Detection of a specific mitochondrial DNA deletion in tissues of older humans

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Received August 31, 1990; Revised and Accepted October 25, 1990

ABSTRACT

Using PCR, we found that normal heart muscle and brain from adult human individuals contain low levels of a specific mitochondrial DNA deletion, previously found only in patients affected with certain types of neuromuscular disease. This deletion was not observed in fetal heart or brain. Experimental tests support the idea that the deletion exists in vivo in adult mitochondria and is not an in vitro artifact of PCR. Our data provide direct experimental support for the idea that accumulation of mitochondrial DNA deletions may be important in aging.

INTRODUCTION

Specific deletions of mitochondrial DNA (dmtDNA) are present at high levels in skeletal muscle and other tissues of patients with the rare and sporadically occurring neuromuscular diseases Kearns-Sayre syndrome (KSS) and progressive external opthalmoplegia (PEO) (1-8).

It has been proposed that somatic mtDNA mutations might occur in the general population and result in lowered cell and tissue function characteristic of advanced age (9-12). However dmtDNA has not been found in blood or skeletal muscle of normal control individuals either related or unrelated to the patients with KSS and PEO (1-3, 6-8). This failure to detect dmtDNA in normal individuals may have been the result of the lack of sensitivity of the methods used or the choice of tissues examined.

Using a combination of PCR strategies, we examined tissues from individuals who did not have neuromuscular disease for the presence of dmtDNA. Each patient with KSS and PEO has characteristic deletion end points which vary among individuals. The most frequently observed deletion is about 5 kb in length (1, 13-16) and occurs at a presumed deletion 'hot spot' involving two 13 base direct repeats beginning at positions 8470 and 13,447 in the mitochondrial genomic sequence. We examined normal individuals for the presence of this specific deletion. Our PCR assay for dmtDNA (4, 7, 15) relied on a large deletion to bring two primers that lie just outside the 13 bp repeats close enough together to efficiently produce a product. We found that selective amplification of dmtDNA was enhanced by significantly decreasing the PCR cycle time used by other groups. These tactics allow much more efficient amplification of deleted than undeleted mitochondrial genomes. Unlike previous studies of normal individuals, we focused on autopsy tissues rich in non-dividing cells, rather than on more easily obtained dividing cell populations such as those found in blood.

MATERIALS AND METHODS

DNA Samples

Human tissue samples consisting of brain, heart and spleen from deceased individuals were collected at autopsy according to standard procedures at LAC/USC Medical Center, and immediately frozen. Seven of the individuals studied were adults. The cause of death, age and sex of these individuals were: lung cancer, 58 years, male; gastrointestinal bleeding, 53 years, male; bacterial endocarditis, 45 years, male; intracranial hemorrhage, 33 years, male and trauma (4 cases); three males 21, 22 and 40 years and one female, 27 years. Two individuals were stillborn males at 34 and 29 weeks with prematurity (male) and trisomy 13 (female), respectively. A fifth female child lived for 4 days and died from trisomy 18.

In addition to the above samples, cerebellar material from 9 brains from individuals ranging in age from 44—104 years was kindly provided by Steve Johnson and the Alzheimer's Disease Research Consortium of Southern California. These samples came from individuals who upon death were found to have no neuropathology.

DNA Purification and PCR analysis

Total DNA was extracted by standard methods. PCR analysis (17-19) was carried out using standard buffers and Taq polymerase (Perkin-Elmer Cetus). The primer and nucleotide concentrations were 1 μM and 200 μM per dNTP respectively. The PCR primers covered the following regions of the Cambridge sequence (43): MT1 (8225-8247), MT2 (13176-13198), MT3 (13551-13574) and MT4 (13707-13729). The sequence GAATTTC was appended to the 5' end of primers MT1 and MT4 to facilitate cloning.

To detect the 5 kb product from undeleted mtDNA using MT1 and MT4, the cycle times were 1 min denaturation at 94°, 1 min annealing at 59° and 5 min extension at 72° for 30 cycles in a Perkin-Elmer thermal cycler. In order to assay for the shorter and rarer dmtDNA products using these same primers the denaturation time was cut to 20 seconds and a single annealing and extension time of 20 seconds at 60° was used. In some cases, an aliquot of the PCR reaction was amplified further using MT4 and a third 'nested' (20) primer, MT3. The short product
conditions were also used with primers MT2 and MT4 to amplify a small fragment present in normal mitochondrial genomes but absent in deleted ones.

