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Age-related Human mtDNA Deletions: A Heterogeneous Set of Deletions Arising at a Single Pair of Directly Repeated Sequences

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Summary

Deletions in mtDNA accumulate during the human aging process, arising from either intramolecular illegitimate recombination or strand slippage during replication, which results in subgenomic mtDNA molecules. We identify here two classes of mtDNA deletions—class A deletions, which are homogeneous at the breakpoints, with all subgenomic molecules therefore being identical in size, and class B deletions, which arise from a less stringent process that gives rise to heterogeneity at the breakpoints, with the subgenomic molecules being of slightly different sizes. A novel approach is described that offers a global overview of the populations of different deletions in individual tissues. It is based on PCR cycle-sequencing reactions that are carried out directly on mtDNA segments, amplified by PCR from total cellular DNA. The results show a clear size homogeneity of the subgenomic mtDNA molecules representative of class A, which carry a commonly detected 4,977-bp deletion occurring at a pair of 13-bp directly repeated sequences. In this case, precisely one copy of the repeat is retained in the subgenomic molecules. We then describe a class B situation comprising a family of at least nine closely related 8.04-kb deletions involving the same pair of 5-bp direct repeats. In this situation, the breakpoints differ at the base-pair level (8,037–8,048-bp deletions); the subgenomic molecules retain >1 copy, 1 copy, or <1 copy of the 5-bp repeat. In different tissues from either the same individual or among different individuals, there is a widely variable occurrence of particular deletions in the subgenomic mtDNA population within this class B set. Class B deletions offer a new approach for studying the accumulation of mtDNA deletions, thereby providing insight into the independent somatic origin of mutated mtDNA molecules, both in aging and in mitochondrial diseases. We also report a convenient method for ascertaining whether a given PCR product results from the amplification of a subgenomic mtDNA template, on the basis of the selective degradation of full-length mtDNA molecules prior to PCR.

Introduction

The accumulation of mtDNA mutations leads to derangement of the respiratory chain function of human cells. High levels of subgenomic mtDNA molecules carrying large deletions, detected by Southern blotting to comprise 5%–90% of total mtDNA in tissue extracts, have been observed in patients affected by mitochondrial myopathies (Holt et al. 1988*a*, 1988*b*; Lestienne and Ponsot 1988; Zeviani et al. 1988) and by Pearson marrow/pancreas syndrome (Rötig et al. 1988, 1989). Studies on mtDNA deletions by using PCR methodology have also indicated the occurrence of subgenomic molecules in patients with cardiomyopathy (Ozawa et al. 1990) and Parkinson disease (Ikebe et al. 1990).

A general proposal was recently made by Linnane et al. (1989) that links the accumulation of mtDNA mutations to the aging process in humans. According to this view, the accumulation of mtDNA mutations in aging represents a significant contributory factor to the decline of mitochondrial energy production that characterizes the aging process in many tissues (Trounce et al.

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1989; Yen et al. 1989). The age-associated accumulation of the 5-kb common deletion, detected by PCR techniques, was first reported in a wide range of human tissues by Linnane et al. (1990), Cortopassi and Arnheim (1990), and Yen et al. (1991). Other age-associated deletions in mtDNA have been reported, such as a 7.4-kb deletion in cardiac tissue (Hattori et al. 1991) and a 6-kb deletion in liver (Yen et al. 1992). Indeed, aging is characterized by the accumulation of multiple mtDNA deletions in many tissues (Linnane et al. 1992*b;* Zhang et al. 1992). Nevertheless, the abundance of subgenomic molecules carrying a particular deletion in mtDNA in tissues of aging individuals is low compared with that in tissues of patients with overt mitochondrial diseases.

Most mtDNA deletions in mitochondrial disease and aging are flanked by short direct repeats of 4–13 bp in length (data compiled by Wallace et al. 1991; Wallace 1992*a;* Wei 1992; Zhang et al. 1992). The most frequently encountered deletion, referred to as the "common deletion," occurs between two short directly repeated sequences, 13 bp in length, and spans 4,977 bp of the mitochondrial genome (Schon et al. 1989). In all reports from studies on both aging and mitochondrial diseases, precisely the same length of deleted sequences (i.e., 4,977 bp), together with retention of one copy of the 13-bp direct repeat, has been reported (e.g., see Rötig et al. 1989; Schon et al. 1989; Shoffner et al. 1989; Cortopassi and Arnheim 1990; Linnane et al. 1990; Ozawa et al. 1990; Degoul et al. 1991).

It is already apparent, however, that such homogeneity of excision events does not apply to all deletions in human mtDNA. Thus, a number of slightly different deletions have been found for an 8.04-kb deletion that is based on the same 5-bp direct repeat. In a 69-year-old individual without overt mitochondrial disease, two different 8.04-kb deletions were observed (Zhang et al. 1992)—an 8,041-bp deletion was detected in brain and an 8,044-bp deletion in muscle. A second elderly individual (age 72 years) contained, in muscle, an 8,043-bp mtDNA deletion centered on the same pair of direct repeats (Linnane et al. 1992*b*). Results obtained from a family affected by a mitochondrial myopathy that is generated by an autosomal dominant mutation producing multiple mtDNA deletions (Zeviani et al. 1989) indicated subgenomic mtDNA molecules representing three different breakpoints related to the 8.04-kb deletion—namely, an 8,039-bp deletion detected in the skeletal muscle of one patient and 8,040- and 8,041-bp deletions in skeletal muscle of her paternal aunt.

The central issue addressed in this paper relates to a novel aspect of the multiplicity of mtDNA deletions in human tissues. Specifically, we ask whether the recombination events centered on a particular pair of direct repeat sequences lead to a single homogeneous outcome. We describe a procedure based on DNA sequencing analyses, carried out directly on PCR products amplified from cellular DNA, to determine whether the subgenomic molecules arising from a particular excision site may vary in their size. We demonstrate, in the case of the 8.04-kb deletion, that such heterogeneity is generated by slightly different outcomes of the deletion process.

