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# Variable Levels of a Heteroplasmic Point Mutation in Individual Hair Roots

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## Summary

**During direct sequencing of the first hypervariable segment of the human mitochondrial control region, we identified one individual with a heteroplasmic point mutation at nt 16256. We used primer extension to analyze the proportions of each mitochondrial haplotype in peripheral blood, buccal cells, and single hair roots from this individual and from eight members of his maternal lineage. Significant levels of heteroplasmy were found in only three individuals, and, in these cases, the proportions of each haplotype were similar in both blood and buccal cells. From the changes in mitochondrial haplotypes within mother-offspring pairs, we calculated that the most likely size of a mitochondrial bottleneck during development was 1–27 segregating units. However, highly variable levels of heteroplasmy were found in single hair roots, even among roots from the same individual. We analyzed a large number of hair roots from one individual and found that the proportion of one haplotype was within a range of 9% to >99% in different roots. Roots originating from within a small patch of skin had haplotype proportions as variable as those from different areas of skin.**

## Introduction

The widespread sequence variation in the mtDNA control region, among different individuals from the same species, coupled with widespread homoplasmy, suggests the existence of an unusual mechanism for the fixation of mitochondrial mutations. Using an estimate of a minimum of 100 mtDNA molecules in a mammalian germ cell and a maximum of 50 germ-cell generations, Upholt and Dawid (1977) calculated that, if mtDNA genomes segregate randomly during cell divisions of the developing oocyte, at least 20 generations from a mixed mtDNA population would be required to reach homoplasmy. These observations, together with a study of rapid segregation of heteroplasmic mitochondrial genotypes in cattle, led to the proposal of a bottleneck in mtDNA numbers at some stage of oocyte or early embryonic development (Hauswirth and Laipis 1982).

The bottleneck hypothesis has been studied in several mammalian species by examination of the segregation of mitochondrial genotypes within heteroplasmic maternal lineages. One study of Holstein cattle found that a heteroplasmic point mutation resolved to homoplasmy in two to three generations and concluded that a bottleneck is likely to contain 20–100 segregating units (Ashley et al. 1989). Subsequently, complete genotype shifts in a single generation in cattle led to the suggestion that a bottleneck could consist of only a single segregating unit (Koehler et al. 1991), although the polymorphism described is adjacent to a homopolymeric tract that may be prone to replication errors. In contrast, a recent study of heteroplasmic mice created by embryo fusion estimated that a bottleneck in mice contains ~200 segregating units (Jenuth et al. 1996).

In humans, there have been four detailed analyses of silent heteroplasmic point mutations. One of these concluded that a bottleneck may not always be small (Howell et al. 1992*a*), whereas the second found both rapid and slow resolution of heteroplasmy, within the same pedigree (Howell et al. 1996). In the third study, Parsons et al. (1997) reported rapid shifts in mitochondrial haplotypes within a single generation. Last, from analyses of two pedigrees, we calculated that a bottleneck contains 3–20 segregating units, although in some cases it may be substantially larger (Bendall et al. 1996). Therefore, considerable data have been accumulated to support the existence of a developmental bottleneck, although its size is disputed and may vary among different human mtDNA lineages.

The developmental stage at which a bottleneck occurs is also unknown. The original hypothesis postulated restricted amplification of mtDNA from a subset of templates during oocyte maturation (Hauswirth and Laipis 1982), but subsequently it was suggested that unequal partitioning of mtDNAs, during early embryonic development, also may contribute to an overall bottleneck