For single molecule detection in the 'concordance test' the short cycle times and temperatures were used. Each sample contained approximately 10 pg of total genomic DNA. The 5 preliminary cycles used primers MT1 and MT4. Three aliquots (16 μL) were taken from this preliminary reaction (see Figure 8). Two were amplified with primers MT1 and MT4 for an additional 30 cycles. A 2 μL aliquot from each of these two sister tubes was further amplified with primer MT1 and primer MT3 for yet another 30 cycles. The third control aliquot was amplified after the three-way split for 30 cycles with MT2 and MT4. A 2 μL aliquot of this reaction was added to a second tube, then amplified with MT2 and MT3 for an additional 30 cycles.

RESULTS

Analysis of tissues from normal individuals

Cellular DNA from adult heart muscle was examined using PCR. In our initial experiments (Figure 1, lanes 2 and 3) using primers MT1 and MT4 (Figure 2) and a long PCR cycle time, we detected a product about 5 kb in length, representing amplification of the region between the primers in undeleted mtDNA. When the total PCR cycle time was shortened 10 fold, the 5 kb band was no longer detected. Instead, the adult heart DNA samples produced the 520 base pair product expected if mitochondrial genomes carrying the major KSS deletion were present (lanes 4 and 5). The significantly shorter cycle time presumably enhanced the preferential amplification of the rarer deleted mitochondrial genomes over the longer and more common undeleted ones.

Identification of the deletion junction fragment by restriction mapping and sequencing

The 520 bp PCR fragment from adult DNA was mapped using 7 restriction enzymes (Fig. 2). The results of two of the seven digestions are shown in Figure 3, lanes 6 and 7. In all 7 cases the digestion products had the sizes expected of the major KSS mtDNA deletion junction fragment described by others (1,14,16). The 520 bp PCR product obtained using primers MT1 and MT4 with DNA from a 53 year old adult was sequenced. In the region expected to contain the junction fragment we observed the sequence TACCACCTacctccctaccaTTGGCAG. This sequence contains one 13bp repeat (lower case letters). On one side of this repeat are sequences from the ATPase8 gene while on the other side are sequences from the gene ND5. This junction is identical to the one observed in the majority of patients with KSS and PEO (7, 13–16) and suggests that the deletions we observe in normal individuals have an identical origin. The product of amplification with control primers MT2 and MT4 gave sequence data identical to the Cambridge sequence(43).

Comparison of fetal and adult heart samples

Primers that amplify undeleted mtDNA (MT2 and MT4) produce a strong product in both the fetal and adult samples (Fig 3, lanes 1 and 2). The band characteristic of dmtDNA was not detected when the fetal sample was tested with primers MT1 and MT4 (lane 4) although the adult sample produced a strong product (lane 5).

We compared the amount of deleted product relative to the number of undeleted mtDNA in heart muscle from 5 fetal and 7 adult samples. After determining the amount of total genomic DNA spectrophotometrically, we measured the amount of undeleted mtDNA in each sample using primers MT2 and MT4. The amount of starting material was adjusted so that each sample contained approximately the same concentration of undeleted genomes. Each adjusted sample was subsequently amplified for 20 cycles with primers MT1 and MT4, followed by nesting with primers MT1 and MT3 for an additional 20 cycles. Some of the results are shown in the top row of Figure 4. All of the adult but none of the fetal samples exhibited the 360 bp nested product expected of the major KSS and PEO deletion. Very weak bands
of a larger size were detected in some of the fetal samples. The bottom row of the figure shows the PCR products obtained using the primers specific for the more common undeleted genomes (MT2 and MT4). These results demonstrate that both adult and fetal samples contained approximately the same amount of undeleted mitochondrial genomes, yet only the adult samples exhibited a PCR product characteristic of the most common dmtDNA mutation present in KSS and PEO.