Material and Methods

Human Tissues

Postmortem tissue samples were obtained within 24 h after death and were stored at −80°C.

DNA Isolation

Total cellular DNA was extracted from about 100 mg of human tissue samples, as described by Linnane et al. (1990).

PCR

PCR reactions were carried out in an Innovonics water/oil bath machine for 30 cycles, as described elsewhere (Linnane et al. 1990), comprising an initial denaturation step at 95°C for 300 s, but for 60 s in subsequent cycles; annealing at 56°C for 90 s; and primer extension at 72°C for 150 s. *Taq* polymerase was purchased from Biotech (Western Australia).

Oligonucleotide Primers

Primers were synthesized using an Applied Biosystems model 380A DNA Synthesizer and were purified using Applied Biosystems OPC columns. The designations and positions of primers, according to the nucleotide numbering, for human mtDNA, of Anderson et al. (1981), are as follows: L7901, 7901–7920; L14820, 14820–14842; H8521, 8540–8521; H13631, 13650–13631; H15658, 15680–15658; and H16514, 16540–16514. The prefixes "L" and "H" denote the particular strand sequence (light and heavy strands, respectively) represented in each primer.

DNA Purification, Cloning, and Sequencing

PCR products were treated with Klenow fragment as described by Obermaier-Kusser et al. (1990) in order to remove overhanging A nucleotides added at the 3′ ends by *Taq* polymerase; the products were then separated by gel electrophoresis. The desired DNA products were

excised from gels, purified using GeneClean Kit (Bio 101), and cloned into pUC18. Double-strand sequencing from recombinant plasmid clones was carried out using the Pharmacia T7 sequencing kit according to the manufacturer's instructions. Radiolabeled products were separated on 0.4-mm-thick sequencing gels (6.7% acrylamide, 0.35% N,N'-methylene-bis-acrylamide), under standard conditions (Sanger et al. 1977) in a BioRad sequencing apparatus.

PCR Cycle Sequencing

To purified DNA (0.1–1 μg in 10 μl water) was added the same volume of a mixture containing 150 mM Tris-HCl (pH 9.0), 25 mM $MgCl_2$, a single oligonucleotide primer (4 pmol), 20 μCi[α-^{35}S]dATP (1,500 Ci/mmol), and *Taq* DNA polymerase (2.5 units). Aliquots of 5 μl each were added to four separate tubes containing the appropriate mixes of deoxyribonucleoside triphosphates (Pharmacia) and a dideoxy analog (Boehringer Mannheim), as follows: G-mix—10 μM dGTP, 100 μM ddGTP, 3 μM dATP, 20 μM dTTP, and 20 μM dCTP; A-mix—20 μM dGTP, 3 μM dATP, 180 μM ddATP, 20 μM dTTP, and 20 μM dCTP; T-mix—20 μM dGTP, 3 μM dATP, 10.4 μM dTTP, 1,600 μM ddTTP, and 20 μM dCTP; and C-mix—20 μM dGTP, 3 μM dATP, 20 μM dTTP, 10 μM dCTP, and 800 μM ddCTP. The PCR-amplification cycles and subsequent analysis of products on sequencing gels were carried out as described above.

Bal 31 Treatment of DNA Samples

Aliquots of approximately 3–5 μg of total cellular DNA extracted from human tissues were digested with the appropriate restriction enzymes. The mixtures were then directly diluted to 100 μl in Bal 31 buffer (final concentration of 20 mM Tris-HCl, 100 mM NaCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 1 mM EDTA, and 65% glycerol, pH 7.5) with 3 units Bal 31 (Boehringer-Mannheim). The reactions were carried out at 37°C for variable times and were stopped by incubation at 100°C for 10 min. Small aliquots of treated DNA (0.5–1 μl) were used directly in PCR-amplification reactions.

Results

Age-related Accumulation of a 5-kb and an 8.04-kb Deletion

We have reported elsewhere the age-related accumulation of subgenomic mtDNA molecules bearing the 5-kb deletion detected by PCR amplification of total DNA extracted from human tissues (Linnane et al. 1990, 1992*a*). In that work, a 773-bp product was amplified with primers L7901 and H13631, covering the breakpoint of the 5-kb deletion (these primers would amplify a 5,750-bp segment of normal mtDNA).

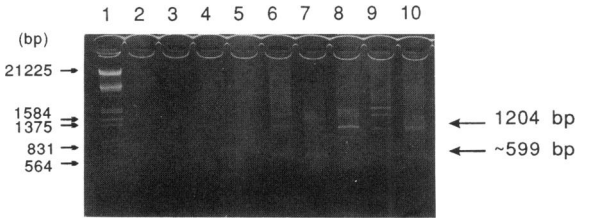

Figure 1 Detection of age-related 8.04-kb deletions. Total cellular DNA from human tissue samples was subjected to PCR amplification with primers L7901 and H16514; products were separated on a 1% agarose gel and photographed after staining in ethidium bromide. Lane 1, Lambda DNA digested with *Eco*RI and *Hin*dIII. The sizes (bp) of some fragments are shown to the left. Lane 2, Liver of an 80-min-old infant. Lanes 3–5, Diaphragm, liver, and heart of a 3-mo-old infant. Lanes 6 and 7, Skeletal muscle and left atrium of a 40-year-old individual. Lane 8, Diaphragm of a 46-year-old individual. Lane 9, Skeletal muscle of a 50-year-old. Lane 10, Skeletal muscle of a 72-year-old. The 8.04-kb deletions are represented by ~599-bp products (indicated by an arrow to the right). A 1,204-bp PCR product representing a 7.4-kb deletion (Zhang et al. 1992) is also indicated.

