The Interorganellar Interaction between Distinct Human Mitochondria with Deletion Mutant mtDNA from a Patient with Mitochondrial Disease and with HeLa mtDNA*

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For the examination of possible intermitochondrial interaction of human mitochondria from different cells, cybrids were constructed by introducing HeLa mitochondria into cells with respiration-deficient (ρ−) mitochondria. Respiration deficiency was due to the predominance of mutant mtDNA with a 5,196-base pair deletion including five tRNA genes (ΔmtDNA5196). The HeLa mtDNA and ΔmtDNA5196 encoded chloramphenicol-resistant (CAPr) and chloramphenicol-sensitive (CAPs) 16S rRNA, respectively. The first evidence for the interaction was that polypeptides exclusively encoded by ΔmtDNA5196 were translated on the introduction of HeLa mitochondria, suggesting supplementation of the missing tRNAs by ρ− mitochondria from HeLa mitochondria. Second, the exchange of mitochondrial rRNAs was observed; even in the presence of CAP, CAPs ΔmtDNA5196-specific polypeptides as well as those encoded by CAPr HeLa mtDNA were translated in the cybrids. These phenomena can be explained assuming that the translation in ρ− mitochondria was restored by tRNAs and CAPs 16S rRNA supplied from HeLa mitochondria, unambiguously indicating interorganellar interaction. These observations introduce a new concept of the dynamics of the mitochondrial genetic system and help in understanding the relationship among mtDNA mutations and expression of human mitochondrial diseases and aging.

Mammalian cells have been proposed to possess hundreds of independent mitochondria, each containing several mitochondrial DNA (mtDNA) molecules (1–3). Variation in the number and morphology of mitochondria in different cell types (4) and even in the same cell type (5) has been suggested. The interaction and fusion between mitochondria in yeast and plant cells have received support from morphological findings (6) and from the molecular and genetic evidence of mtDNA recombination (7, 8). In mammalian species, however, the coexistence of mitochondria and mtDNA from different individuals is completely inhibited by their strictly maternal inheritance (9, 10); sperm-derivered mtDNA was selectively and completely eliminated from fertilized mouse eggs, suggesting that the mtDNA popu-

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1 The abbreviations used are: CAPr, chloramphenicol-sensitive; CAPs, chloramphenicol-resistant; ρ−, mtDNA-less; ρ+, respiration-deficient; ΔmtDNA5196, mutant mtDNA with 5,196-base pair deletion; HAT, hypoxanthine/aminopterin/thymidine; COX, cytochrome c oxidase.
the translation activity of the mitochondria was examined. The results showed that even in the presence of CAP, the fusion proteins exclusively encoded by ΔmtDNA5106 were translated with the help of the tRNAs and CAP+ rRNA supplied from the imported HeLa mitochondria, suggesting unambiguously the occurrence of intermitochondrial cooperation between distinct organelles of ρ0 mitochondria and HeLa mitochondria.

MATERIALS AND METHODS

Cells and Cell Culture—The human skin fibroblast line 197 with 21% ΔmtDNA5106 was isolated from a patient with chronic progressive external ophthalmoplegia, a subgroup of mitochondrial encephalomyopathies. The ρ0 HeLa cells and CAP+ HeLa cells used are resistant to 20 μM 6-thioguanine and 2 mM ouabain. These cell lines were grown in normal medium (RMPI 1640, 0.1 mg/ml pyruvate, 50 μg/ml uridine, 10% fetal bovine serum).

Interellar Transfer of the HeLa Nuclear Genome—Interellar transfer of HeLa nuclei to 197 fibroblasts was achieved by fusion of the fibroblasts with ρ0 HeLa cells using polyethylene glycol 1500 as described previously (18) with slight modifications. Briefly, the fusion mixtures were cultivated in selective medium (RMPI 1640, 0.1 mg/ml pyruvate, 50 μg/ml uridine, 10% fetal bovine serum, hypoxanthine/aminopterin/thymidine (HAT, Sigma), 2 mM ouabain), and on day 10 after fusion colonies growing on selection medium were cloned by the cylinder method. Nuclear hybrids were then derived in normal medium.