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**Table 1**

**Proportions of Heteroplasmy in Different Tissues from Family Members of Individual H5**

| Individual | Proportion of Heteroplasmy [Standard Error][a] (%) | | | |
|---|---|---|---|---|
| | Blood | Mouth Wash | Hair Root 1 | Hair Root 2 |
| II.1 | 25.0, 75.0 [.8] | 31.0, 69.0 [1.4] | <1, >99 | 91.0, 9.0 [1.2] |
| II.2 | 2.5, 97.5 [.2] | 14.0, 86.0 [1.3] | <1, >99 | ND |
| III.1 | 0, 100 | 0, 100 | 0, 100 | ND |
| III.2 (H5) | 27.0, 72.1 [1.1] | 35.7, 64.3 [1.1] | 53.5, 46.5 [.8] | 95.1, 4.9 [.7] |
| III.3 | 3.6, 96.4 [.2] | 6.7, 93.3 [1.1] | <1, >99 | <1, >99 |
| III.4 | 0, 100 | 0, 100 | <1, >99 | <1, >99 |
| III.5 | <1, >99 | 5.9, 94.1 [.8] | <1, >99 | 31.4, 68.6 [4.8] |
| IV.1 | 0, 100 | 2.0, 98.0 | <1, >99 | ND |
| IV.2 | 0, 100 | 0, 100 | <1, >99 | <1, >99 |

[a] The first number is the proportion of haplotypes with the CRS at nt 16256, and the second number is the proportion of haplotypes with a transition at nt 16256. In some cases, the proportion of minor haplotypes was so low that the fainter band visible by eye was below the sensitivity threshold of the densitometer, and, therefore, the proportion is expressed as "<1." ND = not done.

(Hauswirth and Laipis 1985). However, from estimates of the variance among heteroplasmic oogonia at different developmental stages, Jenuth et al. (1996) concluded that in mice the major bottleneck component occurs very early, during differentiation of primordial germ cells to primary oocytes. In humans, the segregation of heteroplasmic pathogenic point mutations has been examined in different tissues. In most cases, levels of wild-type and mutant mtDNA varied in different tissues from the same individual (Lott et al. 1990; Ciafaloni et al. 1991; Yen et al. 1992; Hammans et al. 1993; Howell et al. 1994; Matthews et al. 1994), although Tiranti et al. (1995) reported closely similar levels of heteroplasmy in blood and muscle, and Tatuch et al. (1992) found that several members of one pedigree showed nearly identical levels of heteroplasmy in different tissues. Overall, these results suggest that in humans somatic cell divisions after fertilization probably contribute to mitochondrial segregation.

We examined the levels of a heteroplasmic point mutation in the first hypervariable segment of the control region in three tissues from nine members of a maternal lineage. Only three individuals showed significant levels of heteroplasmy, and, in these cases, the proportions of each haplotype were similar in both blood and buccal cells. However, single hair roots showed highly variable levels of heteroplasmy, even among roots from the same individual. We examined a large number of hair roots from one individual and found that even roots from within a small patch of skin differed widely in their levels of heteroplasmy. Therefore, estimates of heteroplasmy levels from single hair roots may give misleading results, which is relevant to studies of mitochondrial diseases, to forensic investigations, and to population genetics.

## Material and Methods

### *Samples*

Genomic DNA was extracted from peripheral blood and from buccal cells by use of standard techniques. PCR templates from single hair roots were prepared with Chelex (Bio-Rad) (Walsh et al. 1991).

### *Amplification, Sequencing, Cloning, Primer Extension, and Densitometry*

Amplification, sequencing, cloning, primer extension, and densitometry were performed as described in the study by Bendall et al. (1996), with the following modifications. The PCR products from primer extension were generated with the primers conH23 (5′-ACT GTT AAG GGT GGG TAG G-3′) and conLC1B (5′-AAC TAT CAC ACA TCA ACT GC-3′). Primer extension was performed by use of conH256 (5′-TAT CCT AGT GGG TGA GGG G-3′) as an extension primer, with ddA, dG, dC, and dT. Each sample was analyzed with multiple independent PCRs and primer extensions. In tables 1 and 2, the values given for each sample are the mean and standard error of a minimum of eight and six readings, respectively.

