Mitochondrial DNA Heteroplasmy

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ABSTRACT: Heteroplasmy, the presence of more than one type of mitochondrial DNA (mtDNA) in an individual, holds implications for forensic analysis of specimens such as blood, hair, and skeletal material. That is, what can we conclude about the likelihood that heteroplasmic specimens could or could not be from known individuals? Originally believed to be quite rare in healthy individuals, we now know that heteroplasmy exists at some level in all tissues on a predominantly homoplasmic background. A substantial body of general literature covers the biological origins of heteroplasmy, especially its transmission to new offspring and during life, the methodology for its detection, and its distribution in different tissues. In addition, the forensic community has contributed many observations on the characteristic appearance of heteroplasmy in relevant regions of the mtDNA control region and its appropriate treatment in forensic science. As a result of this growing understanding of a relatively simple biological phenomenon, we conclude that heteroplasmy can be expected to play a role in forensic interpretation on a regular basis, and that knowledge of its biological underpinnings contribute to just, conservative, and scientifically appropriate interpretational guidelines.

KEY WORDS: DNA sequencing, forensic DNA interpretation, heteroplasmy, mitochondrial DNA, mtDNA.

INTRODUCTION

Heteroplasmy is defined as the presence of two or more types of mitochondrial DNA (mtDNA) within an individual. In forensic analysis, the presence of heteroplasmy impacts the interpretation of mtDNA matches between evidentiary materials and individuals or their maternal relatives. It is important, therefore, that the mtDNA practitioner have a thorough understanding of the biological underpinnings of heteroplasmy, its frequency, methods of detection, and appropriate interpretational guidelines.

The biology of heteroplasmy has been investigated most often to clarify the origins of serious human mitochondrial DNA disease. A thorough review of mtDNA diseases, which are often characterized by heteroplasmy that affects the functioning of mtDNA products in certain tissues, is beyond our scope and will be covered only peripherally, as their characteristics reveal general biological mechanisms (for more information on heteroplasmy in disease, *see* [39,161,162]). Instead, the focus of this review will be on heteroplasmy in forensic science.

The baseline state of mitochondrial DNA composition in humans, with the exception of individuals with tissue-specific mitochondrial diseases, is homoplasmy. That is, the overwhelming majority of mtDNA-containing cells within an individual contain the same approximately 16569 base pair mtDNA molecule throughout. The exception to this dominant state of uniformity is the frequent occurrence of length heteroplasmy in certain control region homopolymeric C-stretches which changes the length of the molecule. Having stated that homoplasmy is the dominant state of an individual, however, it is also certain that some degree of heteroplasmy exists in all individuals as well. Precisely how detectable and abundant it is becomes the focus of the forensic DNA practitioner's concern.

I. BIOLOGY OF HETEROPLASMY

A. HISTORY OF OBSERVATIONS

The mammalian mitochondrial genome is a tightly constructed circular molecule of approximately 16 kilobases encoding genes for 13 polypeptides involved in cellular respiration, 22 transfer RNAs, and two ribosomal RNAs [2,42,148]. New variation in this small double helix, while constrained to protect against deleterious effects on the organism's survival (via reproductive fitness), is generated by the natural process of mutational change, that is, nucleotide substitution, insertion, and deletion. In fact, this mutational change is the process that generates abundant interindividual mtDNA variation, a characteristic that makes mtDNA a very useful forensic tool. Unlike nuclear DNA, mitochondrial DNA is not wrapped around histone proteins for protection, and the location of the molecule near regions of oxidative phosphorylation exposes it to high levels of mutation-generating oxygen free radicals. The mutation rate in mtDNA is about 10 times faster than that of nuclear DNA, and replication errors such as nucleotide mismatches are much less likely to be repaired [19,96]. Therein lie the basic origins of heteroplasmy. The persistence or loss of heteroplasmy is then determined by forces of natural selection and drift acting on the total population of mtDNA molecules in the individual.

Mitochondrial DNA heteroplasmy has been known to exist in mammalian species for about two decades. In cattle [67], the observation of a rapid shift in mtDNA haplotypes between members of a single maternal lineage led to the investigation and discovery of tissue-specific heteroplasmy in this same species [68]. Subsequently, wild mice were discovered to have a coding region deletion heteroplasmy in liver, kidney, and spleen [21]. Although little recognized, the earliest report of heteroplasmy in humans was in 1983 [60] at the time that the two hypervariable regions of the noncoding D-loop were described through cloning of mtDNA in 11 human placentas. In this report, the authors noted that restriction enzyme cleavage patterns differed among two mtDNA extractions from the same placenta, leading to speculation that heteroplasmy might be present. Previously, restriction studies dating back to the 1970s had indicated that homoplasmy was the rule [124]. Heteroplasmy has also been described in other mammals, from rats and rabbits [13,23] to, more recently, bats [122], opossums [122], and dogs [131].

A comprehensive overview of mtDNA as a forensic tool in humans in 1990 stated that the existence of heteroplasmy could not be ruled out due to observations of heteroplasmy in at least one disease at the time [24]. Originally believed to be quite rare in healthy individuals [110,111], length heteroplasmy and sequence or "site" heteroplasmy in humans have increasingly been both reported as anecdotal observations and studied in depth since a 1995 case report of a single individual with heteroplasmy at positions 16293 and 16311 [43]. An additional study about this time also suggested that certain human tissues, such as the brain, might contain higher levels of nondisease-related heteroplasmy than previously observed [88]. It is now widely believed that due to enormous increases in sensitivity in sequencing methodologies over the last decade, and the generally high quality of sequence data, heteroplasmy will be observed from time to time in routine forensic casework. In addition, a number of studies to examine the mtDNA mutation rate have specifically counted germline heteroplasmy observations in mother-child transmissions.

