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Mitochondrial heteroplasmy among maternally related individuals

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Abstract The second hypervariable segment of the human mtDNA control region contains a homopolymeric tract of cytidines between nucleotides (nt) 303 and 315, interrupted by a thymidine at position 310, according to the Cambridge reference sequence. By direct sequencing, some individuals show blurred sequence chromatograms in this region which are not caused by a sequencing artefact but by high levels of length heteroplasmy. With respect to this length heteroplasmy ten maternally related individuals and two unrelated probands were examined. The relative proportions of length variants in the homopolymeric tract in selected individuals were determined by cloning and sequencing of multiple independent clones. All ten family members examined were heteroplasmic while the proportions of each genotype varied widely in different individuals. The size of a possible mitochondrial bottleneck during embryonic development of the offspring is discussed with respect to the changes in mitochondrial haplotypes within mother-offspring pairs. Our data are consistent with both slow and rapid segregation of mtDNAs between the generations, which would implicate a tight as well as a wide bottleneck. Therefore, a common bottleneck size in all individuals from this lineage seems to be very unlikely.

Key words Mitochondrial DNA · Heteroplasmy · Bottleneck · Non-coding region · D-loop region · Sequencing

Introduction

Mitochondria have a separate, autonomously replicating DNA genome, the inheritance of which is generally considered to be strictly maternal [1]. Proliferation occurs by division after replication of the nucleic acids. Whether partitioning of the existing mitochondrial DNA (mtDNA) molecules occurs by random drift or through a sorting mechanism that determines the distribution of organelles to daughter cells or of mtDNA copies to daughter mitochondria is still unclear. Principally, all individuals appear to be homoplasmic with respect to their mtDNA, i.e. an individual's mtDNA population is ca. 99.9% identical [2]. However, there are reports about individuals that are heteroplasmic with regard to mtDNA [3–15]. Heteroplasmy (the presence of two or more mtDNA haplotypes in the same individual) is conceivable at three levels. These are:

1. The cell: a single cell contains only mitochondria that are homoplasmic, but different cells carry variants.
2. The mitochondrion: one cell carries different mtDNA haplotypes, but each single mitochondrion is homoplasmic.
3. The nucleic acid: an individual mitochondrion carries different mtDNA types.

Compared to genomic DNA, the d-loop-containing region within the mtDNA shows an especially high mutation rate [7]. This establishes the basis for the widespread sequence variations within the mtDNA control region, of which forensic science takes advantage [16–25].

Assuming there are at least ~100,000 mitochondrial genomes in mammalian oocytes, but only 10–50 germ cell divisions per generation available for segregating mitochondrial genotypes [26], a random segregation during cell divisions of the developing oocyte would require at least 20 generations to reach homoplasmy from a mixed mtDNA population [26]. Consequently, the observed tendency to homoplasmy is only explicable by the existence of a fixation mechanism for mtDNA mutations. This raises the question how mtDNA mutations segregate and how they are fixed at the levels of the organelle, the cell, the individual and the population.

On the basis of a study of rapid genotype shifts within a maternal lineage of Holstein cattle, Hauswirth and Laipis [27] formulated the "bottleneck hypothesis" as a conceiv-

Dedicated to Prof. Dr. h.c. B. Brinkmann on the occasion of his 60th birthday

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able mechanism, by which the quantity of mtDNA molecules is reduced to a relatively small number at some stages of oogenesis. In this way, only a few of the large number of existing mtDNA molecules will contribute to the future population. The developmental stage at which the bottleneck occurs is still a matter of debate and the female germline and the early stage of embryogenesis have been proposed [27, 28, 29]. For the molecular mechanism two possibilities are hypothesised. Firstly, a selection/replication event during the maturation of the primary oocyte would be possible. Because of the 100-fold proliferation of the mtDNA molecules at this stage, a rapid shift in the genotype could occur in a single generation by selection and a subsequent amplification of a small subset of DNA templates [27]. The existence of a relaxed control of mtDNA replication permitting a single mtDNA molecule to replicate more than once is known [30, 31].

