BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **232,** 147–152 (1997)
ARTICLE NO. RC976251

Deltoid Human Muscle MTDNA Is Extensively Rearranged in Old Age Subjects

S. A. Kovalenko,[1] G. Kopsidas, J. M. Kelso, and A. W. Linnane
Centre for Molecular Biology and Medicine, Austin and Repatriation Medical Centre, Banksia St., West Heidelberg, Victoria 3084, Australia

Received January 24, 1997

Extra long PCR analysis of mitochondrial DNA (mtDNA) isolated from skeletal muscle of humans of different ages revealed three phenomena: (i) the amount of normal length mtDNA (16.5 kb) was progressively reduced with age, such that the cells of old age individuals (about 90 years) contained little, or undetectable amounts of normal length mtDNA; (ii) the total amount of mtDNA did not appear to be greatly decreased rather the extent of mtDNA deletions greatly increased; (iii) in old age subjects, considerable amounts of over-sized mtDNA (more than 16.5 kb) was observed. Enzyme histochemical analysis of cytochrome-c oxidase (COX) activity in the muscle tissue of all subjects evidenced a cellular bioenergy mosaic with cells ranging from high to zero detectable enzyme activity in the muscle samples. The frequency of COX deficient muscle fibres was highly dependent on the age of the subject. We have found that the extent of the mtDNA mutational changes strongly correlate with the observed progressive decrease in COX activity. Therefore, it was suggested that the total extent of mtDNA mutation is very large in old age subjects and is sufficient to account for the decline in cellular COX activity with age and for a progressive decrease of overall mitochondrial bioenergetic capacity.

Human mtDNA is normally present in healthy young individuals as a closed circular 16,569 nucleotide pair molecule, encoding 2 ribosomal RNAs, 22 transfer RNAs and 13 proteins that are exclusively involved in the generation of bioenergy required for the cell's function. It is now well established that somatic mtDNA mutations (including point mutations and deletions) accumulate with age in human and other mammalian species and are accompanied by the formation of a tissue bioenergy mosaics (1-4). Linnane and colleagues (5) were the first to hypothesise a link between accumulating mtDNA mutations and a concurrent decrease in bioenergy capacity, particularly in post-mitotic tissue. The level of any one mtDNA mutation, be it a point mutation or deletion will be insufficient to result in the gross physiological and pathological changes associated with the decline of bioenergy capacity observed in aged tissues. Thus it has been reported (6) that the occurrence of the 5 kb deletion, so-called "common", in skeletal muscle, accumulates exponentially with age but only up to about 0.1% at age 80-90 years; for the substantia nigra region of the brain the 5 kb deletion has been reported to reach a level of 3 to 12% (7-9), while in the cerebellum it was barely detectable (8,9). At least 26 separate deletions, albeit of low frequency, have been reported to occur in various tissues between repetitive sequence of which are a large number in the mitochondrial genome (2). We have suggested that taken in the aggregate sufficient mutation will occur to explain the decline of cellular bioenergy capacity. However, a high percentage of mutations (deletions) such as occur in human mitochondrial disease patients has yet to be observed. To approach this question we have analysed the mtDNA from skeletal muscle of different age humans by using the extra long PCR (XL-PCR) analysis, that enable the assessment of the entire 16,569 bp human mtDNA sequence. This has allowed the aggregated assessment of all mtDNA mutations (insertions/deletions/duplications) collectively referred to here as rearrangements. The data presented here demonstrates that: the amount of normal length mtDNA (16.5 kb) was progressively reduced with age, such that the cells of old age individuals (about 90 years) contained little, or undetectable amounts of normal length mtDNA; the human skeletal muscle from aged individuals accumulates a wide variety of

[1] Corresponding author's address: Locked Bag 1, West Heidelberg, Melbourne, Victoria 3084 Australia. Fax: 61-3-94964112.

