Am. J. Hum. Genet. 60:1495–1501, 1997

Skewed Segregation of the mtDNA nt 8993 (T→G) Mutation in Human Oocytes

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Summary

Rapid changes in mtDNA variants between generations have led to the bottleneck theory, which proposes a dramatic reduction in mtDNA numbers during early oogenesis. We studied oocytes from a woman with heteroplasmic expression of the mtDNA nt 8993 (T→G) mutation. Of seven oocytes analyzed, one showed no evidence of the mutation, and the remaining six had a mutant load >95%. This skewed expression of the mutation in oocytes is not compatible with the conventional bottleneck theory. A possible explanation is that, during amplification of mtDNA in the developing oocyte, mtDNA from one mitochondrion is preferentially amplified. Thus, subsequent mature oocytes may contain predominantly wild-type or mutant mitochondrial genomes.

Introduction

mtDNA has a high mutation rate, ~10 times greater than that of the nuclear genome. Individual cells contain several thousand copies of mtDNA, and, in normal individuals, nearly all of the mtDNA is thought to be identical (a state termed "homoplasmy") and of maternal origin. However, in some cases, especially in mitochondrial disease, wild-type and variant mtDNA coexist at different levels (a state termed "heteroplasmy"; DiMauro 1993; Shoffner and Wallace 1995).

It might be expected that a large number of generations would be necessary for a mutation or polymorphism that arose in one generation to increase such that it would represent a substantial proportion of cellular mtDNA. However, rapid shifts in mtDNA variant frequency have been observed in Holstein cows and also in human maternal pedigrees with either Leber hereditary optic neuropathy (LHON) (Bolhuis et al. 1990; Lott et al. 1990; Newman et al. 1991) or the nt 8993 (T→G) mutation (Tulinius et al. 1995; de Coo et al. 1996). To explain this rapid shift, it has been suggested that at some stage in oogenesis the number of mitochondrial genomes within any one developing oocyte is reduced to as few as five or less (Hauswirth and Laipis 1982, 1985; Laipis et al. 1988; Ashley et al. 1989; Koehler et al. 1991). Thus, with subsequent generations, some of the progeny of these oocytes may be either homoplasmic for the wild-type DNA or homoplasmic for the mtDNA variant.

Maintenance of a heteroplasmic silent mtDNA polymorphism in a multigeneration family with a homoplasmic LHON mtDNA mutation has been reported elsewhere (Howell et al. 1992). That study concluded that the number of mtDNA genomes at the bottleneck was 36–2,400, suggesting that mitochondrial gene segregation was more complex than predicted by the bottleneck theory.

The studies cited above are based on genetic analysis of mtDNA in white blood cells. It is becoming apparent, however, that the distribution of mtDNA variants in tissues is influenced by a number of factors and that the level of a mtDNA variant in blood does not always predict the distribution of the variant in other tissues (Holt et al. 1989; Poulton and Morten 1993). Thus, to understand the mechanisms that determine mtDNA variation during oogenesis, it is important to study oocytes.

We have analyzed mtDNA in oocytes from the asymptomatic mother of three children with heteroplasmic expression of the mtDNA nt 8993 (T→G) mutation associated with Leigh syndrome (Shoffner et al. 1992; Tatuch et al. 1992). Individual oocytes contained either very high or undetectable levels of mutant mtDNA molecules. We suggest that this may reflect preferential amplification of an mtDNA variant during oogenesis, and we discuss our results in light of the bottleneck theory.

Material and Methods

Oocyte Harvesting and Treatment

Oocyte cryopreservation and investigations not involving fertilization were approved by the Royal Wom-

Received September 25, 1995; accepted for publication March 13, 1997.

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0002-9297/97/6006-0029$02.00

en's Hospital ethics committee, and informed consent was obtained from all patients in this study. Control oocytes were donated by patients undergoing in vitro fertilization at the Royal Women's Hospital.