In order to compare the levels of deleted genomes in fetal and adult tissue, we made serial dilutions of several samples which were first normalized with respect to the total amount of mtDNA (Figure 5). Adult heart samples must be diluted more than 100 fold before the 520 bp product is no longer visible. Using a more sensitive detection system (data not shown) adult samples must be diluted 100,000 before the 520 bp band disappears, whereas at the same concentrations, fetal DNA showed no product. Thus, there must be 100,000 fold times less dmtDNA in fetal than adult heart tissue.

Test for fetal PCR inhibitors

The absence of a deletion product in fetal heart could be explained if these samples contained a PCR inhibitor. Two observations would seem to rule out this explanation. All the fetal samples exhibited the PCR product characteristic of undeleted mitochondrial genomes using primers MT2 and MT4 at a total DNA concentration similar to that of the adult samples (bottom row of Figure 4). Secondly, mixing adult with fetal DNA does not prevent the appearance of the adult deletion product (Figure 5).
Ratio of dmtDNA to normal mitochondrial genomes in adult heart

We estimated the ratio of dmtDNA to normal mt genomes in adult heart by testing serial dilutions with primers for both normal and dmtDNA (data not shown). The limiting dilution for the detection of normal mtDNA was 1000 times less concentrated than the limiting dilution that allowed detection of dmtDNA. Thus we estimate that there is approximately 1 dmtDNA molecule for every 1000 undeleted ones in heart tissue of middle-aged adult individuals.

dmtDNA in adult brain

Figure 6 shows the result of a PCR dilution experiment on an adult brain DNA sample using primers MT1 and MT4. All 15 additional adult brain samples, but none of the 5 fetal samples (data not shown) produced a strong 520 bp product. Thus dmtDNA mutations of the most common type found in KSS and PEO are also found in the brain of individuals who died from causes unrelated to neuromuscular disease. The level of product appears similar to that in the heart. Fetal brain DNAs were negative for dmtDNA and were not found to be inhibitory in mixing experiments (data not shown).

Tests of the in vivo origin of dmtDNA

Our data support the conclusion that normal adult heart and brain tissues contain both normal and deleted mtDNA, and are therefore heteroplasmic. The validity of our interpretation is dependent upon two issues: our experiments must be free of contamination by previously amplified PCR deletion products, and the amplification products characteristic of the deletion must not be artifacts of PCR itself.

Three observations support the idea that deletion products are not the result of contamination. 1) No fetal DNA samples produced a deletion product using our PCR conditions. If deletion PCR products were the spurious result of contamination, one would expect contamination in fetal as well as adult samples. 2) Controls without any added DNA were also negative for deletion product. 3) A restriction fragment length polymorphism was detected between 520 bp deletion products from different individuals. Only a few polymorphic sites are known within the sequence of the junction product made by our primers. A rare Hae III site known to be polymorphic (21) was present in the 520 bp product from heart and brain of one individual (Figure 2). Figure 7 shows the mobility difference detected by Hae III digestion of two adult heart samples.

An unlikely but formal possibility is that PCR itself might generate deleted molecules from undeleted ones. This cannot be discounted a priori since a phenomenon known as 'jumping PCR' can occur on DNA if it contains damage that terminates polymerase extension (22). If the extension product of one primer on undeleted mtDNA terminated prematurely within one 13 bp repeat it could, in a later PCR cycle, act as a primer with its 3' end annealed to the other 13 bp repeat. If extended, it could eventually result in the appearance of a PCR product identical in size to one that came from mtDNA deleted between the repeats.

A test (Figure 8) was devised to determine if the deleted mitochondrial PCR products are the result of a preexisting in vivo mutation (Model I) or an in vitro artifact of PCR of normal mtDNA (Model II). If the deletions preexist in a stock sample, and PCR does not generate them (Model I), then dilutions of the stock can be made so that deleted molecules are Poisson distributed; some of the tubes will receive one or more dmtDNA molecules and the rest none, although each sample receives hundreds of normal mitochondrial genomes. Each sample was amplified for 5 cycles (using primers MT1 and MT4) and then divided equally into three reactions. Two of the three reactions (sister tubes) were further amplified using the deletion-specific protocol under conditions sufficient to detect single DNA molecules (20,23,24). The third tube of the three-way split was amplified with primers specific for undeleted mtDNA, as a control. We would have expected that after the 5 initial cycles, the two sister tubes derived from a sample that originally received a single dmtDNA would contain on the average 10.6 copies (32/3) of the dmtDNA sequence, in addition to normal mtDNA. Sister tubes derived from samples that did not originally receive a dmtDNA molecule will have zero dmtDNA product. Further amplification with deletion-specific primers using conditions that are capable of detecting a single DNA molecule should produce concordant results; the two sister tubes will either both produce (+ +) or both lack (− −) the deletion product (Figure 8).