0006-291X/97 $25.00

mtDNA rearrangements and that the extensive mtDNA changes are correlated with cytochrome-*c* oxidase (COX) activity decline in the same tissue. Based on the data, we propose that the total extent of age associated accumulation of mtDNA mutations (in conjunction with a decline in normal length mtDNA) are sufficient to account for the observed bioenergy decline associated with the ageing process in skeletal muscle, thereby establishing the overall proposal set out by Linnane and colleques (5).

MATERIAL AND METHODS

Material. Human deltoid skeletal muscle autopsies were obtained from individuals of different ages (one subject from each age group) within two hours following their death. The medical history of these individuals did not indicate any mitochondrial disorders or myopathies. The muscle specimens were immediately embedded into Ames OCT compound (Miles Inc. IN U.S.A.) and frozen in isopentane cooled by liquid nitrogen. To assess gross tissue morphology and to verify the state of preservation of each tissue sample, hemotoxylin-eosin staining was performed on 7 μm tissue sections and assessed microscopically for autolytic and putrefaction changes.

Cytochrome-c oxidase (COX) staining. Cytochrome-*c* oxidase activity was assessed on skeletal muscle tissue sections and used as a general indicator of the overall capacity of the oxidative phosphorylation system. COX activity was examined using the method outlined by Müller-Höcker (10).

Isolation of total genomic DNA. To obtain the high quality template for PCR analysis, the total DNA (final concentration 100 ng/μl) was isolated from muscle autopsies using QIAamp Tissue Kit (QIAGEN, Chatsworth, CA, U.S.A.) according to the manufacturer's recommendations.

Extra long PCR (XL-PCR). Primers were designed with balanced melting temperatures in excess of 60°C, with minimisation of secondary structure and dimer formation and with amplification in opposite directions, overlapping by one base at their 5′-ends. Primer sequences were as follows: XLL 14932: 5′ CTT TTC ATC AAT CGC CCA CAT CAC TCG 3′ and XLH 14905: 5′ GCG GTT GAG GCG TCT GGT GAG TAG TGC 3′. A 23kb fragment of the human β-globin gene was also routinely amplified and served as an internal control to assess the quality and relative concentration of individual human DNA templates. XL-PCR was performed with the Expand^{TM} 20kb ^{Plus} PCR system (Boehringer Mannheim, Germany) which utilises a mixture of polymerases and specific buffers to allow amplification of DNA sequences up to 20 kb in length. A hot start amplification was employed using PCR AmpliWax PCR Gem 50 (Perkin-Elmer, Foster City, CA, U.S.A.) to separate the components of the PCR reaction into an upper and lower layers. The lower layer (30 ml total volume) contained 500 mM each of dATP, dTTP, dCTP and dGTP, 400 nM of each of the primers and 100 ng template DNA while the upper mix (20ml total volume) contained Expand Long Template buffer and 2.6 units of Expand Long Template enzyme mix. The PCR profile was run on a Perkin-Elmer 2400. The profile was initiated with a 60 sec denaturation period at 94°C followed by 20 cycles at 94°C for 10 sec and 68°C for 30 min. This was followed by a further 16 cycles incorporating 94°C for 10 sec and 68°C for 30 min with a 20 sec elongation consecutively added to each extension cycle. The profile was completed with a 30 min extension at 72°C. PCR products were visualised on a UV transilluminator following electrophoresis in solidified (0.8% agarose) Tris-acetate-EDTA buffer and following staining with ethidium bromide solution (0.5μg/ml). To test the reproducibility of procedure, all PCR reactions were repeated at least twice. The detection of the oversized mtDNA species depends critically on the experimental conditions used which need to be optimised in regard to the amount of tissues extracted (less than 10 mg), the method of mtDNA preparation (use of anion exchange column) and the XL-PCR conditions (15-30 min extention time).

