The unusual structures of the hot-regions flanking large-scale deletions in human mitochondrial DNA

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Large-scale deletions of mitochondrial DNA (mtDNA) are common events that have been found to occur in human ageing and in patients with mitochondrial myopathies. The mechanisms by which these deletions occur remain unclear, but several mechanisms have been proposed, such as slipped-mispairing, illegitimate recombination, and oxidative reactions elicited by free radicals. In addition, the DNA topological stress and local DNA structures have been suggested as the important factors in eliciting the recombinational events. Upon examination of 128 breakpoints of human mtDNA deletions that have been published in the past 8 years, we found that these large-scale deletions often occur at some 'hot-regions'. We thus hypothesized that there exist unusual structures in these regions of human mtDNA that are important for eliciting the deletions. To test this hypothesis, we used PCR techniques to amplify the sequences of the so-called hot-regions and analysed the PCR

products by two-dimensional gel electrophoresis. We found that the sequences of nucleotide position (np) 5221–5988, np 6928–7493, np 7901–8732 and np 15327–16228 exhibited retarded mobilities like bent DNA structures; np 5989–6750, np 13282–13653 and np 13282–14850 showed increased mobilities like anti-bent DNA structures. Moreover, except for the sequences of np 1175–1766 found in 12 S and 16 S rRNA genes exhibiting abnormal mobility like bent DNA structures, we did not observe significant mobility abnormalities in the np 499–5545 region where deletions rarely occurred. We thus conclude that these hot-regions assume some kinds of unusual DNA structures, which may render these regions more sensitive to the attack of free radicals or serve as recognition motifs for certain recombination machinery that is involved in the large-scale deletions of human mtDNA.

INTRODUCTION

A variety of chronic progressive diseases and common degenerative disorders have been shown to associate with mutations in mitochondrial DNA (mtDNA) [1]. Once mitochondria inherit or sporadically harbour and/or accumulate these mutations, including point mutations and length mutations (e.g. deletions, duplications and insertions), they often display a deficiency in the respiratory function. Such impairment is more harmful to the tissues with a high energy demand (e.g. muscle, brain and heart) [1,2], and results in typical clinical features (e.g. myopathy and encephalopathy) of mitochondrial diseases and degenerative disorders.

Free radicals (e.g. superoxide anion and hydroxyl radical) seem to be the major 'mutators' that may potentially cause mutations of mtDNA. The activities of free radical scavenging enzymes might decline during the aging process, which in turn elevate the levels of free radicals and thus increase the frequency of free radical damage to mtDNA. In the past few years, several types of age-associated damage to mtDNA, including modified base (e.g. 8-hydroxydeoxyguanosine) [3], some disease-associated point mutations [4,5], and large-scale deletions have been identified in elderly subjects [6–8]. Recent studies of Alzheimer's disease (AD) and Parkinson's disease (PD) indicated that neuronal cells are damaged via elevated oxidative stress within cells and that mtDNA mutations are more prevalent in AD and PD patients as compared with age-matched controls [9,10]. These findings suggest that free radicals and/or reactive oxygen species

may play some important roles in the process of mtDNA mutations, mitochondrial genome evolution and sequence variation.

Other than free radical damages, recombination has also been proposed to be a possible mechanism that causes large-scale deletions and duplications of mtDNA [11–14]. Although no clearly identified recombination machinery has been found in mammalian mitochondria, recombination of mtDNA has been proposed on the basis of results from the analysis of interspecific somatic cell hybrids [15,16] and the finding of a unique endonuclease associated with recombination in mouse mitochondria [17].

DNA sequencing of deletion boundaries has revealed three classes of mtDNA deletions in patients with mitochondrial myopathies or old humans [13]. The sequence characteristics of the breakpoints of the mtDNA deletions suggest that they may occur via different mechanisms [13]. One of the mechanisms may be related to recombination occurring at the direct repeat region [11,12]. In addition to sequence homology, several recombination events have also been reported to associate with the formation of non-B DNA structures [17a]. Such structural changes of DNA may activate the recombination machinery and result in recombination events in these regions.

