Mitochondrial DNA rearrangements: intracellular information system

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Abstract The extent of mtDNA rearrangements has been analyzed in nDNA preparations of rat and human with a statistically representative group of oligonucleotides directed to two regions of mtDNA: genes for cytochrome oxidase subunits I and III. Human PCR preparations generated with oligonucleotides directed 'normally' showed the expected fragment for mtDNA and the presence of a plethora of fragments with rearrangements (deletions and insertions), in contrast to rat PCR preparations under the same reaction conditions in which these kinds of rearranged fragments were rarely observed. Both human and rat PCR preparations generated with oligonucleotides directed 'inversely' showed numerous fragments, some of which showed differences in copy number correlating with distinct phases during development/aging. Sequence analysis of some normal and rearranged fragments demonstrated in all cases DNA sequences 99% homologous to mtDNA that had been either deleted or inversely ligated with other mtDNA sequences at rearranged fragments. No evidence of nuclear DNA sequences was found. The following scheme is proposed for mtDNA rearrangements during the lifetime of an organism: variation in copy number of some fragments with inversions of mtDNA depends on the specific developmental/aging period; in old cells there is an increase in higher molecular weight mtDNA deletions. These findings strongly suggest that the mtDNA rearrangements play a role as an intracellular 'information system'.

Key words: Mitochondrial DNA; Development; Aging

1. Introduction

Rearrangement of mtDNA has been known for almost a decade as an important process in senescence in filamentous fungi [1], maternally inherited male sterility in plants [2], and recently, mitochondrial myopathies in humans [3,4]. Only in the fungus *Podospora anserina* has a direct correlation to senescence been shown [5], where mtDNA rearrangements are directly measurable as a cause of the process [6]. Recent evidence has shown the presence of large-scale mtDNA deletions in human mitochondrial myopathies [7,8] and some other neuromuscular [9,10] and hematologic diseases [11]. In the present study, we have tried to find evidence for rearranged mtDNA sequences in two mammalian species, rat and man, using a statistically representative group of oligonucleotides for two regions of mtDNA – genes for cytochrome oxidase subunit I and III, including flanking regions (see Fig. 1), and also to estimate their importance for the normal functioning of the cell. Oligonucleotides in several possible combinations have been used, i.e. not only generating small fragments inside these two areas (directed 'normally'), but also directed inversely.

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2. Materials and methods

All buffers and solutions were prepared according to the laboratory manual [19]. Human biopsies (liver) were obtained from the Biocenter in Basel, and total nDNA was isolated according to Blin and Stafford [20]. For the control, some nDNA preparations were further purified with columns, but the PCR results were identical. Wistar rats (liver and brain) were obtained from the ETH, Zürich, and WAG/Rij rats from TNO Institute of Aging and Vascular Research, Leiden, The Netherlands. The study was done at the Laboratory of Biochemistry, ETH, Zürich, Switzerland; and at the Laboratory of Molecular and Cellular Biology, ENS, Lyon, France.

PCR was done according to the Perkin-Elmer manual with 25 cycles (60 s 94°C, 120 s 37°C, 180 s 72°C); to test the reproducibility of, in some cases, small differences in copy number, all PCR reactions were repeated at least twice. All the PCR-generated fragments were purified and analysed by restriction enzyme analysis. The most interesting ones were subcloned and sequenced. Oligonucleotides (average length of 22 base pairs) were obtained from Microsynth, Switzerland, and from TIB Molbiol GmbH, Berlin, Germany. nDNA concentrations in the PCR assays with rat nDNA were approximately 10 times lower compared to the corresponding assays done with human nDNA (about 10 ng, as estimated from gel-electrophoresis). Two or more animals were used per time point (not all results are shown). Nucleotide sequencing was done by PCR according to [21], and partially according to Sanger [22]. The cDNA clone of poly(ADP-ribose) polymerase gene, together with a set of oligonucleotides specific for the highly conserved areas of the gene, were generously provided by Dr. J.-Heiner Küpper, Inst. für Virusforschung, Heidelberg, Germany.