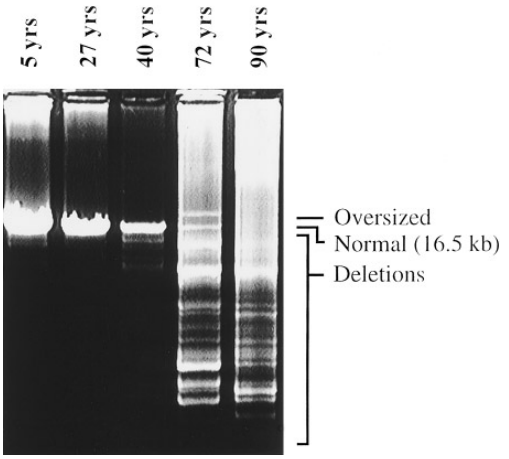

FIG. 1. XL-PCR of mtDNA from human deltoid muscle taken from individuals aged 5, 27, 40, 72 and 90 years. All samples demonstrated rearrangements to varying degrees and an age related decline in the copy number of full length mtDNA. The 40, 72 and 90 year old subjects presented multiple deletions with oversized mtDNA visible in the 72 year old and full length mtDNA absent in the 90 year old.

Southern blotting. Tris-acetate-EDTA agarose gels (0.8%) containing DNA of interest were HCl neutralized (0.25M for 30 min) then capillary blotted and fixed onto nylon Hybond-N+ membranes (Amersham) using 0.4 M NaOH. Digoxigenin-labeled (DIG) mitochondrial probes were generated by conventional PCR amplification of agarose gel purified DNA isolated from human deltoid muscle of a 1hr old individual. Primer pairs were chosen to embrace the entire mtDNA sequence. These DIG-labelled nucleic acid probes were hybridized with mitochondrial target DNA bound to Hybond-N+ membranes and subsequently detected by a DIG labelling and detection kit (Boehringer Mannheim), according to manufacturers instructions.

RESULTS AND DISCUSSION

We have previously suggested (5) that the progressive accumulation of mtDNA mutations with age would lead to a decline in the bioenergetic capacity of tissue/organs. Further that the mutations would be heterogenous and occur randomly to produce a tissue bioenergetic mosaic with individual cells ranging from normal to grossly defective in bioenergy capacity (11). The occurrence of a tissue bioenergetic mosaic in old age as evidenced by the occurrence of cytochrome oxidase activity is well-established, but whether there is sufficient mtDNA mutation to account for the mosaic or whether nu-

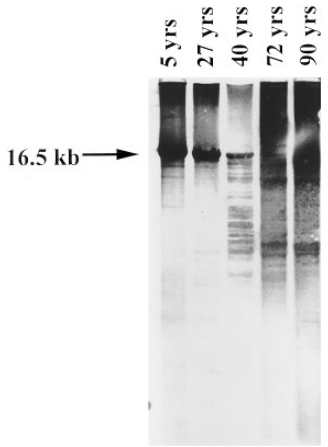

FIG. 2. Southern blot analysis confirming the mitochondrial origin of XL-PCR products. XL-PCR was performed on mtDNA extracted from human deltoid muscle from individuals aged 5, 27, 40, 72 and 90 years.

clear mutation is involved has remained open to question(12,13). The data presented here strongly support the concept of mitochondrial DNA mutation as being the primary cause of the bioenergetic decline and the mosaic.

