Transcomplementation between Different Types of Respiration-deficient Mitochondria with Different Pathogenic Mutant Mitochondrial DNAs*

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Two cell lines were used for determination of whether interaction occurred between different types of respiration-deficient mitochondria. One was a respiration-deficient r− cell line having mutant mitochondrial DNA (mtDNA) with a 5,196-base pair deletion including five tRNA genes (tRNA(Gly), tRNA(Glu), tRNA(Ash), tRNA(Leu(CUN)), tRNA(His)), ΔmtDNA5196, causing Kearns-Sayre syndrome. The other was a respiration-deficient syn− cell line having mutant mtDNA with an A to G substitution at 4,269 in the tRNAIle gene, mtDNA4269, causing fatal cardiomyopathy. The occurrence of mitochondrial interaction was examined by determining whether cybrids constructed by fusion of enucleated r− cells with syn− cells became respiration competent by exchanging their tRNAs. No cybrids were isolated in selection medium, where only respiration-competent cells could survive, suggesting that no interaction occurred, or that it occurred so slowly that sufficient recovery of mitochondrial respiratory function was not attained by the time of selection. The latter possibility was confirmed by the observations that heteroplasmic cybrids with both mutant mtDNA4269 and ΔmtDNA5196 isolated without selection showed restored mitochondrial respiratory activity. This demonstration of transcomplementation between different respiration-deficient mitochondria will help in understanding the relationship between somatic mutant mtDNAs and the roles of such mutations in aging processes.

In yeast and plant cells, the occurrence of mitochondrial interaction has been suggested by the presence of recombinant mitochondrial DNA (mtDNA)1 molecules from two parental cells (1, 2). In mammalian species, however, it has been shown that the mtDNA population is very homoplasmic in a given individual (3, 4) probably because of inhibition of the coexistence of mtDNAs from both parents by their strictly maternal inheritance (5, 6). For example, sperm-derived mtDNA is selectively and completely eliminated from fertilized mouse eggs before the late pronuclear stage, suggesting that the mtDNA population of all individuals is derived exclusively from the eggs (5, 6). Therefore, it is difficult to prove occurrence of mitochondrial interaction by the presence of recombinant mtDNA molecules in mammalian species because of the difficulty in identification of recombinant molecules between mtDNAs that have very similar sequences, even if mtDNA recombination occurs.

However, when cell fusion techniques are applied to mammalian somatic cells, mtDNAs with significant sequence divergences derived from different individuals of the same species or even from those of different species can be coexisted within the same somatic cells, which allow us to examine the occurrence of mtDNA recombination and mitochondrial interaction. Using somatic cell fusion techniques, the occurrence of interaction of the mammalian mitochondrial genetic system was proved in heteroplasmic cells by translational complementation of mitochondrial rRNA between mitochondria with chloramphenicol-sensitive and -resistant mtDNAs (7, 8), and by translational complementation or competition of mitochondrial tRNAs between mitochondria with wild-type and pathogenic deletion mutant mtDNAs (8, 9), although extensive recombination was not observed in heteroplasmic cells with rat mtDNAs or rat and mouse mtDNAs (10). Subsequently, we found rapid penetration of HeLa mtDNA and/or its products into mitochondria of mtDNA-less (ρ0) HeLa cells in cybrids isolated by the fusion of enucleated HeLa cells with ρ0 HeLa cells (11).

A recent contradictory report proposed that there is no mitochondrial interaction between distinct organelles derived from different cells, because the coexistence of mitochondria containing different pathogenic mutant mtDNAs deriving from different patients in single cells did not restore reduced mitochondrial respiratory function (12). On the other hand, we found convincing evidence for the presence of interaction between mitochondria originating from different cell lines (8). We constructed cybrids by introducing chloramphenicol-resistant HeLa mitochondria into cells with respiration-deficient ρ− mitochondria containing deletion mutant mtDNA (ΔmtDNA5196) with a 5,196-base pair deletion including five tRNA genes and seven structural genes derived from a patient with Kearns-Sayre syndrome (9) and found that fusion genes newly formed around the deletion break point in ΔmtDNA5196 could be translated even in the presence of chloramphenicol with the help of both chloramphenicol-resistant rRNA and five kinds of tRNAs lacking in ρ− mitochondria transcribed from HeLa mtDNA. The apparent discrepancy on the presence or absence of mitochondrial interaction might be explained by supposing that there is no interaction when different types of respiration-deficient mitochondria coexist in the same cells, probably

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1 The abbreviations used are: mtDNA, mitochondrial DNA; bp, base pair; kbp, kilobase pair.
because of lack of sufficient energy supply from respiration-deficient mitochondria required for mitochondrial fusion, subsequent mixing of their contents, and peptide synthesis.