Upon examination of 128 breakpoints of the human mtDNA deletions that have been published in the past 8 years, we found that these large-scale deletions often occurred at three localized regions that encompass np 5000–6500, np 7000–10000, and np 12000–16500, respectively. We thus hypothesized that

Abbreviations used: AD, Alzheimer's disease; FIS, factor for Inversion stimulation; mtDNA, mitochondrial DNA; np, nucleotide position; PD, Parkinson's disease.

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these regions assume some kinds of 'deletion-prone' DNA structures, and such structural distortions may render these regions more sensitive to free radicals attack or serve as a recognition motif for certain recombination machinery.

To test this hypothesis, we used PCR techniques to amplify the sequences of the so-called hot-regions and a control region, respectively, of mtDNA and then analysed these PCR products by two-dimensional gel electrophoresis to search for non-B DNA structures in these hot-regions. We found that some stretches of DNA sequences in these three hot-regions exhibit mobility abnormalities in the two-dimensional gel analysis. These results suggest that some DNA sequences within these three hot-regions can assume a bent or anti-bent DNA structure.

MATERIALS AND METHODS

Cell cultures and preparation of intact mitochondria

HeLa cells were cultured with the DMEM (Dulbecco's modified Eagle's medium) and harvested with trypsinization and centrifugation at 800 *g* for 5 min. The cell pellet was suspended with ice-cold SEH buffer (0.25 M sucrose, 0.5 mM EDTA, 3 mM Hepes, pH 7.2) and gently homogenized using a glass homogenizer and a loose-fitting Teflon pestle. The homogenate was centrifuged at 1300 *g* for 10 min and the supernatant was centrifuged again at 7900 *g* for 15 min. The mitochondrial pellet was resuspended in ice-cold SEH buffer.

Preparation of DNA

Total DNA (mostly mtDNA) was extracted from mitochondria by alkaline/SDS lysis. An aliquot of mitochondrial suspension was added with 0.1 ml of 10% (w/v) SDS and the mixture was incubated at 37 °C for 10 min. The mixture was subjected to phenol/chloroform extraction. After centrifugation, the aqueous phase was collected and sodium acetate (pH 5.2) was added to a final concentration of 0.3 M and DNA was precipitated by 0.6 vol. of propan-2-ol at -20 °C for 2 h.

Extensive PCR

Extensive PCR was carried out the same as conventional PCR, except that the Takara *Taq* DNA polymerase (Takara Shuzo Co., Ltd., Shiga, Japan) was used. The first cycle was performed by denaturation at 94 °C for 5 min, annealing at 58 °C for 5 min, and extension at 72 °C for 10 min. After completion of the first cycle, the following program was then executed for another 25 cycles: denaturation at 95 °C for 1 min, annealing at 58 °C for 1 min, and elongation at 72 °C for 6 min. The thermal profile of the last 10 cycles was similar to that of the first 15 cycles, except that the elongation step was extended to 6 min and 20 s for full extension and compensation for the possibility of loss-of-function of Takara *Taq* DNA polymerase during the reaction.

Two-dimensional gel electrophoresis

The DNA sequences of the three hot-regions were amplified by PCR and then analysed for the presence of unusual DNA structures by two-dimensional gel electrophoresis [19]. Briefly, in the first dimension, the purified PCR product of each hot-region was separated with or without the presence of the 123 bp ladder DNA size markers (BRL, Life Technologies, Gaithersburg, MD, U.S.A.) in a 1.5% (w/v) agarose tube gel at 300 V for 1 h at room temperature. The agarose tube gel was then recovered and reoriented by 90° on to an 8% (v/v) polyacrylamide gel slab (0.15 cm × 15 cm × 17 cm; acrylamide:bisacrylamide = 29:1, v/v). Electrophoresis in the second dimension was carried out at

4 V/cm for 20 h at 4 °C. The two-dimensional gel electrophoresis performed without the 123 bp ladder markers was used to distinguish those tested DNA fragments that overlapped with the 123 bp ladder markers. After electrophoresis, the gel was subjected to silver staining. The electrophoretic mobilities of the PCR-amplified or restriction-enzyme-digested DNA fragments were analysed and compared with the mobilities of the 123 bp ladder markers.