Ovarian stimulation regimens have been reported elsewhere (Gook et al. 1994). Oocyte maturation was triggered by an injection of human chorionic gonadotrophin (HCG; 10,000 IU; Profasi; Serono) administered when three follicles >18 mm were detected on ultrasound. Eight oocyte-cumulus complexes were retrieved transvaginally under ultrasound guidance 36 h after HCG administration. Oocyte-cumulus complexes were transferred to Earles balanced salt solution supplemented with synthetic serum replacement, pyruvate, and 1% (w/v) human serum albumin (Medicult). At no time were these oocytes exposed to sperm.

Individual oocytes were prepared, frozen, and thawed as described elsewhere (Gook et al. 1993). Complete removal of cumulus and corona cells from the oocytes was achieved by brief exposure to hyaluronidase (50 IU/ml), followed by mechanical disruption. The zona pellucida was partially or completely removed by digestion with 0.125% (w/v) pronase (Boehringer Mannheim) in 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid–buffered human tubular fluid medium (Irvine) supplemented with 20% (v/v) serum substitute (Irvine).

DNA Extraction

Total DNA was extracted from whole blood, cultured skin fibroblasts, tissue samples, and Guthrie blood spots, as described elsewhere (Blok et al. 1995*b*).

PCR Studies

1. Total genome amplification.—Pronase-treated oocytes (or 1 drop of pronase solution) were placed into 20 μl of sterile distilled water and stored at −20°C until required. Prior to PCR analysis, the samples were incubated at 95°C for 15 min to lyse the oocytes. Total genome amplification was performed in a total volume of 50 μl, as described elsewhere (Schmitt et al. 1994), by use of a thermal cycler (model FTS-320; Corbett Research), with conditions of 95°C for 3 min; 49 cycles of 95°C for 1 min, 37°C for 2 min, and 55°C for 4 min; and 1 cycle of 95°C for 1 min, 37°C for 2 min, and 72°C for 7 min.

2. Gene-specific PCR.—Either 2 μl of the first-round PCR described above or 20 ng of total DNA extracted from patient or control samples was used in a total reaction volume of 50 μl, for mtDNA gene-specific amplification. Reactions were as described above, except that, in place of the random 15mer primers, the mtDNA-specific primer set used was 8961F (5′ 8961–8980 3′) and 9050R (5′ 9050–9031 3′), each at a concentration of 10 μg/ml. Numbering of mtDNA nucleotide positions follows that of Anderson et al. (1981). The nt 8993 (T→G) substitution introduces a novel *Hpa*II restriction site at nt 8993, which is identified after digestion of the PCR product described above. PCR amplifications were performed for 35 cycles of 95°C for 1 min, 56°C for 1 min, and 72°C for 2 min. Ten-microliter aliquots of the PCR reactions were digested in a total reaction volume of 20 μl containing 20 units of restriction endonuclease *Hpa*II, at 37°C for 3 h. The samples were electrophoresed through a 3.0% [w/v] NuSieve agarose gel containing ethidium bromide. PCR products were visualized by use of a 320-nm UV transilluminator.

3. PCR from Guthrie blood spots.—To each of the eluted samples was added 10 μl of 2 mM dNTPs, 1 μl of primer 8961F (1 mg/ml stock), 1 μl of primer 9050R (1 mg/ml stock), and 0.5 μl of *Taq* polymerase (5 U/ml). PCR amplifications were performed for 35 cycles of 95°C for 1 min, 56°C for 1 min, and 72°C for 2 min). *Hpa*II restriction digests were performed as described above.

4. Radioactive (quantitative) PCR.—Either ~20 ng of total DNA or 2 μl of the first-round whole-genome amplification of the oocytes was amplified in a standard 50-μl PCR with the mtDNA primer set 8961F and 9050R. After 24 cycles of 95°C for 1 min, 56°C for 1 min, and 72°C for 2 min, 10 μCi of α^{32}P-dCTP was added to each sample during the denaturation step of the final cycle: 95°C for 3 min, 56°C for 1 min, and 72°C for 2 min.

Endonuclease *Hpa*II digestion was as described above. The products were analyzed after electrophoresis through a 15% nondenaturing polyacrylamide gel. The dried gel was exposed to x-ray film at room temperature, and the relative proportions of wild-type and mutant DNA were estimated after densitometry of the autoradiogram by use of a Bio-Rad® imaging densitometer (model GS-670).