In this study, we examined this possibility by isolating cells with syn− mitochondria, which are respiration-deficient because of pathogenic mutant mtDNA with a point mutation in the trNAle gene at 4,269 derived from a patient with fatal cardiomyopathy (13, 14) and then creating a chance for interaction between syn− mitochondria and ρ− mitochondria. In this combination, because fusion peptides were exclusively encoded by ΔmtDNA5196 whereas ATP8, ATP6, COIII ND3, ND4L, ND4, and ND5 were exclusively encoded by mtDNA4269, the interaction could be unambiguously proved by identification of these peptides. The results suggested the occurrence of mitochondrial interaction even between distinct respiration-deficient organelles with different types of pathogenic mutant mtDNAs derived from patients with different mitochondrial diseases.

**EXPERIMENTAL PROCEDURES**

*Cells and Cell Culture—*Respiration-deficient ρ− H5 cells (8) contain only mutant ΔmtDNA5196 with a large-scale deletion from nucleotide positions 8,563 to 13,758 including seven structural genes and five tRNA genes, which was derived from a patient with Kearns-Sayre syndrome (9). Respiration-deficient syn− CM114a and normal CM1-9 cells were isolated by the fusion of ρ− HeLa cells with enucleated fibroblasts from a patient with fatal cardiomyopathy (13). Therefore, the nuclear genomes of CM114a and CM1-9 clones were from HeLa cells, and mtDNAs were from the patient. CM114a cells contain only mutant mtDNA with an A to G substitution at 4,269 in trNAle gene, mtDNA4269 whereas CM1-9 cells possessed predominantly wild-type mtDNA from the patient. As these respiration-deficient cell lines are auxotrophic for uridine and pyruvate, cells were grown in medium (RPMI 1640 + pyruvate (0.1 mg/ml) + uridine (50 μg/ml) + 10% fetal bovine serum).

*Intercellular Transfer of mtDNA—*Intercellular transfer of mtDNA was carried out as described previously (8) by fusion of enucleated H5 cells with CM114a cells in the presence of polyethylene glycol 1500 (Boehringer Mannheim).

*Southern Blot and Polymerase Chain Reaction Analyses of mtDNA—*For identification and determination of the content of ΔmtDNA5196, total DNA (2 μg) extracted from 2 × 10^6 cells was digested with XhoI, and the fragments were separated by 1% agarose gel electrophoresis. After blotting onto a NYTRAN membrane, the DNA fragments were hybridized with [α-32P]dATP-labeled HeLa mtDNA. The radioactivities of the fragments were measured with a BAS2000 instrument (Fuji Photo Film, Tokyo). The mutant mtDNA4269 was identified by the mismatch polymerase chain reaction method as described previously (13). The products digested with SspI were separated by 4% agarose X gel electrophoresis in the presence of ethidium bromide.

*Analysis of Mitochondrial Translation Activity and Cytochrome c Oxidase Activity—*Analysis of mitochondrial translation products labeled with [35S]methionine was carried out as described previously (8). Cytochrome c oxidase activity was measured as the rate of cyanide-sensitive oxidation of reduced cytochrome c as described before (15).

**RESULTS AND DISCUSSION**

We examined mitochondrial interaction by isolation of cybrids using the nutritional requirements of two different types of respiration-deficient cell lines as parental cells. One was ρ− H5 cells (8), which are respiration-deficient because of a large-scale deletion mutant ΔmtDNA5196 derived from a patient with Kearns-Sayre syndrome (9). The other was syn− CM114a cells, which are respiration-deficient because of a mutant ΔmtDNA4269 (13) derived from a patient with fatal cardiomyopathy. The CM114a cells without mitochondrial translation activity (Fig. 1) were isolated by repeated recloning of CM114a cells that showed slight translation activity (14). These two lines are auxotrophic for pyruvate and uridine because of the complete absence of oxidative phosphorylation activity in their mitochondria (8, 14).

