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Genetics

A pattern of accumulation of a somatic deletion of mitochondrial DNA in aging human tissues

(PCR/somatic mutation/degenerative disease/heart/brain)

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ABSTRACT **An assay that selectively amplifies a specific deletion of the mitochondrial genome has been used to study the extent of the deletion's accumulation in a variety of human tissues. The deletion occurs at much higher levels in nervous and muscle tissues than in all other tissues studied. The variation in deletion level between the same tissues in different persons of similar age appears to be less than the variation among tissues within an individual. Tests for artifactual explanations of the level differences were each negative. Three cellular parameters that are correlated with the level of the deletion are identified. The preferential accumulation of deleterious mitochondrial mutations in a restricted subset of aging human tissues may compound deficiencies of function in those tissues that accrue with age.**

It has been suggested that an accumulation of somatic DNA mutations with time may underlie the deleterious cellular changes that increase with age in mammals (1–6). In the nuclear genome, inappropriate mutational inactivation of nuclear genes occurs with age (7), as well as inappropriate reactivation of silent genes (8–10). The accumulation of deleterious mutations in the mitochondrial genome has also been proposed to be important in aging (2–5). It was known that some types of DNA damage occur at higher levels in mitochondrial DNA (mtDNA) and that mitochondria are deficient for some types of DNA repair; some repeated nuclear sequences also show such a deficiency (11–14). But assays with the sensitivity to detect the basal level of some types of mitochondrial mutation in normal human tissues have only recently been developed (5, 15, 16). One of these methods involves the selective amplification of deleted mitochondrial genomes using a short-cycle PCR regimen (5, 15). Assays were developed for one specific type of deletion (the "common deletion" or mtDNA^{4977}), the most frequent deleted form found among patients with the rare mitochondrial diseases Kearns–Sayres syndrome and progressive external ophthalmoplegia. This deletion occurs between two 13-base-pair (bp) direct repeats that are separated by 4977 bp in normal mtDNA (17–20). We reported earlier that levels of mtDNA^{4977} increase at least 100-fold with age in human heart and brain tissue in individuals without heart or neurological disease (5). Studies by other groups have also shown that mtDNA deletions increase with age in heart muscle (21, 22). In the current study, levels of the mtDNA^{4977} deletion mutation in many additional human tissues were assayed to gain insight into the cellular forces that influence the accumulation of mitochondrial deletions.

MATERIALS AND METHODS

Autopsy Materials. Autopsy tissues were supplied through the Los Angeles County Hospital/University of Southern California Medical Center. The age at autopsy, sex, and cause of death of the nine individuals appear in Table 1.

PCR Methods. All methods and primers have been described (5). A schematic representation of the assay is presented in Fig. 1. Primers 1 and 2 are designed to flank the 13-bp repeats, and cycle time is shortened so that deleted template molecules that yield a 520-bp product are more likely to be amplified than normal templates that yield a 5.5-kilobase (kb) product (i.e., a selection based on short PCR cycles).

Control Amplifications. Studies of deletion level differences between tissues include a separate reaction that amplifies undeleted mtDNA genomes as an "internal" measurement of total mtDNA present (5). First, samples are normalized for total nucleic acid content by absorption at 260 nm. Then, each sample is amplified with primers specific for undeleted genomes (Fig. 1, primers 2 and 3), and the product intensities on ethidium bromide-stained agarose gels are compared. Samples with strong product intensities are diluted, the samples are reamplified, and the product intensities are compared. This process is iterated until the control signals from each sample appear similar, at which time the identical dilution of DNA extract is amplified with the primers specific for the deletion (1 and 2). Because the number of normal mtDNA genomes does vary with tissue type, this procedure is an important control for the comparison of deletion levels in different samples. For semiquantitative comparisons between tissues, samples with the same levels of control signal were serially diluted 1:2 and amplified with primers 1 and 2. The ratio of the dilution factors required to give deletion products of similar intensity is assumed to be the ratio of the amount of deleted mtDNA between the two samples.

