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Multiple mitochondrial DNA deletions in an elderly human individual

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We have used the polymerase chain reaction (PCR) to study deletions in the mitochondrial DNA (mtDNA) of an elderly human individual. An extended set of PCR primers has been utilised to identify 10 mitochondrial DNA deletions in a 69-year-old female subject with no known mitochondrial disease. The particular deletions visualised as PCR products depended on the primer pairs used, such that the more distantly separated PCR primers enabled visualisation of larger deletions. Some deletions were common to the heart, brain and skeletal muscle, whereas others were apparently specific to individual tissues. DNA sequencing analysis of PCR products showed that short direct repeat sequences (5 to 13 bp) flanked all deletion breakpoints; in most cases one copy of the repeat was deleted. It is proposed that the accumulation of such multiple deletions is a general phenomenon during the ageing process.

Somatic gene mutation; DNA deletion; Mitochondrial DNA; Ageing; Polymerase chain reaction; Human

1. INTRODUCTION

It has been proposed that the accumulation of somatic gene mutations in the mitochondrial DNA (mtDNA) during life is an important contributor to ageing and diseases [1]. In support of this hypothesis, our laboratory [2] and others [3,4] have utilised the polymerase chain reaction (PCR) to demonstrate the age-related occurrence of a particular 4977 bp deletion in mtDNA of various human tissues including skeletal muscle, heart, brain and several internal organs. This same deletion had been previously shown to be present in tissues of patients with mitochondrial diseases [5–7]. Other deletions affecting mtDNA have been reported in patients, suffering from mitochondrial myopathy [8–11] and cardiomyopathy [12]. Among these reported mtDNA deletions, a 7436 bp deletion, which was originally detected in the cardiomyocytes of cardiomyopathy patients [12], was subsequently shown to occur in an age-related manner in the cardiomyocytes of subjects without manifest cardiac disease [13].

Such deletions in mtDNA generally occur in the longer region between the physically separated origins of replication of the heavy and light DNA strands. Since the heavy strand is exposed as a single strand for a considerable time during replication and most of the deletions have short direct repeat sequences in the vicinity of the breakpoint, a slipped mispairing mechanism has been suggested to generate deletions [6]. An examination of the human mitochondrial genome indicates that there are numerous direct repeats of 4 bp or more. Since direct repeats as short as 4 bp have been shown to be involved in mtDNA deletion in mitochondrial myopathies [8,9], we predict that a wide spectrum of further mtDNA deletions should be found in humans as part of the ageing process. Yet it is a common experience that use of one given pair of PCR primers picks up only one predominant deletion [2,3,14].

We therefore have undertaken a systematic study of primers in various combinations to see if further deletions of human mtDNA could be observed. We report here that in fact there are multiple mtDNA deletions evident in an aged human subject. Our results indicate that any particular deletion could be detected with only a subset of primer pairs. While some deletions were common in the three tissues tested (heart, brain and skeletal muscle), other deletions were accumulated in skeletal muscle only.

Abbreviations: PCR, polymerase chain reaction; mtDNA, mitochondrial DNA; bp, base pairs; kb, kilobase pairs.

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2. MATERIALS AND METHODS

2.1. *Source of tissues*

Heart, brain and skeletal muscle were taken at autopsy from a 69-year-old female who had died of primary carcinoma of splenic flexure of bowel. She had no symptoms of mitochondrial myopathies upon death.

2.2. *Preparation of DNA*

Total cellular DNA was isolated from 100 mg tissue samples by the rapid DNA preparation procedure described by Davis et al. [15], with the following modifications. Proteinase K (Boehringer Mannheim) was included in the SDS extraction step at a final concentration of 200

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μg/ml followed by extended overnight incubation at 37°C. The subsequent potassium acetate precipitation of SDS was also omitted.

