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Determination of Normal Ranges of Mitochondrial Respiratory Activities by mtDNA Transfer from 54 Human Subjects to mtDNA-less HeLa Cells for Identification of the Pathogenicities of Mutated mtDNAs

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To determine the pathogenicities of mutated mtDNAs in patients with respiration defects, the possible involvement of nuclear DNA mutations has to be excluded, since respiratory function is controlled by both nuclear DNA and mtDNA. This was achieved by showing that the mutated mtDNAs and respiration defects were co-transferred from patients to mtDNA-less human cells, and the resultant cybrid clones carrying mutated mtDNAs expressed respiration defects. To decide whether the cybrid clones expressed respiration defects, in this study the lowest limits of normal respiratory function were evaluated by transfer of mtDNAs from 54 normal subjects to mtDNA-less HeLa cells. The resultant cybrid clones showed that 71% respiratory function was the lowest limit of mtDNAs from normal subjects. On the other hand, cybrid clones carrying pathogenic mtDNAs from patients with mitochondrial diseases showed 0–64% respiratory function, suggesting that less than 71% respiratory function in cybrid clones should be a reliable indicator of whether the mutated mtDNAs of the patients were pathogenic.

Key words: mtDNA-less HeLa cells, mtDNA transfer, mitochondrial diseases, pathogenic mtDNA mutations, respiratory function.

Mitochondrial diseases expressing respiration defects in various tissues were shown to be closely associated with mutated mtDNAs (*1*, *2*). Although respiratory function is controlled by both nuclear DNA and mtDNA, the possible involvement of nuclear DNA mutations in the respiration defects was excluded by examination of cybrid clones that resulted from cytoplasmic transfer of heteroplasmic mtDNAs with and without mutations from patients to mtDNA-less human cell lines (*3*–*7*). In these studies, cytoplasmic transfer of respiration defects was observed exclusively in cybrid clones carrying mutated mtDNAs, but not in cybrid clones carrying wild-type mtDNAs from the same patients. This co-transfer of the mutated mtDNAs and respiration defects suggested that accumulation of these mutated mtDNAs without the nuclear DNA mutations is responsible for the expression of respiration defects. Moreover, we provided direct evidence that the respiration defects induced by mutated mtDNAs were sufficient for expression of disease phenotypes in mitomice generated by introduction of mutated mouse mtDNA into fertilized mouse eggs (*8*, *9*).

On the other hand, the presence of these pathogenic mtDNAs was not restricted to patients with mitochondrial diseases, but extended to patients with common age-associated disorders including diabetes, deafness and neurodegenerative diseases, and even to all aged normal subjects (*1*, *2*). Moreover, many other mutations in mtDNAs expressing much weaker pathogenicities were also observed in patients with these age-associated disorders (*1*, *2*). In the latter, however, there were two problems in the determination of pathogenicities. One was that the reductions of respiratory enzyme activities in cybrid clones with mutated mtDNAs were too slight for unambiguous determination of their pathogenicities (*10*–*12*). The other was that patients carrying the mutated mtDNAs with weak pathogenicities showed homoplasmy and did not possess wild-type mtDNAs, so that cybrid clones with mtDNAs from different subjects had to be used as normal controls (*10*–*12*). Since variations in respiratory enzyme activities can be expected even in cybrid clones with mtDNAs from normal subjects, it was difficult to determine whether such slight reduction in cybrid clones with mtDNAs from patients was within the normal range of variation.

These problems could be solved by determining the normal range of respiratory function in cybrid clones with imported mtDNAs from many normal subjects in

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Table 1. **Characterization of cybrid clones with exogenous mtDNAs from various individuals.**