RESULTS

A Pattern of Accumulation of the mtDNA^{4977} Deletion in Human Tissues. The levels of the mtDNA^{4977} deletion in different tissues of adults and children were studied. Autopsy tissues from up to nine individuals were examined for deletion levels in heart muscle, brain, diaphragm muscle, kidney, spleen, skin, and liver; and the results appear in Table 1. A representative sample of the data from two individuals is shown in Fig. 2. Adult heart muscle, brainstem, brain cortex, psoas muscle, and diaphragm muscle (Fig. 2, lanes 1–5) have higher levels of the deletion than liver, kidney, skin, spleen, and lung (Fig. 2, lanes 6–10). Every muscle or brain sample from other adult individuals (data not shown) also produced a strong deletion signal, but the samples from children were negative. Nonbrain and nonmuscle samples from both adults and children produced little or no deletion signal. To estimate the quantitative difference in relative deletion amounts among adult tissues, extracts from available samples were

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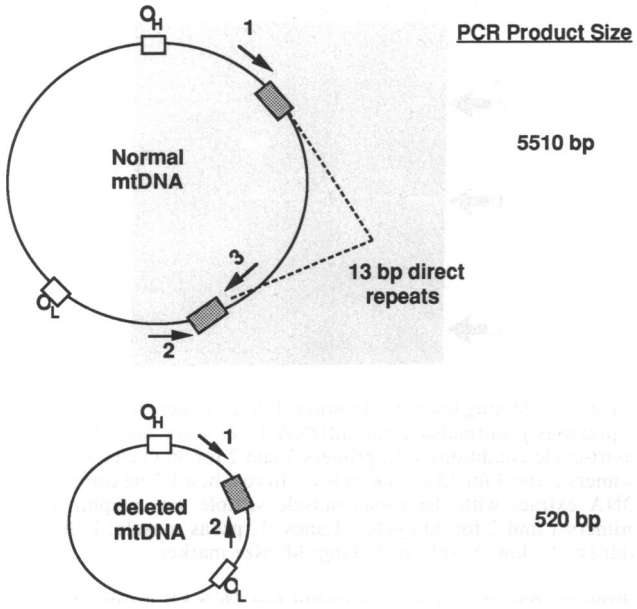

FIG. 1. Schematic diagram of the technique to selectively amplify deleted mtDNA genomes. Arrows denote approximate primer binding sites. Open boxes designate origins of mitochondrial replication. Shaded boxes denote 13-bp direct repeats. The schematic diagram is not drawn to scale.

individually normalized with respect to undeleted mtDNA and then pooled; the pools were serially diluted and amplified, and deletion product signals were compared to quantify levels of the deletion. The pooling experiment confirmed the existence of two groups of tissues (Table 2): one group with a relatively high deletion level (skeletal muscle, cardiac muscle, and brain) and one with a relatively low deletion level (kidney, spleen, skin, and liver). The high deletion level tissues contained from 16 to 64 times the deletion frequency of the other samples.

Analysis of Additional Tissues. A wider series of tissues was assayed for the level of the deletion from single samples. The tissues in Fig. 3 also include representatives of the urogenital and alimentary systems. Tongue muscle, psoas muscle, diaphragm muscle, and ocular muscle (Fig. 3, lanes 1–4) had relatively high deletion levels, whereas other tissues had significantly lower levels (Fig. 3, lanes 5–15), including liver, kidney, spleen, prostate, bladder, stomach, testis, lung,

Table 1. mtDNA^{4977} level in human autopsy tissues

	Individual								
	1	2	3	4	5	6	7	8	9
Age, years	80	78	77	49	42	38	30	15	4
L. ventricle	+	+	+	+	+	+	+	−	−
F. cortex	ND	+	+	+	+	+	+	−	−
Diaphragm	ND	+	+	+	ND	+	+	ND	−
Kidney	−	−	−	−	−	−	−	ND	−
Spleen	ND	−	−	−	−	−	−	ND	−
Skin	ND	−	−	−	−	−	−	ND	−
Liver	−	−	−	−	−	−	−	ND	−

Sex and cause of death of individuals 1–9 follow: 1, male, coronary artery disease (CAD); 2, male, CAD; 3, female, CAD; 4, male, CAD; 5, female, trauma; 6, male, cardiomyopathy; 7, reference number not available, male, asthma attack; 8, male, trauma; 9, male, trauma. +, relatively high deletion frequency; −, relatively low deletion frequency; ND, not done; L., left; F., frontal.

adrenal gland, cervix, and fatty (adipose) tissue. Ovary, skin, small intestine, breast, and fallopian tube tissue exhibited relatively low levels (data not shown). In other experiments, five distinct regions of heart tissue (septum, left atrium, right atrium, left ventricle, and right ventricle) from individuals 1–5 were found to contain relatively high levels of the deletion. We note that mtDNA^{4977} occurs at high levels in ischemic hearts (21). Although autopsy records from individuals 1–4 and 6 indicated cardiovascular disease, the individual samples we studied were not acutely involved.