Secondly, an unequal partitioning event during cytokinesis would also be conceivable. At the early blastocyst stage, a small number of cells (10–20) contributes to the inner cell mass which develops to the three germ layers, while the residual cells of the blastocyst form the extraembryonic tissues. At this stage of embryogenesis, an unequal division of the mtDNA molecules in the inner cell mass could add to a rapid segregation of the mtDNA [28, 29].

Whether segregation occurs by random drift or through a complex sampling process is not yet understood, but hints of a sorting mechanism were recently found in yeast [32].

The segregation rate of the mtDNA mutations within the generations could be influenced by the size of the bottleneck. The larger the bottleneck, the more likely it is that the descendants resemble the mother. This could result in a slow segregation but does not exclude rapid changes by genetic drift. In turn, the narrower the bottleneck, the more likely is a clonal descendant population regarding mtDNA, which would support a rapid segregation mechanism.

Concerning the size of the bottleneck, several groups have analysed the distribution of the mtDNA genotypes within heteroplasmic lineages of different animals and contradictory results were obtained

A study in Holstein cattle which shows the change of a heteroplasmic point mutation to homoplasmy within two or three generations implicated a bottleneck size of 20–100 segregating units [28]. Subsequently, Koehler et al. [33] observed such a shift within a single generation and conclude the possibility that the bottleneck size comprises only one segregating unit. By contrast, in other animals, the estimates range from 200 (in mice) [34] to 370 (in Drosophila) [35].

Most of the publications about heteroplasmy in humans concern disease-associated mtDNA mutations, which is probable because homoplasmy would be lethal for the majority of these pathogenic mutants [36, 37]. Such polymorphisms are not ideal for examination of the molecular mechanisms of mtDNA distribution, since the role of selection in the segregation is uncertain. There are a few observations of silent heteroplasmic point mutations in human mitochondrial DNA. The scant reports available for heteroplasmy within the non-coding region give either evidence for slow [3, 8, 38] or rapid [4, 5, 14] or for both slow and rapid segregation events [9].

By direct sequencing of the human d-loop region, we found abortion of the sequencing reaction behind the homopolymeric C-tracts at positions 16184–16193 and 303–309 (according to Anderson et al. [39]) in some cases. These monotonous runs of the same base at three or more positions in succession have been implicated as hot spots for mutations, likely caused by replication slippage [40]. By analysing ten maternally related family members (FM) and two unrelated persons (UP), we examined the occurrence of length heteroplasmy behind the C-tract at positions 303–309.

Materials and methods

Nucleotides in the mtDNA d-loop sequence are numbered according to the reference sequence reported by Anderson et al. [39].

Samples

Samples were taken from ten family members, related in their maternal lineage and two unrelated probands (Fig. 1). Besides sequencing the entire mtDNA d-loop, the relationships were also controlled by genomic short tandem repeat (STR) analyses (data not shown). Either peripheral blood (FM I.1, II.1-II.4, III.1,2,5; UP 1,2) or buccal cells (FM III.3,4) were used for DNA extraction.

DNA extraction

DNA was extracted from peripheral blood and buccal cells using standard techniques as previously described [20].

Amplification of mtDNA

The PCR primers used to amplify a 477 bp mtDNA fragment encompassing positions 303–309 were L182 (nt 162–182) and H619

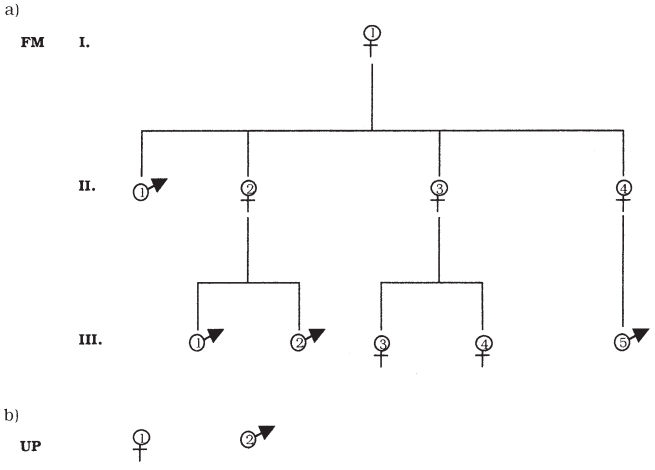

Fig. 1a, b Genealogical tree indicating: **a** Maternally related individuals. FM family member; *I, II, III* the different generations. Individual persons within the generations are indicated by arabic numerals. **b** Unrelated individuals (*UP*)