Silver staining of DNA fragments

The polyacrylamide gel was soaked in a mixture of acetic acid and ethanol (1:9, v/v) for 15 min, and then transferred to 10% acetic acid and soaked for another 15 min. After fixation, the gel was dried with 95% ethanol and recovered three times with distilled water in order to dilute the salt concentration in the polyacrylamide gel. The gel was then transferred to the developing solution (6 mM AgNO₃, 0.15% formaldehyde) and soaked with gentle shaking for 2 h. After developing, the gel was washed with distilled water three times and soaked with 0.3 M Na₂CO₃ until the DNA fragments became visible, the Na₂CO₃ solution was discarded and the gel was reimmersed in 10% acetic acid to decay the remaining Na₂CO₃.

RESULTS

MtDNA deletions occur at some localized regions

We analysed 128 breakpoints of large-scale deletions of human mtDNA and found that most of these mtDNA deletions occurred at three localized regions (Figure 1), which encompass the sequences of np 5000–6500, np 7000–10000, np 12000–16500, respectively. We thus examined whether some kinds of non-B DNA structures exist in these regions by amplification of the DNA sequences with PCR followed by analysis for mobility abnormality in two-dimensional gel electrophoresis.

Some sequences of the hot-regions can assume DNA curvatures

Since some kinds of curvature DNA structures have been identified in the region upstream of the O_L (np 5000–6500, so-called hot-region I) [20], we first searched for curvature DNA

Figure 1 Summary of the distribution of the 128 breakpoints of large-scale mtDNA deletions

We have analysed 128 breakpoints of large-scale deletions that have been reported to occur in human mtDNA. From this figure, we can roughly group the hot-regions of mtDNA into three groups, np 5000–6500, np 7000–10000, and np 12000–16500. This figure was constructed using the data from WWW address: <http://infinity.gen.emory.edu/mitomap.html> and [18].

Figure 2 The two-dimensional gel electrophoretic patterns of the DNA fragments amplified from hot-regions of human mtDNA

The arrow 'S' points to the 492 bp DNA fragment of 123 bp ladder markers. (A) shows the PCR products amplified from the hot-region II; the arrows 1, 2, 3 and 4 refer to the DNA fragments of np 8285–8676, np 8211–8676, np 8149–8676, and np 7901–8732, respectively. (B) shows the PCR products amplified from the hot-region III; and arrows 1, 2 and 3 refer to the DNA fragments of np 13282–13653, np 14125–14580, and np 13282–14580, respectively. (C) shows the PCR products amplified from the sequences of 15327–16228 of hot-region III; and arrows 1, 2, 3 and 4 refer to the DNA fragments of np 15504–15896, np 14645–16228, np 15504–16228 and 15327–16228 respectively. The details of length and nucleotide position (np) of the PCR products amplified from each of the hot-regions are shown in Table 1. In all two-dimensional gels, the direction of first-dimension gels is from right to left, and that of the second dimension is from top to bottom.

structures in the other two hot-regions. The DNA fragment of each hot-region was PCR-amplified and separated by two-dimensional gel electrophoresis. After silver staining of the polyacrylamide gel, we tracked the bands of DNA fragments of the 123 bp ladder markers as the standard curve, and measured the distance of each tested fragment from the top of the gel (T; tested). Most of the PCR fragments moved in the two-dimensional gel in the track of the standard curve, but some of them exhibited an obvious mobility shift. We lined each of these fragments from the top of the gel and extended this line to cross the standard curve of the 123 bp ladder, we could then measure

Table 1 Summary of the two-dimensional gel electrophoretic behaviours of the PCR-amplified DNA fragments in the 'hot regions' of HeLa mtDNA

The *K* value of each PCR-amplified DNA fragment was calculated as described in the Materials and methods section. The sign '–' means no significant mobility difference between tested DNA fragment and standard.