To confirm complete *Hpa*II digestion, the primer set 3130F (5′ 3130–3149 3′) and 3558R (5′ 3558–3539 3′) was included in the PCR described above. This primer set amplifies the region of the mitochondrial genome either side of the *Hpa*II restriction site at nt 3246. Multiplex PCR products were digested with *Hpa*II and were analyzed as described above.

To determine the reproducibility of mtDNA mutant-load estimations from single oocytes, we performed PCR analysis on four samples of patient DNA (isolated from blood), with different loads of the nt 8993 (T→G) mutation. The mutant load was determined by six independent whole-genome amplifications of 350, 35, 3.5, and 0.35 pg of lymphocyte DNA of each sample, followed by radioactive gene-specific PCR analysis as described above. However, the number of PCR cycles was increased to 32, and the intensity of radioactive mutant and wild-type *Hpa*II bands was determined on a Molec-

ular Dynamics Phosphorimager. The mutant load of each sample was also estimated by direct gene-specific PCR analysis (without whole-genome amplification) of 20 ng of DNA samples.

5. DNA sequence analysis.—PCR-amplified DNA was sequenced as described elsewhere (Blok et al. 1995*a*). The mtDNA D-loop region was amplified by use of primer set 15981F (5′ 15981–16000 3′) and 57R (5′ 57–38 3′) and primer set 16541F (5′ 16541–16560 3′) and 630R (5′ 630–611 3′). The DNAs were sequenced by use of the same primers plus primer set 420R (5′ 420–401 3′) and 16400F (5′ 16400–16419 3′).

Results

Maternal Transmission of the mtDNA nt 8993 (T→G) Mutation

We identified the nt 8993 (T→G) mutation in two male siblings with Leigh syndrome (IV-3 and IV-1; fig. 1 [patient 23 and sibling, respectively, in Rahman et al. 1996]), a third male sibling who died at 3 mo of age with a diagnosis of sudden infant death syndrome (SIDS) (IV-2; fig. 1), and the asymptomatic mother (III-1; fig. 1). The mother has a mutant load of 50% in her blood, consistent with both her being a carrier of the pathogenic mtDNA point mutation and her lack of symptoms. Generally, expression of the nt 8993 (T→G) mutation at a level >70% is required for clinical symptoms (Santorelli et al. 1993; Shoffner and Wallace 1995). In contrast, the three progeny of this woman had levels >85% in tissues analyzed. The mutation was not detected in blood from other maternal relatives, when such was available. This suggests that the mutation either arose in individual III-1's mother or resulted from a spontaneous mutagenic event during embryogenesis in III-1 (fig. 1).

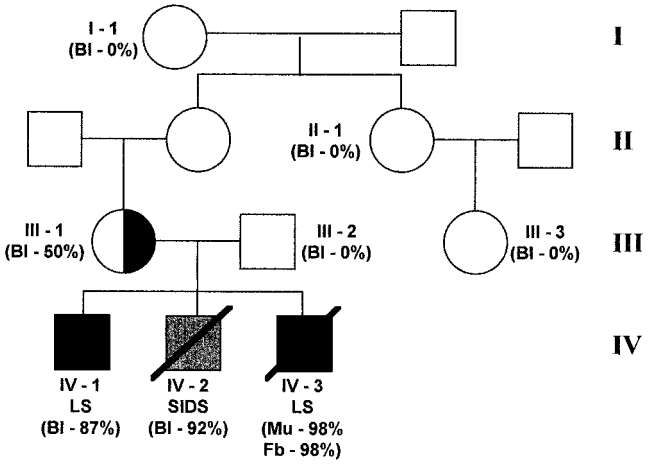

Figure 1 Familial expression of the nt 8993 (T→G) mtDNA mutation. When available, mtDNA from blood (Bl), muscle (Mu), or fibroblasts (Fb) from indicated symptomatic members (Leigh syndrome [LS] and sudden infant-death syndrome [SIDS]) and asymptomatic members of the pedigree was analyzed, by PCR, for presence of the nt 8993 (T→G) mutation. Mutant loads, which are shown in parentheses, were estimated after radioactive PCR and densitometry.