(nt 638–619). The primers are numbered according to the location of the 3′-ends in the reference sequence and, "L" and "H" designate the light and the heavy strands of the mtDNA molecule, respectively. Amplification was performed in a total volume of 50 µl using a Biozym PCT-200 thermal cycler (Biozym, Germany). Each reaction contained 50 mM KCl, 10 mM Tris/HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 µM of each dNTP, 5 pmol of each primer and 1.25 U Taq DNA polymerase (PE Applied Biosystems, USA). The products were separated from residual primers, dNTPs and buffer using the QIAquick PCR purification kit (Qiagen, Germany).

Cloning

PCR products were cloned into the pCR2.1 TOPO-vector, using the Topo-TA-Cloning kit (Invitrogen, USA). The plasmids were isolated and purified by the Plasmid Mini Kit (Qiagen, Germany).

Cycle sequencing and electrophoresis

The purified PCR products and plasmids were sequenced by the dideoxy chain-termination procedure of Sanger et al. [41] using the cycle sequencing method which was performed according to the instructions provided by the manufacturer in the Dye Primer Cycle Sequencing Core Kit manual (PE Applied Biosystems, USA). PCR products were sequenced with fluorescence-labelled primer L182 and plasmids were sequenced with fluorescence-labelled reverse primer (5′ GGAAACAGCTATGACCATG 3′). Electrophoresis and detection of the fluorescence-labelled chain termination products were performed with an Applied Biosystems DNA sequencer model 373A.

Results

In most of the maternally related individuals, direct sequencing of the d-loop region produced ambiguous sequences behind the polycytidine tract between nt 303–309. Repeated sequencing reactions with independently produced amplification products always provided identical results. However, the sequence ambiguities differed in intensity between the tested persons. The highest background was always seen in FM I.1 and FM II.2, the lowest in FM III.5, UP 1 and UP 2 and the remaining individuals were intermediate. Figure 2 shows the sequence electropherograms of three family members (FMI.1, III.3, III.5) and those of the two unrelated probands for comparison (UP1,2).

In order to demonstrate that deterioration of the sequence chromatograms beyond the C-stretch is caused by length heteroplasmy, we cloned seven independently produced PCR products, sequenced approximately 100 clones for each person (12–18 clones/PCR product) and the results are summarised in Table 1. The findings were consistent for the independently produced PCR products demonstrating that errors of the Taq DNA polymerase in the first cycles of the amplification are unlikely to be the cause of the polymorphism (data not shown). Figure 3 shows the frequency distribution of the different length variants illustrated as pie charts in a genealogical tree.

Following the definition of Monnat and Loeb [2], which describes homoplasmy as a population that is 99.9% identical, all investigated individuals would have to be considered heteroplasmic with respect to the length polymorphism examined. However, the number and the extent of the different lineages occurring in each case varies among the individuals. Whereas FM I.1 showed four length variants in a ratio of 4 : 60 : 29 : 6, individual UP 2 showed only two length variants in a ratio of 13 : 81. Moreover, the proportions of each genotype varied widely among different maternally related individuals. FM II.2, III.1 and III.2 as well as FM II.3, III.3 and III.4 showed very similar proportions of the length variants but there was a complete switch of the mtDNA type between two generations (compare FM I.1 and II.2 and also FM II.4 and III.5).