2.3. *Polymerase chain reaction (PCR)*

PCR was carried out in an Innovonics Gene Machine, in which reaction tubes are automatically transferred between three water/oil baths. Total cellular DNA (1 μg) was amplified in 100 μl reaction mixture containing 200 μM of each dNTP, 40 pmol of each primer, 2.5 U of Taq DNA polymerase (Perkin-Elmer/Cetus), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin (Sigma-Porcine Skin type I) and 0.1% Triton X-100 (BDH). The mixture was overlaid with 100 μl paraffin oil (Fluka) and the reaction carried out for 30 cycles under the following conditions: denaturation, 60 s at 94°C; annealing, 90 s at 55°C; primer extension, 150 s at 72°C (in the initial cycle, the denaturation step was extended to 300 s). The amplified products were separated electrophoretically on a 1% agarose gel and the DNA detected after staining with ethidium bromide and irradiating under ultraviolet light.

2.4. *Oligonucleotide primers*

Oligonucleotide primers were synthesized using an Applied Biosystems model 380A DNA synthesizer and purified using Applied Biosystems OPC cartridges. Their sequence and corresponding position on the human mtDNA genome are described in Table I.

2.5. *DNA cloning and sequencing*

The PCR product was treated with Klenow fragment as described by Obermaier-Kusser et al. [7] to eliminate artefactually polymerised deoxyadenylic acid at the 3′ end. The desired product bearing a deletion was purified with the Geneclean Kit (Bio 101 Inc.) after agarose gel electrophoresis and cloned by blunt-end ligation into pUC13 cut with *Sma*I. Double strand sequencing of the mtDNA fragments was performed using a Pharmacia T7 Sequencing Kit according to the manufacturer's instructions. The primers used for DNA sequencing were the universal sequencing primer, L7901 or L8316.

3. RESULTS

The primers L7901 and H13631 have been routinely used in PCR analysis for the detection of the 4977 bp deletion [2,4,14]. The full size segment amplified with this pair of primers is 5.75 kb. An additional 0.77 kb product was consistently amplified from DNA of adult tissues, indicating the presence of mtDNA templates bearing the 4977 bp deletion [2]. This primer pair was used here for PCR amplification from DNA extracted from the heart, brain and skeletal muscle of a 69-year-old female. The 0.77 kb fragment was amplified from each tissue (Fig. 1, lanes b, c and d). In subsequent PCR analyses, L7901 was paired with three more primers, each shifted a different distance further away from H13631 (Table II). In general, the products amplified did not always correspond to the expected size after the 4977 bp deletion (data in Fig. 1 and summarized in Table II). Furthermore, multiple bands were observed in some samples.

Primers pairs L7901 + H13905, which shifted 278 bp from L7901 + H13631, gave the expected 1.05 kb product corresponding to the 4977 bp deletion in all three tissues tested (Fig. 1, lanes e, f and g). In addition, a 0.20 kb molecule was amplified in the skeletal muscle (but not heart and brain) suggesting a larger deletion of about 5.83 kb (Fig. 1, lane g). L7901 was combined with H14850 which shifted 1223 bp from H13631. The 1996 bp expected product predicted for the 4977 bp deletion was not seen (Fig. 1, lanes h, i and j); instead, a 0.64 kb molecule was amplified in the skeletal muscle, predicting a 6.33 kb deletion (Fig. 1, lane j). When L7901 + H16514 were used as the primers, which shifted 2890 bp from L7901 + H13631, there was no product of 3663 bp corresponding to the 4977 bp deletion (Fig. 1, lanes k, l and m). In its place, two products of 1.20 and 0.60 kb were amplified from all tissues (Fig. 1, lanes k, l and m), suggesting the presence of two deletions of 7.44 and 8.04 kb respectively. Moreover, three additional products of 1.01, 0.90 and 0.73 kb, indicative of further deletions, were amplified from the skeletal muscle (Fig. 1, lane m). H13631 was then combined with L7293 which shifted 608 bp from L7901 towards the other direction. The 1.38 kb product expected for the 4977 bp deletion was amplified from all tissues (Fig. 1, lanes n, o and p), and an additional 0.60 kb molecule was also amplified from the skeletal muscle (Fig. 1, lane p), corresponding to a deletion of about 5.76 kb.