The most well-known forensic germline heteroplasmy observation was that of the two Romanov brothers, Czar Nicholas and Grand Duke Georgij, who shared heteroplasmic position 16169, thereby strengthening the putative identification of the czar's remains [58,84]. Beyond this kind of simple case reporting of individual heteroplasmy was the more comprehensive study of a mother, daughter, and son trio with heteroplasmy at position 16355 in hair, blood, and buccal samples in a forensic context [168]. Anecdotal reports of individuals with multiple sequence heteroplasmies have appeared in the literature [1,8,9,12,28,155], while a report of high levels of multiple sequence heteroplasmies in hair has been extensively challenged [25,26,49,61]. Interpretationally relevant heteroplasmy in forensic analyses is usually observed at a single nucleotide site in the region analyzed, where the appearance of two alternative nucleotides indicates that two different populations of mtDNA molecules co-amplified from the sample that was tested.

B. GERMLINE HETEROPLASMY AND INHERITANCE

Complex mathematical population genetics models for haploid organellar genetic systems (mitochondria and chloroplasts) have evaluated the effect of such variables as gene diversity, number of germ cell line divisions, effective number of segregating genomes, degree of paternal transmission, selection, and mutation rate [15,16,41]. These variables are different according to the mode of inheritance, and in a practical sense we need consider only two forms of transmission of mtDNA. Inheritance of heteroplasmy can be at the level of the germline, where different populations of mtDNA molecules are passed from mother to offspring, or at the level of somatic cells such as blood, hair, bone, muscle, or epithelium, where mtDNA replication and cellular mitosis disseminate heteroplasmy during growth or maintenance of tissues. In both cases, sampling effects determine the ultimate proportion of the different populations of molecules in the tissues that are forensically analyzed, and therefore impact the likelihood of forensic detection.

Mutational change is the obvious generator of new mtDNA variation in humans, and selectively neutral heteroplasmy can be thought of as the transitional state between fixed homoplasmic states. The simplest models of germline transmission of heteroplasmy assume that random drift but not selection is acting on the new variant, while many disease models, of necessity, must weigh selective effects because observed large-scale deletions and nonsilent substitutions negatively affect the functioning of the mtDNA molecule. Germline transmission of heteroplasmy has now been observed in a number of human pedigrees that are not specifically disease-associated [8,59, 78,102,121,168]; the list of disease-linked mitochondrial pedigrees is lengthy and beyond the scope of this paper.

In Hauswirth and Laipis's 1982 study of Holstein cattle, a rapid shift between genotypes differing at a single nucleotide was observed to occur within two generations, creating offspring that were homoplasmic for a new haplotype [67] (*see* also [5,94,121]). Because the number of mtDNA molecules in the mature bovine oocyte is large

(100,000; 100-fold greater than in somatic cells), a "bottleneck" was proposed, or an intermediate stage in oocyte development where the number of segregating molecules must be reduced to some very small number to probabilistically segregate the new variant, presumably present at low frequency after a single mutational event, into a nearly fixed homoplasmic state (*see* also [106]). In this sampling scenario, the replication of this small number of partitioned molecules containing the new variant would repopulate the new organism with a high proportion of the new variant. Genetic drift would then soon fix the new variant in subsequent offspring. This mechanism appeared consistent with relatively rapid replacement of an old variant with a new variant, with a complete change within two or three generations. In fact, using the Wright/Solignac model of genetic drift [138,170], the number of segregating units (mtDNA molecules) at the bottleneck in cattle was estimated at between 20 and 100 [5].

Studies of the so-called "bottleneck" in mtDNA inheritance are complicated by the fact that heteroplasmic pedigrees have been examined from somatic tissue samples, and oocyte development cannot easily be studied in humans [125]. The number of germline cell divisions is also critical to estimates of the bottleneck size, but in mammals has been only estimated at between 10 and 50 [5,158]. However, a study in mice has shed light on possible mechanisms of germline heteroplasmy transmission [89]. Heteroplasmic mouse oocytes were quantitatively genotyped for their heteroplasmic variants at all stages of development, from primordial germ cells through mature ova. When the variances between the cells were compared, the lowest intercellular variance in haplotype copy number was observed among the primordial germ cells, the progenitors of oogonia, and the highest variance was observed among the primary oocytes and mature oocytes within an individual. This suggested that the segregation of heteroplasmic variants must be occurring during a period of low copy number during the development of primordial germ cells in the mouse embryo and prior to the overwhelming increase in mtDNAs that occurs during oocyte maturation. The mature human oocyte contains approximately 100,000 mtDNA genomes [32], while mice have about the same number [123], and, using the same Wright/Solignac genetic drift equation applied to cattle, approximately 200 mtDNA molecules have been estimated to be present at the bottleneck in a mouse oogonium [89]. It is still unclear whether mouse models are a good starting point for speculation about what happens in human mtDNA inheritance. A study of mature human oocytes showed a highly skewed distribution of two variants in seven oocytes, with one oocyte containing only the wild-type variant, and the other six being heteroplasmic for the wild-type and mutant variants, with a 95% mutant load [17]. This suggested a bottleneck at some stage with $\geq$20 mtDNA genomes. However, generalizing to all mammalian species, a bottleneck as low as a single molecule may be required to explain some observations [94].