Parents and cybrids	Fusion combination	Genotype
Parent cells		
Nuclear donors (mtDNA recipients)		
mtDNA-less HeLa cells (ref)		without mtDNA
mtDNA donors (tissues)		
HeLa cytoplasts (enucleated HeLa cell)		HeLa mtDNA
N1-54 (platelets from 54 normal subjects)		wild-types
P4269 (enucleated fibroblasts from a CM patient)		A4269G in $tRNA^{Ile}$
P3243 (enucleated fibroblasts from a MELAS patient)		A3243G in $tRNA^{Leu(UUR)}$
P3394 (platelets from a CM patient)		T3394C in *ND1*
P14577 (platelets from a DM patient)		T14577C in *ND6*
P-DHCM (platelets from a DHCM patient)		mutations not assigned
Cybrid clones[a]		
Group 1 cybrid clones		
CyHeLa-1	mtDNA-less HeLa cells × HeLa cytoplasts	HeLa mtDNA
-2		
-3		
Group 2 cybrid clones with mtDNA from a normal subject		
CyN54-1	mtDNA-less HeLa cells × N54	wild-type
CyN54-2	mtDNA-less HeLa cells × N54	wild-type
Group 3 cybrid clones with mtDNAs from three normal brothers		
CyN51	mtDNA-less HeLa cells × N51	wild-type
CyN52	mtDNA-less HeLa cells × N52	wild-type
CyN53	mtDNA-less HeLa cells × N53	wild-type
Cybrid clones with mtDNAs from normal subjects		
CyN1–N50	mtDNA-less HeLa cells × N1–N50	wild-types
Cybrid clones with mutated mtDNAs from patients		
CyP4269(9)	mtDNA-less HeLa cells × P4269	A4269G in $tRNA^{Ile}$
CyP3243(9)	mtDNA-less HeLa cells × P3243	A3243G in $tRNA^{Leu(UUR)}$
CyP3394(10)	mtDNA-less HeLa cells × P3394	T3394C in *ND1*
CyP14577(11)	mtDNA-less HeLa cells × P14577	T14577C in *ND6*
CyP-DHCM	mtDNA-less HeLa cells × P-DHCM	mutations not assigned

[a]Group 1 consisted of CyHeLa cybrid clones with exogenously-introduced HeLa mtDNA. Group 2 consisted of CyN54-1 and CyN54-2 cybrid clones with mtDNA from the same individual in the normal population, but blood collection and fusion experiments were carried out at different times. Group 3 consisted of CyN51, N52, and N53 cybrid clones with mtDNAs from three brothers with the same mother in the normal population. In this case, although mtDNAs were derived from three different individuals, all three cybrid clones share the same mtDNA as their mother.

the general population. However, no precise data is available on the normal limit of reduced respiratory function that might be used to determine whether mutated mtDNAs in patients possess pathogenic or polymorphic mutations.

We addressed this issue by introducing platelet mtDNAs from 54 normal subjects into mtDNA-less HeLa cells. The resultant cybrid clones with various wild-type mtDNAs and negative control cybrid clones with mutated mtDNAs from patients with mitochondrial diseases were used to determine the normal range of variation of mitochondrial respiratory function.

MATERIALS AND METHODS

Cells and Cell Culture—Parental mtDNA-less HeLa cells and their cybrid clones with mtDNAs from different subjects (Table 1) were used. The mtDNA-less HeLa cells (*3*) and their cybrid clones were grown in normal medium: RPMI1640 (Nissui Seiyaku, Tokyo) containing 10% fetal calf serum, 50 mg/ml uridine and 0.1 mg/ml pyruvate. Cybrid clones CyN1–N54 with mtDNAs from 54 subjects in the normal population were used for determination of normal ranges of respiratory function. Cybrid clones CyP3243 and CyP4269 showing complete respiration defects (*9*) were used as pathogenic controls. Cybrid clones CyP3394 with a T3394C mutation (*12*) and CyP14577 with a T14577C mutation (*11*), both showing low respiratory enzyme activities, were also used as pathogenic controls. CyP3243 and CyP4269 cybrid clones carried mtDNAs with an A3243G mutation in the $tRNA^{Leu(UUR)}$ gene and with an A4269G mutation in the $tRNA^{Ile}$ gene from a patient with mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) and a patient with cardiomyopathy (CM), respectively (*9*). Cybrid clones CyP3394 and CyP14577 carried mtDNAs with a T3394C and a T14577C mutation in the *ND1* gene and *ND6* gene from a patient with cardiomyopathy (*10*) and type II diabetes mellitus (*11*), respectively.