Figure 6. Amplification of 1 μg of an adult brain DNA sample with primers MT1 and MT4 for 30 cycles. Lanes 1, 2 size markers; lanes 3–7, serial 10-fold dilutions of the brain DNA beginning with 1 μg. The arrowhead shows the position of the 520 bp product.

Figure 7. Gel electrophoresis of polymorphic mtDNA amplified from two different adults with primers MT1 and MT4. The 520 bp products were digested with HaeIII, which detects a polymorphism between some individuals (see text). The polymorphic Hae III site is designated by an asterisk in Figure 2. The remaining 20 bp fragment expected from this digestion migrated out of the gel.
In Model II, no mitochondrial deletions preexist, and all deletion products result from jumping PCR on normal mtDNA after the three-way split. It is unlikely that any jumping PCR occurs during the five preliminary cycles because the concentration of the extension products is extremely low. If jumping PCR were to occur, it is much more likely to happen during the subsequent 60 cycles. Since the probability of a jumping PCR event in one tube is independent of its occurrence in a sister tube, the pair could be either concordant (+ + or − −) or discordant (+ − or − +).

Figure 9 shows the results of such an experiment. Because of the nested primer step, the size of the fragment characteristic of the common KSS deletion is 360 bp. The duplicate samples are concordant; 5 are both positive and 10 are both negative for the specific KSS deletion product (in some rare cases bands of a different size appeared and are discussed in the legend to Table I). All together 7 experimental trials consisting of 105 pairs of sister tubes were carried out (Table I). For each trial, the observed frequency of concordant and discordant pairs for the 360 bp band was compared to the expected frequency of such pairs assuming an independent artificial origin after splitting (Model II). Thus we expect \( p^2 + q^2 \) concordant and \( 2pq \) discordant pairs, where \( p \) and \( q \) are the total number of deletion product positive and negative tubes respectively, divided by the total number of tubes. In only one case did we observe discordance for the 360 bp band. As can be seen in Table I, our data strongly reject the hypothesis of independence, and support the idea that the dmtDNA type characteristic of the majority of KSS and PEO cases exists in tissues of normal adults.

**DISCUSSION**

The classic somatic mutational theory of aging, inspired by radiation biology experiments, held that aging is the result of an accumulation of mutational hits in nuclear DNA over time (25). Harman and Ames extended the somatic mutation theory to mitochondrial DNA, with oxygen radicals as the destructive force (9–11). mtDNA contains higher levels of oxidatively damaged bases than nuclear DNA (26), and certain chemical modifications of mtDNA, as well as large deletions, increase with advancing age in rodents and some fungi (27–29). The rodent work (29) was based upon the analysis of heteroduplexes using EM and showed that 5% of liver mtDNA of old mice contain deletions and/or insertions and that the breakpoints may not be randomly distributed. mtDNA is not repaired (30) and is the preferred target of some known toxins and carcinogens (31,32). The rate of mtDNA evolution is faster than that of nuclear DNA, which may reflect its increased susceptibility to mutation (33–34). The recent evidence that mitochondrial mutations cause disease in humans (cited above and reviewed in 35) stimulated others (12,36) to propose that the accumulation of damage to mtDNA is a major cause of age-related degenerative human disease.

Our results show that a specific dmtDNA, which is known to

![Diagram](image-url)

**Figure 8.** Scheme for carrying out a concordance test to determine if the PCR products expected from dmtDNA are artifacts of the amplification process. Assuming that model I is true, that amplification efficiency is 100%, and that the three-way split divides the PCR products equally, we expect only two types of sister tube observations (+ +) and (− −). In model II, 4 possibilities exist (+ +), (− −) (− +) and (− +), but for clarity of the drawing, only two are shown.
be pathogenic, is present in heart and brain of older individuals who died from causes unrelated to neuromuscular disease. We failed to detect dmtDNA in fetal heart and brain, and estimate that fetal tissues must have from 1/100 to 1/100,000 times less of the deletion than adults.