These models, however, are predicated on rapid replacement of haplotypes within a few generations, with a resulting transitory heteroplasmic state [38,98]. Persistent heteroplasmy has been observed in human pedigrees that would not be consistent with small bottlenecks or dramatic genetic drift [78]. A silent substitution in the ND6 gene persisted in a family spanning three generations and eight individuals, while the proportions of the two variants changed little between generations. This slow segregation phenomenon needs a more complex model to explain it, perhaps one invoking the actual organizational structure of the cell. Here, in theory, clusters of membrane-bound mtDNA molecules called nucleoids [86,130] segregate as an organellar unit, rather than as a panmictic, independently segregating population within the whole cell [78]. However, the overall intracellular organization of mtDNAs is unresolved, as more recent studies suggest that because transcomplementarity appears to be occurring intracellularly for some heteroplasmy diseases, extensive intermixing of wild-type and mutant mtDNAs is the norm, as highlighted by histochemical staining techniques [69, 156].

Persistent heteroplasmy is often found in families harboring mitochondrial DNA diseases [34,38]. In most of these diseases, the mutant deleterious form of the molecule tags along through growth and development because enough wild-type or normal molecules preserve adequate cellular functioning [148]. The severity of the disease phenotype is associated with the dose of mutant molecule inherited by the affected individual, and positive or negative selection may operate at the level of the affected tissues to either increase or decrease the mutation load during the individual's lifetime [38]. What is currently not clear is whether selection is operating on these phenotypes during oocyte development, because affected individuals and their matrilines have only been studied at the level of blood sampling. However, the simplest deleterious mutations usually take the form of single site changes that alter the transcribed gene products involved in oxidative metabolism. DiMauro [50] notes that the "morbidity map" of mtDNA, that is, known mutational changes responsible for disease, has grown from one point mutation described in 1988 [163] to 115 point mutations by 2001, which are associated with a panoply of sometimes serious human diseases. Of these, it is rare to observe one originating in the noncoding region (but *see* [7]), although certain haplogroups, or groups of similar mitochondrial

control region profiles, have been correlated with disease phenotypes in some populations [134,162]. In addition to point mutations and single-site deletions, numerous larger-scale deletions and rearrangements are associated with mtDNA diseases; the first of such disease phenotypes related to this form of mutation was reported in 1988 [76]. While deleted molecules are not usually passed from clinically affected women to their children [35], duplications or other rearrangements may be inherited [6].

A recent report on an individual with lifelong severe exercise intolerance who was heteroplasmic for both paternal and maternal forms of mtDNA was a startling example of heteroplasmy in disease ([132], and also *see* [140]). The phenomenon of strict maternal inheritance of mitochondrial DNA, which is due to the ubiquitination of paternal mtDNAs and their subsequent destruction after fertilization, is still, however, believed to be the norm among mammalian species [145,146]. In a disease state such as the one described above, heteroplasmy might be evident in some tissues but not all, as in this case, where a minor maternal component was observed on the background of the father's haplotype in muscle, but blood, hair, and cultured fibroblasts were maternal in origin.

Despite the frequency of mutational change in mtDNA, homoplasmy remains the baseline state for individuals and tissues throughout life, and researchers are trying to find out why this is true. Population genetics predicts the steady accumulation of deleterious mutations in haploid genetic systems where there is no recombination, which could in theory result in the demise of a species ("Muller's rachet", [116]). However, mitochondria may have evolved the bottleneck as a protective mechanism to restore homoplasmy in the germline [11]; this mechanism may then be enhanced by a phenomenon known as germ cell atresia. In this process, female mammalian germ cell populations are drastically reduced through programmed cell death during gestation and neonatal life; some authors believe these measurable events to be evidence of deliberate reduction of poorly functioning mtDNAs [95]. Between atresia and the bottleneck, mitochondrial numbers are reduced to a level that reveals the functioning of each cell [128] and propels outstanding performers to the next stage of development: full oocyte maturation.

C. SOMATIC HETEROPLASMY AND TISSUE SPECIFICITY

Beyond germline inheritance, mtDNA heteroplasmy has been closely examined in somatic tissues. A distinction must be made here between somatic heteroplasmy that has been present since birth and may or may not be partitioned into various tissues during early embryogenesis, and heteroplasmy that arises spontaneously at any time during an individual's life within one or more tissues. Both kinds of heteroplasmy hold implications for forensic analysis. At a basic level, the former impacts estimates of the mtDNA mutation rate as it relates to human evolutionary studies and studies of maternal pedigrees, and may be particularly relevant in forensic casework that involves comparison between individuals of a maternal lineage. The latter has more relevance in studies of intraindividual forensic comparisons, for example, comparison of different kinds of tissue samples taken from the same person.