3. Results

In almost all PCR reactions in which a combination of oligonucleotides directed normally was used, an expected fragment for mtDNA was recognised as the most intense band, which showed very small variation in copy number during aging. Interestingly, additional fragments were seen in some PCR reactions. Fig. 2A shows the results of a PCR reaction with oligonucleotides 6 and 8. The 1000 bp fragment is present only from PCR reactions using nDNA material isolated from liver biopsies of old patients. Both fragments from Fig. 2 were isolated, purified and sequenced to completion or, at least, partially. The smaller fragment of 300 bp was recognised as the expected fragment of mtDNA (with a single nucleotide difference to the published sequence [12] that does not change the amino acid sequence). The partial nucleotide sequence (about 400 bp) of the 1000 bp fragment contained mtDNA sequences (99% homology) that had been rearranged and inverted, as shown in Fig. 2B.

PCR reactions with oligonucleotides directed 'inversely' produced very interesting results: e.g. Fig. 3 shows nDNAs isolated from human liver biopsies in a PCR reaction with oligonucleotides 5 and 4. In three samples the fragment has almost disappeared. Interestingly, these samples were obtained from the oldest patients (more than 44 years). The nucleotide sequence of this fragment (500 bp; after sequencing about 370 bp)

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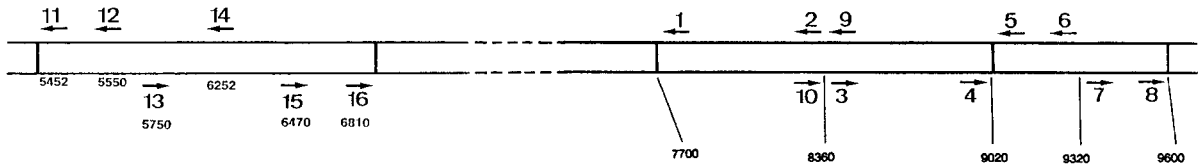

Fig. 1. Schematic representation of the oligonucleotides used in the PCR assay for the analysis of the rat nDNA preparations. Numbers on the mtDNA are according to the published sequence [18]. Exact positions of the oligonucleotides on the DNA are as follows: oligonucleotide 1, 7689–7713; 2, 8331–8355; 3, 8354–8378; 4, 8986–9010; 5, 9027–9045; 6, 9297–9321; 7, 9325–9349; 8, 9578–9596; 9, 8360–8384; 10, 8331–8355; 11, 5353–5377; 12, 5451–5475; 13, 5751–5775; 14, 6253–6277; 15, 6471–6495; 16, 6811–6835. Synthesis of the DNA fragment begins on the end of the arrow.

demonstrated the presence of the rearranged (spliced and inverted) mtDNA sequences (99% homology).

As a second part of the study, we looked for the same phenomena in nDNA isolates from rat. We expected to obtain a clearer picture about the importance of the mtDNA rearrangement process, starting with a simple definition of aging as a species-independent event, beginning already with a birth: the rat as a species, based on the same physiology as humans but with approximately only 1/30 of the human middle-life expectancy, offers better possibilities for almost all purposes. The study was targeted to the same region as in the human mtDNA. Preparations from liver and brain were used but brain preparations were preferred because of higher purity.

The results were in some ways unexpected: oligonucleotides directed normally showed the expected fragment of mtDNA but almost no additional fragments (rat equivalents of oligonucleotides 6 and 8 showed an additional fragment of 1100 bp only with nDNA preparations from cancerous cells; data not shown). On the other hand, rat oligonucleotides 5 and 4 directed inversely recognised a fragment (450 bp, Fig. 4A) of comparable length to the one seen in human nDNA preparations, which showed almost the same pattern of rearrangements (shown in Fig. 4B). Additionally to this fragment, a larger fragment (2700 bp) was present in all preparations. An expected correlation of the smaller fragment with aging, taken as a parallel to the human mtDNA rearrangements, can not be observed, rather the opposite in fact: a decrease in intensity after several weeks of age was observed in reactions with several other oligonucleotides directed inversely, e.g. oligonucleotides 13 and 5 (Fig. 5). The lower band (800 bp) is present in all lanes (lane 12, an 8-week-old animal, shows a uniform smear in all reactions because of impurities in the preparation) with a maximum intensity at 3 weeks (lanes 5 and 6). The upper two bands of low intensity have no correlation with the aging phases.

The situation shown in Fig. 6 (obtained with oligonucleotides 13 and 2) is even more complicated: the first two samples (2 days before birth) show a band of 500 bp; the following two samples from 1-week-old animals show a smear. In samples from animals of 3 weeks to 3 years there is a band of 800 bp. The last sample (3 years) shows an additional band at 1800 bp.