One possible explanation for the difference between adult and fetal tissues is that dmtDNA accumulates with age. Because it is found at high levels in KSS and PEO, dmtDNA is probably replicated, and consistent with this notion, no dmtDNA has been described where either or both origins of replication (37) are absent. Some have proposed that dmtDNA might have an intracellular advantage over normal mtDNA either because shorter molecules are replicated faster than longer ones (35), or sequences involved in down-regulating mtDNA replication are lost in the deletion event (38). While the hypothesis of a cumulative increase of dmtDNA in normal individuals is appealing, many more samples from intermediate age groups will need to be looked at for confirmation.

dmtDNA has not been found in human tissue culture cells or in normal spleen (39, and our unpublished data). This suggests that intercellular selection of the kind proposed by Holt et al. (1), may be acting. If dmtDNA is even slightly deleterious in low copy number to cells that carry it, those cells would be expected to produce fewer daughters than cells with less dmtDNA. This type of intercellular selection could act in populations of dividing cells but could not act in adult tissues composed primarily of non-dividing cells such as terminally differentiated heart muscle and brain.

We estimate that in the heart of middle aged adults there is approximately 1 dmtDNA molecule of the common KSS type for every 1000 undeleted ones. Because we found dmtDNA in all 16 adult brains and all 7 adult hearts we tested, it is likely that most adult humans carry this somatic mutation. The fraction of dmtDNA we measure in normal individuals is about 0.1%, and contrasts with the proportion of dmtDNA seen in skeletal muscle of patients with KSS and PEO, that ranges from 20–90%.

Could the dmtDNA levels we observe contribute to the progress of age-related degenerative diseases? The number of mitochondrial genomes per cell is on the order of a thousand (40). Therefore, a dmtDNA level of 0.1% translates into about 1 dmtDNA molecule of this type per cell on average. At this time we do not know if the deletions we have described are distributed randomly among the cells of a tissue or whether some cells have very high proportions of dmtDNA.

Whether or not dmtDNA at low levels has an effect on tissue function is unknown. At high levels such as in skeletal muscle of patients with KSS, the presence of dmtDNA can have a dominant deleterious physiological effect (38). The threshold level necessary for dmtDNA to produce such effects is unknown. We have examined tissues that are functioning normally, and do not yet know if tissues exhibiting age related degenerative changes have even higher dmtDNA levels. If dmtDNA levels reached a threshold level in a particular tissue during the course of an individuals' life pathological effects similar to those seen in KSS could arise.

In addition to essential tRNA genes (41), a critical part of the enzyme cytochrome oxidase (subunit III) is included in the common deletion detected by our primers. Cytochrome oxidase is the major site of reduction of molecular oxygen in normal cells. The presence of a deficient cytochrome oxidase complex could lead to increased levels of cellular oxidative damage.

The loss of cytochrome oxidase activity occurs mosaically among muscle fibers in KSS (38). A mosaic pattern of loss of cytochrome oxidase activity is also found to increase with age, in cardiomyocytes of normal older humans (42). In the former case, the coexistence of dmtDNA and normal mtDNA is associated with this loss, which may be due to the dmtDNA acting in a dominant fashion (38). Age-related loss of this enzyme...
activity in normal older individuals could have the same explanation.

The reviewers of this manuscript brought to our attention the paper of Ikebe et al. (44) which was published after our work was completed. They used PCR to detect the common KSS and PEO mtDNA deletion in brain tissue of patients with Parkinson’s disease. They found the deletion in all 5 Parkinson patients but not all of their 6 adult control individuals although they carried out only 20 PCR cycles. Additional studies will be needed to confirm their interesting observation.

ACKNOWLEDGEMENTS

We thank C.E. Finch, S. Johnson, W. Navidi, D. Shibata, M. Stoneking, W.K. Thomas, J. Valentine and A.C. Wilson for helpful comments and DNA samples. This work was supported in part by NIH grants GM36745 and GM42942 (NA) and NIA training grant AG00093 (C.E. Finch).

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