Interellar Transfer of the HeLa Mitochondrial Genome—Interellar transfer of HeLa mitochondria was carried out as described previously (18) by fusion of encapsulated CAP+ HeLa cells with the nuclear hybrid clone H5 containing only ΔmtDNA5106, and cybrid clones were isolated in selective medium (DM170 + HAT). Briefly, CAP+ HeLa cells grown on round glass dishes were encapsulated by centrifugation (23,000 × g, at 34 °C for 10 min) in the presence of cytochalasin B (Sigma; 10 μg/ml). The resulting cytoplasts were mixed with H5 cells, and fusion was carried out in the presence of 50% (v/v) polyethylene glycol 1500 (Boehringer Mannheim). The fusion mixture was cultivated in selection medium without glucose, pyruvate, and uridine (DM170, Kyokuto Kagaku, Tokyo) supplemented with HAT (9). On day 14–20 after fusion, cybrid clones grown in selective medium were harvested and cloned by the cylinder method. Cybrids were cultivated in DM170 medium without HAT.

Southern Blot Analyses of mtDNA—For determination of the content of ΔmtDNA5106, total DNA (2 μg) extracted from 2 × 105 cells was digested with a single cut restriction enzyme, PvuII, and the restriction fragments were separated by 0.6% agarose gel electrophoresis. After blotting onto a Nytran membrane, the DNA fragments were hybridized with [32P]dATP-labeled HeLa mtDNA. The contents of HeLa mtDNA in cybrid clones were determined from the HeLa mtDNA-specific polymorphisms of HaeIII restriction patterns, which can distinguish HeLa mtDNA from other human mtDNAs (19). The total DNA (2 μg) extracted from 2 × 105 cells was digested with HaeIII, and the fragments were separated by 2% agarose gel electrophoresis. After blotting onto a Nytran membrane, the DNA fragments were hybridized with [32P]dATP-labeled polymerase chain reaction products amplified using two primers (15,756–15,775 and 15,916–15,897). The 1,284-base product was used as a probe.

RESULTS

Isolation of ρ0 Nuclear Hybrid Clones Containing Predominantly ΔmtDNA5106—to determine whether there is intermitochondrial cooperation between distinct organelles derived from different cells, we first isolated ρ0 nuclear hybrid cells, which show no mitochondrial translation or oxidative phosphorylation activities due to predominance of patient-derived ΔmtDNA5106 with a deletion of five tRNA genes (14). Previously, cybrid clones were isolated by fusion of ρ0 HeLa cells with encapsulated fibroblasts from a chronic progressive external ophthalmoplegia patient with ΔmtDNA5106 in selective medium without pyruvate and uridine to remove ρ0 HeLa cells (14). In this selective medium, however, ρ0 cybrids containing ΔmtDNA5106 predominantly must also be removed because of their overall respiratory deficiency. Therefore, we tried to isolate ρ0 nuclear hybrid clones by fusing ρ0 HeLa cells with the fibroblasts followed by HAT selection, which does not have any selective pressure upon growth of ρ0 cells but can remove ρ0 HeLa cells.

Southern blot analysis showed that of the 12 hybrid clones isolated, one (H5) had ΔmtDNA5106 predominantly, four (H2, H8, H10, and H12) had both ΔmtDNA5106 and wild type mtDNA, and seven (H1, H3, H4, H6, H7, H9, and H11) had only wild type mtDNA (Table I). Using three clones, H9 without ΔmtDNA5106, H8 with 41% ΔmtDNA5106, and H5 with only ΔmtDNA5106, we analyzed the influence of the amount of ΔmtDNA5106 on the activity of mitochondrial translation using [35S]methionine labeling.

No mitochondrial translation was observed in H5 cells with only ΔmtDNA5106, suggesting that they are ρ0 cells (Fig. 1). On the other hand, quantitative estimation of mtDNA translation products showed that the overall translation capacity of H8 cells with 59% ΔmtDNA5106 was comparable to that of H9 cells without ΔmtDNA5106. Moreover, because of the 5,196-base pair long deletion with a breakpoint between 8,563 and 13,788, ΔmtDNA5106 newly acquired a unique fusion gene that encoded two fusion proteins, ATP8/ND5 (FA) and ATP6/ND5 (FB) (14), which were exclusively translated in H8 cells (Fig. 1). These features indicate that polypeptides encoded by ΔmtDNA5106 were translated using the tRNAs supplied from the endogenous wild type mtDNA of the patient, suggesting intermitochondrial cooperation.
Cooperation of Human mtDNA from Different Cell Types

Analysis of Intermitochondrial Cooperation between Distinct Organelles Derived from Different Cells—For determination of whether intermitochondrial cooperation occurs even between distinct organelles from different cell types, the chance of interaction between \( \rho^- \) mitochondria from H5 cells and \( \rho^+ \) mitochondria from HeLa cells was created by fusion of enucleated CAP\(^r\) HeLa cells with H5 cells. All cybrid clones isolated in selective HAT medium without pyruvate and uridine contained both \( \Delta m \text{mtDNA}^{5196} \) and HeLa mtDNA (Table II).