Fig. 7 shows the same samples in reaction with oligonucleotides 16 and 1. A fragment of 4000 bp can be observed for the two samples of 1 week and 2 years with higher intensity. It is also present in low intensity in the samples from 4 and 5 weeks. No DNA was detected in the other samples.

Oligonucleotides 6 and 10 directed inversely gave a single fragment of 3500 bp, which was decreased in copy number specifically in the preparations from 1-year-old animals; the same situation was seen with the fragments obtained with the pair 5 and 10 (not shown). Oligonucleotide 3, in combination with oligonucleotides 5 or 6, gave no fragments at all, despite its near proximity to oligonucleotide 10. This indicates that the

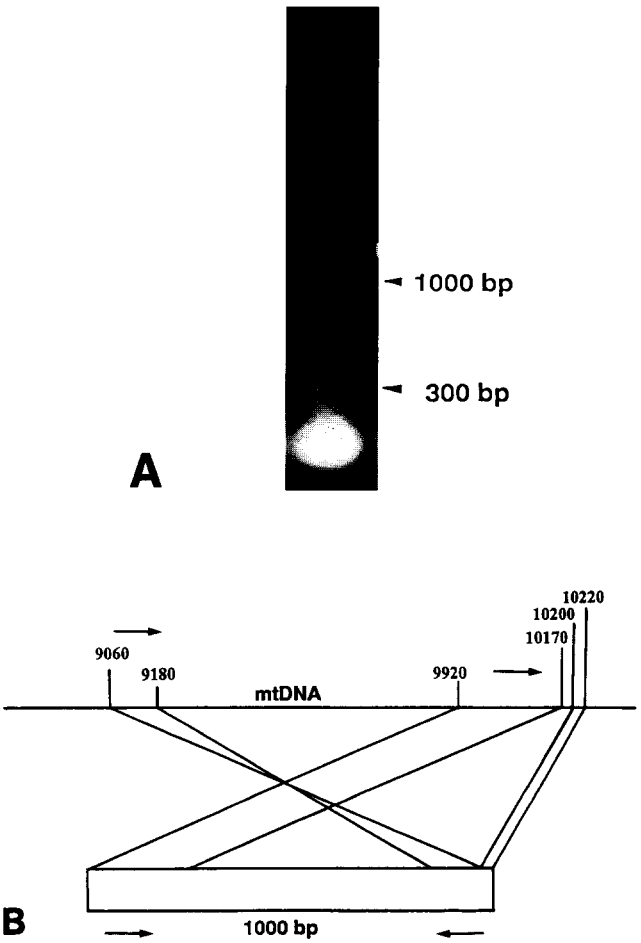

Fig. 2. (A) Results of a PCR reaction using oligonucleotides 6 and 8. The expected fragment for mtDNA is at 300 bp, and an additional fragment of lower copy number at 1000 bp. The PCR reaction was done with nDNA isolated from liver biopsy of an older patient. (B) Schematic representation of the sequence data obtained by direct PCR sequencing, using [^{35}S]dATP. Sequences from one end show the unexpected nucleotide sequence from the other part of the human mtDNA, that has been integrated in the inverse orientation. Homology tests in this area of mtDNA (around nucleotide 9060) do not show any significant homology to the oligonucleotide used for PCR, thus excluding the possibility of PCR 'jumping'. Arrows show the orientation of the corresponding sequences in mtDNA and in the 1000 bp fragment. Numbering of mtDNA is according to the published sequence [12].

Fig. 3. Results of a PCR reaction using oligonucleotides 5 and 4 on nDNA isolated from human liver biopsies. Lanes 2, 3 and 6 from the right are preparations from patients of age 44, 54 and 64 years, respectively.