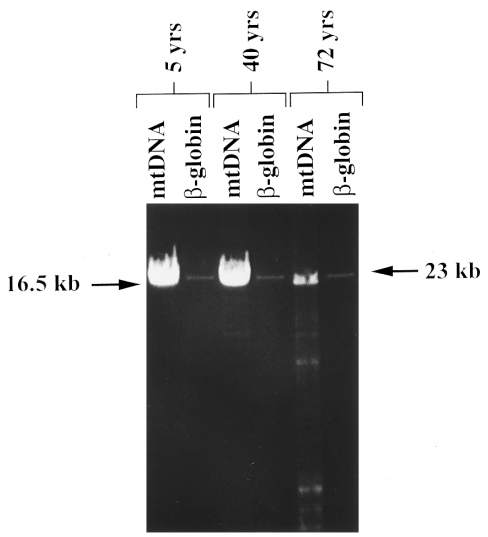

FIG. 3. XL-PCR amplification of the mtDNA genome and the β-globin gene. DNA isolated from human deltoid muscle from individuals aged 5, 40 and 72 years was amplified in two separate PCR reactions performed concurrently. One reaction incorporated primers to amplifying the entire mtDNA chromosome whilst the other reaction utilised primers to amplify a 23 kb segment of the nuclear β-globin gene. Lane 1 (mtDNA) and Lane 2 (β-globin gene) amplified from the same DNA sample isolated from a 5 year old; Lane 3 (mtDNA) and Lane 4 (β-globin gene) amplified from the same DNA sample isolated from a 40 year old individual; Lane 5 (mtDNA) and Lane 6 (β-globin gene) amplified from the same DNA sample isolated from a 72 year old subject.

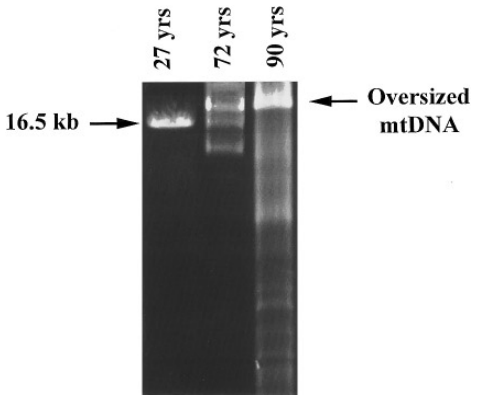

FIG. 4. Occurrence of oversized mtDNA demonstrated by extra long PCR on human deltoid muscles of different age.

XL-PCR has been recently used to demonstrate the accumulation with age of a wide range of mtDNA deletions in a number of tissues (14,15). However, the procedures adopted while appropriate for the detection of full length 16.5 kb mtDNA were not suitable for the detection of extra large (greater than 16.5 kb) abnormal mtDNA species. In the present study we have made use of the Expand 20 kb ^{Plus} PCR system (see methods) to assess the full range of mtDNA mutations associated with age. Amplification of mtDNA isolated from human deltoid muscle from individuals aged 5, 27, 40, 72 and 90 years shows (Fig.1) the progressive accumulation with age of a spectrum of changes, including changes in mtDNA copy number, deletions and extra large mtDNA. The XL-PCR profile of the 5 and 90 year old individuals illustrate the striking global picture with respect to the state of the mtDNA. The mtDNA from the 5 year old subject showed a small number of deleted molecules, but essentially gave only a full-length 16.5 kb product. As the age of the sampled individuals increased, the copy number of full-length mtDNA decreased to the point where, the 72 year old tissue sample still clearly contained a small amount of full length mtDNA but remarkably, it was doubtful that there was any full length mtDNA detected in the muscle sample of the 90 year old subject. The progressive decrease of full length mtDNA with age was accompanied by a corresponding increase of mtDNA deletions. The data indicates that the mitochondrial genome of skeletal muscle in aged subjects is grossly rearranged to the extent that full length mtDNA is no longer present or is present in only very small amounts (within the limits of our detection procedure). Southern blot analyses as shown in Fig.2 has confirmed the mitochondrial origin of the PCR products illustrated in Fig.1. The occurrence of the observed decrease of full length

