 1983). Since cells do not exchange mtDNA, heteroplasmmy would not appear again once cells become homoplasmic by stochastic segregation. If there were no exchange of wt- and Δ-mtDNA between mitochondria, as in the case between cells, their "intermitochondrial" stochastic segregation should occur much faster than their "intercellular" stochastic segregation, because mitochondria are as-
have thousands (Clayton, 1984; Attardi and Schatz, 1988). Therefore, in this case mitochondria possess either wt- or Δ-mtDNA predominantly, and the ratio of COX+ and COX- mitochondria in a cell should be proportional to the ratio of wt- and Δ-mtDNA molecules. As predicted, however, the results in Fig. 7 show the uniform distribution of either COX+ or COX- mitochondria within single cells, which can be explained by sufficiently rapid diffusion of wt- and Δ-mtDNA molecules throughout the mitochondria to overcome their intermitochondrial stochastic segregation.

Accordingly, the results in Figs. 3 and 5 provide evidence for frequent mitochondrial fusion and subsequent rapid diffusion of mtDNA and its transcripts among all the mitochondria in living cells. This rapid diffusion explains the complementation of mitochondrial rRNA observed in heteroplasmic cells with chloramphenicol-resistant and -sensitive mtDNA (Olivey and Wallace, 1982; Gillespie et al., 1986), and the observations that deletion mutants are dominant over wt-mtDNA in muscle fiber segments in which the mutations are predominant (Shoubridge et al., 1990). The results are not inconsistent with biochemical studies on mtDNA in muscle L cells (Berk and Clayton, 1976). Moreover, the observations in Figs. 6 and 7 suggest that wt- and Δ-mtDNA molecules, or at least their transcripts diffuse rapidly enough to ensure their homogeneous distribution throughout the mitochondria in a cell. This means that mitochondria in a living human cell have lost their individuality, and thus function as a single dynamic unit.

This functional concept is consistent with structural features of human mitochondria: on R123 staining, the mitochondria of both HeLa cells and skin fibroblasts appear as long-filamentous and highly branched network structures (Fig. 1 B and H) although sometimes they may not be strictly single (Johnson et al., 1980; Posakony et al., 1975). Moreover, the structural continuity of mitochondria was also confirmed at the ultrastructural level in thick sections by high-voltage electron microscopy (Fig. 6 C), a method that shows their three-dimensional distribution much better than conventional electron microscopy (Fig. 6 A). The presence of a single mitochondrion per cell was first observed in a yeast strain (Hoffmann and Avers, 1973), but subsequent studies demonstrated that the number and morphology of mitochondria in yeast and mammalian cells vary depending on the cell type and stage in the life cycle (Johnson et al., 1980; Stevens, 1981; Miyakawa, 1984). Moreover, Posakony et al. (1975) showed that the number and morphology of mitochondria vary in HeLa cells. However, even if mitochondria in HeLa cells are not structurally single, rapid diffusion of mtDNA and/or its transcripts throughout the mitochondrion in a cell should be structurally attainable assuming that the mitochondrial network frequently and repeatedly breaks off into fragments and reassembles into a continuous network, as suggested in the case of yeast mitochondria (Stevens, 1981; Miyakawa, 1984).

Mammalian cells have been thought to contain hundreds of independent mitochondria. However, our observations provide the total different view that in living cells mitochondria and the mitochondrial genetic system have a fluid nature and function as a single dynamic cellular unit, indicating that they are virtually single in dividing cells. These observations indicate the need for reassessment of the conventional, classical concept of the vital organization of mitochondria, and introduce the new concepts of dynamics of the mitochondrial genetic system and of organelle biogenesis in living human cells. These concepts help in understanding the relationship between mtDNA mutations and expression of human mitochondrial diseases (Holt et al., 1988; Zeviani et al., 1989; Gotoh et al., 1990; Lander and Lodish, 1990; Shoffner et al., 1990; Hess et al., 1991; Chomyn et al., 1992; Wallace, 1992).

Moreover, the swollen structure of mitochondria observed in Δ HeLa cells was not observed in all other human cells with normal mtDNA we tested including myoblast cells, but was always observed in various syn® human cells with pathogenic mtDNA mutations (J-I. Hayashi, manuscript in preparation). Therefore, this structural abnormality of mitochondria can be used as an indicator to determine whether mitochondria possess mtDNA with pathogenic mutations or with simple polymorphic mutations.

We are grateful to K. Nakachi and O. Gotoh for discussion and critical reading of the manuscript. We thank T. Arii, M. Sakamoto, and S. Kuwahara for taking the micrographs.

This work was partly supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan and University of Tsukuba Research Projects.

Received for publication 20 September 1993 and in revised form 14 December 1993.

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