Introduction of Platelet mtDNA into mtDNA-less HeLa Cells—Fifty-four subjects including 51 males and 3 females (18 to 75 years; average 31.3 ± 17.7 years) were used as normal subjects. None showed any clinical symp-

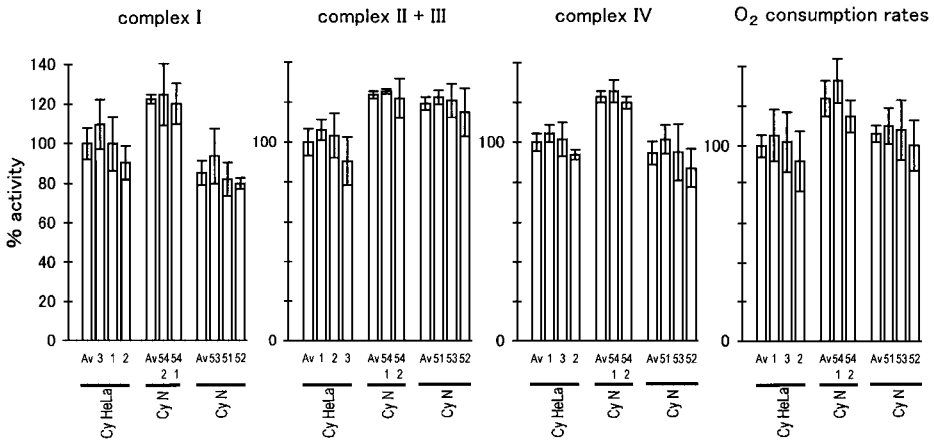

Fig. 1. **Activities of respiratory enzyme complexes and O_2 consumption rates in cybrid clones sharing the same nuclear and the same mtDNA background.** Av, average values; CyHeLa-1, -2, -3, group 1 cybrid clones with mtDNA from HeLa cells; CyN54-1, CyN54-2, group 2 cybrid clones with mtDNA from the same individual; CyN51, N52, N53, group 3 cybrid clones with mtDNAs from three brothers with the same mother. Cybrid clones are lined up according to the values. Average values of group 1 cybrid clones with mtDNA from HeLa cells (CyHeLa-1, -2, -3) were taken as 100%. Variations of complexes I, II+III, and IV activities and O_2 consumption rates were 90–108, 91–106, 94–105, and 92–105% in the group 1, 120–125, 122–126, 120–125, and 115–133% in the group 2, and 80–94, 115–123, 87–101, and 101–111% in the group 3.

toms suggestive of mitochondrial diseases. Blood samples provided with informed consent by the 54 subjects and a patient with the sporadic dilated phase of hypertrophic cardiomyopathy (DHCM) were used as sources of mtDNA. Transfer of mtDNA to mtDNA-less HeLa cells by cell fusion techniques and isolation of resultant cybrid clones were carried out as described previously (*14*). Briefly, platelets were fused with mtDNA-less HeLa cells in the presence of 50% (w/v) polyethylene glycol 1500 (Boehringer Mannheim, Germany). The fusion mixture was cultivated in selection medium RPMI1640 without pyruvate and uridine. On day 10–14 after fusion, the cybrid clones growing in the medium were isolated clonally by the cylinder method.

Measurements of Respiratory Enzyme Activities and O2 Consumption Rates—To avoid the effects of cultivation time on respiration phenotypes, we used cybrid clones cultivated for 2–4 months after the fusion. Cybrid clones in log-phase growth were harvested, and complex I, complex II+III, and complex IV activities were measured as described before (*11*, *13*). The rate of oxygen consumption was measured by trypsinizing cells, incubating the suspension in phosphate-buffered saline, and recording oxygen consumption in a polarographic cell (1.0 ml) at 37°C with a Clark-type oxygen electrode (Yellow Springs Instruments, OH, USA) (*11*, *13*). The respiratory enzyme activities were standardized by the weight of mitochondrial proteins, and the O_2 consumption was standardized by the number of cells. Based on the results of respiratory enzyme activities, we also calculated summed activity of complex I, complex II+III and complex IV, which were normalized by summed activity of CyHeLa cybrid clones. Average values of CyHeLa cybrid clones with HeLa mtDNA were used as 100%. Statistical analysis was performed with Fisher's exact propability test, and a P value below 0.05 was considered statistically significant.