Search for an Inhibitor of Deletion Amplification. One possible artifactual explanation of differences in deletion levels between human tissues is that certain tissues have higher levels of some inhibitor of the deletion amplification reaction than other tissues. Such an inhibitor would have to be specific for deletion amplification since the samples are all normalized by PCR for total mtDNA. In earlier experiments (5), we found no evidence to support the specific inhibitor hypothesis in comparisons of deletion level of the same tissue (heart) at different ages (fetal and adult). In this study, the inhibitor hypothesis was tested by mixing psoas muscle DNA (relatively high deletion levels) with liver, kidney, skin, spleen, or lung extracts (Fig. 4). These latter five extracts in which the deletion signal is initially weak have increased deletion signals after mixing with the psoas extract. Although the signal is not always as intense as the psoas itself, it appears as if the low deletion levels cannot be solely explained by an inhibitor of deletion amplification.

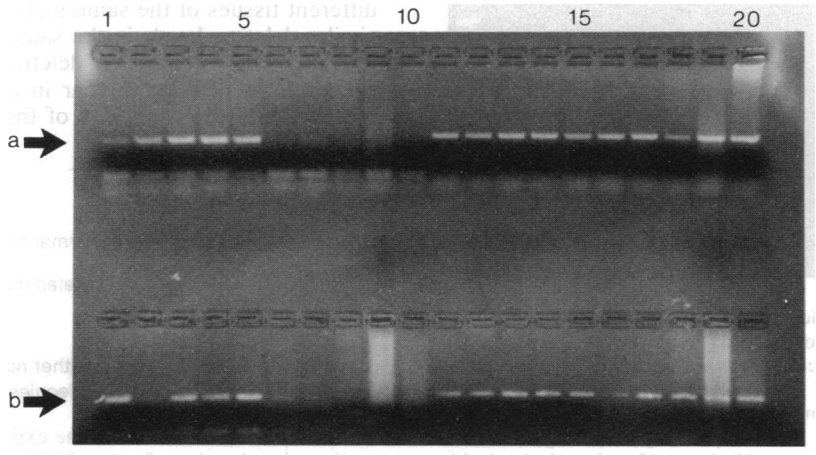

FIG. 2. Comparison of deletion levels among 10 tissues from two individuals. DNA was extracted from several tissues from individual 3 (row a) and individual 2 (row b). Samples were normalized for the amount of undeleted mtDNA by amplification with primers 2 and 3 for 15 cycles (lanes 11–20). Lanes 1–10 are the results of amplification under short-cycle conditions (9) using the same amount of DNA for normalization with primers 1 and 2 for 30 cycles. Lanes: 1 and 11, heart (left ventricle); 2 and 12, brainstem; 3 and 13, brain cortex; 4 and 14, psoas muscle; 5 and 15, diaphragm muscle; 6 and 16, liver; 7 and 17, kidney; 8 and 18, skin; 9 and 19, spleen; 10 and 20, lung.

Table 2. Quantitation of relative deletion levels among adult tissues

Tissue	Signal	Individuals
Diaphragm	64	2, 3, 4, 6
L. ventricle	16	1, 2, 3, 4, 5, 6
F. cortex	16	2, 3, 4, 5, 6
Kidney	1	1, 2, 3, 4, 5, 6
Spleen	1	2, 3, 4, 5, 6
Skin	1	2, 3, 4, 5, 6
Liver	1	1, 2, 3, 4, 5, 6

Normalized samples were pooled from the indicated individuals. Signals were determined by dilutions of the pooled samples. L., left; F., frontal.