Studies of somatic tissue heteroplasmy in mitochondrial diseases cannot be considered strictly illustrative of the biology at play in forensic heteroplasmy evaluation. Obviously, mutations that severely impact the phenotype of a tissue may be operating under directional selection; there is evidence that some deleterious point mutations or deletions may even result in a replicative advantage for those mtDNAs carrying them [115,171]. Research in this area has been examining whether, after maternal inheritance has occurred, subsequent distribution of deleteriously mutated mtDNA into different tissues in the offspring is random or not [48,75]. Studies of pathological tissue have given clues to the dynamics of all tissue heteroplasmy and its distribution. Within five individuals with the 3242 point mutation that is responsible for MELAS (mitochondrial encephalopathy with lactic acidosis and strokelike episodes), Chinnery et al. [40] reported the highest level of mutant DNA in skeletal muscle, followed by hair follicles and buccal mucosa, and the lowest levels in blood. Because this precise pattern of distribution was also then observed in an unaffected individual with low mutation loads throughout these same tissues, the authors suggested that this nonrandom dosage hierarchy is correlated with the rate of cell turnover in different tissues, with the cells having the highest rate of turnover having the lowest levels of heteroplasmy. In fact, the lowest levels of heteroplasmy have consistently been found in blood with its freely circulating panmictic cell population. This may be why blood is not usually a satisfactory sentinel tissue for diagnosis of mitochondrial diseases that most affect other tissues such as skeletal muscle or the nervous system [151].

In addition, in all cell types mtDNA is constantly being degraded and replaced, even in nondividing cells such as central neurons and skeletal muscle [37]. Mathematical modeling of this "relaxed replication" [14] in a neutral-alleles scenario was performed for a two-haplotype model with a 75:25 haplotype proportion. This modeling assumed that the number of mtDNAs remained fairly constant over the life of the cell, that each mtDNA was copied only one time when it replicated, and that no

cell partitioning was operating [36]. While the individual cells' proportions of the two haplotypes varied widely over the 20-year simulation period, with loss or fixation of haplotypes in some cells, the overall proportions of each haplotype in the group of cells remained highly constant. These results suggested that sampling large numbers of cells at multiple, distant points in time (longitudinally) would be better at uncovering the mechanisms of heteroplasmy distribution than sampling small numbers of cells at single points of time. In this same study, among other observations, the mean time to fixation of an allele was proportional to the number of mtDNAs in the cell, and the proportion of cells that became fixed for an allele was equal to the initial allele frequency. Because mtDNA copy number is so closely related to probability of fixation of an allele, the authors of this study propose that human cells contain high numbers of mtDNAs for protection against accumulation of mutations during long life (Muller's rachet). This, in turn, may explain why short-lived cells such as sperm and leukocytes [3,172] have vastly smaller numbers of mtDNAs than cells with long life spans such as skeletal muscle, neurons, and oocytes [98]. A related hypothesis is that cells with high energy demands, such as brain and skeletal muscle, have more mtDNAs to provide for larger cellular respiratory needs, and these cells are exposed to high levels of free radicals, leading to higher mutation rates and therefore higher levels of heteroplasmy [63,74,135].

A study by Jenuth et al. [90] of genetically engineered heteroplasmic mice with two mtDNA genomes differing by 0.6% (neutral polymorphisms) gave a mixed picture of the forces that may be operating on tissue-specific heteroplasmy. These authors first genotyped cells from the mouse colonic crypts, where cells are constantly replenished from a small population of stem cells at the base of each crypt. These groupings of clonal cells would be highly analogous to hair shafts, a similarly generated tissue. Here, the observed proportions of the two heteroplasmic variants were consistent with a random genetic drift model of mtDNA segregation acting throughout the life of the animal. However, liver, kidney, spleen, and blood in one-month-old mice showed significantly different proportions of genotypes. In all the mice, the two genotypes segregated in opposite directions as blood/spleen vs. liver/kidney, and the rate of segregation was faster in liver and spleen than in kidney. In contrast to the crypt experiment, this result was highly consistent with selection acting on the different tissues, either because of replicative advantage, advantageous respiratory chain function, or enhanced cell turnover rate due to one of the genotypes. In addition, because two different nuclear backgrounds were operational in conjunction with the heteroplasmy as a part of the study design, resulting in different rates of cell turnover in the liver, the authors proposed that nuclear-mitochondrial interactions might be playing a large role in regulating the heteroplasmy genotypes (*see* also [51]). Hence, tissue-specific heteroplasmy may be determined by very complex, and as yet not well understood mechanisms.

Whether mitochondrial DNA mutation, and by extension, heteroplasmy, increases with age is currently being debated. This is part of a larger debate on the role of mitochondria in senescence, cancer, and disease [4,37, 99]. While some groups have not observed age-related mutational change [118], others have [44,45,93]. The so-called "common deletion", often found in patients with Kearns-Sayres syndrome and progressive external opthalmoplegia, or mtDNA^{4977}, was also observed to increase with age as a heteroplasmic fraction in heart and brain tissue in individuals without any known disease ([46,107], but *see* [159]), and the variation in deletion level between the same tissues in different persons of similar age appeared to be less than the variation among tissues within an individual [47]. This same pattern of tissue variability and age-related change was observed in a study of the noncoding hypervariable region 2 (HV2) from heart, skeletal muscle, brain, and blood from autopsy cases [28]. Using sequence-specific oligonucleotide (SSO) probes, sequence heteroplasmy was detected in 11.6% of the sample (5 of 43 individuals) and the highest frequency of heteroplasmy was found in skeletal muscle of individuals over 60 years of age. These authors suggested that the age of an individual might need to be considered in making forensic comparisons and that skeletal muscle should perhaps be avoided where possible when doing forensic testing. Both inherited and novel somatic heteroplasmies were observed in the sampled individuals. The examination of multiple tissue types allowed speculation about origins: where the heteroplasmy was pervasive among several tissues, a maternal source was postulated. Using cloning, Jazin et al. [88] also noted age-related increases in heteroplasmy in brain tissue of older individuals typed for the noncoding control region.