Skewed Expression of the mtDNA nt 8993 (T→G) Mutation in Oocytes

After genetic counseling, III-1 decided to have a tubal ligation to prevent any subsequent pregnancies. At her request, and after informed consent was obtained, her ovaries were hyperstimulated, and eight oocytes were harvested for analysis of the mtDNA mutation. Seven of the eight oocytes gave a result when analyzed for the nt 8993 (T→G) mutation (fig. 2). One oocyte (number 1) had only normal mtDNA, six oocytes (numbers 2–7) had a mutant load >95% (fig. 2), and one oocyte (number 8) failed to yield an identifiable PCR gene product, suggesting that this oocyte was probably not recovered after the pronase-treatment step. It is possible that the oocyte (number 1) that did not have a detectable load of the nt 8993 (T→G) mutation may have contained the mutation at a level below the sensitivity of detection in our study. Control oocytes from an unrelated female were also negative for the mutation. Aliquots of first-round no-DNA negative controls were included in the second-round mtDNA-specific PCR. These and second-round negative (no DNA) controls did not yield a PCR product, suggesting the absence of contaminating mtDNA in the reactions (fig. 2, lanes pri-1 and pri-2).

To confirm that the low levels of wild-type mtDNA that we consistently observed in oocytes containing the mutation were not artifacts due to incomplete digestion, multiplex PCRs were performed, in which the second-round PCR described above included primers for amplification of a nonrelated mtDNA gene product with a single homoplasmic *Hpa*II site. The control *Hpa*II site was cleaved to completion under conditions in which the mtDNA in the oocytes was not (data not shown). Sequence analysis of the mtDNA nt 8993 region in oocyte 1 showed a wild-type sequence, making it unlikely that the absence of a *Hpa*II site is due to a second mutation introduced during PCR amplification.

To ensure that there was not preferential amplification of one of the mtDNA variants, an aliquot of first-round whole genome–amplification PCR, from oocyte 1, was mixed with that of mutation-containing oocytes prior to the mtDNA-specific PCR. The presence of both mtDNA variants in the second-round PCR did not influence the efficiency of amplification of both sequence variants (fig. 3). We also performed whole-genome and gene-specific amplification on DNA from four samples containing various proportions of the nt 8993 (T→G) mutation. Assuming that a lymphocyte contains 7 pg of total DNA and has ~2,000 mtDNA copies (Vaillant and Nagley 1995), we calculate that 350 pg of lymphocyte DNA contains 100,000 mtDNA molecules, equiva-