In these cybrids, the fusion proteins (FA and FB) can be used as specific markers of the translation of \( \Delta m \text{mtDNA}^{5196} \). On the other hand, since ND3 of HeLa cells, ND3\(^r\), migrated slightly faster in SDS-polyacrylamide gel than the corresponding ND3 of other human cells (13, 17, 22) because of a single amino acid exchange (Asp to Asn) (23), ND3\(^r\) could be used as a specific marker of the translation of HeLa mtDNA.

First, as a negative control of intermitochondrial interaction, we examined mitochondrial translation in a simple mixture of H5 and HeLa cells with 53\% \( \Delta m \text{mtDNA}^{5196} \). In this case, as \( \rho^- \) mitochondria in H5 cells and \( \rho^+ \) mitochondria in HeLa cells were completely separated and confined to each type of cell, cooperation of the \( \rho^- \) and \( \rho^+ \) mitochondria could not occur, and thus their translation should be limited to mitochondria in HeLa cells. As expected, the results showed that ND3\(^r\) but not fusion proteins was translated and that the total amount of \[^{[35]}\text{S} \text{methionine} \] incorporation corresponded exactly to the amount of HeLa mtDNA (Figs. 2 and 3).

Then mitochondrial translation activity was examined by \[^{[35]}\text{S} \text{methionine} \] labeling using a cybrid clone CH5-2 with 53\% \( \Delta m \text{mtDNA}^{5196} \) (Fig. 2A), in which the \( \rho^- \) and \( \rho^+ \) mitochondria coexisted by cell fusion. Figs. 2B and 3 show that the total amount of \[^{[35]}\text{S} \text{methionine} \] incorporation into mtDNA-encoded polypeptides in CH5-2 cells was comparable to that in HeLa cells. Furthermore, the fusion proteins FA and FB exclusively encoded on \( \Delta m \text{mtDNA}^{5196} \) were observed in the CH5-2 cells (Fig. 2B). Thus, in contrast to the case in the simple mixture, \( \Delta m \text{mtDNA}^{5196} \)-encoded polypeptides were translated using the tRNAs supplied from the imported HeLa mitochondria in CH5-2 cells, showing unambiguously the occurrence of intermitochondrial cooperation between distinct organelles of the \( \rho^- \) and \( \rho^+ \) mitochondria derived from H5 cells and HeLa cells, respectively.

Effects of CAP on the Mitochondrial Translation Activity in CH5-2 Cells—By use of CH5-2 cells, the occurrence of intermitochondrial cooperation could be confirmed by examining the effects of CAP on the translations of both CAP\(^r\) \( \Delta m \text{mtDNA}^{5196} \) and CAP\(^r\) HeLa mtDNA. If intermitochondrial cooperation occurred, the fusion proteins encoded by CAP\(^r\) \( \Delta m \text{mtDNA}^{5196} \) should be translated by the use of CAP\(^r\) 16 S rRNA transcribed from CAP\(^r\) HeLa mtDNA.

First, we carried out a negative control experiment by testing the effect of CAP on translation of mitochondria in a simple mixture of 53\% H5 and 47\% HeLa cells, in which no intermitochondrial interaction occurred. In the presence of CAP, translation in CAP\(^r\) HeLa mitochondria was slightly inhibited (Fig. 2B). The amount of mitochondrial translation in the mixture of H5 and HeLa cells corresponded exactly to that expected from the proportion of HeLa cells in the mixture (Fig. 2B), and no fusion proteins were translated (Fig. 2B), reflecting the absence of cooperation.

If there is no interaction between mitochondria from different cells, the mitochondrial translation profiles in CH5-2 cells with 47\% HeLa mtDNA must correspond to those in the simple parental cell mixture with 47\% HeLa mtDNA. However, the translation of the fusion proteins was observed in CH5-2 cells even in the presence of CAP (Fig. 2B). Furthermore, the amount of translation of ND3\(^r\) exclusively encoded by CAP\(^r\) HeLa mtDNA, as well as those of CAP\(^r\) \( \Delta m \text{mtDNA}^{5196} \)-specific fusion proteins, was reduced simultaneously to much lower levels than those in the simple parental cell mixture with 47\% HeLa mtDNA (Figs. 2B and 3).