RESULTS

mtDNAs were transferred from normal human subjects or from patients with mitochondrial diseases to mtDNA-less HeLa cells, and 3 cybrid clones were isolated from each mtDNA donor. We isolated 162 cybrid clones carrying imported mtDNAs from 54 different subjects (CyN1–CyN54), and 12 cybrid clones carrying imported mtDNAs from four patients with mitochondrial diseases as pathogenic controls expressing respiration defects (CyP) (Table 1). To deduce overall respiratory function of these cybrid clones, we examined the activities of respiratory complexes I, II+III, IV, and O_2 consumption rates, which may be affected by mutations in mtDNAs.

To test the reliability of our procedures for estimating respiratory function, we isolated three groups of cybrid clones that shared the same mtDNA as well as the same HeLa nuclear background (Table 1), and thus theoretically should show the same respiratory function. The first group consisted of CyHeLa cybrid clones with exogenously-introduced HeLa mtDNA. This group was clonal variations established to examine in respiratory function. The second group consisted of CyN54–1 and CyN54–2 cybrid clones carrying mtDNA from the same individual, but collection of blood and mtDNA transfer to mtDNA-less HeLa cells were carried out at different times. The third group consisted of CyN51, CyN52, and CyN53 cybrid clones carrying mtDNAs from three brothers with the same mother. In this case, although mtDNAs were derived from three different individuals, all cybrid clones shared the same mtDNA as their mother. The latter two groups were established to examine experimental variations.

The results on the first group of cybrid clones with HeLa mtDNA showed that clonal variations in activities of respiratory complexes and O_2 consumption rates were extremely small and limited to 90–110% of normal (Fig. 1). The average values in the first group were taken as 100% in this and following experiments (Figs. 1 and 2). The results on the second and third groups also showed small experimental variations in activities of respiratory complexes and O_2 consumption rates (Fig. 1).

To obtain the normal ranges of respiratory function of cybrid clones with mtDNAs from normal subjects, we isolated 3 cybrid clones from each subject, and 162 cybrid clones in total, by mtDNA transfer from 54 normal sub-