Similarly, for the detection of the 8.04-kb deletion, which involves two 5-bp direct repeats, PCR amplification with primers L7901 and H16514 was used to generate a product of 0.6 kb (the binding sites for this primer pair are 8,640 bp apart on normal human mtDNA). As illustrated in figure 1 (lanes 6–10), by using these primers for PCR amplification of mtDNA from adult tissues, multiple PCR products representing a number of other mtDNA deletions were coamplified together with the 0.6-kb product. This primer pair did not yield any products from DNA in tissue extracts of infants (lanes 2–5). It was in such PCR amplifications, using the primer pair L7901 and H16514, on DNA in tissue samples of adult individuals, that we previously reported (Linnane et al. 1992*b*; Zhang et al. 1992) individual deletions, in the 0.6-kb band, of 8,041, 8,043, and 8,044 bp in length. Furthermore, we reported (Zhang et al. 1992) that the 1,204-bp PCR product corresponded to a deletion of 7.4 kb centered on a pair of 12-bp direct repeats.

We first deal here with the age relationship of the 8.04-kb deletion. A variety of tissues from two infants and seven adults were analyzed from the set of tissues described by Linnane et al. (1990, 1992*a*). To summarize the data (not shown in detail here), the 8.04-kb deletion was detected after 30 cycles of PCR amplifica-

tion only in DNA samples from adults and mainly in skeletal muscle, cardiac muscle, and brain. This deletion was less prevalent in liver and kidney. The 8.04-kb deletion was not detected in any tissues from infants, even after 60 cycles of PCR amplification. However, in ~50% of the tissues of adults in which the 8.04-kb deletion was not initially detected after 30 cycles of PCR, the relevant PCR product of 0.6 kb was detectable after 60 cycles of amplification (data not shown). This emphasizes the variable levels of the subgenomic mtDNA molecules carrying the 8.04-kb deletion in different tissues of adults.

Principles of Determining Heterogeneity at the Breakpoints of mtDNA Deletions

A method was developed to test directly the potentially heterogenous nature of the breakpoints of deletions. It avoids the sampling errors inherent in sequence analysis of independently cloned DNA segments from a PCR product. In the new method, PCR products containing the deletion breakpoints are amplified, as usual, from total cellular DNA extracted from tissue samples. After gel electrophoresis, the relevant PCR products are purified, before digestion with the appropriate restriction enzyme. These smaller fragments are used as templates for a PCR reaction using a single primer only. Such amplification conditions generate products of just one strand, extending from the primer itself to the terminal position determined by the restriction enzyme cleavage. The site of priming and the restriction enzyme cleavage site are chosen to encompass the breakpoint of the deletion (illustrated in two particular cases in figs. 2*A* and 3*A*).

With a single primer, PCR cycle-sequencing reactions are carried out using mixes of deoxyribonucleoside triphosphates and dideoxy analogs. The products are then separated on sequencing gels, so as to visualize both the complete extension products and the nucleotide sequence up to and past the breakpoints. With *Taq* polymerase, complete extension products of such a PCR cycle-sequencing reaction give rise to a double band, as an additional adenine nucleotide is added by *Taq* polymerase at the 3′ end of about half of the newly synthesized DNA strands (Clark 1988). This feature of *Taq* polymerase does not affect dideoxy-terminated intermediates that generate readable DNA sequence. Further, as is shown below, the complete extension product doublet can be avoided by use of *Vent* polymerase for PCR amplification.

On the basis of the foregoing, class A deletions representing a completely homogeneous set of deletions would give rise only to a double band for the complete extension products obtained from PCR cycle-sequencing reactions with *Taq* polymerase. In the case of the 5-kb deletion, this doublet is 225 and 226 nt in size (fig. 2*A*). For a set of heterogeneous breakpoints, predicted for a class B family of deletions, an overlapping set of doublet bands of slightly different sizes is expected. In the case of the 8.04-kb putative family, the median lengths are expected to be represented by a doublet of 188 and 189 nt (fig. 3*A*). With regard to the sequence ladders generated by this PCR cycle-sequencing technique, a class A deletion should generate a coherent readable single sequence all the way up to the complete extension product, since the outcome of the deletion events at the breakpoint is homogeneous. By contrast, in class B deletions, the sequence should be uniform and readable up to the breakpoint region but, depending on the extent of heterogeneity, may represent two or more overlapping sequences beyond the first position at which heterogeneity is encountered.

Homogeneity at the Breakpoint of the 5-kb Deletion

The sequence homogeneity at the breakpoints of the 5-kb deletion, in a number of different tissues, was empirically demonstrated using the new method (fig. 2). The 0.77-kb products obtained from human tissue DNA samples by an initial PCR amplification with primers L7901 and H13631, using *Taq* polymerase, were digested with *Ssp*I and gave rise to 225-bp fragments containing the breakpoints of the 5-kb deletion. The 225-bp fragments were used as templates in the subsequent PCR cycle-sequencing reactions using primer H13631. Figure 2*B* (panel 1) shows the results obtained with cloned template DNA, which was constructed by inserting the 0.77-kb PCR product into the *Sma*I site of pUC18 and which was used to provide a control homogeneous template. A double band was detected for the complete extension products, with the lower band representing the length of the template (225 nt) and the upper band (226 nt) representing molecules with an additional A nucleotide at the 3′ end. Figure 2*B* (panel 2) shows the separation of the complete extension products (225 and 226 nt) in the sequencing reactions, obtained from the analyses of DNA from a brain sample of an 87-year-old individual. A doublet is observed that is identical in size to that generated by cloned control template in panel 1, indicating the homogeneity of the 5-kb deletion in this tissue sample.