Estimates of the frequency of point-mutation heteroplasmy in different tissues, also known as sequence heteroplasmy, in nonpathological states have been offered by different groups. The following reports do not include evaluations of length heteroplasmy, which will be covered in another section. Using SSO typing, Calloway et al. [28] observed HV2 sequence heteroplasmy in muscle tissue in 9.3%, in blood in 4.7%, in brain in 4.7%, and in heart in 4. 7% of individuals. In addition, sequence heteroplasmy at additional positions in the control region in the muscle tissue of several of these individuals was detected by

sequencing. Using sequencing, Melton and Nelson [108] reported frequencies of 8.6% in muscle tissue, bone, or organ tissue, 1.7% in whole blood, and 9.7% in hairs in a review of 105 forensic cases that analyzed 336 samples.

Attempts to estimate the mitochondrial DNA control region mutation rate have led to fairly numerous and consistent estimates of the frequency of heteroplasmy in blood, at least with sequencing methods currently used in forensic laboratories. A direct sequencing study of cloned DNAs from blood samples from 180 twin pairs revealed that 1.7% of the twins were heteroplasmic; two twin pairs shared their heteroplasmic positions, while in two other pairs, one twin was heteroplasmic and the other was not [8]. In an examination of blood from mother-child pairs by direct sequencing, four of the children were observed to have sequence heteroplasmy (2.6%) [81]. A sequencing study of blood designed to estimate the mutation rate in the control region that also was specifically looking for heteroplasmy confirmed sequence heteroplasmy in three of 357 individuals (0.84%), and could not rule it out in "several other lineages" where electropherograms were suggestive of low levels of heteroplasmy [121]. Four of 162 unrelated Japanese individuals sampled by sequencing for a database were noted to have sequence heteroplasmy in blood (2.5%) [83]. Using single-strand conformation polymorphism (SSCP) screening, a frequency of 2.5% was reported in samples from CEPH lymphoblast cell lines or blood from 119 individuals [59]. Using SSO typing on blood samples, Reynolds et al. [127] reported a frequency of 0.73% and observed no additional heteroplasmic positions in these individuals after HV2 region sequencing. Taken as a group, these studies appear to indicate that heteroplasmy in blood is a relatively uncommon phenomenon, but most of these studies relied on direct sequencing as a detection method, where the level of sensitivity for detection of proportion of a second variant of a sequence has been estimated to be about 20% in mixture studies [167]. This consensus could be contrasted with a DGGE (denaturing gradient gel electrophoresis) study of blood samples from 253 individuals showing an overall level of 13.8% heteroplasmy; this method is capable of detecting sequence heteroplasmy at levels of 1% [155]. Three of the 37 heteroplasmic positions in this study were detectable via direct sequencing, but most of the other heteroplasmic positions were found at very low levels.

In contrast to measures of frequency in blood, the level of sequence heteroplasmy in hairs, a frequently used forensic tissue, appears to be higher with current forensic detection methods, although there have been conflicting reports. Hühne et al. [82] observed no heteroplasmy in 150 hair shafts sequenced from 10 unrelated individuals, and no intraindividual differences, while Melton and Nelson have reported a frequency of 9.7% in hairs from casework [108]. A report by Grzybowski [61] reported 24 different heteroplasmic positions in 100 hair roots from 35 individuals, some in combinations of up to six sites in a single individual, but this report was challenged and debated on the basis of methodology, especially an excessively high number of PCR cycles, and faulty data [25,26,49,154]. A subsequent re-examination by the original author [62] using 38 cycles of direct PCR instead of nested PCR showed that none of the 13 hairs in question exhibited more than two heteroplasmic positions.

Reynolds and Calloway have carried out several unpublished studies on heteroplasmy in hairs (personal communications, R. Reynolds). Sequencing of mtDNA from over 500 hairs from 24 different individuals showed that 37.5% (n = 9) of these individuals had sequence heteroplasmy in one or more hairs. The frequency of heteroplasmic hairs observed within those heteroplasmic individuals varied from 2 to 28% but usually was less than 10%. These authors also have observed that the variant proportions of a heteroplasmic site may shift along the length of the hair shaft. Similarly, a collaborative project organized by the Spanish and Portuguese working group of the International Society for Forensic Genetics (GEP-ISFG) to study hair heteroplasmy in a single donor [1] observed the same phenomenon. In this study, the variation in proportions of the two sequence alternatives along fragments of a single hair shaft was similar to that observed in different hairs. As always, frequency estimates of heteroplasmy in any tissue are reliant on the methods used for detection.

The mechanisms for the development of heteroplasmy in hair may be more complex than those in other tissues [100], and invoke some degree of preliminary segregation of mtDNA types during tissue differentiation of the embryo followed by a secondary bottleneck at the hair shaft development stage. Although the exact number of precursor stem cells in this ectoderm-derived tissue that gives rise to the single keratinized hair shaft is not known, it is presumed to be small [114], and the number of mtDNAs in a hair stem cell is really not known but is not presumed to be different from the number in other stem cells. However, interesting microscopic observations of mitochondria in hairs are found in Montagna [113]. This author notes that the number of mitochondria in hairs is never as high as that seen in cells of the surface epidermis, and that for a rapidly proliferating tissue, the number of mitochondria is surprisingly small. This observation may raise questions about the potential size of the bottleneck in hair development.