If mitochondrial interaction occurs between the respiration-deficient ρ− and syn− mitochondria, mitochondrial respiratory function should be restored by their coexistence in a cell, because the missing five tRNAs in the ρ− mitochondria and the missing wild-type trNAle in the syn− mitochondria should be supplemented by the syn− and ρ− mitochondria, respectively. Because syn− CM114a cells are resistant to 6-thioguanine, the coexistence of syn− CM114a mitochondria and ρ− H5 mitochondria could be attained by the fusion of enucleated H5 cells with CM114a cells followed by selection in medium with 6-thioguanine but without pyruvate and uridine. In this nutrition-deficient selection medium, only cybrids, i.e. CM114a cells that had acquired respiration competence by introduction of exogenous ρ− H5 mitochondria, could survive. The results showed that no colonies grew in this selective medium, suggesting that the cybrids were not respiration competent, possibly because of the absence of mitochondrial interaction between syn− and ρ− mitochondria.

However, the failure to isolate cybrids by nutritional selection could not necessarily be direct evidence for the absence of interaction. For example, our results did not completely exclude the possibility that transcomplementation between syn− and ρ− mitochondria occurred very slowly because of the absence of an energy supply necessary for rapid interaction, and so sufficient restoration of mitochondrial translation by transcomplementation was not accomplished by the time of nutritional selection that exclude respiration deficient cells. For examination of this possibility, cybrids with both syn− and ρ− mitochondria must be isolated to study whether restoration of mitochondrial respiratory function could be attained in the heteroplasmic cybrids due to prolonged cultivation after fusion. However, we could not use effective selection to remove host CM114a cells from fusion mixtures of CM114a cells and enucleated H5 cells (Table I). As selection with 6-thioguanine was incomplete and could exclude only parental H5 cells, most of the colonies that grew in the selective medium would be either the host CM114a cells without ρ− mitochondria or cy-

![FIG. 1. Analyses of mtDNA genotypes and phenotypic expression of mitochondrial translation in cybrids. CM114a contained only mtDNA4269, whereas CM1-9 contained predominantly wild-type mtDNA. H5 contained only ΔmtDNA5196. Clones 0A4 and 8B3 are cybrid clones. A, identification of the amount of mtDNA4269 by SspI digestion of the polymerase chain reaction products. Wild type mtDNA and ΔmtDNA5196 gave a 153-bp fragment, whereas mtDNA4269 gave a 184-bp fragment because of the loss of an SspI site by an A to G substitution at 4,269 (13). B, identification of the amount of ΔmtDNA5196 by Southern blot analysis of XhoI digests. Wild type mtDNA and mtDNA4269 gave a 16-kbp fragment, whereas ΔmtDNA5196 gave an 11-kbp fragment (8). C, analysis of mitochondrial translation products by SDS-polyacrylamide gel electrophoresis. ND5, COI, ND4, Cyb, ND2, ND1, COI, COII, ATP6, ND6, ND3, ATP8, and ND4L are polypeptides assigned to mtDNA. Genes encoding ATP8, ATP6, COIII, ND3, ND4L, ND4, and ND5 are missing in ΔmtDNA5196, whereas fusion polypeptides (shown by an arrowhead) were exclusively encoded by ΔmtDNA5196.]
briggs, i.e. CM114a cells with imported \( r^2 \) mitochondria.

Therefore, on day 14–20 after fusion, we picked up 98 growing colonies randomly without using nutritional selection to exclude respiration-deficient cells and analyzed their mtDNA compositions to find cybrid clones. Most of these colonies did not contain even a slight amount of \( D^\text{mtDNA5196} \), and both parental mtDNAs were detectable only in 2 of 98 clones, suggesting that two heteroplasmic clones, named 0A4 and 8B3, were cybrids but that the others were parental CM114a cells. Cybrid clone 0A4 had 60% \( D^\text{mtDNA5196} \) imported from \( r^2 \)H5 cells, whereas cybrid clone 8B3 had 18% \( D^\text{mtDNA5196} \), the remaining mtDNA being host mtDNA4269 (Fig. 1 and Table I).