Hot region	Amplified sequences	No. of bp	<i>K</i> value	Fragment number	Figure
II (7000–10000)	8285–8676	392	–	1	2(A)
	8211–8676	466	–	2	2(A)
	8149–8676	528	1.05	3	2(A)
	7901–8732	832	1.23	4	2(A)
III (12000–15000)	13282–13653	372	0.94	1	2(B)
	14125–14580	456	–	2	2(B)
	13282–14580	1299	0.90	3	2(B)
IV (15000–16500)	15504–15896	393	–	1	2(C)
	14645–16228	584	–	2	2(C)
	15504–16228	725	1.06	3	2(C)
	15327–16228	902	1.10	4	2(C)

Figure 3 The two-dimensional gel electrophoretic patterns of *StylI*-restricted DNA fragments of extensive PCR products of human mtDNA encompassing np 5042–7493

The sequences of np 5042–7493 include the region of O_L (hot-region I) and part of the sequences of hot-region II. The arrow 'S' points to the 492 bp DNA fragment of the 123 bp ladder markers. The details of length, np and *K* values of the *StylI*-restricted fragments are summarized in Table 2.

the distance of this crossover point (S; standard). The ratio of S/T (*K* value; mobility reduction value) was used to indicate the bending potential of each DNA fragment [19]. The fragments carrying bent DNA sequences have been demonstrated to exhibit retarded mobility in the polyacrylamide gel [21]; the fragments which moved faster than the standards were thought to result from a succession of bends cancelling each other out, producing a rod-like DNA structure [22].

Figure 2 shows the two-dimensional gel patterns of the sequences in hot-regions II and III of HeLa mtDNA; the sequences and position of each PCR product are shown in Table 1. The arrow S points to the 492 bp fragment of the 123 bp ladder. Figure 2(A) shows the two-dimensional gel patterns of the mtDNA fragments in hot-region II. The arrow 4 of Figure

Figure 4 The two-dimensional gel electrophoretic patterns of restriction enzyme-digested fragments of extensive PCR products of human mtDNA encompassing np 499–5545

The sequences of np 499–5545 of human mtDNA in which large-scale deletions rarely occurred were amplified by extensive PCR and digested by *CfoI* (A) and *BstNI* (B), respectively. The arrow 'S' points to the 492 bp DNA fragment of 123 bp ladder markers. The details of length, np and *K* values of the *CfoI*- and *BstNI*-restricted fragments are summarized in Table 2.

2(A) (fragment A4) showed mobility retardation (the *K* value of this DNA fragment was 1.23) similar to a DNA fragment carrying the bent structure [21]. Figures 2(B) and 2(C) show the two-dimensional patterns of the sequences of np 13282–14580 and np 15327–16228 of hot-region III, respectively. The fragment 1 of Figure 2(B) (fragment B1) showed mobility abnormality like an anti-bent DNA, since fragment B1 is a 372 bp DNA fragment and it moved faster than the 369 bp fragment of the 123 bp ladder in the polyacrylamide gel (*K* value of fragment B1 was 0.94). Fragment 3 of Figure 2(B) (fragment B3, np 13282–14580), including the sequence of fragment B1 (np 13282–13653), also showed increased mobility and the *K* value of this fragment was calculated as 0.90. We conjectured that the DNA fragments that exhibited increased mobility might carry at least two bent DNA elements which cancelled each other out and rendered this DNA fragment more compact than B-DNA. Among the DNA fragments shown in Figure 2(C), fragments 3 and 4 behaved like a weak bent DNA and bent DNA, respectively (the *K* values were 1.00, 1.00, 1.06, and 1.10 for fragments C1, C2, C3 and C4, respectively). Other than bent DNA structures, 4-way junctions can also exhibit mobility reduction in the polyacrylamide gel

Table 2 Summary of the two-dimensional gel electrophoretic behaviour of the restriction fragments of extensive PCR-amplified HeLa mtDNA

Extensive PCR was carried out by using HeLa mtDNA as template and the *K* value of each restriction DNA fragment was calculated as described in the Materials and methods section. The sign '–' means no significant mobility difference between tested DNA fragment and standard.