Further Support of the Deletion's Existence *in Vivo*. We have presented extensive evidence (5) that the short-cycle PCR method selectively amplifies a lesion that existed before the PCR was initiated, a lesion that increases by >100-fold with age in some tissues. The lesion was assumed to be the deletion itself, but one possibility not eliminated by those earlier tests is that another type of DNA damage might exist in a 13-bp repeat of an undeleted mtDNA that promoted "jumping PCR" (23), producing a deletion *in vitro*, even though no deletion existed in the DNA sample. A test was designed to eliminate this possibility (Fig. 5). In the test, brain DNA from individual 1 was digested with two enzymes (*Xho* I and *Cla* I) that cut uniquely in the nondeleted area of the mitochondrial genome. The expected distance of migration after restriction digestion is thus different for mitochondrial genomes bearing the deletion (11.5 kb) versus normal mitochondrial genomes (16.5 kb). If the deletion existed before the PCR (model I), one expects the gel fraction containing the 11.5-kb molecules to contain the highest ratio of deletion to normal mtDNA. On the other hand, if deletions are the result of some *in vitro* PCR artifact (model II), one expects the ratio of deletion to normal mtDNA to be similar for all size fractions. In Fig. 6, row c, the results of amplification with control primers 2 and 3 of the three gel fractions from *Xho* I-cut, *Cla* I-cut, and uncut DNA are shown. As expected, the slices containing the cut 16.5-kb molecules (lanes 1 and 4) produce the highest control signal. Dilution experiments

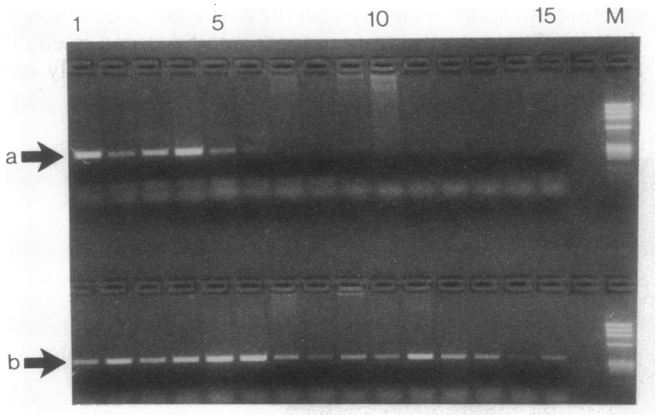

FIG. 3. Comparison of additional tissues for deletion level. DNA was extracted from tissues, normalized for total mtDNA content, and amplified under short-cycle conditions with primers 1 and 2 (row a) for 30 cycles or with control primers 2 and 3 (row b) for 15 cycles. Lanes: 1, tongue; 2, psoas muscle; 3, diaphragm muscle; 4, ocular (extensor) muscle; 5, liver; 6, kidney; 7, spleen; 8, prostate; 9, bladder; 10, stomach; 11, testis; 12, lung; 13, adrenal gland; 14, cervix; 15, fatty (adipose) tissue. Samples in lanes 1 and 13 came from individual 3. Samples for lanes 2, 3, 5–7, 11, and 12 came from individual 2. Samples in lanes 4 and 14 came from individual 6. Samples from lanes 8–10 came from individual 7. The remaining sample in lane 15 came from individual 4. M, size markers.

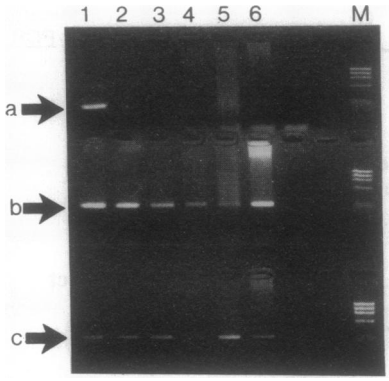

FIG. 4. Mixing test for inhibition. DNA extracts from individual 3 previously normalized for mtDNA level were amplified under short-cycle conditions with primers 1 and 2 for 30 cycles (row a) or primers 2 and 3 for 15 cycles (row c). In row b, a 1:1 mixture of each DNA extract with the psoas muscle sample was amplified with primers 1 and 2 for 30 cycles. Lanes: 1, psoas muscle; 2, liver; 3, kidney; 4, skin; 5, spleen; 6, lung; M, size markers.

showed that the slice containing the 16.5-kb molecule class had to be diluted ≈1:100 to match the control signals of the fractions containing the 11.5- and 7.0-kb bands. We infer that the concentration of normal mitochondrial sequences in the three fractions was approximately 100:1:1, for the three fractions of 16.5 kb, 11.5 kb, and 7.0 kb, respectively. After a 1:100 dilution of the 16.5-kb fraction, the amount of undeleted mtDNA product is approximately equal in all three fractions (row b). In row a, the samples normalized in row b were subjected to deletion amplification. The highest levels of the deletion product occur in lanes 2 and 5, the fractions that contain cut mtDNA of 11.5 kb, consistent with expectations of model I, that mtDNA^{4977} molecules existed before the PCR. If model II were true, that deletions are the artifactual result of amplification of normal mtDNA, one would expect equivalent deletion signals from the three size fractions.