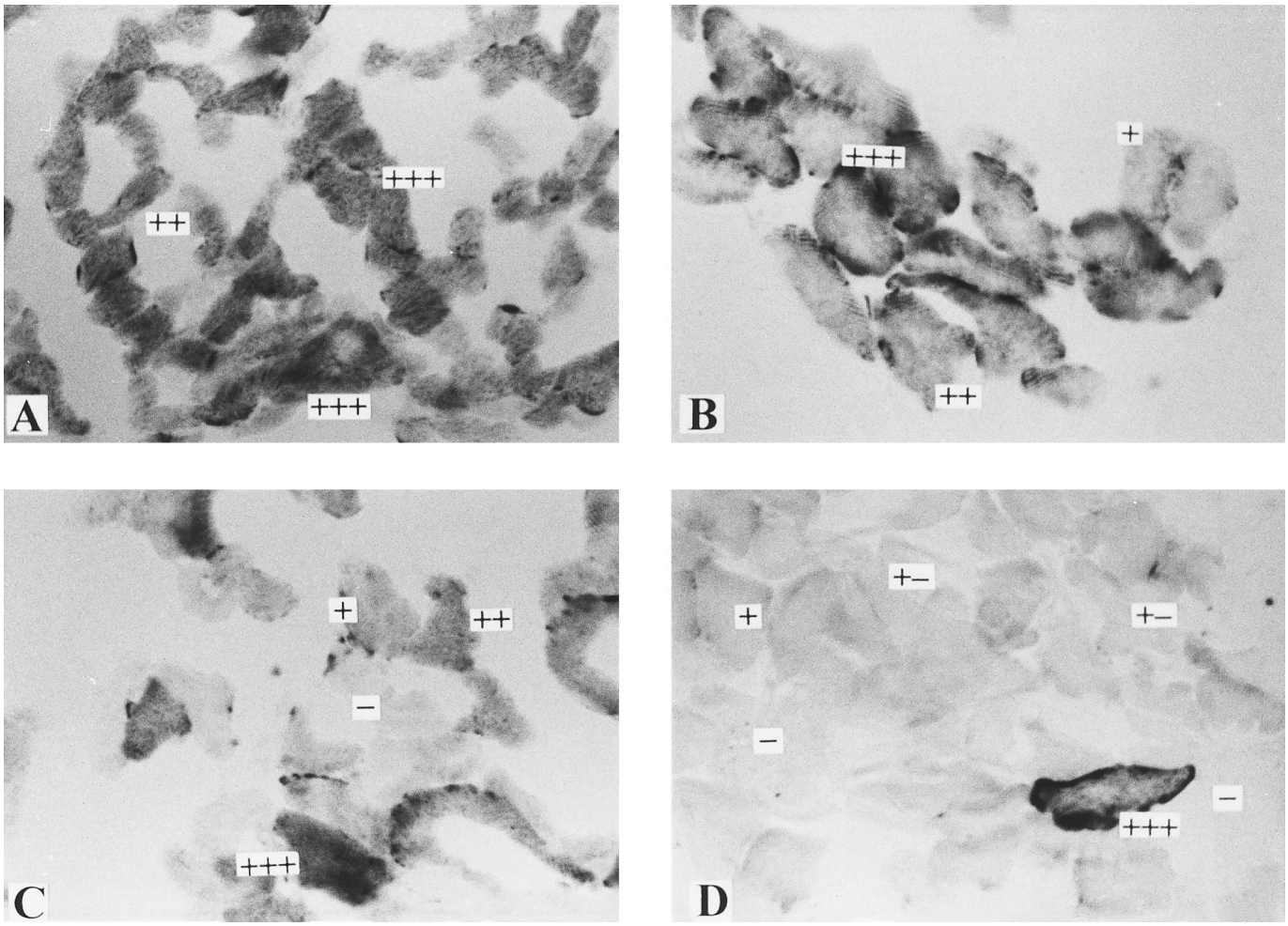

FIG. 5. COX staining of human deltoid muscle from individuals aged 5 (panel A), 27 (panel B), 72 (panel C) and 90 years (panel D). Cytochrome-*c* oxidase activity is indicated by dark deposits with the intensity of the deposits correlating to COX activity. The 5 and 27 year old subjects show predominantly COX positive fibres whist the 72 and 90 year old individuals show a prevalence of intermediate and negative fibres. All panels are at a magnification of 200×.