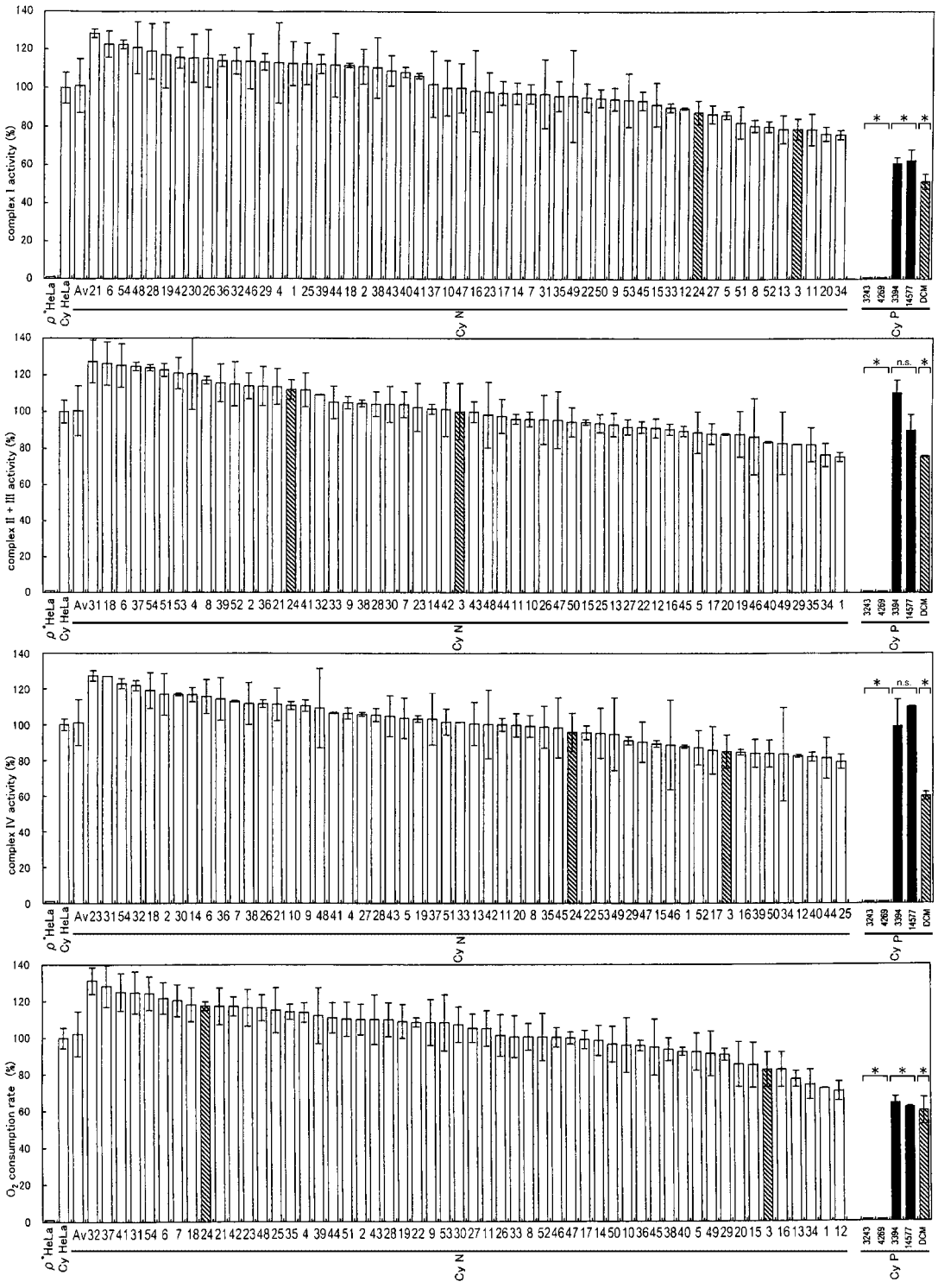

Fig. 2. **Activities of respiratory enzyme complexes and O_2 consumption rates in cybrid clones with mtDNAs from normal subjects and from patients with mitochondrial diseases.** Average values of group 1 cybrid clones with mtDNA from HeLa cells (CyHeLa-1, -2, -3) were taken as 100% (open bars); Av, average values of CyN1-N54 (open bars); CyN1–N54, cybrid clones with mtDNAs from 54 subjects in the normal population. All CyN1–N54 except for CyN3 and CyN24 are shown by open bars, while CyN3 and CyN24 with 16189 mutated mtDNAs are shown by hatched bars. CyN1–N54 cybrid clones are lined up in order of higher values. CyP3243, CyP4269, CyP3394, CyP14577, cybrid clones with mtDNAs from patients with mitochondrial diseases (solid bars); CyP-DHCM, cybrid clones with T16189C mutated mtDNA from a patient with DHCM (hatched bars); ρ^0 HeLa, mtDNA-less HeLa cells used as mtDNA recipients. Variations of complex I, complex II+III, and complex IV enzyme activities and O_2 consumption rates in 54 cybrid clones were 76–128, 76–127, 79–128, and 71–131%, respectively, when average values of CyHeLa cybrid clones with HeLa mtDNA (Fig. 1) were taken as 100%. * and n.s. indicate a *P* value less than 0.05 and no significant difference between normal subjects and pathogenic group, respectively.

jects to mtDNA-less HeLa cells (Table 1). Average values of all respiratory complex activities and O_2 consumption rates in cybrid clones with mtDNAs from 54 subjects (CyN1–CyN54) were comparable to those in cybrid clones with HeLa mtDNA (CyHeLa) (Fig. 2). Therefore, the values of respiratory function in CyHeLa cybrid clones could be used as normal standards. On the other hand, significant variations in respiratory function, particularly in O_2 consumption rates, were observed in CyN1–CyN54 cybrid clones (Fig. 2). For example, CyN12 and CyN1 cybrid clones showed 71% of the normal O_2 consumption rate, suggesting that their 29% reduction was within the normal range.

As pathogenic controls with proven pathogenic mtDNA mutations (*9–11*), we used two groups of respiration-deficient cybrid clones. One consisted of completely respiration-deficient CyP3243 and CyP4269 cybrid clones carrying imported mtDNAs with a homoplasmic A3243G mutation and A4269G mutation in the tRNA genes from patients with MELAS and cardiomyopathy, respectively (*9*). The other consisted of intermediately respiration-deficient CyP3394 and CyP14577 cybrid clones carrying imported mtDNAs with homoplasmic T3394C and T14577C mutations in the *ND1* and *ND6* genes encoding subunits for complex I, from patients with cardiac disease (*10*) and with type 2 diabetes mellitus (*11*), respectively (Table 1).