Amplified sequences	Restriction fragment	No. of bp	<i>K</i> value	Fragment number	Figure
5042–7493 (<i>StyI</i> digestion)	6751–6927	177	–	1	3
	5042–5220	178	–	1	3
	6928–7493	567	1.10	2	3
	5989–6750	762	0.88	3	3
	5221–5988	768	1.23	4	3
499–5545 (<i>CfoI</i> digestion)	1305–1475	171	–	1	4(A)
	2945–3164	220	–	2	4(A)
	1476–1771	296	–	3	4(A)
	3165–3701	537	–	4	4(A)
	499–1304	806	–	5	4(A)
	3702–4533	832	–	6	4(A)
	4534–5545	1012	–	7	4(A)
	1772–2944	1173	–	8	4(A)
	1767–2007	241	–	1	4(B)
	872–1174	303	–	2	4(B)
499–5545 (<i>BstNI</i> digestion)	499–871	373	–	3	4(B)
	3090–3590	501	–	4	4(B)
	1175–1766	592	1.10	5	4(B)
	3591–4480	890	–	6	4(B)
	4481–5545	1065	–	7	4(B)
	2008–3089	1082	–	8	4(B)

[23]. Since we used PCR products for the two-dimensional gel electrophoresis, we can rule out the possibilities of formation of 4-way junctions in the DNA fragments.

Figure 3 shows the two-dimensional gel electrophoretic pattern of the *StyI*-restricted DNA fragments of the sequences of np 5042–7493, the region that includes the sequences of O_L and part of the sequences of hot-region II. Other than arrow 1 (including a 177 bp and a 178 bp *StyI*-restricted fragment), the rest of the restriction fragments all showed mobility abnormalities. Arrows 2 (*K* = 1.10) and 4 (*K* = 1.23), representing the *StyI*-restricted fragments of 567 bp and 768 bp with retarded mobilities, are considered as typical bent structures. Arrow 3 which points to the restriction fragment of 762 bp with an increased mobility is considered as an anti-bent DNA structure (*K* = 0.88). Some kinds of curvature DNA have been shown to exist in front of O_L [20], we have thus confirmed this by the demonstration of mobility abnormality in two-dimensional gel electrophoresis.

The regions outside the hot-regions exhibit normal mobility

We also amplified the mtDNA sequences of np 499–5545, the region in which large-scale deletions rarely occurred, by extensive PCR and then digestion with the restriction enzymes *CfoI* and *BstNI*. Except for the sequences of np 1175–1766 of *BstNI*-restricted DNA, fragments exhibited retarded mobility (fragment 5 of Figure 4B); none of these restriction fragments showed obvious mobility abnormalities, since every DNA fragment generated by the restriction enzymes moved along the track of the 123 bp ladder markers (Figure 4). The *K* values and nucleotide positions of *CfoI*- and *BstNI*-restricted fragments are shown in Table 2. Apparently, except for the region encompassing 12 S

and 16 S rRNA genes, there were no obvious bent DNA structures outside the hot-regions of human mtDNA.