Generalized heteroplasmy that has been inherited from the mother may result in all or most of an offspring's

hairs, as well as other tissues, being heteroplasmic for her variants. Bendall et al. [9] studied an individual with sequence heteroplasmy at nucleotide position 16256 in hair, blood, and buccal tissue. Additional members of this subject's maternal lineage also displayed heteroplasmy at the same position in hair, blood, and buccal cells, indicating that the heteroplasmy was pervasive and maternal in origin. Although the proportions of the two variants remained approximately the same in blood and buccal samples of the subject, the proportions differed widely in individual hair roots with the wild-type variant ranging from 9 to > 99% of the total; this is a good example of inherited heteroplasmy followed by segregation of haplotypes in hairs that have undergone a secondary bottleneck. In addition, hairs from adjacent follicles showed equally variable proportions of the variants as did hairs from different locations on the body, indicating that the segregation of variants in the hairs may have been following random drift models in all locations. Similar results were observed in a study by Wilson et al. [168] where a heteroplasmic site in hairs of three family members demonstrated widely varying proportions of the two pyrimidines C and T.

In a follicle's germinal bulb matrix, cells leaving to become the hair shaft never divide again, but differentiate to become cuticle cells, medullary cells, and cortical cells. Heteroplasmy that arises at any stage of a single hair's development will be present in only that hair, and the variant may not be observed in any other cell population in that individual. It follows then that different hairs from the same individual also may have different heteroplasmic variants. However, with an empirical observation rate of under 10% [108], heteroplasmy does not appear to be present in a majority of hairs sampled for forensic testing. The higher observed rate of heteroplasmy in hair is due to the peculiar sampling strategy of hair analysis: a single hair from a clonal precursor population that has passed through a small bottleneck. This may be contrasted with the observed low levels in blood from sequencing, which has been sampled from a large panmictic population of peripheral blood cells derived from many stem cell sources. Interestingly, based on the DGGE blood study by Tully et al. [155] it seems possible that the level of heteroplasmy may actually be similar in blood but is less detectable due to sampling strategy and detection methods.

D. MUTATION RATE AND POLYMORPHIC HOTSPOTS

Estimation of the mutation rate in the mitochondrial DNA molecule, particularly the noncoding control region, has been covered extensively elsewhere (in addition to this volume, among others *see* [80,109,117,121,136,150]). What is clear is that not all polymorphisms behave the same way biologically: certain nucleotide positions mutate more frequently than others [52,65,87,109,143,160]. Statistical analyses by Malyarchuk et al. [105] on mutational spectra of HV1 and HV2 suggest that sequence contexts, particularly neighboring polytracts, play an active role in the frequency of mutagenesis at these polymorphic "hot spots" during replication events [see also 77]. Whatever the mechanism, some of these substitutions have also been observed to appear more often as heteroplasmic sites in noncoding control region studies, which would be expected since heteroplasmy is the transitional state between fixed homoplasmic states.

Those positions mentioned as frequently heteroplasmic have been positions 16093 and 16129 in hypervariable region 1 [74,108,155] and 73, 152, and 189 in hypervariable region 2 [28] although other positions have been observed by these and other authors at lower frequencies. Heteroplasmy at position 16093 was observed 13 times in 35 heteroplasmic individuals in one study [155]; in all but two cases the T was the minority component whereas C, the majority component, is the variant from CRS (6% frequency in population databases). To these authors, this phenomenon suggested strong directionality of this mutation from T to C with a return to the T repeatedly occurring during a bottleneck. Of 13 observations of sequence heteroplasmy in forensic casework in one lab [108], six were at position 16093, with one each at positions 72, 152, 189, 207, 279, 16166, and 16286. Meyer et al. [109] point out that sites 16093, 152, 189, and 207, among others, have been observed as substitutions within family lineages [80,121] and also have some of the highest relative mutation rates in the control region. These authors report that the proportion of nonmutating sites is quite low (54% in HV1 and 28% in HV2), so in fact it would be possible to observe heteroplasmy at virtually any site within these regions. For example, Hühne et al. [81] report sequence heteroplasmy at positions 16205 and 16309, only one of which (16309) is on the lists of fast sites [109, 160] and Parsons et al. [121] report 16092, 94, and 234, all of which have relatively low rates of mutation [109]. Therefore, even if a position has never previously been categorized as a "fast site", heteroplasmy may well be observed in that location.