The former pathogenic group (CyP3243 and CyP4269) showed overall and complete reduction of respiratory function (Fig. 2) as expected, since mutations in the tRNA genes resulted in inhibition of overall protein synthesis in mitochondria. The latter pathogenic group (CyP3394 and CyP14577) showed normal activities of complexes II+III and IV, but reduced activities of complex I of 60–62% of normal (Fig. 2). Preferential reduction of complex I activity was expected, since the mtDNA mutations were created in the *ND1* or *ND6* gene encoding subunits for complex I. We also observed reduction to 62–64% in O_2 consumption rates, which was a reflection of reduced complex I activity. Thus, O_2 consumption rates can be used to represent overall respiratory function of the cybrid clones.

A frequency histogram showed that all cybrid clones with mtDNAs from normal subjects had O_2 consumption rates of 71 to 131%, whereas pathogenic cybrid clones carrying mtDNAs from patients with mitochondrial diseases had O_2 consumption rates of less than 64% (Fig. 3A). In contrast to O_2 consumption rates, no clear differences between normal and pathogenic cybrid clones were observed in the summed activities of the respiration complexes tested (Fig. 3B). These observations suggested that O_2 consumption rate, but not summed activities of respiratory complexes, could provide a good indicator of whether mtDNAs in cybrid clones possess pathogenic or polymorphic mutations.

This idea was applied to determine the pathogenicity of mtDNA from a patient expressing sporadic DHCM. mtDNA was transferred from the patient to mtDNA-less HeLa cells, and three resultant cybrid clones (CyP-DHCM) were examined for respiratory function. The relative activities of the respiratory enzyme complexes I, II+III, and IV, and O_2 consumption rates were 51, 76, 60, and 60%, respectively (Fig. 2). Their significant and overall reduction suggested the presence of pathogenic mutations in the tRNA genes or in the control region of mtDNA.

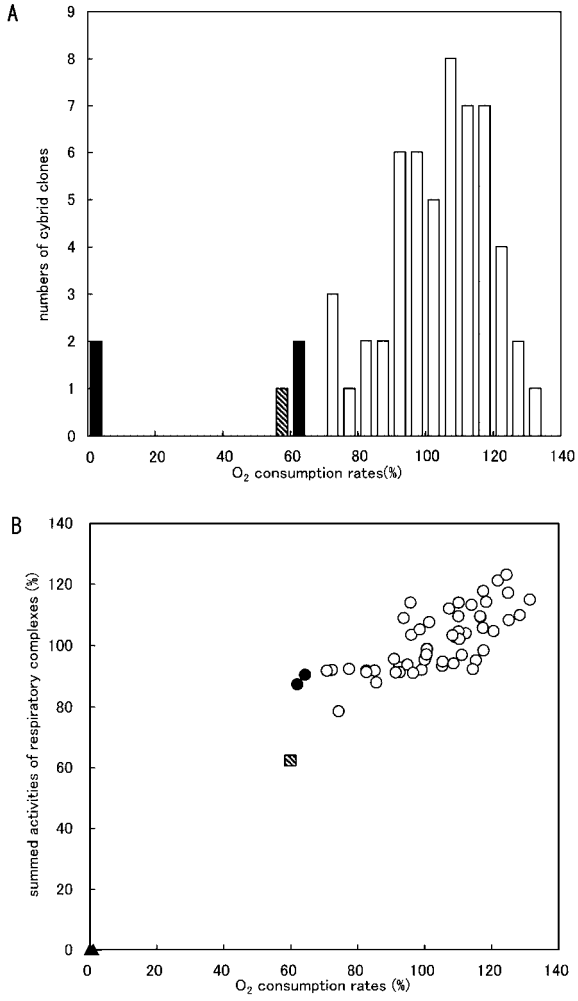

Fig. 3. **Normal and pathogenic ranges of respiratory function using cybrid clones with mtDNAs from normal subjects and from patients expressing mitochondrial diseases.** A: Frequency histogram of distribution of cybrid clones expressing various O_2 consumption rates. Open bars are CyN1-N54 cybrid clones with mtDNAs from normal subjects. Solid bars are CyP3243, CyP4269, CyP3394, and CyP14577 cybrid clones with mtDNAs from patients with mitochondrial diseases. The hatched bar represents CyP-DHCM cybrid clones with mutated mtDNA from a patient with DHCM. B: Correlation of O_2 consumption rates and summed activities of all respiration complexes examined. Open circles, 54 cybrid clones with mtDNAs from 54 subjects in the normal population (CyN1-N54); closed triangles, cybrid clones with mutated 3243 and 4269 mtDNAs (CyP3243 and CyP4269, respectively); closed circles, two cybrid clones with mutated 3394 and 14577 mtDNAs (CyP3394 and CyP14577); hatched square, cybrid clones with mtDNA from DHCM (CyP-DHCM).