Discussion

In a previous study, a heteroplasmic length polymorphism at position 16189 of the human mtDNA genome was identified by Bendall and Sykes [3]. This mutation is characterised by heteroplasmic variations from 9 to 13 bp in the length of a C tract due to the loss of the T residue at position 16189. The authors found that the length of this C stretch differed between but not within maternal lineages. In contrast, here we discuss a heteroplasmic length polymorphism at positions 303–309, which exhibits great variability among maternally related individuals.

Unrelated persons

Both unrelated persons showed clearly resolved sequencing bands before, in and after the homopolymeric tract at positions 303–309 (Fig. 2 UP1,2). At first sight, they seem to be homoplasmic; on comparison, however, the sequence of UP 1 is more unequivocal. This observation was reflected by the distribution of the lengths of the sequenced clones (cf. Table 1). An examination of 100 clones from UP 1 provided three types of mtDNA haplotypes whereas the distribution showed a clear preference for one type. In contrast, UP 2 showed only two different mtDNA haplotypes, but the second type was present in 14% of the clones.

This result confirms the finding of Bendall et al. [4] that a minimum contribution of 20% is required for detection of heteroplasmy by direct sequencing. Therefore, heteroplasmy might be more frequent than generally assumed. The lack of heteroplasmy observed in many population studies may have resulted from difficulties in detection by direct sequencing. Moreover, the cloning result of UP1 represents another piece of evidence to exclude the possibility of a systematic error such as slippage of the Taq DNA polymerase during PCR amplification. Among 99 clones only 3 (C)-8 clones and 1 (C)-6 clone were found.

Family members

The sequencing peaks of all family members, except for FM III.5, were clearly resolved before and in the homopolymeric tract, but were poorly resolved beyond it. Moreover, the extent of superimposition varied widely in different individuals. FM III.5 showed a sequence electro-