### *Statistical Analysis of Bottleneck Size*

On the assumption of a simple model of the bottleneck process as a single sampling of a small number of units of segregation from a large pool, we determined, for a mother-offspring pair, the posterior probability distribution of the number of units, given the observed levels of heteroplasmy (with knowledge of the likely experi-

**Table 2**

**Levels of Heteroplasmy Present in Each Hair Root**

| Origin of Hairs | Proportion of Heteroplasmy [Standard Error][a] (%) | Mean Level of CRS (%) | SD (%) |
|---|---|---|---|
| Group A | 61.9 [3.8], >99, >99, 65.3 [3.8], 77.5 [2.3], 38.0 [1.4], 92.0 [0.6] | 76.1 | 21.0 |
| Group B | 51.8 [3.0], 10.7 [1.6], 9.1 [0.7], 74.9 [3.2], 71.8 [3.9], 44.4 [2.6], 80.1 [2.0], 12.8 [1.6], 72.2 [1.5], 20.6 [2.9], 42.1 [4.4] | 44.6 | 26.5 |
| Group C | 55.8 [3.4], 32.4 [4.2], 42.6 [6.4], 80.1 [3.0], 75.5 [4.5], 17.1 [3.5], 42.5 [2.0], 51.3 [4.1], 51.2 [5.7] | 49.8 | 18.5 |

[a] Each number is the proportion of the CRS, measured in an individual root. The standard error is given for repeated measurements.

mental error) in both individuals, as described elsewhere (Bendall et al. 1996). From this distribution, we quote the most probable size and a central 95%-credible region. We assumed a uniform prior distribution for the bottleneck size, up to a maximum size of 100 segregating units: for the cases described below, the results were insensitive to increases in this cutoff, since the data constrained the bottleneck to well below this value.

## Results

In a search for heteroplasmic point mutations in the human mitochondrial control region, DNA samples extracted from peripheral blood from 473 unrelated individuals were directly sequenced between nt 16050 and nt 16362 (Bendall et al. 1996). One individual, H5, revealed homoplasmic transitions at nt 16179 (C→T) and nt 16356 (T→C), relative to the Cambridge Reference Sequence (CRS) (Anderson et al. 1981), and a heteroplasmic C→T transition at nt 16256. The presence of heteroplasmy at this site was confirmed by sequencing clones (data not shown).

In order to examine the segregation of a noncoding heteroplasmic point mutation in different somatic tissues, primer extension was used to measure the levels of each mitochondrial haplotype at nt 16256 in peripheral blood, buccal cells, and single hair roots from individual H5 and from eight members of his maternal lineage (see figs. 1 and 2). The primer-extension results are shown in table 1. Accuracy of the results was verified by direct sequencing of 67 clones originating from the peripheral blood of individual H5, 18 (27%) of which had the CRS at nt 16256, which is in good agreement with the measurement from primer extension.

In both peripheral blood and buccal cells, a marked change in levels of heteroplasmy between a mother and her offspring was seen in only two pairs (II.1/III.1 and II.1/III.2 [H5]). The changes in proportions within these pairs were used to calculate the possible sizes of developmental bottlenecks in each tissue, and the results are shown in table 3. The similar 95% confidence limits in both blood and buccal cells within each pair suggest that an effective developmental bottleneck may have been of a comparable size in both tissues.

A number of other interesting observations arose from this pedigree. First, in contrast with the heteroplasmic pedigrees described elsewhere (Bendall et al. 1996), only two individuals (II.1 and III.2 [H5]) showed significant levels of heteroplasmy in peripheral blood, and the remaining seven maternal relatives were all either fixed or very nearly fixed for a transitional variant at nt 16256, relative to the CRS. Second, within each individual, levels of heteroplasmy were comparable between blood and buccal cells, with the greatest difference being 11.5% in