Figure 2*C* shows a more extensive gel display of the sequencing reactions carried out on preparations from five other tissues: skeletal muscle from a 40-, a 50-, and a 72-year-old individual; right ventricle from a 46-year-

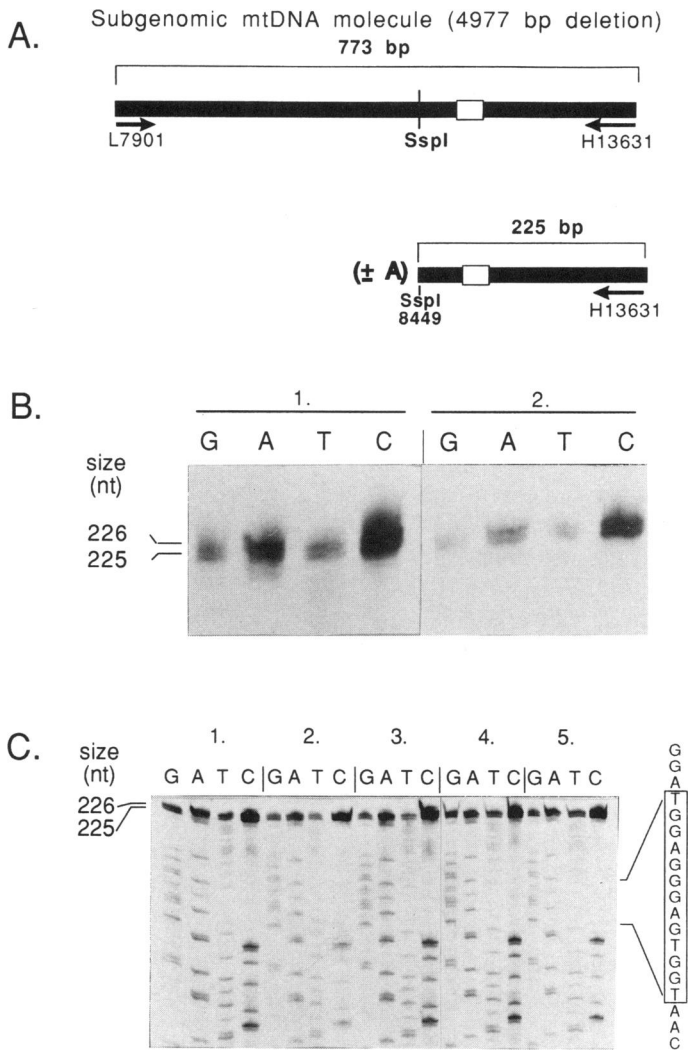

Figure 2 Homogeneity of the 5-kb deletion in different tissues. *A*, Schematic representation of the strategy used to analyze the breakpoint of the 5-kb deletion. The 225-bp H13631-*Ssp*I fragments (nucleotide positions 13650–8449), obtained from digestion of the 773-bp PCR products of subgenomic mtDNA templates amplified with primers L7901 and H13631 from human tissue samples, were subjected to PCR cycle-sequencing reactions using primer H13631. *B*, Homogeneity of the 5-kb deletion in (lanes 1) a 0.77-kb PCR product cloned into pUC18 and (lanes 2) a brain sample of an 87-year-old individual. The complete extension products corresponding to the length of the templates and products with an additional A nucleotide at the 3′ terminus are shown (225 nt and 226 nt, respectively). *C*, Homogeneity of the 5-kb deletion in five other human DNA samples. Sequencing data for PCR cycle-sequencing reactions on DNA templates (prepared as H13631-*Ssp*I fragments) are shown for the following tissue samples (age, tissue). Lanes 1, 40 years, skeletal muscle. Lanes 2, 46 years, right ventricle. Lanes 3, 50 years, skeletal muscle. Lanes 4, 72 years, muscle. Lanes 5, 80 years, brain. To the right, the inferred sequence is shown, covering the breakpoint and encompassing the residual single copy of the 13-bp direct repeat (*boxed*). In this partial sequence, the uppermost G is nt 8467, and the lowermost C is nt 13462.

old; and brain from an 80-year-old. In all samples, a double band was detected at the position of the complete extension products (225 and 226 nt). The sequencing ladders for each tissue indicate an unambiguous unique sequence encompassing the precise breakpoint of the 5-kb deletion. These data thus reflect homogeneity at the breakpoint of the 5-kb deletion (class A deletion) within each tissue.

Heterogeneity at the Breakpoint of the 8.04-kb Deletion

The population of 8.04-kb deletions in individual tissues was analyzed by the same procedure—namely, a