Because hot spots for mutational change are well acknowledged in the scientific literature, there has been speculation about the susceptibility of human tissues to mutational change when exposed to external environmental mutagens, such as ultraviolet light or radioactivity. Early studies on retinal tissue, which was suspected to be a likely target for mutation because of a lifetime of light exposure,

did not show a high rate of mutational change or evidence of heteroplasmy, and in fact was one of the earliest indications that homoplasmy was the baseline state of mtDNA in tissues [18]. This study was carried out using the older, less-sensitive manual sequencing method using radioactive labeling, which could have missed heteroplasmy. A recent study of buccal swabs from a coastal Indian population of Kerala, a geographic region exposed to the world's highest levels of naturally occurring radioactivity, showed significantly higher numbers of germline point mutations than in a control population [55]. A total of 22 heteroplasmic mutations was observed in 595 mother-offspring high-radioactivity transmissions, whereas only one was observed in the low-radioactivity population (200 transmissions), suggesting that the mutation rate might be higher in individuals experiencing unusual exposure to radioactivity. The most common sites for heteroplasmy in this study included 152 ($n = 3$), 215 ($n = 3$), 16189 ($n = 3$), and 16093 ($n = 2$), with a number of other sites observed one time each; some of these sites are clearly recognizable mutational hot spots. This quite interesting study raises questions about the forensic implications of testing populations such as those exposed to Chernobyl fallout, even though a recent study indicates that mice exposed to the low-level radioactive Chernobyl environment had no increased levels of heteroplasmy [164]. As previously noted, the "4977 common deletion" has been observed by some authors to increase (and be heteroplasmic) in aging tissues. Recently, a study of smokers noted that the incidence of this deletion was also significantly higher in hair follicles of those subjects who had smoking indices of greater than 5 pack-years [101]. As a known mutagen, tobacco should be further studied as a causative factor in sequence heteroplasmy in hairs of smokers versus nonsmokers.

Mitochondrial DNA mutational hot spots are also those sites most likely to undergo postmortem damage, at least in ancient skeletal remains over several centuries old [56,57]. These damaged sites are observed in individual clones on the homoplasmic background of the sample (since they are not replicated after death). These changes could in theory be detected in very old or environmentally compromised forensic samples where amplification of a molecule with a postmortem mutation occurs during the earliest rounds of PCR, yielding a high proportion of the variant type in sequencing. In this sense, an analysis detecting a postmortem-generated heteroplasmy in this way would not be different from an analysis detecting one generated during life. A byproduct of the aforementioned ancient DNA studies is that there appears to be high fidelity of the *taq* polymerase enzymes used for PCR amplification, indicating that sequence heteroplasmy is unlikely to be a byproduct of nucleotide misincorporation during PCR amplification in the laboratory.

E. LENGTH HETEROPLASMY

The most common form of heteroplasmy observed in the mitochondrial DNA control region is length heteroplasmy. An individual with this condition has multiple species of the mtDNA genome that differ in length by single nucleotides, usually cytosine (C) residues in homopolymeric tracts (as characterized by determination on the light strand; the heavy strand would be characterized by the complementary base). Depending on its extent, length heteroplasmy may result in an inability to read or interpret sequence data downstream and must be compensated for with alternative sequencing strategies when severe [126,147]. Length heteroplasmy is observed primarily in two regions, each of which has been studied in depth. Each is believed to occur because strand slippage occurs during replication of the molecule, and because fidelity of the DNA polymerase in reproducing the original number of C residues declines as the number of Cs increases beyond eight [66,104]. In addition, a third C-stretch region has been reported around positions 568-573 that has interesting evolutionary implications due to its history of expansion and contraction in some populations [79]. Other infrequent length heteroplasmy has been observed in the G-stretch downstream from position 66 in HV2 and at position 249 in HV2 [30,108]; both of these relatively mild phenomena are caused by a single base-pair deletion resulting in mtDNA templates that are one base shorter than the Cambridge Reference Sequence (CRS)[2].

The first major form of length heteroplasmy is observed at moderate frequency approximately equal to the frequency of the 16189 T to C transition (~20%) [74]. Comprehensively described by Bendall and Sykes in 1995 [10], this length heteroplasmy is observed near a homopolymeric C-stretch containing five Cs (C_5) upstream and four Cs (C_4) downstream of position 16189 in HV1, which in most individuals is a thymine (T). However, in individuals where the T has been replaced by a C, the C-stretch beginning at positions 16182, 16183, or 16184 does not terminate with a finite number of residues, but has multiple templates such as C_9, C_{10}, C_{11}, and C_{12}. If one or more of the four adenine (A) residues that typically precede the C-stretch are also substituted to Cs (or deleted; "substitution" is the conventional description where the biology may actually be more complex), the number of Cs in the tract may reach 14. Length heteroplasmy of this A_4 tract has also been observed [10]. Interestingly, when there have been additional substitutions within this C-stretch, for example, a C to T transition at 16186, the

length heteroplasmy is usually absent, indicating that a critical number of Cs, perhaps eight, is necessary to create the replication slippage. With a DGGE analysis, the frequency of this length heteroplasmy was reported to be about 12% in one study [155]. Some individuals with length heteroplasmy in this region may also have sequence heteroplasmy at position 16189 (C/T), which results in an even more complex pattern of heteroplasmy in electropherogram traces.