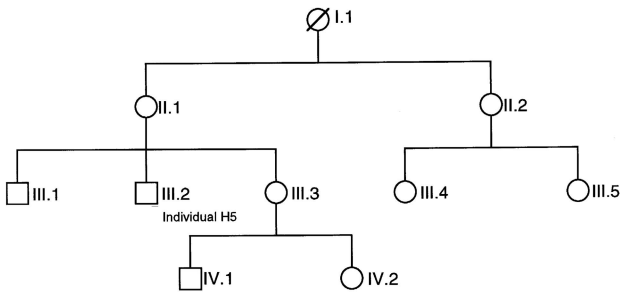


**Figure 1** Maternal lineage heteroplasmic at nt 16256

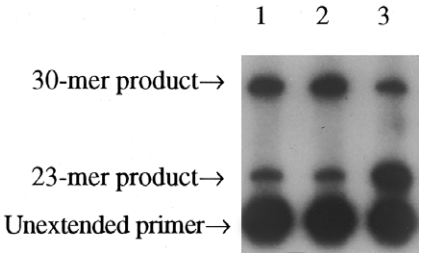


**Figure 2** Example of primer extension to quantify levels of heteroplasmy at nt 16256. Each lane shows an individual root from group B. Lane 1, 71.8% CRS. Lane 2, 80.1% CRS. Lane 3, 20.6% CRS.

**Table 3**

**Estimated Sizes of Developmental Bottlenecks in Two Mother-Offspring Pairs**

| Mother | Offspring | Tissue | Most Probable Bottleneck Size (Segregating Units) | Lower Limit on Probable Bottleneck Size (Segregating Units) | Upper Limit on Probable Bottleneck Size (Segregating Units) |
|---|---|---|---|---|---|
| II.1 | III.1 | Blood | 1 | 1 | 13 |
| II.1 | III.1 | Buccal cells | 1 | 1 | 10 |
| II.1 | III.3 | Blood | 27 | 24 | 31 |
| II.1 | III.3 | Buccal cells | 14 | 10 | 35 |

individual II.2. Third, levels of heteroplasmy in single hair roots from three individuals (II.1, III.2 [H5], and III.5) differed substantially from the levels in blood and buccal cells. Last, and most unexpectedly, in these three individuals, levels of heteroplasmy differed widely, even among hair roots from the same individual.

In order to investigate further the differing levels of heteroplasmy in single hair roots from one individual, we obtained a large number of roots from individual H5. Roots in group A were taken from a patch on the skull ~1 cm across and ~2 cm behind the right ear; roots in group B were from an ~1-cm patch on the skull, ~2 cm behind the left ear; and roots in group C were taken from the thumb side of the left wrist. Levels of heteroplasmy in each root are shown in table 2 and figure 3. The levels varied widely in different roots and were within a range of 9.1% to >99% CRS. The mean and SD of the proportions of the CRS haplotype show that roots within a localized patch were no more similar in their levels of heteroplasmy than were widely dispersed roots. The roots from the wrist showed a level of divergence as high as that for the roots from the skull.

## Discussion

In contrast with our earlier observations (Bendall et al. 1996), most members of this maternal lineage are fixed or nearly fixed for a variant at nt 16256 in peripheral blood and in buccal cells. The fluctuating proportions of heteroplasmy in members of generations II and III are compatible with the effects of random genetic drift operating through a small developmental bottleneck. The members of generation IV are effectively fixed for a variant at nt 16256. This is expected, since their mother (III.3) also is close to fixation for the 16256 variant: if bottleneck sizes are very small and the levels of heteroplasmy within the germ line of an individual are near fixation, the proportions of heteroplasmy in that individual's offspring are statistically likely to drift closer to fixation (V. A. Macaulay, unpublished data). The variant at nt 16256, therefore, is probably in the final stages of fixation within this lineage, although it also is possible that the variant was fixed within the lineage many generations ago and that a reversion to the CRS arose but is in the process of being eliminated.