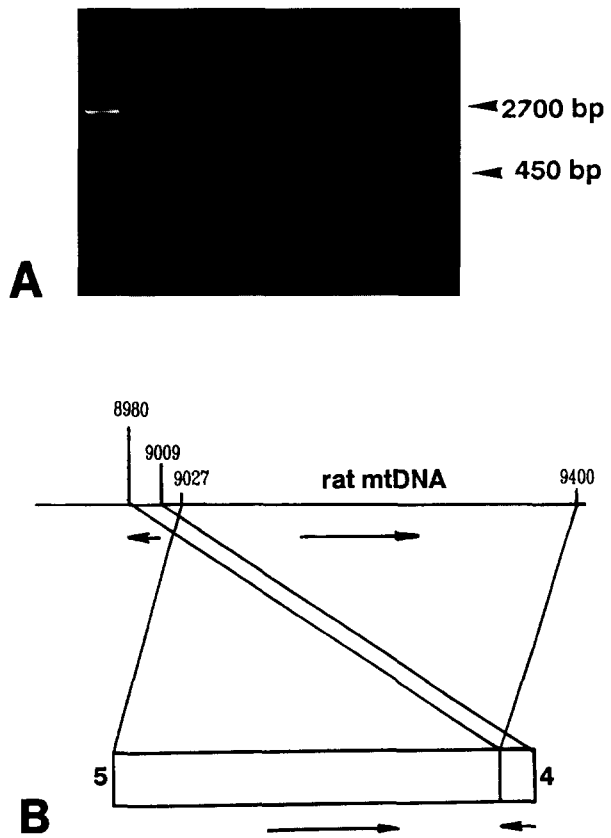

Fig. 4. (A) Results of a PCR reaction using oligonucleotides 5 and 4 directed against rat nDNA preparations. The first lane is from animals aged 1 week, the second aged 3.5 months, lanes 3–5 aged 1 year, and the last two aged 2 years. (B) Schematic representations of the sequence data analysis of the smaller fragment generated with oligonucleotides 5 and 4 (see A).

rearrangement pattern is very precise and, obviously, very complicated. The summary of all correlations obtained is shown in Table 1.

4. Discussion

DNA rearrangements have been known for a long time as a natural process occurring normally at a slow rate in all organisms. Only recently [13] has it been pointed out that inversions and repetitions can contain information. A similar interpretation can be drawn from this study. In both species an increase in which progressive aging is observed only in one reaction, although this would be expected from the numerous studies on mtDNA deletions [14,15]: it is worth mentioning that the same rat samples in the reaction with oligonucleotides 8 and 11 show the presence of deleted mtDNA that increases in copy number with age. In contrary, a decrease in copy number, beginning already in young organisms for some fragments, or an even more complicated situation showing dependence of copy number on the specific developmental/aging period, is observed. The copy number of all the fragments generated with oligonucleotides directed inversely never reached the levels of mtDNA (some fragments are on the border of detectability). Knowing that mtDNA is present at least, in several hundred copies in cells, this suggests the presence of a few copies of these fragments per cell, sometimes even less, suggesting that not all cells are generating all fragments. This possibility has been checked with oligonucleotides directed to the gene of poly ADP-ribose polymerase, a single copy nuclear gene [16]. Under the same reaction conditions, the intensities of the obtained DNA fragments were almost the same for all samples (data not shown). This reaction has also been used as an internal control of contamination of samples, found only in a preparation from 8-week-old rat.

There are several theoretical explanations for these findings, but in general it can be said that the precision of the mtDNA

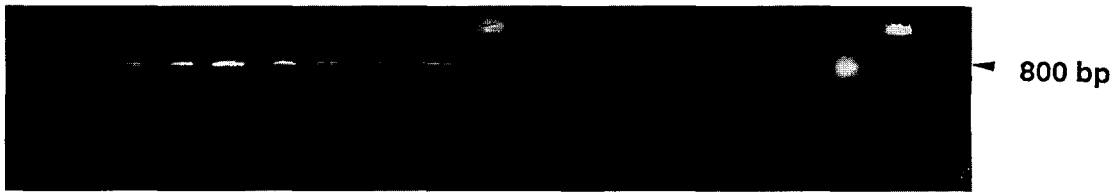

Fig. 5. Results of PCR reactions obtained with oligonucleotides 13 and 5. Three fragments have been generated, of 5000 bp, 3500 bp and 800 bp (most intense band). Lanes 1, 2, preparations of rat brains 2 days before birth; lanes 3, 4, brains of 1 week old animals; lanes 5, 6, 3 weeks; lanes 7, 8, 1 month; lane 9, 5 weeks; lanes 10, 11, 6 weeks; lane 12, 8 weeks; lane 13, 3.5 months; lanes 14, 15, 1 year; lanes 16–18, 2 years; and lane 19, 3 years.