DISCUSSION

The Accumulation of Deleted mtDNA Genomes in Aging Individuals Is Tissue-Specific. There is wide variation in levels of the mtDNA^{4977} deletion in different tissues of older individuals. This variation does not appear to be random. Although extensive variation in deletion level exists between different tissues of the same individual, there appears to be similar deletion levels in the same tissues of adults—i.e., a tissue-specific "pattern" of deletion accumulation. The highest levels of deletion appear in a restricted set of human tissues. With the exception of the cerebellum, all samples

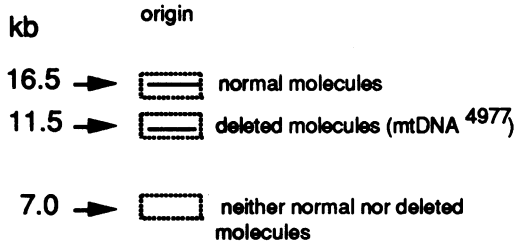

FIG. 5. Schematic view of the expectation for distance of electrophoretic migration of cut mtDNA molecules if mtDNA^{4977} preexisted the PCR. Bars denote the expected distance of migration of normal and deleted mtDNA. Dotted boxes indicate schematically which gel fractions were dissected for amplification in Fig. 6. The 7.0-kb fraction was included as a control for sheared or contaminating mtDNA fragments.

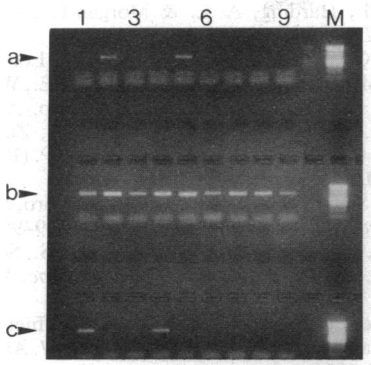

FIG. 6. Amplification of gel-fractionated mtDNA. Five micrograms per lane of total DNA from brain of individual 1 was cut with restriction enzymes and electrophoresed in a 2% agarose gel. Slices that contained the three size classes shown in Fig. 5 were dissected and melted, and a PCR was performed. Each series of three lanes contained, in order, the size fractions 16.5 kb, 11.5 kb, and 7.0 kb. Lanes: 1–3, size fractions from *Xho* I-cut DNA; 4–6, *Cla* I-cut DNA; 7–9, uncut DNA. In row c, the samples were amplified with control primers 2 and 3 for 20 cycles. A strong signal is only visible in the 16.5-kb class for cut samples. In row b, the results after normalization for normal mtDNA and amplification with control primers 2 and 3 for 15 cycles is shown. In row a, the identical amount of sample was used as in row b, except that primers 1 and 2 were used for 30 cycles of amplification. A strong signal for the deletion appears only in lanes 2 and 4, samples containing cut mtDNA of 11.5 kb.

from muscle or nerve cells have high deletion levels relative to other tissues tested. The difference in deletion level between tissues of adult individuals can be substantial, as high as 64-fold (Table 2). Deletion levels were relatively high in every muscle tissue tested and in every brain area surveyed including the medulla (Table 1 and other data not shown). In contrast to other brain areas tested, only cerebellar samples had relatively low deletion levels (ref. 15 and data not shown).

mtDNA^{4977} Occurs at Much Higher Frequency than Nuclear Gene Deletions. In normal human cells, deletions and rearrangements of the nuclear *HPRT* locus occur at a frequency of about 1 in 1,000,000 normal copies (24). No particular *HPRT* deletion appears to predominate. By contrast, the frequency of the mtDNA^{4977} deletion alone per normal mtDNA chromosome can occur at about a 1000-fold higher frequency, up to 0.1%, in heart and brain tissues of middle-aged humans (5, 21, 22); the permissiveness for mitochondrial somatic mutations may be a result of the redundancy of the mtDNA genes.

mtDNA^{4977} Is Presumably Generated by Slipped-Strand Mispairing. One molecular explanation for mitochondrial deletions is a slipped-strand mispairing event that could occur during mitochondrial replication (20). Because mitochondrial replication occurs continuously in all cells (25), this mechanism could explain the origin and replication of deletions in somatic cells. If the deleted genomes replicate faster than normal genomes, their number should gradually increase over time (26). An increase in deleted mtDNA over time has been demonstrated in a Kearns–Sayres syndrome patient (27).