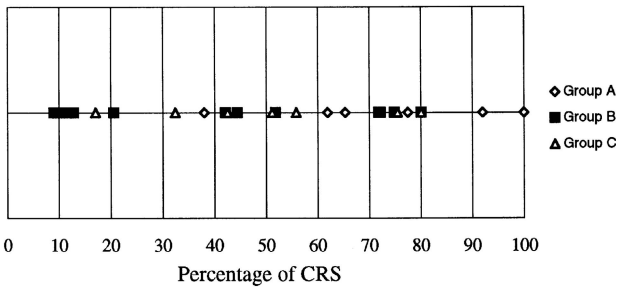


**Figure 3** Proportions of mitochondrial haplotypes in individual hair roots from different skin patches.

The variation in levels of heteroplasmy in different hair roots from one individual is probably a result of the developmental origins of hair follicles. Hair follicles originate in a crowding of cell nuclei in the basal layer of the epidermis, during the 2d or 3d mo of fetal life (Pinkus 1958). Some invagination of neighboring epidermal cells occurs in the early stages of follicle development, but most cells are produced by mitotic divisions of existing follicle cells, which differentiate to produce the various cell types seen in mature hair follicles. In a fully developed hair follicle, hair formation is thought to be induced by a small group of cells, the dermal papilla, in the base of the follicle (Montagna and Van Scott 1958). Hair roots and shafts are produced by rapid mitotic divisions of the matrix, which is a ring of cells surrounding the dermal papilla. Cells from the matrix move into the upper part of the hair bulb, increase in volume, and elongate vertically, eventually becoming keratinized.

Therefore, the variable levels of heteroplasmy in individual hair roots reflect the formation of each root from discrete groups of stem cells. In contrast, peripheral-blood samples consist of lymphocytes produced from very large numbers of hemopoietic stem cells in the bone marrow, and, if proportions differ in individual stem cells, the heteroplasmy measured is an averaged value of the proportions in many stem cells. Analyses of heteroplasmy levels in single lymphocytes from a heteroplasmic person might give proportions of the two

alleles that are as variable as those described here for individual hair roots.

We analyzed hair roots from discrete regions on the skull in order to investigate whether hair stem cells originating from common precursors showed more similar levels of heteroplasmy than stem cells originating from different precursors. However, the variation among roots within a 1-cm patch of skin was as high as that among roots from different patches. Presumably, stochastic segregation of mtDNA haplotypes between daughter cells occurs at every mitotic division, and the rapid cell divisions in developing hair follicles result in highly variable levels of heteroplasmy, even among follicles in close proximity.

These results suggest that in humans somatic cell divisions of the developing embryo contribute to the segregation of mtDNA haplotypes and that somatic segregation is occurring at least until the 3d mo of fetal life. The results are in agreement with a brief study of another human family, which also reported variable levels of heteroplasmy in single hair roots from one individual (Wilson et al. 1997). In contrast, a study of heteroplasmic mice concluded that mitochondrial segregation occurs only during early oogenesis (Jenuth et al. 1996). Our results also differ from the results of Jenuth et al. (1997), who examined levels of mtDNA haplotypes in the colonic crypts of heteroplasmic mice. They found variable levels of heteroplasmy among different crypts in mice at 4 mo of age. However, in mice 15 mo old, the crypts had segregated into two distinct populations, the majority of crypts having completely lost one genotype, whereas a minority contained predominantly that genotype. The mean proportion of heteroplasmy remained unchanged, in accordance with predictions from a random-drift model. In contrast, we did not observe segregation to homoplasmy in individual hair follicles. Since each hair follicle originates from a group of stem cells, rather than from a single cell, it is possible that successive mitotic divisions produced homoplasmic stem cells and that the variable levels of heteroplasmy within different follicles resulted from different ratios of homoplasmic cells within each follicle. However, the number of stem cells within each follicle cannot be very large, since in that case the differences, in levels of heteroplasmy, among individual follicles would be eliminated.

Estimation of levels of heteroplasmy within an individual, from a single hair root, is almost certain to give an inaccurate representation of the proportions in the zygote. In exceptional cases, individual hair roots may appear to be homoplasmic for different mitochondrial haplotypes within the same individual. Therefore, for forensic investigations, there very occasionally may be a discordance between the haplotypes derived from a single hair root and from blood, from the same individual, even though neither is demonstrably heteroplasmic. The probability of this happening is very low and would effectively vanish if two or more hairs were analyzed, even if the hairs were from the same skin patch.

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