Sequence analysis of all mitochondrial tRNA genes showed no reported pathogenic mutations, whereas its control region possessed a T16189C mutation, which was reported to be polymorphic and frequently present in the normal population, but to be associated with the pathogeneses of dilated cardiomyopathy (DHCM) (*15*) and type II diabetes (*16*, *17*). Then, we randomly selected cybrid

clones with mtDNAs from nine normal subjects for sequence analysis of the control region. Two cybrid clones, CyN3 and CyN24, possessed the mutation, but did not show preferential reduction of respiratory function (Fig. 2). These observations suggested that a T16189C mutation alone was not responsible for expression of the respiration defects, and that, in addition to a T16189C mutation, the mtDNA derived from the patient possessed other mutations, which were responsible for the respiration defects.

DISCUSSION

Mitochondrial respiratory function is controlled by nuclear DNA and mtDNA, and four respiratory enzyme complexes I, III, IV, and V are consisting with subunits encoded by both DNAs (*1*, *2*). Therefore, to determine the pathogenicities of mutated mtDNAs in patients, the influence of nuclear DNA mutations has to be excluded by mtDNA transfer from patients to mtDNA-less human cells and isolation of cybrid clones. In previous studies, cybrid clones with wild-type and mutated mtDNAs were isolated from the same patients with typical mitochondrial diseases, and the pathogenicities of the mtDNA mutations were determined by co-transfer of the mutated mtDNAs and respiration defects from the patients to cybrid clones (*3*–*7*). In these studies, heteroplasmy of mutated and wild-type mtDNAs was always observed in the patients, since homoplasmy of the mutated mtDNAs was lethal. Thus, cybrid clones with only mutated mtDNA and with only wild-type mtDNA from the same patients can be isolated for comparison of respiratory function. Moreover, since mutated mtDNAs induced complete and overall respiration defects in the cybrid clones, it was very easy to determine the pathogenicities of the mutated mtDNAs.

On the other hand, when the pathogenicities of the candidate mutated mtDNAs were weak, patients showed very slight reduction of respiratory function and carried the mutated mtDNAs in homoplasmy. Therefore, it was difficult to obtain cybrid clones with wild-type mtDNAs from the same patients for comparison of their respiratory function. Moreover, it was also difficult to determine whether the slight reduction of respiratory function was involved in the pathogeneses. Limits have not been reported for the reduction of respiratory function when disease phenotypes are not expressed. This study, therefore, addressed this issue. For this we transferred mtDNAs from 54 normal subjects in the general population to mtDNA-less HeLa cells. The resultant cybrid clones showed that an O_2 consumption rate of 71% was the lowest limit of cybrid clones with mtDNAs from normal subjects. On the other hand, cybrid clones with mutated mtDNAs from patients expressing mitochondrial diseases gave values of less than 64%. Therefore, an O_2 consumption rate of less than 71% in cybrid clones should be a reliable indicator that the mtDNAs from patients possess pathogenic mutations.

The ranges of normal respiratory function in our cybrid clones could be used effectively to determine the pathogenicities of mtDNA from a patient expressing DHCM (Fig. 3). This study showed co-transfer of the mtDNA and respiration defects to CyPDHCM cybrid clones, suggesting the presence of pathogenic mutations in the mtDNA of the patient. Sequence analysis of its control region showed the presence of T16189C mutation, which was reported to be associated with susceptibility to dilated cardiomyopathy (*15*) and to type II diabetes mellitus (*16*, *17*). Although this mutation was present in about 10% or more of the general population, and thus concluded to be polymorphic (*16*), these observations suggested its involvement in the pathogenesis of DHCM of the patient. However, CyN3 and CyN24 cybrid clones with mtDNAs possessing the T16189C mutation from two normal subjects did not show reduced respiratory function beyond the lower limit of the normal range (Fig. 2), suggesting that the T16189C mutation alone was not responsible for respiration defects.