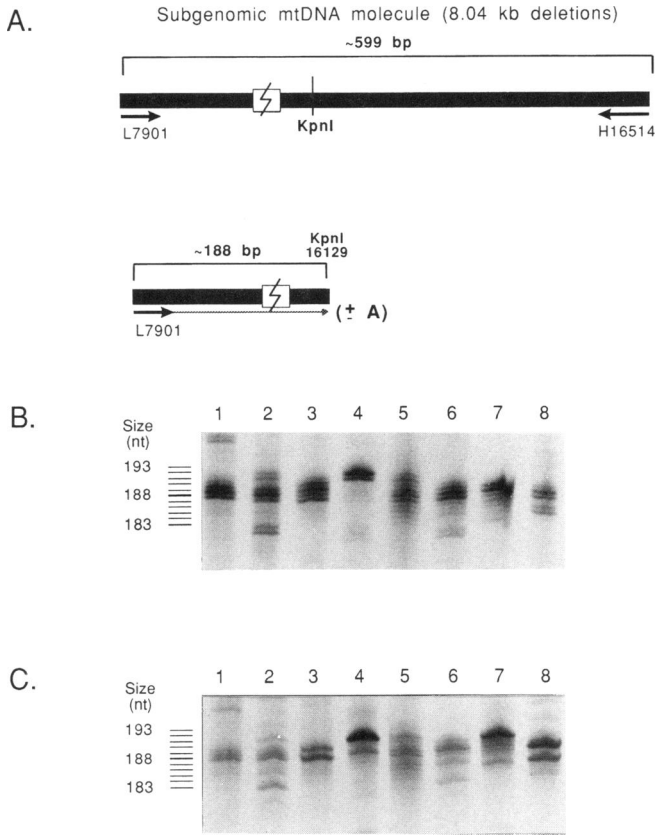

Figure 3 Heterogeneity of the 8.04-kb deletions in different tissues. *A*, Schematic representation of the analyses of the breakpoints of the 8.04-kb deletions by PCR cycle sequencing of the ~188-bp L7901-*Kpn*I fragments (nucleotide positions 7901–16129), obtained from digestion of the 0.6-kb PCR products of subgenomic mtDNA templates amplified with primers L7901 and H16514. These fragments were subjected to PCR cycle sequencing using primer L7901. *B*, Heterogeneity of the 8.04-kb deletions in a number of human tissues. The G-tracks of the sequencing products, obtained with *Taq* polymerase, corresponding to the complete extension products on DNA templates (prepared as L7901-*Kpn*I fragments) are shown for the following tissue samples (age, tissue). Lane 1, 40 years, left ventricle. Lane 2, 40 years, right ventricle. Lane 3, 46 years, right atrium. Lane 4, 50 years, skeletal muscle. Lane 5, 69 years, brain. Lane 6, 69 years, heart. Lane 7, 69 years, skeletal muscle. Lane 8, 72 years, muscle. *C*, Products obtained in PCR cycle-sequencing reactions (G-tracks, complete extension products) carried out as for panel *B* but using *Vent* polymerase instead of *Taq* polymerase. Each lane corresponds to the same tissue as described in panel *B*.

number of DNA extracts from different human autopsy tissue samples were initially subjected to PCR amplification with primers L7901 and H16514. The 0.6-kb PCR products were digested with *Kpn*I, which gave rise to ~188-bp fragments containing the breakpoints of the subgenomic mtDNA molecules representing the 8.04-kb deletions and the priming site for the PCR primer L7901. The ~188-bp L7901-*Kpn*I fragments were used as templates for PCR cycle sequencing, using *Taq* polymerase and L7901 as the sequencing primer (fig. 3*A*). Electrophoretic separation on sequencing gels revealed the variability in size, at a level of resolution corresponding to single base pairs, of individual members of the family of 8.04-kb deletions.

The heterogeneity at the breakpoint of the 8.04-kb deletion is shown in figure 3*B*, where the overlapping of doublets representing different size variants is evident (as predicted for a class B family of deletions). The photograph shows the complete extension products of the G-tracks of the PCR cycle-sequencing reactions using the ~188-bp fragments as templates and presents a representative sample of tissues with a range of heterogeneity at the breakpoints of the 8.04-kb deletions. In some tissues, the results obtained from PCR cycle sequencing showed that several different 8.04-kb deletions were found to be present in the same tissues; for example, at least six different 8.04-kb deletions were detected in the 69-year-old brain and heart samples (lanes 5 and 6).

Table 1

Occurrence of Different 8.04-kb Deletions in Various Tissues from Individual Human Subjects

Age (years)	Tissue	Sizes (bp) of 8.04-kb Deletions Detected by PCR Cycle Sequencing	
		Major population	Minor Population[a]
40	Left atrium	8039	nd
	Left ventricle	8040, 8041	8039
	Right ventricle	8037, 8040, 8041, 8047	
	Skeletal muscle	8039	nd
46	Diaphragm	8037	nd
	Right atrium	8039, 8040, 8041	
50	Skeletal muscle	8037	8040, 8041, 8048
69	Brain	8041	8037, 8038, 8039, 8040, 8043
	Heart	8040	8038, 8039, 8041, 8043, 8047
	Muscle	8038	8039, 8040, 8044
72	Muscle	8040, 8043	8039
	Heart	8040, 8041	nd

Note.—Data from fig. 3*B* and other data not shown.
[a] nd = not detected.

The number of different deletions in other tissues (e.g., muscle of a 72-year-old) amounted to two or three (lane 8). In others a clear predominance of one member of the family of 8.04-kb deletions was detected (e.g., lane 4, which is skeletal muscle of a 50-year-old individual).

The PCR cycle-sequencing results were repeated several times on the tissues, using *Taq* polymerase, and results were essentially identical to those displayed in figure 3*A*. In a few experiments, PCR cycle sequencing was carried out with *Vent* polymerase in place of *Taq* polymerase. The former enzyme gave rise to a single complete extension product per DNA template, such that the rate of nontemplated terminal addition of nucleotides is apparently minimal. Use of *Vent* polymerase on a subset of tissue DNA samples (fig. 3*C*) revealed a similar proportion of the variously sized templates to that inferred from the *Taq*-generated results (compare lanes 1–8 of fig. 3*B* with corresponding lanes in fig. 3*C*). For example, the major doublet in lane 4 of figure 3*B* is replaced by a single predominent band in lane 4 of figure 3*C*.

Determination of the exact sizes of the different 8.04-kb deletions was achieved by precise measurement of the lengths of the complete extension products of PCR cycle-sequencing reactions, with reference to the mobilities of products from known templates, run alongside the samples on the sequencing gels. Table 1 summarizes a range of tissues in which the 8.04-kb deletion was detected. The deletions in each tissue are subdivided in major or minor populations according to the intensities of the complete extension products detected by PCR cycle sequencing. The number of different-sized deletions, their relative abundance, and the representation of particular deletions each vary between individuals. These parameters may also vary markedly in tissues from the same individual.

Characterization of the 8.04-kb Deletion Breakpoints

DNA sequences of the breakpoints of the individual mtDNA molecules bearing the 8.04-kb deletions were characterized, where possible, using PCR cycle-sequencing reactions. Useful sequence data (not visible in figure 3*B* or 3*C* because of the faintness of the bands in photographs, although unambiguous in the original autoradiograms which contained all four tracks—G, A, T, and C) could only be achieved where a single deletion strongly predominated in a particular tissue extract. In other cases, where the overlapping of mixed populations hindered the reading of the DNA sequence, the sequence information was obtained by double-strand sequencing of cloned 0.6-kb PCR-amplified products. In these cases, not all variants present in a given tissue sample would necessarily be detected because of random selection of a small number of individual clones for sequencing.