Thus, the coexistence of respiration-deficient synth2 and \( r^2 \) mitochondria was attained in these cybrid clones.

Then, by \([35S]\)methionine labeling we examined whether mitochondrial translation activity was restored in these cybrid clones. Fig. 1 shows that mitochondrial translation activity was observed in 0A4 cybrids with 60% \( D^\text{mtDNA5196} \) but not in 8B3 cybrids with 18% \( D^\text{mtDNA5196} \). The apparent discrepancy on the presence or absence of mitochondrial translation activity in cybrid clones 0A4 and 8B3 could be explained by supposing that the 8B3 clone was not a cybrid clone but was derived from parental CM114a cells contaminated with H5 cells. Such contamination could occur, if we had failed to isolate a single clone, and some H5 cells, which happen to survive in the presence of 6-thioguanine during the selection period (14–20 days), were included in the cell population of CM114a line, although we confirmed before fusion that 6-thioguanine killed all parental H5 cells during the 14-day cultivation period.

For examination of this possibility, five subclones were isolated from the 8B3 clone by recloning, and their mtDNA compositions were studied. As shown in Fig. 2, A, B, and C, all the subclones had both mutant mtDNA4269 and \( D^\text{mtDNA5196} \), suggesting that the 8B3 cells were a simple mixture of the parental CM114a and H5 cells, but were heteroplasmic cybrids with both \( D^\text{mtDNA5196} \) and \( r^2 \) mitochondria. However, during cultivation for about 30 passages for isolation and examination of subclones, the amount of \( D^\text{mtDNA5196} \) in all the subclones progressively increased to 40–55%. Considering that the amount of \( D^\text{mtDNA5196} \) in the original 8B3 clone was 18% (Fig. 1A and Table I), its increase was not because of random segregation but to a propagational advantage of \( D^\text{mtDNA5196} \) of smaller size than mutant mtDNA4269. These observations are consistent with our previous observations that \( D^\text{mtDNA5196} \) was propagated preferentially over wild-type mtDNA in following generations of cybrids (9). Then, we examined the activities of mitochondrial translation and respiratory enzymes in these

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**Table I**

| Cell line                  | Drug resistance          | \( \Delta \)mtDNA5196 content |
|----------------------------|--------------------------|-------------------------------|
| Parent cell line           |                          |                               |
| mtDNA donor line H5        | 6-thioguanine-sensitive  | 100                           |
| mtDNA recipient line CM114a| 6-thioguanine-resistant  | 0                             |
| Cybrid clone               |                          |                               |
| 0A4                        | 60                       |                               |
| 8B3                        | 18                       |                               |
| 8B3 subclones              |                          |                               |
| 8B3-1                      | 55                       |                               |
| 8B3-2                      | 48                       |                               |
| 8B3-3                      | 40                       |                               |
| 8B3-4                      | 54                       |                               |
| 8B3-5                      | 53                       |                               |

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**Fig. 2. Analyses of mtDNA genotypes and phenotypic expression of mitochondrial translation in subclones of 8B3.** Subclones 8B3-1, -2, -3, -4, and -5 were isolated by recloning the cybrid clone 8B3. A, identification of the amount of mtDNA4269 by \( SspI \) digestion of the polymerase chain reaction products. Wild type mtDNA and \( D^\text{mtDNA5196} \) gave a 153-bp fragment, whereas mtDNA4269 gave a 184-bp fragment. B, identification of the amount of \( \Delta \)mtDNA5196 by Southern blot analysis of \( XhoI \) digests. Wild type mtDNA and mtDNA4269 gave a 16-kbp fragment, whereas \( \Delta \)mtDNA5196 gave an 11-kbp fragment (8). C, analysis of mitochondrial translation products by SDS-polyacrylamide gel electrophoresis. The arrowhead shows fusion polypeptides exclusively encoded by \( D^\text{mtDNA5196} \).
subclones. All subclones showed mitochondrial translation and cytochrome c oxidase activities restored to 40–70% of those of cybrids repopulated with wild-type mtDNA (Figs. 2 and 3), suggesting the occurrence of interaction between syn⁻ and ρ⁻ mitochondria.