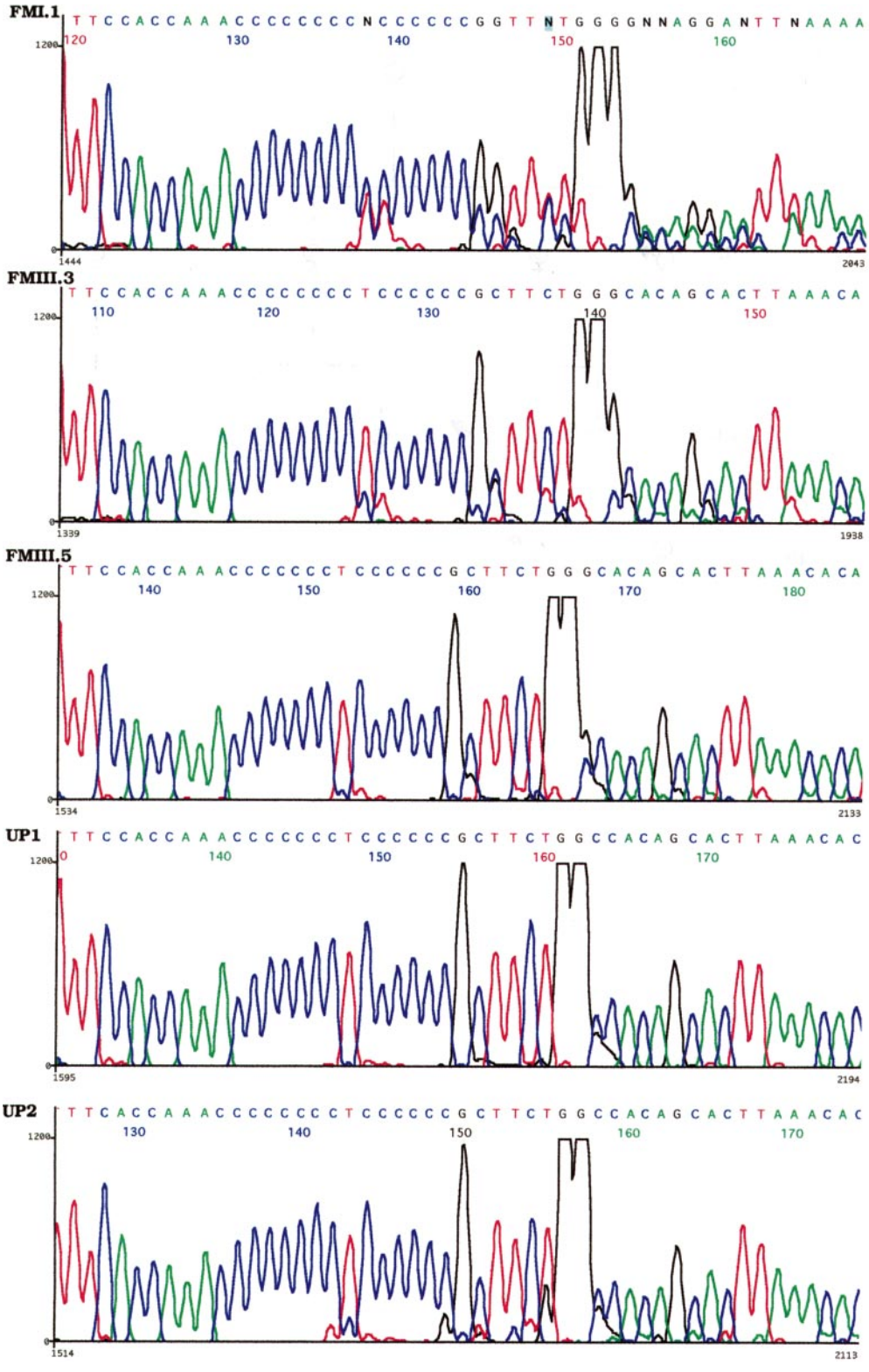

Fig. 2 Representative sequence electropherograms of three maternally related individuals (*FMI.1, III.3, III.5*) and two unrelated persons (*UP1,2*) (numbering system as in Fig. 1) showing the sequence around the homopolymeric tract between positions 303 and 315. FM I.1 and FM III.3 show particularly high background after the C-stretch caused by length heteroplasmy but in contrast, FM III.5 shows only low background. In direct comparison, UP 1 seems to possess a lower level of heteroplasmy than UP 2

pherogram with practically no background. The situation was also reflected by the distribution of the lengths of the analysed clones from the different individuals.

All family members have to be considered as heteroplasmic. Especially FM I.1, who showed a very blurred sequence chromatogram, possessed a highly heterogeneous pool of mtDNA fragment lengths. In contrast, the electropherogram of FM III.5 showed only low background and the results of the cloning analysis revealed only a low level of heteroplasmy.

Considering the transmission through the female germline, the family examined showed a very rapid segregation, i.e. a switch between two mtDNA haplotypes within one generation (cf. Fig. 3 a, FM I.1 and II.2 as well as FM II.4

Table 1 Distribution of mtDNA length variants within the C-stretch at position 303–309 in ten maternally related family members (FM) and two unrelated persons (UP). "Tract length" means the number of cytidine residues before the thymidine residue at position 310. "total number" indicate the total number of analysed clones

Number of clones												
Tract length	FM I.1	FM II.1	FM II.2	FM III.1	FM III.2	FM II.3	FM III.3	FM III.4	FM II.4	FM III.5	UPI	UPII
6	0	0	0	1	0	0	0	0	0	1	1	0
7	4	2	0	1	2	3	4	2	2	95	95	13
8	60	82	10	5	18	81	76	78	73	3	3	81
9	29	12	78	87	73	14	18	18	24	1	0	0
10	6	0	10	6	8	1	2	0	1	0	0	0
11	0	0	1	0	1	0	0	0	0	0	0	0
Total number	99	96	99	100	102	99	100	98	100	100	99	94