In the tissues examined, the lengths of the PCR prod-

Table 2

Breakpoints of Eight Different 8.04-kb Deletions

Deletion Size (bp)	Deletion Breakpoint	Size of PCR Product (bp)	Comment
8,037	TTGAAGCCCCCA(*TTCG . . . A*)CCCATCAACAACCG	603	More than one equivalent repeat retained
8,038	TTGAAGCCCCCA(*TTCG . . . AC*)CCATCAACAACCG	602	More than one equivalent repeat retained
8,039	TTGAAGCCCCC(*ATTCG . . . AC*)CCATCAACAACCG	601	More than one equivalent repeat retained
8,040	TTGAAGCCCCC(*ATTCG . . . ACC*)CATCAACAACCG	600	More than one equivalent repeat retained
8,041	TTGAAGCCCCCAT(*TCG . . . ACCCAT*)CAACAACCG	599	One equivalent repeat retained
8,043	TTGAAGCCCC(*CATTCG . . . ACCCA*)TCAACAACCG	597	Less than one equivalent repeat retained
8,044	TTGAAGCCC(*CCATTCG . . . ACCCA*)TCAACAACCG	596	Less than one equivalent repeat retained
8,047	TTGAAGCCC(*CCATTCG . . . ACCCATCA*)ACAACCG	593	Less than one equivalent repeat retained
	8030 16071		

NOTE.—The breakpoints of the 8.04-kb deletions were determined by PCR cycle sequencing of the 0.6-kb PCR products (fig. 3*A*) and double-strand sequencing of cloned PCR-amplified fragments (see text). In the sequence details of deletion breakpoints, the 5-bp direct repeats involved in the deletion are underlined (numbers below the sequences indicate the first nucleotide of the repeats), and the deleted regions are shown in italics within the parentheses.

ucts derived from subgenomic mtDNA molecules corresponded to deletions of 8,037–8,048 bp. The DNA sequences at the breakpoint of eight different 8.04-kb deletions are listed in table 2. The results show the close relatedness of the 0.6-kb PCR products, as well as indicate that the size variability of such products is due to deletion events occurring at the same 5-bp repeats. The size of the deletion in which only one full copy of the 5-bp repeat is maintained is 8,041 bp. In four of the characterized deletions (i.e., deletions of 8,037, 8,038, 8,039, and 8,040 bp), only part of one repeat is deleted. In the cases of the members of this family carrying the two largest deletions for which data have been obtained (i.e., deletions of 8,044 and 8,047 bp), most of the sequences of both repeats are deleted.

Tests Showing Whether PCR Products Are Generated from Subgenomic mtDNA Molecules Carrying Deletions

An important aspect of these studies is the elimination of the possible artifactual origin for the PCR products interpreted to represent subgenomic mtDNA molecules occurring in vivo at very low abundance. In the course of our work on mtDNA deletions, we have observed that a number of amplified mtDNA molecules appeared not to be age related and are not, in fact, amplification products of genuine deleted mtDNA templates. Thus, the PCR system used in these experiments has resulted, at times, in the amplification of nonspecific products because of false priming or in vitro recombinations (data not shown here). It was, therefore, necessary to confirm the nature of the templates that gave rise to the PCR products analyzed here, even though they do satisfy the primary criterion of being age related.

A method was devised in order to prove the in vivo origin of the subgenomic mtDNA templates extracted from human tissues. The principle of the method is based on the selective degradation of normal mtDNA molecules before PCR amplification; in contrast, circular subgenomic mtDNA molecules bearing deletions are not degraded and are subsequently amplified by PCR. This was achieved by digesting DNA samples with restriction enzymes that have recognition sites only within the deleted DNA segments; subgenomic molecules are not cleaved, whereas full-length mtDNA molecules are linearized. Treatment with exonuclease Bal 31 then caused degradation of normal mtDNA but not of subgenomic mtDNA, since this enzyme selectively attacks linear DNA but spares circular DNA. The method was used to prove that the relevant PCR products were derived from the commonly detected 5-kb deletion and the family of 8.04-kb deletions described in this study. Furthermore, this test was also applied to a further PCR product, representing a subgenomic mtDNA molecule coamplified with the 0.6-kb products representing the 8.04-kb deletions. Such coamplified molecules arise from subgenomic mtDNA carrying a 7.4-kb deletion (Zhang et al. 1992), which, as shown in figure 1, was detected as a 1.2-kb PCR product by using primers L7901 and H16514.

Figure 4*A* shows the PCR products obtained from the analyses of the 5-kb deletion. In this case, digestion

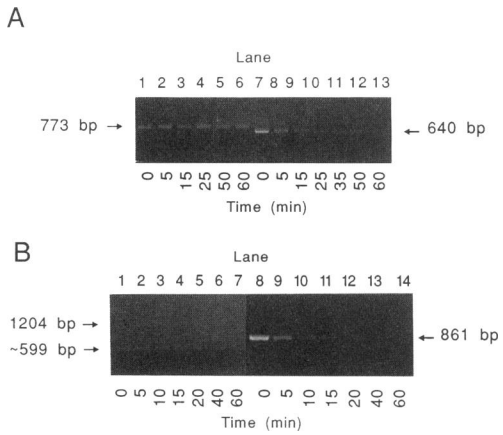

Figure 4 Confirmation that PCR products arise from subgenomic mtDNA molecules. *A*, Tests on the PCR products covering the 5-kb deletion. Total DNA extracted from skeletal muscle sample of a 40-year-old individual was subjected to *Sna*BI digestion (cleavage site at nucleotide position 10734), with subsequent Bal 31 treatment for up to 60 min. After the indicated incubation time (min), PCR amplifications were carried out. Primers for PCR were L7901 and H13631 (lanes 1–6), which amplify a 773-bp product across the breakpoint of the 5-kb deletion (fig. 2*A*), and L7901 and H8521 (lanes 7–13) which amplify a 640-bp product of wild-type mtDNA, the sequences of which extend into the segment of mtDNA deleted in the putative subgenomic molecules. *B*, Tests on PCR products representing the subgenomic mtDNA molecules bearing the 8.04-kb and 7.4-kb deletions. Total cellular DNA extracted from skeletal muscle of a 40-year-old was digested with *Bam*HI (cleavage site at position 14258), followed by Bal 31 treatment for up to 60 min. After the indicated incubation time (min), PCR was carried out. Primers for PCR were L7901 and H16514 (lanes 1–7), which amplify an ~599-bp product across the breakpoint of the 8.04-kb deletion (fig. 3*A*) and a 1,204-bp product (see fig. 1) corresponding to a 7.4-kb deletion (Zhang et al. 1992), and L14820 and H15658 (lanes 8–14), which amplify an 861-bp product of wild-type mtDNA, the sequences of which lie entirely within the segments of mtDNA deleted from both types of putative subgenomic mtDNA molecules.