The translation of a small amount of fusion proteins in the presence of CAP represents the occurrence of intermitochondrial cooperation, since CAP\(^r\) 16 S rRNA transcribed from the imported CAP\(^r\) HeLa mtDNA should be necessary for translation of the fusion protein in the presence of CAP, suggesting rRNA complementation. The progressive inhibition of the translation of CAP\(^r\) HeLa mtDNA-encoded polypeptides including ND3\(^r\) in CH5-2 cells by CAP might be explained by
supposing that the CAPs allele is dominant over CAPr in mammalian cells. This phenomenon could not occur when mitochondria with CAPs mtDNA5196 and with CAPr HeLa mtDNA did not cooperate with each other as in the simple mixture of mitochondria prepared from the cells (Figs. 2 and 3).

Analysis of COX Activity in Individual Mitochondria in Single CH5-2 Cells Using COX Electron Microscopy—The observed complementation of mitochondrial tRNA and rRNA excludes the possibility that ΔmtDNA5196 and HeLa mtDNA remained separated in respective mitochondria. Accordingly, the ΔmtDNA5196 and HeLa mtDNA in cells of the cybrid clone CH5-2 should mix homogeneously throughout the mitochondria. This prediction can be tested by electron microscopic analysis of COX activity in mitochondria using CH5-2 cells with 53% ΔmtDNA5196, since this technique clearly identifies COX activity of individual mitochondria in single cells.

No individual mitochondria with predominantly ΔmtDNA5196 in H5 cells showed COX activity, whereas most mitochondria in HeLa cells showed clear COX activity (Fig. 4). Therefore, the COX activity of individual mitochondria could be used as a probe to identify mitochondria with ΔmtDNA5196 and with HeLa mtDNA. If they do not interact with each other and remain unfused in CH5-2 cells after the import of HeLa mitochondria, they can be distinguished unambiguously by COX electron microscopy, and the ratio of COX-negative to COX-positive mitochondria in a cell should be proportional to the ratio of ΔmtDNA5196 to HeLa mtDNA (53:47).

COX activity of individual mitochondria in single CH5-2 cells was analyzed immediately after clonal isolation to minimize intercellular variations in the ΔmtDNA5196 contents of different cells in the clone. Fig. 4 shows that most mitochondria in CH5-2 cells were COX-positive, suggesting that mitochondria from H5 cells and from HeLa cells did not remain segregated, but interacted with each other in the CH5-2 cells. This uniform distribution of COX-positive mitochondria within single CH5-2 cells containing 53% ΔmtDNA5196 can be explained by rapid diffusion of ΔmtDNA5196, HeLa mtDNA, and their products throughout the mitochondria.

Accordingly, all observations in this study support the idea that intermitochondrial interaction occurs even between distinct organelles with ΔmtDNA5196 from a patient with mitochondrial disease and wild type mtDNA of HeLa cells.

DISCUSSION

In this study, to provide direct evidence for the presence of interaction between distinct mitochondria originating in different cells, we created using cell fusion technique the chance of their interaction by isolating CH5-2 cells containing both ρ−.
mitochondria with ΔmtDNA5196 derived from a patient with a mitochondrial disease and ρ− mitochondria with CAPr mtDNA derived from HeLa cells. We demonstrated intermitochondrial interaction unambiguously in three different ways.

First, we demonstrated complementation of mitochondrial tRNAs between mitochondria originating from different cells. Polypeptides encoded by ΔmtDNA5196, which has a deletion of five tRNA genes, were translated when exogenous normal mitochondria imported from HeLa cells coexisted within CH5-2 cells (Fig. 2). This suggests the mitochondrial fusion and subsequent diffusion of the tRNAs transcribed from the imported HeLa mitochondria into the host ρ− mitochondria with ΔmtDNA5196.

Second, we observed complementation of mitochondrial rRNAs. Even in the presence of CAP, fusion proteins exclusively encoded by CAPr ΔmtDNA5196 were translated in the CH5-2 cells. Furthermore, the mitochondrial translations of all polypeptides including the CAPr HeLa mtDNA-specific polypeptide ND3 in CH5-2 cells were reduced simultaneously to much lower levels than those in a simple mixture of the parental cells with an equivalent amount of HeLa mtDNA (47%) (Figs. 2B and 3). These results could be explained by supposing that intermitochondrial interaction, i.e. complementation of mitochondrial 16 S rRNAs, occurs between mitochondria with CAPr and CAPr mtDNA derived from different cells and that the CAPr allele is dominant over the CAPr allele.