DISCUSSION

We have demonstrated in this study that the DNA sequences near or within the deletion-prone regions in human mtDNA can assume some kinds of curvature DNA structures. The sequences of np 5221–5988, np 6928–7493 (Table 2), np 7901–8732 and np 15327–16228 (Table 1) of human mtDNA exhibited retarded mobility abnormalities in the two-dimensional gel just like bent DNA, whereas the sequences of np 5989–6750 (Table 2), np 13282–13653 and np 13282–14580 (Table 1) showed increased mobilities like anti-bent DNA structures. However, most of the DNA fragments shown in the two-dimensional gel electrophoretic patterns of the *Cfo*I- and *Bst*NI-restricted PCR products encompassing np 499–5545 did not reveal any mobility abnormality. The two restriction enzymes had different cutting sites in the np 499–5545 region of mtDNA and all except one *Bst*NI-restricted DNA fragment (np 1175–1766) showed normal mobility in two-dimensional gel electrophoresis (Figure 4B). The sequences of np 1175–1766 included part of the 12 S and 16 S rRNA genes and the whole tRNA^{Val} gene. Since the two rRNAs are vital for the gene expression and biogenesis of mitochondria, large-scale deletions are rarely found in this region.

Several primary and higher-order structures of DNA [16a,25] have been suggested to play important roles in recombination events, such as sequence homology in the homologous recombination, the structural homology [17a,27], local distortion [22a,28] and negative supercoiling [30]. It is well established that bent DNA structures play important roles in regulating several recombination events. Hubner et al. [23] argued that a third equivalent of the FIS (factor for inversion stimulation) protein can interact with the already bound FIS proteins in the enhancer if a pre-existing curve, lying between the two primary FIS-binding sites, exists in the enhancer. They postulated that bent DNA structure is one of the important factors for the site-specific recombination of the *Cin* system. Milot et al. [31] characterized several junctions of illegitimate recombination and found that these junctions shared the same 'bent structure' rather than sequence homology. Such bent DNA structures had formed before the recombination events occurred. Mazin et al. [32] found a new mouse nuclear protein, KIN17, that cross-reacts with the antibodies directed against RecA and binds to the curved DNA fragments present at illegitimate recombination sites. Bent DNA structures can also serve as recognition motifs for binding of several DNA-binding proteins and enzymes, such as topoisomerase I [33], topoisomerase II [34], endonuclease [35], and transcription factors [36,37]. Since topoisomerases and nicking enzymes can interact with the intrinsic bent DNA structure, topoisomerases can mediate DNA strand breakage [38], DNA strand transfer reaction [39] and illegitimate recombination [40,41], and DNA strand breakage can induce illegitimate recombination [42], thus the interaction between bent DNA structures and these enzymes may also play some role in the recombination events.

In the past few years, several disease-associated and age-dependent large-scale mtDNA deletions have been found in various tissues of old humans [1,2]. These observations suggest that mtDNA deletions may be due to the inefficiency of cellular defence systems (e.g. superoxide dismutase, catalase and glutathione peroxidase) or regulatory systems. On the basis of these findings, we propose that mtDNA deletions may occur via two possible mechanisms. First, the free radical level is elevated in tissue cells during the aging process and thus increases the

frequency of oxidative damage to mtDNA. Since free radical attack is a random process, yet mtDNA deletions often occur at some specific regions, we thus propose that there may exist some 'deletion-prone' regions in mtDNA. Such structurally labile regions can be easily attacked by free radicals and elicit several kinds of deletions in mtDNA. Secondly, recombination within mammalian mtDNA must be under stringent control, otherwise these events will cause severe alterations in organization and gene expression of the mitochondrial genome. These mitochondria will be selected out under evolutionary pressure. Thus during the aging process, the regulatory stringency of recombination machinery may be gradually declined and the frequency of recombination is increased, and that may result in specific mtDNA deletions or duplications in these so-called hot-regions.

In conclusion, by using two-dimensional gel electrophoresis to analyse the PCR-amplified and/or restriction-enzyme-digested DNA fragments, we clearly showed that there do exist unusual DNA structures in the so-called 'hot-regions' of human mtDNA. Taking these results and recent findings that bent DNA structures are involved in DNA recombination and other genetic processes, and that several enzymes such as endonuclease and topoisomerases exist in mammalian mitochondria, we suggest that bent or anti-bent DNA structures of these hot-regions of human mtDNA may play important roles in the generation of large-scale deletions.

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