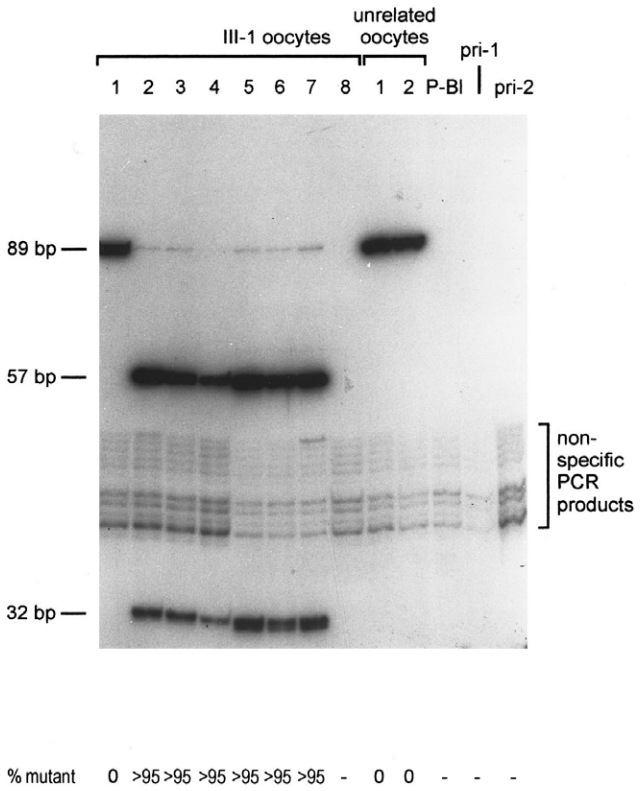

Figure 2 Oocytes displaying skewed levels of wild-type and mutant mtDNA. Whole-genome amplification of total DNA of lysed eggs from individual III-1 (lanes 1–8), control oocytes (unrelated oocytes 1 and 2), and negative PCR controls was performed simultaneously in a volume of 50 µl. mtDNA gene-specific amplification and quantitation of the nt 8993 (T→G) mutation utilized 1/25th of the whole-genome PCR in a standard second-round PCR with the mtDNA-specific primer set 8961F and 9050R, followed by *Hpa*II digestion. The nt 8993 (T→G) mutation introduces a *Hpa*II restriction site, so the expected digestion products are 89 bp (wild-type and uncut mtDNA) or 57 and 32 bp (nt 8993 [T→G] mutation present). The percentage of mutant mtDNA also is indicated. Lanes P-Bl, pri-1, and pri-2 show results in negative controls: P-Bl = pronase solution amplified in the presence of primers in both rounds of PCR; pri-1 = no-DNA/primers-only sample amplified in both rounds of PCR; and pri-2 = second-round mtDNA-specific PCR no-DNA/primers-only sample.

lent to that of a human oocyte. The four DNA samples (A–D) contained 0%, 19%, 30%, and 89% mutant mtDNA, as determined by direct gene-specific PCR analysis of 20 ng of DNA. Six independent whole-genome amplifications of 350, 35, 3.5, and 0.35 pg of DNA of each sample, followed by radioactive gene-specific PCR analysis, gave consistent and reproducible results, similar to those obtained by the direct analysis of 20 ng of DNA (table 1). The most-dilute samples gave the weakest signals and, consequently, the poorest signal-to-noise ratios. Nevertheless, even for samples containing only 0.1% of the mtDNA content of a single oocyte, there was equivalent amplification of both mtDNA species. Thus, preferential amplification of a single mtDNA species would be unlikely even if 99.9% of the mtDNA content of a single oocyte had been accidentally lost.

We sequenced the mtDNA D-loop region (a 1,119-bp fragment spanning nt 16031–580) of maternal blood and oocyte 1 and confirmed, on the basis of the identity of the DNA sequences, that the DNA in oocyte 1 was maternally derived and not due to external contamination. Both sequences differed from that reported by Anderson et al. (1981), at nt 263 (T→G) and at nt 303–309 (eight C's) and 311–315 (six C's). This D-loop sequence was not among 52 D-loop groups determined in 525 individuals, including 142 Caucasians (Stoneking et al. 1991). On the basis of the frequencies of D-loop variations, the sequence in the mother and the oocyte should be found in <3% of Caucasians, making it unlikely that the result from oocyte 1 is caused by contamination by "foreign" mtDNA.

To further exclude maternal or other contamination, X-chromosome short-tandem-repeat mapping was performed on the intron 45 short tandem $(CA)_n$ repeat of the X chromosome–encoded Duchenne muscular dystrophy gene (Clemens et al. 1991). Both analysis of relevant family members and aliquots of the first-round whole-genome amplification PCR of selected mutation-