The Paradox of Differential Accumulation in Cells and Tissues. If mitochondrial deletions are being generated in all human cells, forces at the level of the cell may determine the disparity in deletion levels between cell types. At the present time, it is not known what forces allow the greater accumulation of mtDNA^{4977} in some cell types and not others. We find correlations of mtDNA^{4977} level with three particular cellular characteristics (presented below), which may explain the differential accumulation of this deletion in human tissues.

Levels of mtDNA^{4977} Are High in Tissues with Low Mitotic Activity. Tissues with the lowest deletion levels are made up of highly mitotic cells, and tissues with the highest deletion levels are made up primarily of nonmitotic cells. Since mtDNA^{4977} deletions are deleterious in Kearns–Sayres and progressive external ophthalmoplegia syndromes, it is possible that lower levels are also deleterious and are lost as a result of negative (purifying) selection in rapidly dividing cells (5, 17).

Deletion Levels Are Higher in Cells of Larger Size. If the deletion mutation is initially absent but occurs stochastically as a function of numbers of mitochondrial replications within a somatic cell, cells with more mitochondrial genomes are more likely to experience a deletion event than cells with fewer mitochondrial genomes. Because of its small size, the mtDNA^{4977} genome may have a selective replicative intracellular advantage over normal mitochondrial genomes (26). Thus, large cells with more mitochondrial genomes might be expected to have higher levels of mtDNA^{4977} than smaller cells in older individuals.

Deletion Levels Are High in Tissues with a High Metabolic Rate. The tissues in which we observe higher levels of deletion, brain and muscle, are also known to have a high requirement for ATP, which is generated by an oxidative process. The brain for example uses about 20% of the total oxygen in the body (28). Because damaging oxygen species (superoxide radical, hydroxyl radical, and singlet oxygen) are thought to be produced in mitochondria as side reactions of electron transport (2, 11, 29), it is possible that tissues with elevated oxygen consumption have higher levels of oxidative damage to mtDNA and, consequently, increased deletion mutation frequency. Thus the relatively high metabolic activity of brain and muscle tissues could accelerate the production of several types of mtDNA damage, of which mtDNA^{4977} is perhaps just one representative. If mitochondrial mutation events induce dysregulation of electron transport and further oxidative damage to mtDNA, such events could be "catastrophic" (30).

A Stochastic Model of Deletion Formation and Its Implications for Mitochondrial Disease in Normal Humans. Presumably, the mtDNA^{4977} and other mitochondrial deletions occur during development as stochastic phenomena. As such, rare early mutations (early jackpots) could explain the existence of Kearns–Sayres syndrome and progressive external ophthalmoplegia individuals (17), who carry mtDNA deletions systemically, that is, in all tissues. It has been found that mtDNA deletions in Kearns–Sayres syndrome patients are low in blood and liver, but high in skeletal muscle, consistent with our results in normal humans (17, 31, 32). Therefore, the same cellular forces that constrain deletion levels may be shared between Kearns–Sayres syndrome patients and normal humans.

The expectation of such a stochastic model is that most (normal) humans will not have systemic early jackpots to mutant mtDNA, but the possibility exists of later jackpots in the founder cells of specific organs. Such organ-specific jackpots might be responsible for some organ-specific diseases that are currently idiopathic. The recent development of more quantitative PCR methods (33, 34), as have been applied to the heart (21, 22), should help define the levels of mtDNA^{4977} and other mutations in a broader range of tissues.

The Effect of Mitochondrial Damage on Cellular Physiology. At present, it is not known if mitochondrial deletions occurring at a frequency of 0.1% are sufficient to produce a deleterious physiological effect *in vivo*, as has been discussed (5). Levels of 60–80% mutant mtDNA are required *in vitro* to observe deleterious biochemical effects (35, 36). On the other hand, the mtDNA^{4977} deletion may be representative of other types of mitochondrial genetic damage that accumulate with age, including other deletions and point mutations. If

mtDNA^{4977} is representative of other mitochondrial genetic damage, one would expect brain and muscle tissues to have the highest levels of such damage. If mitochondrial damage has deleterious physiological consequences, then these organs are most likely to be affected. It is intriguing that deficits of brain and heart muscle function are among the most important health risks facing aged humans.

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