Fig. 6. Results of PCR reactions with oligonucleotides 13 and 2. The age of the nDNA preparations from rat brains in each lane is the same as in Fig. 4.

Fig. 7. Results of the PCR reactions with oligonucleotides 16 and 1. The age of the nDNA preparations from rat brains in each lane is the same as in Fig. 4.

Table 1
Summary of all correlations (either decrease or increase) of rat mtDNA-like fragments with different age groups obtained with the oligonucleotides directed inversely

Oligopair	Distance	Length	Age correlation
5, 4	20	2700	
		450	
6, 4	300	2500	
6, 3	980		
5, 3	680		
5, 10	700	4000	1 year
		400	
6, 10	1000	3300	1 year
13, 14	500	200	
15, 6	2800	2500	1 year
		1400	1 year
		100	
16, 6	2500	1400	1–3 weeks
13, 5	3300	5000	
		3500	
		850	3 weeks
16, 5	2200	2500	
15, 5	2500	3000	3.5 month–1 year
		1900	
		500	
13, 1	2000	5000	1 week
		2500	1–5 weeks
13, 2	2600	1800	3 years
		800	3 weeks–3 years
		500	2 days before birth
13, 6	3600	5000	1 week
		600	
15, 1	1200	1800	1 year
16, 1	900	4000	1 week and 2 years
16, 2	1500	3400	
15, 2	1900	4000	1 week and 3.5 months
		2400	1 week and 3.5 months
		1000	

The lengths of the fragments vary from 50 bp to 5000 bp, showing no correlation with age dependency or distance between oligonucleotides used for PCR.

rearrangement events and its specificity are strongly suggestive of these DNA fragments bearing information relating to aging and development (either passive or active information). It means that specific mtDNA rearrangement enzymes are imported into the mitochondrion where they generate specific mtDNA fragments from parts of mtDNA (specific recognition of the mtDNA sequence, cutting at precise sites, inversion and ligation of mtDNA fragments). One can propose that these fragments are metabolized at the same rate as the mtDNA, so that their presence is dependent on the presence of mtDNA rearrangement enzymes imported from the cytoplasm. This suggests that the nuclear genome uses mtDNA as an information storage system – a role comparable to the computer diskette in the micro-universe. This seems logical, bearing in mind the semi-independent role of the mtDNA genome and its isolation from the rest of the cytoplasm by the mitochondrial membrane. It also means that mtDNA places an additional evolutionary constraint on the genome organisation because of the rearrangement process.

mtDNA genome analysis of many species shows mtDNA as a very compact genome with small or no introns in the animal kingdom, and as a genome of varying length in the plant kingdom, even though almost the same genes are expressed. These facts suggest that there is an additional evolutionary constraint on the organisation of the mtDNA genome that does not exist in the nuclear genome. Previous studies have shown that mtDNA is less protected against damage and rearrangement [17], thus also making the rearrangement events possible. The findings of many rearranged mtDNA fragments in the first few weeks of the rat life can be explained as a direct connection to the synthesis of higher mtRNA and proteins (in rat, brain mt protein synthesis per unit amount of mtDNA increases at 10–13 days after birth, and declines sharply in the 3rd week, reaching a level that remains constant over a 2 year period [23]). The fact that some fragments are generated at very low levels, sometimes

less than a single copy per cell, can be explained by the fact that not all cells contain information for aging (one cell with the signs of aging in the 100 normal cells is enough to cause a malfunction in heart muscle). This suggests that aging is coded and expressed in a small proportion of cells in almost all organs, and not caused by the action of hormones or similar factors – a logical conclusion, consider that aging is a characteristic of all multicellular organisms, and as a process is probably evolutionarily older than complex neuronal networks. Of most interest are, certainly, the rearranged mtDNA fragments that correlate with the last phase of life, in this case, with 3-year-old rats. If one assumes an active role for these DNA fragments, the question is how the information is recognised, and also how it is transported back into the cytoplasm (there are several reports indicating the influence of changes in mtDNA on outer surface proteins, making a role for rearranged mtDNA fragments logical and possible). As a conclusion, it can be concluded that these findings open up the possibility of prolongation and manipulation of the aging process, the last unbroken frontier of human knowledge; however, until this task is even theoretically reached, a lot of additional experimental work will be necessary.

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