with *Sna*BI (cleavage site at nucleotide position 10734) preceded Bal 31 treatment. The yield of the 640-bp PCR products, amplified with primers L7901 and H8521 from full-length mtDNA molecules, decreased with increasing Bal 31 treatment time; in contrast, the yield of the 773-bp PCR products representing the 5-kb deletion, amplified with primers L7901 and H13631, was unaffected by the treatment. The same results were obtained for the analyses of products representing the 8.04- and 7.4-kb deletions (fig. 4*B*); here digestion with *Bam*HI (cleavage site at position 14258) was utilized. The yield of the 861-bp PCR products amplified with primers L14820 and H15658, corresponding to full-length mtDNA, decreased with the time of the Bal 31 treatment; in contrast, the yields of the ~599-bp and 1,204-bp PCR products, corresponding to the circular subgenomic mtDNA molecules bearing the 8.04-kb and 7.4-kb deletions, respectively, were unaffected by the nuclease treatment. The different susceptibility of full-length mtDNA and subgenomic mtDNA molecules to the treatment with Bal 31 demonstrates that the subgenomic molecules are present in the original DNA sample and do not arise artifactually during PCR amplifications. By using a less direct and more time-consuming procedure, Cortopassi et al. (1992) have also obtained evidence that subgenomic molecules carrying the 5-kb deletion are responsible for generating the relevant PCR product on amplification of tissue DNA from adult human subjects.

Further Deletions Represented in the 0.6-kb PCR Product Amplified with Primers L7901 and H16514

In addition to the family of 8.04-kb deletions represented in the 0.6-kb PCR product amplified with primers L7901 and H16514, we have detected further deletions at low abundance. These were detected among cloned DNA segments generated from the 0.6-kb band. Two types of deletions have been encountered so far (data not shown). One represents a deletion of 8,021 bp, occurring between positions 8033 and 16055 and flanked by a pair of 4-bp direct repeats (i.e., CCCA). The second deletion generated an mtDNA product in which the *Kpn*I site at position 16128 was within the deleted segment. Using other primers, we have also encountered this phenomenon of a single PCR product on a gel containing amplification products from two or more different deletions (C. Zhang, A. Quigley, and P. Nagley, unpublished data).

Discussion

The Multiplicity and Heterogeneity of Human mtDNA Deletions

Our findings indicate that single DNA bands of PCR products on agarose gels, representing human mtDNA deletions, may show sequence heterogeneity at the breakpoints when subjected to more detailed analysis. For the 8.04-kb deletions, we have identified eight sequence variants (table 2); one further size variant (corresponding to an 8,048-bp deletion) was detected as a complete extension product (table 1; 50-year-old skeletal muscle), but the breakpoint was not characterized. Further analysis may reveal more members of this family of deletions, especially in tissues that show a

mixture of different 8.04-kb deletions. The question thus arises as to whether there is heterogeneity of other mtDNA deletions. In patients with mitochondrial disorders, heterogeneity at the breakpoints of mtDNA deletions has been reported in a limited number of cases (Zeviani et al. 1989; Degoul et al. 1991). The method described in the present study can provide the means for the detection of other class B deletions (i.e., heterogeneous breakpoints).

Furthermore, we have reported here that a single PCR product (0.6 kb) separated by agarose gel electrophoresis could indeed represent subgenomic mtDNA molecules with similarly sized deletions but with breakpoints based on different direct repeats. In this light, the range of different deletions in human mtDNA, accumulating during the aging process, is certainly larger than that indicated by the initial PCR analysis.

The number of different mtDNA deletions thus far reported exceeds 100, when deletions encountered in mitochondrial disease cases (compiled by Wallace et al. 1991) and normal human aging (compilations of Wallace 1992*b;* Wei 1992; Zhang et al. 1992; Nagley et al. 1993; present data; A. Baumer and C. Zhang, unpublished observations) are taken into account. This emphasizes the propensity of human mtDNA to undergo deletion, a phenomenon determined by the large number of short direct-repeat sequences in mtDNA (Schon et al. 1989; Nagley et al. 1993). Shorter deletions/insertions of the type encountered in the D-loop region of human mtDNA (Hauswirth and Clayton 1985) have also been encountered among mtDNA molecules in tissues of mitochondrially diseased and aging human subjects (Wallace et al. 1991; A. Baumer and C. Zhang, unpublished data).

Possible Mechanisms of Generation of Class A and Class B Deletions

A possible mechanism for the generation of large deletions in mtDNA molecules, the "slip-replication model," was proposed by Shoffner et al. (1989) to explain the 5-kb deletion on the basis of 13-bp direct repeats. According to an alternative model we have developed (to be elaborated in detail, elsewhere), the deletion events are suggested to arise by template switching by the DNA polymerase during replication. Deletion events, in both cases, are envisaged to take place at direct-repeat sequences, which are presumed to interact, thus providing stability for an intermediate complex that leads to the generation of subgenomic molecules containing precisely specified deletions.