mtDNA with age was validated by also amplifying a 23 kb portion of the β-globin nuclear gene present in the total cellular DNA fraction, extracted for the mtDNA analyses, as shown in Fig.3. Thus, the amplified β-globin gene fragment remained constant in amount under the conditions used while the amounts of full length mtDNA progressively decreased with samples obtained from the 5, 40 and 72 years old subjects. To obtain the data shown in Fig.1 the XL-PCR conditions (5-10 min extention time) were selected to favour the detection of the full length mtDNA and deletions. Fig.4 illustrates another striking feature of the old age tissue samples (72 and 90 years) which is the occurrence of extra large mtDNA species (>16.5 kb), presumably, the result of insertions and duplications in mtDNA. The phenomenon of the age related occurrence of extra large mtDNA was firstly described by Piko et al. in 1978 (16) using electron microscopic technique.

An old age-associated decline of muscle mitochondrial respiration is well-established (12,17) but some workers have questioned whether the extent of mtDNA changes are sufficient to account for the observed bioenergy capacity decrease. The results presented herein establish that the changes in mtDNA in old age individuals (72, 90 years) are so extensive that little normal full length mtDNA remains in human deltoid muscle. Enzyme-histochemical analysis for the occurrence of cytochrome oxidase activity in muscle fibre samples from 5, 27, 72 and 90 year old subjects are shown in Fig.5. The 5 year old subjects tissue has predominantly strongly COX positive fibres with a few fibres of low COX activity, in contrast to the fibre samples from the 90 year old subject. The

TABLE 1

Cytochrome-c Oxidase Analysis of the Human Deltoid Muscle from Different Age Individuals

Age (years)	Number of fibres counted	Staining intensity (% fibres stained) Negative −/+−	Positive +	Positive ++	Positive +++
5	174	4	16	25	55
27	237	19	17	22	42
72	214	29	8	40	23
90	425	38	43	16	3

Note. The estimation of COX activity from −/+− to +++ and the assignment of a value has been scored as illustrated in Fig. 5.

90 year muscle fibre samples show predominantly fibres of low COX activity with some fibres showing very low or no detectable activity. The COX activity of the samples from the 27 and 72 year subjects fall in between those obtained with the 5 and 90 year material. The COX data may be interpreted to suggest that the oversized mtDNA is transcriptionally/translationally inactive. A semi-quantitative estimate of the relative occurrence of COX activity in the samples obtained from different age subjects is set out in TABLE 1. We suggest that there is a strong correlation between the observed COX activities, which reflect the bioenergy status of the tissues, and the extensive age associated progressive mtDNA changes as described herein.

The question arises as to how many intact mtDNA 16.5 kb copies per senescent cell are required to enable it to survive? Data from mitochondrial disease patients suggest that 5-10% intact mtDNA molecules per cell may be sufficient to maintain viable cellular bioenergetic function. However, the results obtained particularly with the 90 year old subject suggest that the intact mtDNA accounts for less than 5% of the total mtDNA of the extracted tissue, and that based on the COX activity some cells will contain perhaps no full length functional mtDNA while others will contain amounts sufficient to support COX activity ranging from fully functional to intermediate levels. The question then arises, as to how do senescent cells with perhaps no capacity for mitochondrial oxidative phosphorylation survive? We have earlier reported that human cell lines can be grown anaerobically, fully maintained by glycolytic activity provided a redox sink such as excess pyruvate is added to the culture medium (18). Also that Rho^0 (no mtDNA) cells can be grown aerobically dependent only on the presence of a suitable redox sink such as pyruvate or coenzyme Q10 or ferricyanide (19). The aerobically grown Rho^0 cells generate their energy requirement via the interaction of glycolytic and plasma membrane NADH oxido-reductase activity; plasma membrane NADH oxido-reductase is up-regulated in order to maintain an adequate supply of NAD required for continued glycolysis activity (20). Similarly, we suggest by analogy, that some deltoid cells can survive, albeit with limited functionality without the need for mitochondrial bioenergy activity rather and that their ATP needs are derived from an interactive glycolytic and plasma membrane oxido-reductase system. Perhaps too much attention has been focused on the extent of mtDNA mutations and/or nuclear mutations affecting the mitochondrial system, to explain mitochondrial disease and some of the phenomena of bioenergy decline with ageing. With the establishment of the gross mtDNA mutations associated with ageing, other interactive cellular bioenergy systems require increased attention.