Since it was not possible to obtain cybrid clones with wild-type mtDNA from the same patient due to homoplasmy of the mutated mtDNA of the patient, candidate pathogenic mutations have to be determined by comparison with Cambridge sequences (*18*). However, there are too many sequence differences between them to determine a pathogenic mutation. Moreover, it was also possible that combination of several polymorphic mutations functioned as pathogenic mutations to express respiration defects. In the case of mtDNA in CyPDHCM, the T16189C mutation and other mutations in the mtDNA may be involved to some degree in the pathogenesis of DHCM. Considering that no maternal inheritance of DHCM was observed in the patient, mutations in nuclear DNA were responsible for the pathogenesis, and respiration defects induced by the mutated mtDNA modified the pathogenesis by accelerating the onset of the diseases.

Similar coordination of both nuclear and mtDNA mutations in pathogenesis has been reported in patients, particularly those carrying weakly pathogenic mtDNA mutations (*10*, *12*). In one case (*10*), a homoplasmic T3394C mutation in the *ND1* gene of mtDNA, which was first reported in a patient expressing sporadic diabetes (*19*), was observed in a pedigree expressing maternally inherited cardiac abnormalities, but not expressing diabetes. Transfer of the T3394C mutated mtDNA from the patient to mtDNA-less HeLa cells and resultant CyP3394 cybrid clones showed 64% O_2 consumption rates (Fig. 2). Moreover, mutations of the *ether-a-go-go* related gene in nuclear DNA were also suggested to be responsible for the pathogenesis (*10*). In another case (*12*), association of nuclear DNA mutations in the calcium-sensing receptor (*CaSR*) gene and an A4833G mtDNA mutation in the *ND2* gene were reported in a pedigree expressing type II diabetes mellitus. Recently, Johnson *et al.* (*20*) reported that the auditory-brain response thresholds of A/J strain mice possessing nuclear DNA mutations in the age-related hearing loss (*ahl*) locus were slightly increased by addition of a mutation in the mtDNA-encoded $tRNA^{Arg}$ gene, suggesting that expression of hearing loss caused by nuclear gene mutation was enhanced by the mtDNA mutation. In this case, however, it was uncertain whether mtDNA mutation in the $tRNA^{Arg}$ gene induced respiration defects. These observations suggested that the specificity of disease phenotypes was determined and controlled by mutations in nuclear DNA, and mtDNAs carrying weakly pathogenic mutations were involved in

the pathogenesis by enhancing the onset or severity of disease phenotypes.

On the other hand, the mutated mtDNAs inducing 100% reduction of respiratory function in the cybrid clones were suggested to be responsible for typical mitochondrial diseases (*3*–*7*). Mito-mice provided direct evidence that mutated mtDNA alone was responsible for pathogenesis without the help of mutations in nuclear DNA (*8*, *9*). However, when the mutated mtDNAs induced 64% values of respiratory function in the cybrid clones, mutations in nuclear DNA would be required for expression of disease phenotypes. Then, what about the mtDNAs inducing 71% respiratory function in the cybrid clones with mtDNAs from normal subjects? CyN-12 and CyN-1 cybrid clones with mtDNAs from 2 of 54 normal subjects showed O_2 consumption rates of 71% (Fig. 2). Considering that O_2 consumption rates of 64% were involved in pathogenesis, it is possible that 71% values are also involved in the pathogeneses of age-associated disorders with the help of some mutations in nuclear DNA.

These observations suggested that there should be polymorphic mutations in mtDNAs of normal subjects that could be risk factors for early onset of age-associated disorders by inducing about 30% reduction of respiratory function. On the other hand, there should be other polymorphic mtDNA mutations that conversely induce about 30% activation of respiratory function (Figs. 2 and 3) and may contribute to maintaining health or enhancing trainability. We are investigating the physiological roles of these polymorphic mutations, and also investigating the possibility that apparent reduction or activation of respiratory function in cybrid clones is due to incompatibility between mtDNAs and HeLa nuclear genome by the use of human cell lines carrying different nuclear genomes as recipients of mtDNAs of normal subjects.

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