Initially, products of the same size amplified from different tissues with the same primer combination were assumed to be the same and were given a single deletion code number (Table II). One fragment of each size was cloned into pUC13 and the deletion breakpoint sequenced. Nine deletions were found to be represented amongst the 11 fragments amplified (Table III). Deletions 1, 2 and 10 represent the same 4977 bp deletion. In most other cases here, direct repeat sequences (5 to 12 bp) were found at the boundaries of the deletions and one copy of the repeat was deleted. An exception is deletion 8 which retained both repeats. However, when DNA bearing deletion 9 amplified from the skeletal muscle was sequenced (deletion 9-2 of Table III), it was

Table I

Oligonucleotide primers for PCR

Primer	Sequence 5′–3′	Nucleotide position
L7293	GCAGTAATATTAA-TAATTTTCATG	7293 to 7316
L7901	TGAACCTACGAGTACACCGA	7901 to 7920
L8316	TTAACCTTTTAAGTTAAA-GATTAAGAGAAC	8316 to 8345
H13631	GGGGAAGC-GAGGTTGACCTG	13650 to 13631
H13905	CTAGGGTAGAATCC-GAGTATGTTG	13928 to 13905
H14850	GGATCAGGCAGGCGC-CAAGGAGTG	14873 to 14850
H16514	GTGGGCTATTTAGGCTT-TATGACCCTG	16540 to 16514

Primers L7293 and L7901 were used to amplify the light strand whereas primers H13631, H13905, H14850 and H16514 were used for the amplification of the heavy strand of mtDNA. Primers L7901 and L8316 were used for DNA sequencing. Nucleotide numbering of mtDNA is according to Anderson et al. [16].

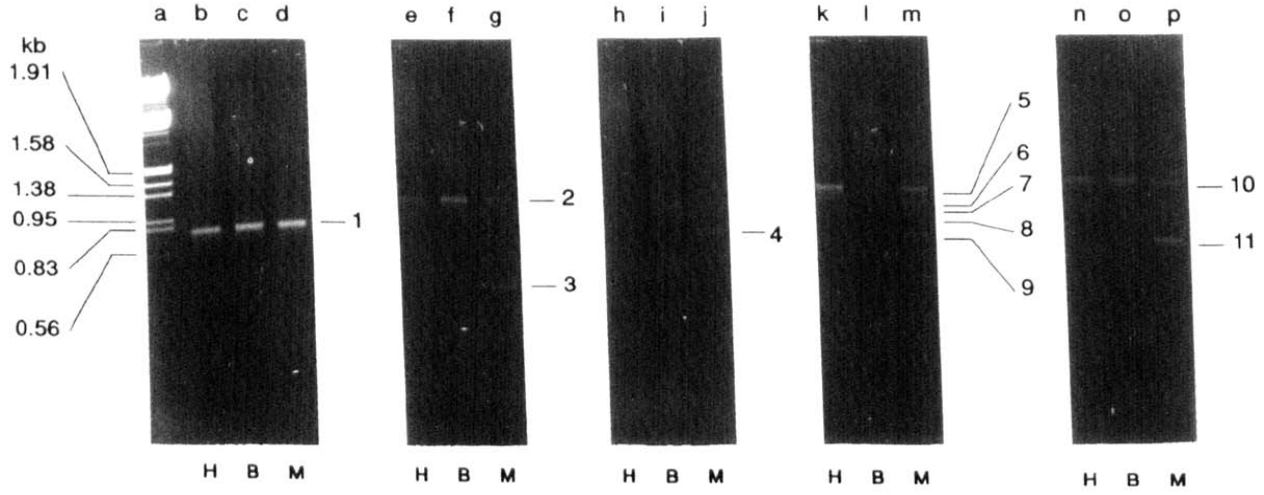

Fig. 1. Detection of multiple mtDNA deletions in a single individual. DNA was amplified by PCR from heart (H), brain (B) and skeletal muscle (M), and the products visualised using ethidium bromide following separation by agarose gel electrophoresis. The following primer combinations were used: lanes b, c and d: L7901 and H13631; lanes e, f and g: L7901 and H13905; lanes h, i and j: L7901 and H14850; lanes k, l and m: L7901 and H16514; lanes n, o and p: L7293 and H13631. Lane a contains λ DNA digested with *Eco*R1 and *Hin*dIII; sizes of some fragments (in kb) are shown at the left. For each group of lanes, corresponding to a particular primer combination, are indicated the positions and code numbers of PCR products containing deletions (Table II).