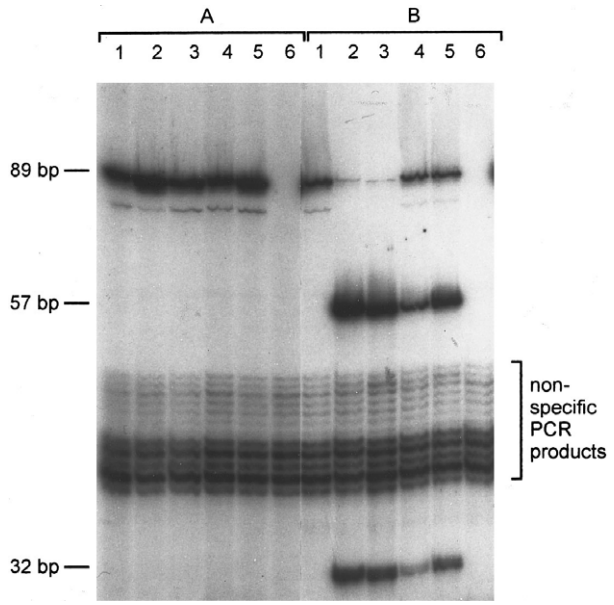

Figure 3 mtDNA amplification shown to be not variant specific. Aliquots (2 µl total) of the first-round whole-genome amplification of indicated oocytes were mixed and amplified in a second-round mtDNA gene-specific PCR with the primer set 8961F and 9050R. After radioactive PCR, 10-µl aliquots of the PCR reaction were digested with *Hpa*II. Undigested (*A*) and digested (*B*) products were electrophoresed through a 15% nondenaturing polyacrylamide gel. PCR products were visualized after autoradiography. Lane 1, Oocyte 1. Lane 2, Oocyte 2. Lane 3, Oocyte 3. Lane 4, Oocytes 1 and 2. Lane 5, Oocytes 1 and 3. Lane 6, No-DNA/primers-only control. *Hpa*II digestion products are as described in the legend to figure 2.

Table 1

Reproducibility of PCR Amplifications of Lymphocyte mtDNA

Sample[a] and DNA (pg)	Average Mutant Load (SD)[b] (%)
A (0%):	
350	1 (±1)
35	1 (±0)
3.5	3 (±1)
.35	5 (±3)
B (18%):	
350	16 (±1)
35	17 (±5)
3.5	15 (±4)
.35	16 (±6)
C (30%):	
350	31 (±3)
35	30 (±4)
3.5	28 (±2)
.35	23 (±4)
D (89%):	
350	88 (±2)
35	87 (±6)
3.5	85 (±3)
.35	83 (±5)

[a] Percentages shown in parentheses are the nt 8993 (T→G) mutant loads, determined by direct gene-specific PCR analysis of 20 ng DNA (without prior whole-genome amplification).

[b] Six independent whole-genome amplifications followed by specific PCR amplification of the mtDNA nt 8993 region were performed on each of the DNA samples.

containing oocytes and of the nonmutant oocyte of III-1 were consistent with the origin of the oocytes. Each oocyte tested had only one of the mother's X-chromosome alleles, indicating that there was no contamination of the oocyte mtDNA by maternal or other DNA (data not shown).

Discussion

It has not previously been possible to measure levels of an mtDNA variant in human oocytes. The frequencies of mtDNA variants in human tissues therefore have been determined in somatic tissues in which the variant DNA has been subjected to postfertilization segregation events and, possibly, tissue-specific selection. When the formula used to calculate mtDNA numbers at the bottleneck (Ashley et al. 1989; Howell et al. 1992) is applied to the blood mutant loads in our family, it yields an estimate of 800–4,000, whereas the data from the oocytes and from the mother's blood predicts a range of 15–70. The exact number of mitochondrial genomes at the time of the bottleneck is unclear. However, during maturation from the primordial oocyte to the mature oocyte, which contains ~100,000 mtDNA, the number of mitochondrial genomes increases by ≥50-fold, and the number of genomes per mitochondrion falls to an average of approximately two (Hauswirth and Laipis 1985).

It is striking that we saw only extreme levels of wild-type and mutant mtDNA in the oocytes. If there is random segregation of mutant and normal mtDNA after a "bottleneck," the presence of ~5% wild-type mtDNA in each oocyte carrying the mutation implies that there must be ≥20 genomes present at the bottleneck. In this case, however, we would expect to find some oocytes with intermediate degrees of heteroplasmy. Instead, the skewed mtDNA expression patterns suggest that additional factors influence the distribution of normal and mutant mtDNA. The simplest model that would explain our data would be a preferential, but not exclusive, amplification of mtDNA in perhaps just one organelle early in oogenesis.