Moreover, these cybrid clones synthesized fusion peptides exclusively encoded by ΔmtDNA5196 and seven peptides ATP8, ATP6, COIII, ND3, ND4L, ND4, and ND5 missing in ΔmtDNA5196, although their amounts seem to be slightly lower than those of the other peptides (Figs. 1 and 2). As ΔmtDNA5196 had lost seven structural genes but gained new fusion genes because of 5,196-bp deletion with a break point between 8,563 and 13,758 (9), these results suggest the recoveries of gene expressions of both ΔmtDNA5196 and mtDNA4269 by their coexistence in the cybrids. Thus, these observations provided unambiguous evidence for the presence of complementation between different types of respiration-deficient mitochondria.

There seem to be two possible explanations for the apparent discrepancy between the findings that all subclones showed restored mitochondrial translation activity, whereas the original cybrid clone 8B3 did not (Figs. 1 and 2). One is that interaction did occur in clone 8B3, but that the amount of ΔmtDNA5196 (18%) was not enough to provide sufficient normal tRNAleu for restoring mitochondrial translation activity in syn⁻ mitochondria. The other is that interaction occurred, but that a long expression time was required to restore mitochondrial translation activity particularly between respiration-deficient mitochondria. Because it seems impossible to keep the amount of ΔmtDNA5196 at lower levels because of its propagational advantage over mtDNA of normal size, other mtDNA with a pathogenic point mutation, that is expected to be neutral with respect to propagational advantage, must be used to examine the above possibility.

The discrepancy with respect to the presence (this study) and absence (12) of interaction between respiration-deficient mitochondria and our inconsistent findings that cybrids were not isolated in selective medium, whereas heteroplasmic cybrids with syn⁻ and ρ⁻ mitochondria restored mitochondrial respiratory function might also be explained by supposing as follows: a long expression time is required to restore mitochondrial respiratory function in cybrids isolated by the fusion of syn⁻ cells with nucleated ρ⁻ cells, or the amount of exogenuously imported mtDNA required to restore respiration deficiency in host cells might be limited to about 40–60%, particularly when imported mtDNA is not wild-type but possesses a different type of pathogenic mutation. It is also possible that the different types of pathogenic mtDNA mutations used in the supplementation assay allows for a different conclusion. We are testing these possibilities by isolating cybrids with the same pathogenic mtDNA mutations as those used by Yoneda et al. (12).

In any case, this study showed the occurrence of interaction even between different types of respiration-deficient mitochondria and provided two important aspects of mitochondrial biogenesis. One was the individuality of each mitochondrion within a cell. Mammalian cells have been thought to contain thousands of independent mitochondria (16). However, our previous observations provided the totally different view that in living cells mitochondria function as a single dynamic cellular unit, indicating that they lose individuality (11). In this study, we again provided convincing evidence to support this idea by showing the presence of transcomplementation between respiration-deficient mitochondria originated from different cell types.

The other important aspect of mitochondrial biogenesis provided from our findings was with respect to the relationship between age-associated mitochondrial dysfunction and age-associated accumulation of somatic mutations in mtDNA. It is generally thought that somatic mutations are more likely to accumulate in mtDNA than in nuclear DNA, because mtDNA is a target of most carcinogens and mutagens and is continuously exposed to oxygen-free radicals produced in mitochondria (17–19). If there were no mitochondrial interaction, it would be much easier to induce reduction of mitochondrial respiratory function by progressive accumulation of somatic mtDNA mutations during aging, particularly in postmitotic oxidative tissues, such as brain and muscles (20, 21). On the other hand, if transcomplementation occurred continuously, mitochondrial respiration activity could be maintained without significant reduction, even when various kinds of somatic mutant mtDNAs were accumulated. In fact, we recently found that mtDNA in autopsied brain tissues from aged subjects could be rescued in ρ² HeLa cells by fusion of brain synaptosomal fractions with ρ² HeLa cells, and that this mtDNA transfer resulted in complete restoration of mitochondrial respiratory function, even though the brain tissues and their cybrids possessed mtDNAs with various pathogenic mutations, suggesting functional integrity of the brain mtDNA from aged subjects (22). These observations can be explained by occurrence of transcomplementation between mitochondria. Thus, even though somatic mutations are more likely to accumulate in mtDNA than in nuclear DNA, the occurrence of transcomplementation between mitochondria could provide stable respiratory function in cells during aging.

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