found to be different from deletion 9-1 that was initially assumed to be the same deletion in the brain. Deletions 9-1 and 9-2 represent deletions of 8041 bp and 8044 bp in brain and muscle, respectively (Table III). The same 5 bp direct repeat (CCCAT) was found in the vicinity of the deletion breakpoint in each case. However, one copy of the repeat was deleted in the brain, but both copies were partially deleted in the skeletal muscle.

4. DISCUSSION

As predicted on the basis of the general theory that mutations in the mtDNA accumulate with age, multiple mtDNA deletions were found in an elderly human individual with no known mitochondrial disease. Using PCR, at least 10 mtDNA deletions have been detected in a 69-year-old female subject. Tissue-specific deletions

Table II

Detection of multiple mtDNA deletions in a 69-year-old female subject

Primer pair	Distance between two primers (bp)	Expected size after 4977 bp deletion (bp)	Amplified fragments (bp)			Predicted deletion size (kb)	Deletion code number
			Heart	Brain	Muscle		
L7901 H13631	5750	773	0.77	0.77	0.77	4.98	1
L7901 H13905	6028	1051	1.05	1.05	1.05	4.98	2
					0.20	5.83	3
L7901 H14850	6973	1996			0.64	6.33	4
L7901 H16514	8640	3663	1.20	1.20	1.20	7.44	5
					1.01	7.63	6
					0.90	7.74	7
					0.78	7.86	8
			0.60	0.60	0.60	8.04	9
L7293 H13631	6358	1381	1.38	1.38	1.38	4.98	10
					0.60	5.76	11

The table lists bands visible amongs PCR products in Fig. 1. Other faint bands could be seen in various lanes but are not listed here.

were also demonstrated, suggesting that the accumulation of deletions in individual tissues may occur independently, emphasising the stochastic nature of the processes leading to mtDNA deletions. We have not quantitated the abundance of each deletion as a proportion of normal mtDNA. Cortopassi and Arnheim [3] estimated the 4977 bp deletion to account for about 0.1% of mtDNA in the samples they studied. It is further not known if these age-related deletions are distributed evenly between cells of a tissue, or concentrated in a limited fraction of cells.

One feature of the results presented here is that not all deletions present between a given pair of primers are represented in the final product. In general, the further apart the primers are on mtDNA, the larger are the deletions observed (Table II). It is a well established feature of PCR that when a population of differentially sized templates are present, smaller products tend to be amplified preferentially. The largest amplified product in Table II is 1.38 kb, although larger products were predicted on the basis of deletions that were already identified using more closely apposed primers. One might generalise that each pair of primers would preferentially amplify mtDNA carrying the longest available deletions between the priming sites. This apparently holds in some instances, for example L7901 + H16514 combination in Table II. Whilst for the primer combination L 7901 + H14850 a 0.64 kb product is observed in muscle (Table II), no clear bands were observed in heart or brain, although there were faint bands