Disease-causing mtDNA point mutations are frequently inherited, whereas single deletions of mtDNA are rarely inherited and thus appear as spontaneous mutations (Shoffner and Wallace 1995). Furthermore, there is an accumulation of mtDNA deletions and point mutations in somatic cells with age (Trounce et al. 1989; Münscher et al. 1993; Nagley et al. 1993; Shoffner and Wallace 1995). A mechanism must exist to minimize inheritance of deleterious mtDNA mutations. Chen et al. (1995) have suggested that this could be achieved if mutations did not arise in the female germ line, were actively removed in oocytes, or were only rarely transmitted through the mitochondrial bottleneck. Recently, Jenuth et al. (1996) have created a heteroplasmic mouse model that enabled them to study mtDNA distribution in mature and progenitor oocytes. It was concluded that random segregation and a bottleneck of ~200 mtDNA in mouse progenitor oocytes could explain the distribution of two polymorphic mtDNAs in heteroplasmic mice. It is difficult to envisage how such a model could explain our results and the rapid changes in nt 8993 (T→G) mutation load between generations in some human pedigrees (Tulinius et al. 1995; de Coo et al. 1996). Mice may segregate mtDNA variants less rapidly than humans do.

Two factors seem most relevant to the heritability of mtDNA mutations. First, if a mutation is present in only a small proportion of genomes in the primordial oocyte, as would be the case with age-associated mutations, then preferential transmission of a single genome usually will prevent transfer to the primary oocyte. Second, there is the functional consequence of a particular mtDNA mutation. Studies, at the cellular level, of pathogenic mtDNA point mutations suggest that these usually need to be expressed at a level >95% in order to show a biochemical defect (Chomyn et al. 1992; King et al. 1992; Trounce et al. 1994; Shoffner and Wallace 1995). These point mutations are unlikely to compromise en-

ergy production severely in the early embryo, which is not highly dependent on aerobic metabolism (Piko and Chase 1973). In contrast, deletions seriously affect energy production and cell function in cybrids when present at much lower levels (<60%) (Hayashi et al. 1991). Only mtDNA mutations that allow survival of the oocyte even when they are present at very high levels, will be passed on. A skewed replication process therefore could be an important mechanism for the removal of deleterious mtDNA deletions and severe point mutations, while still being compatible with maternal inheritance of milder point mutations. This also can explain the marked differences, in mtDNA mutant load, between siblings and generations (Larsson et al. 1992; Penisson-Besnier et al. 1992; Morten et al. 1993; Suomalainen et al. 1993; Tulinius et al. 1995; de Coo et al. 1996). The maintenance of a heteroplasmic silent polymorphism in one pedigree with LHON (Howell et al. 1992) might be explained by heteroplasmic mitochondria being the units of segregation.

The data obtained in our study have implications for the genetic counseling and prenatal testing of families with mtDNA point mutations. Although one might infer that there is an association between high maternal blood level of the nt 8993 (T→G) mutation and increased transmission risk, our model does not support the concept of a specific maternal blood threshold level distinguishing between high- and low-risk pregnancies.

If mtDNA mutations accumulate in the female germ line as a result of aging, then mitochondrial genetics must explain how many of these mutations are filtered out between generations. It also must explain the rapid segregation of polymorphisms and certain pathogenic point mutations, as well as the lack of inheritance of, for example, mtDNA deletions. We suggest that selective amplification of mtDNA in a randomly chosen mitochondrion during oogenesis may account for the observed skewed patterns and explain other unusual aspects of mtDNA genetics.

Acknowledgments

We express our gratitude to individual III-1 for providing us with the possibility for the genetic analysis of her oocytes; Dr. A. Trenholme (Middlemore Hospital, New Zealand), who managed the care of III-1 and her family; and Drs. R. Fisher and F. Graham (Fertility Associates Ltd., Auckland). We gratefully acknowledge helpful discussions with Prof. D. Danks, Prof. R. Williamson, and Dr. S. Forrest, as well as technical assistance from W. Hutchison, S. Nasioulas, and J. Shaw. This work was supported in part by an Institute Grant from the National Health and Medical Research Council (NHMRC) of Australia. R.B.B. was the Helen M. Schutt postdoctoral fellow at the Murdoch Institute, and H.-H.M.D. is an NHMRC Senior Research Fellow.

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