The third line of evidence for intermitochondrial cooperation was obtained by COX electron microscopy. The observed mitochondrial tRNA and rRNA complementation could not occur if ΔmtDNA5196 and HeLa mtDNA remained confined to their respective mitochondria after the introduction of HeLa mitochondria. This interpretation predicts that coexisting ΔmtDNA5196 and HeLa mtDNA in single CH5-2 cells should mix homogeneously throughout the mitochondria. This prediction was examined by electron microscopic analysis of COX activity in individual mitochondria within CH5-2 cells containing 53% ΔmtDNA5196, and the results demonstrated that all of the individual mitochondria showed COX activity (Fig. 4).

These observations consistently suggest the coexistence and cooperation of ΔmtDNA5196 and HeLa mtDNA within the same organelles, supporting the occurrence of interaction between distinct organelles. Such intermitochondrial interaction could not occur if mitochondria did not fuse and exchange mtDNA and its transcripts between fused mitochondria. Therefore, the presence of intermitochondrial interaction provides evidence not only for the mitochondrial fusion but also for subsequent mixing of their contents and supports the idea we reported previously that mitochondria and mitochondrial genome function as a single dynamic cellular unit in living human cells (16). Moreover, this concept will help in understanding the relationship between mtDNA mutations and expression of human mitochondrial diseases and aging. For example, although age-associated accumulations of various somatic deletion mutant mtDNAs and age-associated mitochondrial dysfunction were observed in mammalian tissues (3, 18, 24), our results show that mtDNA mutations can complement each other, and thus they do not have a serious influence on age-associated mitochondrial dysfunction.

With respect to the dominant behavior of the CAPr allele in CH5-2 cells, however, it has been proposed that the CAPr allele could not be dominant for the following reasons (13, 17). First, it is reported that in bacteria CAPr ribosomes were able to move along mRNA without forming polypeptides in the presence of CAP (25). However, this simple observation does not exclude the possibility of the dominant behavior of the CAPr allele. The second reason is that both CAPr and CAPr mtDNA-encoded polypeptides can be translated even in the presence of CAP (13, 17). However, the amount of the translation of CAPr mtDNA-encoded polypeptide ND3 in the presence of CAP was reduced progressively to a much lower level than that expected from the amount of CAPr mtDNA in the cells (Fig. 2), suggesting the presence of intermitochondrial cooperation in which the CAPr allele behaves dominantly.

If the CAPr phenotype is dominant, the question arises of why a small amount of CAPr mtDNA-encoded polypeptides, fusion proteins, was translated in the presence of CAP, as observed in Fig. 2B. This question can be explained by supposing that CAPr rRNA and CAPr rRNA are distributed randomly throughout the mitochondria of cells by intermitochondrial interaction. In this case, most polysomes in CH5-2 cells would have more than one CAPr rRNA, and these translation would be inhibited by CAP. On the other hand, a small proportion of polysomes happened to contain no CAPr rRNA, and in these both CAPr and CAPr mtDNA-encoded polypeptides were translated proportionally in the presence of CAP. Accordingly, all of the results in this study are consistent with the occurrence of intermitochondrial cooperation between organelles derived from different cells.

Recently, the absence of intermitochondrial interaction on mixing mitochondria derived from different cells by the cell fusion techniques was reported (17); interactions of mutant and wild type mtDNA molecules occur only when they have been coexisting in the mitochondria from the time of the mutation event, but not when they originate in distinct organelles in different cells. However, these possibilities are unlikely, since originally coexisting mutant and wild type mtDNA within a mitochondrion would eventually segregate stochastically during culture of the cells, if exchange of mtDNA between mitochondria did not occur (26) as in the case between cells. It is well known that coexisting wild type and mutant mtDNA (27–29) and coexisting mtDNAs of different species (30) in a cell segregate in a stochastic way. Furthermore, this study provided direct evidence for the presence of cooperation even between organelles originating from different cells. However, it is still possible that cooperation does not occur between respiratory-deficient mitochondria (17), possibly because of a lack of a sufficient energy supply for mitochondrial fusion and mixing of their contents. We are now investigating this possibility by isolating cells with syn− mitochondria, which are respiration-deficient due to point mutations of the trNALeu(UUR) or trNAHis gene (15), and then creating a chance for interaction between syn− mitochondria or syn− and ρ− mitochondria.
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