In consideration of class B deletions, we propose a modified mechanism for the generation of a heterogeneous family of closely related deletions (e.g., the family of 8.04-kb deletions). In general, this would rely only partially on the involvement of direct-repeat sequences to generate the critical intermediate complex leading to the deletion events. We propose that the formation of strong secondary structures, supplementary to interactions of direct repeats, involving extended stretches of single-stranded mtDNA in the expanded D-loop region (Clayton 1982) holds the relevant pairing sequences in place such that precise hybridizations between direct-repeat sequences are not critical factors leading to deletion events. The secondary structures may be further stabilized by interaction with proteins encoded by nuclear DNA (Zeviani and Tiranti 1993). These relatively ordered structures are proposed to function as clamps, perhaps only transiently, bringing two distant positions of the mtDNA genome together, thereby facilitating deletion events according to either the slip-replication scheme or the template-switching models. In other words, the putative intermediate complex may be less precisely specified than is the case for class A deletions.

These modified schemes for the generation of class B deletions could also be invoked to explain deletions occurring without an apparent involvement of direct repeats at the breakpoints (Degoul et al. 1991; Hattori et al. 1991; Larsson et al. 1992; Linnane et al. 1992*a;* A. Baumer, unpublished results). In such cases, the secondary-structure clamp in the expanded D-loop single-stranded segment would be entirely responsible for the apposition of the relevant H- and L-strand regions at which the deletion occurs. Here, either in the slip replication or in the template-switching scheme, the deletion events could thus occur independently of localized direct-repeat sequences.

The "clamp structure" models are not only invoked by class B deletions involving imprecise template switching, but also by the observation that a substantial number of mtDNA deletions share either one end or both ends of the breakpoints with other mtDNA deletions. An example of an mtDNA region involved in multiple mtDNA deletions in aging is the region 16070–16075. This "half-excision" site is found not only in the family of 8.04-kb deletions that has been described here (in aging subjects) and that was detected in mitochondrial disease patients (Zeviani et al. 1989), but also in a frequently detected 7,436-bp deletion and in a 7,635-bp and a 7,856-bp deletion (A. Baumer and P. Nagley, unpublished data). The upstream end of the

8.04-kb deletions (namely, position 8030–8034) was, in its turn, found also to be implicated in another deletion of 8,021 bp, mentioned above. It is envisaged that deletions occurring at such multiple localized hot-spot regions of mtDNA would be based on clamp structures rather than based solely on short-repeat sequences. The phenomenon of multiple hot-spot regions in the mtDNA genome (Zeviani et al. 1989) is found both in aging and in mitochondrial disorders; and, importantly, some of these mtDNA regions are common to both aging and diseases. Finally, it has been proposed that mtDNA molecules bearing duplications may be intermediates in the formation of deleted mtDNA molecules (Poulton et al. 1993).

Is the 5-kb Class A Deletion Absolutely Homogeneous?

Among >120 reported examples of nucleotide sequences of subgenomic mtDNA molecules carrying the 4,977-bp deletion centered on the pair of 13-bp direct repeats (compiled by Wallace et al. 1991), from both elderly subjects and mitochondrial disease patients, all but three have the identical breakpoint sequence. In these three rare cases, all from mitochondrial disease patients, an alternative sequence is found in which the T nucleotide at position 8469 is replaced by an A in the subgenomic mtDNA molecules; two cases were reported by Degoul et al. (1991) and one by Shoffner et al. (1989). On the basis of these rare sequence variants, Shoffner et al. (1989) have argued as follows in support of their slip-replication scheme: If this A residue did arise from just upstream of the direct repeat at position 13447–13459 (there is an A at nucleotide position 13446), it would indicate a directionality of strand entry into the deletion-generating process that could be accommodated within the slip-replication scheme.

Yet it is not clear from the reports by Degoul et al. (1991) and Shoffner et al. (1989) whether their sequence data, obtained from analyzing cloned DNA segments from subgenomic mtDNA molecules, reflected a nonartifactual variation at the breakpoint. In fact, the rarely encountered junction sequence may reflect a polymorphic sequence change (i.e., T to A at nt 8469) in the parental mtDNA templates or, perhaps, an artifact arising in the cloning or sequencing manipulations. Clearly, the methods developed in the present study for recovery of sequence information directly from PCR products would be highly suitable for resolving these uncertainties, in terms of sequencing both subgenomic and parental mtDNA molecules in given human tissue extracts.

Somatic Origin and Accumulation of Mutated mtDNA Molecules

The range of deletions in a specific region, such as the set of heterogeneous deletions presented in this work, offers the means for the study of a more global overview of the nature of the somatic accumulation of mtDNA deletions in aging and mitochondrial diseases. In principle, deletions with homogeneous sequences at the breakpoint (e.g., 5-kb deletion) cannot indicate whether the accumulated population of subgenomic mtDNA molecules in tissues arises from the propagation of a small number of mutated molecules formed early in ontogeny (or even present in the oocyte before fertilization) or whether this population is augmented by further independent, but identical, mutational events throughout the life span of an individual. Our studies on class B deletions (heterogeneity at the breakpoint) suggest that the accumulation of mtDNA deletions in the life span of normal individuals may be due to new mutational events as such, as well as amplification of subgenomic mtDNA molecules in tissues. In this view, individual deletion events (e.g., marked by a particular member of the 8.04-kb-deletion family) would occur randomly and in an independent somatic manner in different tissues at different stages of development and differentiation. A similar pattern of randomly occurring point mutations can be inferred to occur during aging in humans, as evidenced by the occurrence of particular base substitutions in human mtDNA in different tissues as a function of age (Münscher et al. 1993; Zhang et al. 1993).

Collectively, these mutant mtDNA molecules may contribute to the decline in mitochondrial energy output during human aging. This decline is manifested as tissue bioenergy mosaics (Linnane et al. 1992*b*), namely, the increasing prevalence, with age, of histochemically defined zones of tissues that stain very poorly for cytochrome *c* oxidase activity (Müller-Höcker 1989, 1992). A gross age-related decline in human mitochondrial functions has also been reported (Trounce et al. 1989; Yen et al. 1989).

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