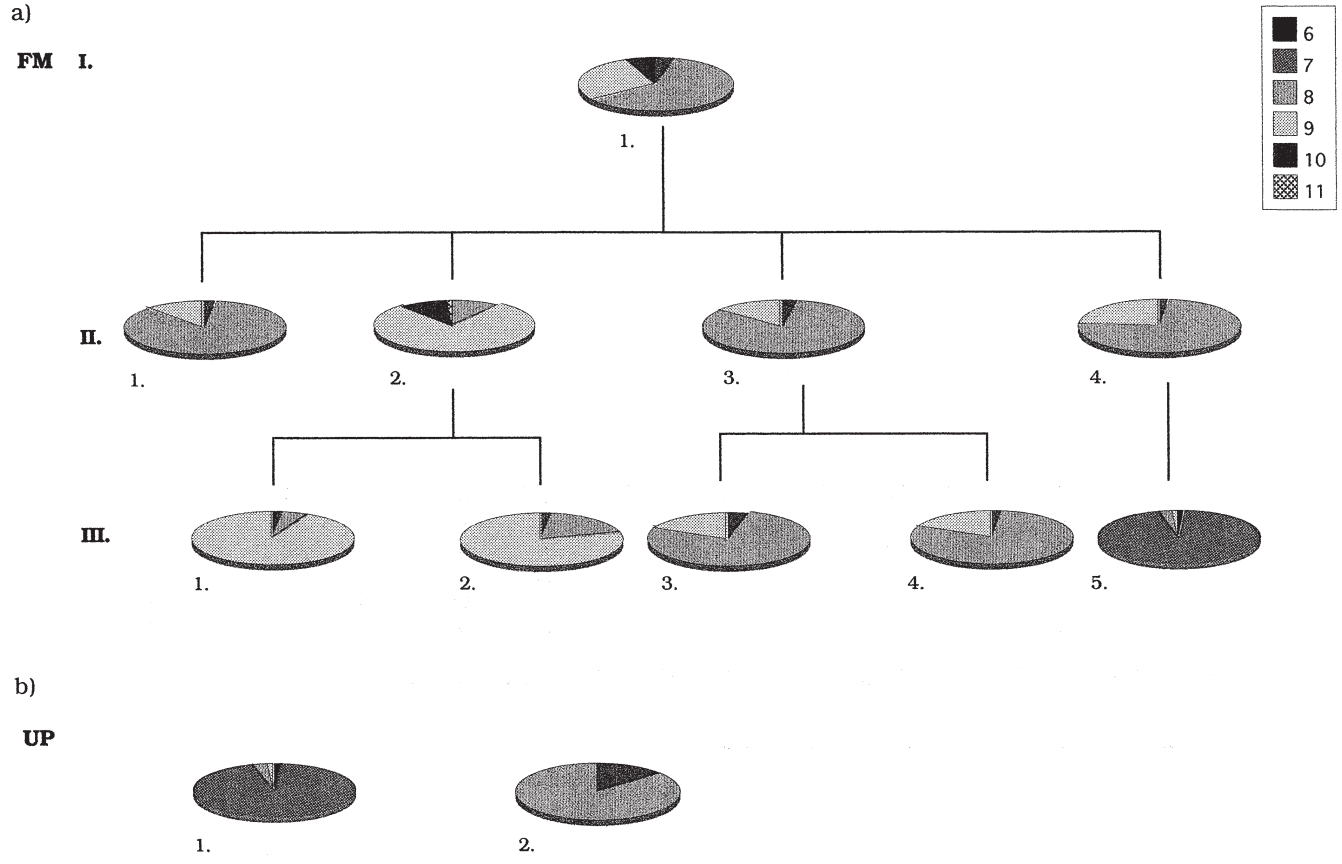

Fig. 3 a, b Genealogical tree indicating distribution of the length variants: **a** within the maternally related individuals; **b** within unrelated individuals. Symbols, numbering system as in Fig. 1. The legend shows the pattern for the number of C-residues

and III.5). This is in accordance with observations made in Holstein cows [27, 33] and with studies on humans, which also reported rapid shifts in haplotypes within one or few generations [4, 5 14].