Table III

Nucleotide sequences of the deletion breakpoint regions

Deletion number	Tissue analyzed	Deletion size (bp)	Sequence
1	Muscle	4977	8470 ACTACCACCTACCTCCCTCACCA (*AAGCC.....CTTCAACCTCCCTCACCA*) 13447 TTGGCAGCCT
2	Brain	4977	8470 ACTACCACCTACCTCCCTCACCA (*AAGCC.....CTTCAACCTCCCTCACCA*) 13447 TTGGCAGCCT
3	Muscle	5827	7954 CCTACATACTTCCCCC (*ATTAT.....AACAATCCCCC*) 13781 TCTACCTAAA
4	Muscle	6335	8470 ACTACCACCTACCTCCC (*TCACC.....CATCGACCTCCC*) 14805 CACCCCATCC
5	Heart	7436	8367 CCAAATATCTCATCAACAACCG (*ACTAA.....TCACCCATCAACAACCG*) 16073 CTATGTATTT
6	Muscle	7635	8433 CTATTCCTCATCACCCA (*ACTAA.....TTGACTCACCCA*) 16068 TCAACAACCG
7	Muscle	7737	7986 GGCGACCTGCGACTCCT (*TGACG.....TTATTGACTCCT*) 15723 AGCCGCAGAC
8	Muscle	7856	8027 CAGCTTCATGCCCATCG (*TCCT.....ACTC*) 16071 ACCCATCAACAACCGCT
9-1	Brain	8041	8030 GATTGAAGCCCCCAT (*TCGTA.....ACTCACCCAT*) 16071 CAACAACCGC
9-2	Muscle	8044	8030 GATTGAAGCCC (*CCATTCGTA.....ACTCACCCA*) 16071 TCAACAACCGC
10	Heart	4977	8470 ACTACCACCTACCTCCCTCACCA (*AAGCC.....CTTCAACCTCCCTCACCA*) 13447 TTGGCAGCCT
11	Muscle	5756	7769 TCAGGAAATAGAAACCG (*TCTGA.....TCATCGAAACCG*) 13525 CAAACATA

Direct repeat sequences are underlined and the numbers above each sequence indicate the position of the first nucleotide of each repeat. The numbering of nucleotides is according to that of Anderson et al. [16]. The breakpoints are indicated by parentheses and the nucleotides shown inside the parentheses (indicated by italics) were deleted.

that could represent further PCR products amplified in place of the 4977 bp deletion known to be present in these tissues.

Among the various deletions detected in the subject of this study, the 4977 bp deletion and the 7436 bp deletion have been previously shown to occur in an age-related manner. In our previous study [2], the 4977 bp deletion was detected in tissues of 8 adults 40 years and older, tested after 30 cycles of PCR amplification, whereas the same deletion could be detected in tissues of two infants up to three months only after 60 cycles of amplification. Cortopassi and Arnheim [3] (studying brain and heart) showed that this deletion was found in adults (21 years and above) but not in fetal samples. Yen et al. [4] (focusing on liver) showed the increasing incidence of this deletion in successive decades of life. Hattori et al. [13] have demonstrated the age-associated occurrence of the 7436 bp deletion in cardiomyocytes, the incidence of this deletion increased steadily through successive life decades. This particular deletion had been previously detected in the cardiomyocytes of cardiomyopathy patients by Ozawa et al. [12], who proposed a close relationship between this deletion and the disease etiology. In the present study, this deletion (deletion 5 in Table III) was detected and its breakpoint sequenced in the heart. On the basis of gel mobility of the PCR product of 1.20 kb (Fig. 1 and Table II) and sequencing data (not shown), we suggest that the same deletion is present in the brain and skeletal muscle of this non-cardiomyopathy patient. The occurrence of this deletion may thus not be limited to the heart, which therefore questions a specific relationship between this deletion and cardiomyopathy.

It has been proposed that mitochondrial diseases are the consequences of an accelerated form of a natural ageing process involving mtDNA damage [2,17]. Multiple mtDNA deletions have been described in patients with mitochondrial diseases [8–11] and cardiomyopathy [12]. Probably, the susceptibility to accumulation of deletions varies in individuals. In some cases, notably mitochondrial disease patients, the accumulation of mtDNA deletions may occur at a fast rate. The threshold to express mitochondrial diseases would thus be reached early in life. On the other hand, most healthy people would accumulate such deletions slowly during the ageing process. These may contribute, along with other mutagenic changes in mtDNA such as free radical-induced damage [18,19], to the general loss of mitochondrial bioenergetic output that is part of the ageing process [1,20].

The accumulation of deletions on the mitochondrial genome during ageing may be a general biological phenomenon in mammals. Piko et al. [21] have reported the accumulation with age of mtDNA deletions in laboratory mice. Moreover, we have observed in laboratory rats an age-related mtDNA deletion of 4834 bp between a pair of 16 bp direct repeat sequences lying within a sector bounded by the heavy and light strand origins of replication (A. Boubolas, R.J. Maxwell, A.W. Linnane and P. Nagley, unpublished).

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