REFERENCES

1. Hayakawa, M., Hattori, K., Sugiyama, S., and Ozawa, T. (1992) *Biochem. Biophys. Res. Commun.* **189,** 979–985.
2. Zhang, C., Baumer, A., Maxwell, R. J., Linnane, A. W., and Nagley, P. (1992) *FEBS Lett.* **297,** 4–8.
3. Wallace, D. (1992) *Science* **256,** 628–632.
4. Linnane, A. W., Zhang, C., Baumer, A., and Nagley, P. (1992) *Mutat. Res.* **275,** 195–208.
5. Linnane, A. W., Marzuki, S., Ozawa, T., and Tanaka, M. (1989) *Lancet* 642–645.
6. Ozawa, T., Tanaka, M., Ikebe, S., Ohno, K., Kondo, T., and Mizuno, Y. (1990) *Biochem. Biophys. Res. Commun.* **172,** 483–489.
7. Ikebe, S., Tanaka, M., Ohno, K., Sato, W., Hattori, K., Kondo, T., Mizuno, Y., and Ozawa, T. (1990) *Biochem. Biophys. Res. Commun.* **170,** 1044–1048.
8. Cortopassi, G. A., and Arnheim, N. (1990) *Nucleic Acids Res.* **18,** 6927–6933.
9. Corral-Debrinski, M., Horton, T., Lott, M. T., Shoffner, J. M., Beal, M. F., and Wallace, D. C. (1992) *Nature Genet.* **2,** 324–329.
10. Müller-Höcker, J. (1989) *Am. J. Pathol.* **134,** 1167–1173.
11. Linnane, A. W., Baumer, A., Maxwell, R. J., Preston, H., Zhang, C. F., and Marzuki, S. (1990) *Biochem. Int.* **22,** 1067–1076.
12. Trounce, I., Byrne, E., and Marzuki, S. (1989) *Lancet* **ii,** 637–639.
13. Hayashi, J.-I., Ohta, S., Kagawa, Y., Kondo, H., Kaneda, H.,

Yonekawa, H., Takai, D., and Miyabayashi, S. (1994) *J. Biol. Chem.* **269,** 6878–6883.

14. Cheng, S., Higuchi, R., and Stoneking, M. (1994) *Nature Genet.* **7,** 350–351.
15. Melov, S., Shoffner, J. M., Kaufman, A., and Wallace, D. C. (1995) *Nucleic Acids Research* **23,** 4122–4126.
16. Piko, L., Meyer, R., Eipe, J., and Costea, N. (1978) *Mech. Ageing Develop.* **3,** 351–365.
17. Wallace, D. (1993) *Trends in Genet.* **9,** 128–133.
18. Vaillant, F., Loveland, B. E., Nagley, P., and Linnane, A. W. (1991) *Biochem. Internat.* **23,** 571–580.
19. Martinus, R. D., Linnane, A. W., and Nagley, P. (1993) *Biochem. Mol. Biol. Internat.* **31,** 997–1005.
20. Larm, J. A., Vaillant, F., Linnane, A. W., and Lawen, A. (1994) *J. Biol. Chem.* **269,** 30097–30100.