Such rapid segregation is hypothesised to reflect a narrow bottleneck. The segregating unit should be very small, because FM II.4 exhibits only 2 fragments with 7 cytosines among 100 clones. In comparable cases, bottleneck sizes from 3 to 20 or 1 to 27 segregating units were calculated [4, 5]. In the present case, it is conceivable that either a population of maternal (C)-7 genomes or a genome which was modified by a mutation event was increased in its relative amount during oocyte maturation or embryogenesis. The exact developmental stage at which the bottleneck occurs is unknown. On the basis of studies by "trimmed PCR" Marchington et al. suggest a replication/amplification event occurring between conception and maturation of oocytes [13]. In mice, the segregation of mtDNA genomes seems to occur during cell divisions between the primordial germ cell stage and the primary oocyte [34].

However, some members of the family show a slowed segregation as fixed or similar proportions of different mtDNA types were maintained between two generations (cf. Fig. 3 a, FM II.2, III.1 and III.2 as well as FM II.3, III.3 and III.4). These results are in agreement with another study, which described individuals with heteroplasmic silent polymorphisms. All maternally related individuals studied showed similar frequencies of the different mtDNA haplotypes [3, 8].

This slow segregation would indicate a wide bottleneck, because fixed proportions of mtDNA genomes were apparently transferred from the mother to the child. It seems that a sufficiently large number of mitochondrial genomes may be inherited in each generation to maintain the same proportions of length variants in a distribution characteristic for each lineage.

One possible explanation of a stable heteroplasmy is the assumption of a structurally implicated joint transmission of two or more mtDNA types. In this respect, the organelle itself (intraorganellar heteroplasmy) or a network of mtDNA molecules (nucleoid formation) is conceivable. However, the published copy numbers of mtDNA genomes per organelle are incompatible with the suggestion of an intraorganellar heteroplasmy. Robin and Wong [42] found 2.3–4.3 genomes per organelle (mean values of 4 mammalian species), but for bovine oocyte 10 genomes per organelle were found [43].

Another explanation for stable heteroplasmy would be the transmission of mtDNA of uniform length and the subsequent de novo formation of length variants during mtDNA replication. This agrees with the fact that the rate of replication slippage within an uninterrupted homopolymeric tract is very high. However, it contrasts the observation that two branches of the maternal pedigree show different heteroplasmic profiles, although the individuals within a branch are similar to each other with respect to mtDNA length polymorphism (cf. Fig. 3 a, FM II.2, III.1 and III.2 as well as FM II.3, III.3 and III.4). Therefore, the formation of a similar distribution pattern of length variants would depend on an unidentified maternally inherited factor. However, this assumption is difficult to bring in accordance with the result of individual FM III.5, whose mtDNA haplotype differs totally from that of the mother.

The data have to be critically evaluated. The results reflect the situation in blood or buccal cells upon taking of the samples. Thus they may not necessarily accurately reflect the situation within the oocyte during oocyte maturation or embryogenesis (i.e. quantitative changes can have occurred during development). Moreover, the data are obtained by PCR-amplified DNA, which is not certain to reflect the in vivo situation in a correct manner. To exclude the possibility of a systematic error due to the inaccuracy of Taq DNA polymerase replication, we included a maternally unrelated, in direct sequencing apparently homoplasmic person (UP1) in the examination. The cloning results reflect a high reading accuracy of the enzyme. Again, we amplified ten clones by PCR and directly sequenced the products. The sequences were always readable, as expected for products of uniform length. Moreover, for each person we cloned seven independent PCR products to minimise the effects of a possible error of the Taq DNA polymerase in the first cycles of the amplification. The resulting clones of the different PCR products of one person show always similar distributions of the fragment lengths.

Examination of heteroplasmy plays an important role for understanding mitochondrial diseases and for the development of causal therapeutic strategies. In addition, examination of heteroplasmy is of great importance for the assessment of the forensic significance of mtDNA polymorphisms and, consequently also for utilisation of mitochondrial markers in phylogenetic studies. For instance, this study underscores the fact that a single sequence deviation is not sufficient for an exclusion in forensic questions, particularly, if the deviation is a length polymorphism [44]. A better understanding of the segregation mechanisms of mtDNA molecules and the occurrence of heteroplasmy will facilitate the correct interpretation of sequences in case work comparisons.

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