Cloned mtDNA from blood of individuals with this HV1 length heteroplasmy showed that while individuals in different maternal lineages usually had quite different proportions of the different length variants, individuals in the same lineage shared the same proportions of the different length templates with their maternal relatives, even those who were distantly related (with only 1/32 nuclear genes shared). Bendall and Sykes [10] observed that this result is not consistent with a small bottleneck effect, and speculate that the proportions could be regenerated *de novo* in each individual. However, this would of necessity invoke some maternally inherited factor that controls the degree of replication slippage within a particular lineage. A recent study by Malik et al. reported evidence for *de novo* regeneration of the site 16189-associated length polymorphism pattern in an experiment that artificially recreates the mtDNA bottleneck in cultured fibroblasts [103]. A subsequent study by the same group showed that nuclear factors do indeed appear to control the development of consistent patterns of the different length variants, and suggests that these factors may belong to nuclear DNA-encoded mtDNA replication factors [104]. If nuclear factors are not invoked, an alternative explanation is that lineage-specific intraorganellar-length heteroplasmy exists and is being preserved in this way in spite of a small bottleneck for the total number of organelles that are inherited. Regardless of the precise mechanism, Bendall and Sykes suggested that because there appears to be a limit on the overall length of this region in HV1 to about 14 or 15 nucleotides (As plus Cs), some selective pressure is preventing its unlimited expansion.

A second more common length heteroplasmy is found in a C-stretch tract downstream from nucleotide position 304 in HV2 [60,142]. This region frequently gains or loses single C residues during replication, resulting in populations of molecules with 7, 8, 9, 10, or more adjacent Cs followed by a T and 6 more Cs. While the CRS has C_7-T-C_5, almost all other individuals have C_7-T-C_6 or C_8-T-C_6, such that the notation "315.1 C" is found in most haplotypes where this C is the last nucleotide in the C_6 stretch. The human population is almost equally divided into those with seven or eight Cs preceding the 310T, but where there are eight, most individuals begin to have some degree of length heteroplasmy, and it is sometimes observed where there are seven Cs [30]. Where nine or more Cs precede the 310T, the heteroplasmy becomes increasingly pronounced and creates the characteristic difficulty in sequence interpretation downstream, particularly where proportions of the different length templates are equal or similar. Interestingly, although this region has an adenine stretch immediately preceding the C-stretch, this group of three A residues is only rarely observed to vary, unlike the 2-4 base adenine group preceding the HV1 C-stretch. Many complex additional variants in this region have also been observed, leading to comprehensive nomenclature guidelines suggested by practitioners in the forensic community [165,166]. Typically, and most simply, the dominant number of C insertions exceeding the CRS C_7 residues preceding the T is reported as 309.1C (C_8), 309.2C (C_9), and so on.

Length heteroplasmy in HV2 is widely recognized as the most common type of heteroplasmy observed in casework, and although good frequency statistics are not available, the frequency could almost certainly estimated according to the frequency of C insertions at 309.1, 309.2, or beyond, since it is present to some degree, albeit minimally, in virtually all individuals with these insertions. For example, 19 of 101 Austrians have clearly designated HV2 length heteroplasmy in a population database (19%; "N"s are present in the data table in this region, indicating difficulty with data interpretation), while an additional 42 individuals have the 309.1 insertion, indicating that the total frequency of length heteroplasmy could be as high as 60% in this group even though data interpretation was not compromised [120]. In formal studies, it was observed at a frequency of 67% in brain tissue of subjects with mitochondrial disorders [92], and 12% in blood samples from normal individuals [126]. A study of this variation within individuals has shown that hairs, blood, and saliva within the same individual can have both widely differing numbers of C residues and proportions of these residues [142]. This observation has led to general consensus within the forensic community that this region cannot generally be used to support an interpretation of exclusion [142].

The biology of this region is not clearly understood. Speculation about whether length heteroplasmy could be an artifact related to *taq* polymerase errors or other factors arising from sequencing or cloning methods, and not due solely to the existence of different intraindividual DNAs, has been laid to rest by study of a unstable "568" cytosine repeat similar to the two common types just presented [79]. In a study of cloned and sequenced blood or buccal swab samples, Lutz et al. [102] showed that 10 maternally

related individuals had heteroplasmy in HV2 downstream from position 304, but the numbers of C residues and their proportions varied widely among the family members, unlike the pattern seen for the 16189 heteroplasmic region in HV1 reported by Bendall and Sykes [10]. However, a longitudinal study of cervical cells from four women over one or two decades, using solid-phase minisequencing, showed highly stable proportions of the length variants in this HV2 region over time, as well as stability for the 16189 HV1 length heteroplasmy [97], so there is a mixed picture of both slow and fast segregation depending on the research project [see also 30]. Also of interest in this study was an observation that one of the women had heteroplasmy in both the HV1 and HV2 C-stretches, in equal proportions (97%:97% for 16189T/309T, and 3%:3% for 16189C/309C), suggesting that the linkage phase was consistent for each variant.

II. DETECTABILITY AND INTERPRETATION

The definition of heteroplasmy as the existence of two types of mitochondrial DNA within an individual can be refined to account for the *in vitro* detectability of this *in vivo* biological state. It is known that the sensitivity of heteroplasmy detection is method-dependent when the same samples are evaluated using different instrumentation and chemistries [28,73,155]. The most fundamental approach to sampling the individual mtDNA molecules present in an individual's cells would be cloning, where single molecules are selected, amplified, and sequenced. With a large enough sample size (in theory, every mtDNA in the body), the cloned population of mtDNAs would represent the distribution of mtDNA variation within every cell or tissue, the proportions of each variant could be quantitated, and the linkage phase of every minor substitution variant could be mapped on its sequence background. However, because cloning is itself nothing more than a sampling of the different mtDNA variants within an individual, this selection is vulnerable to stochastic sampling processes just as forensic sampling is.

In the preceding sections, we have seen that the observed frequencies of heteroplasmy may be correlated with type of tissue, due to the existence of "more clonal" tissues (hair, muscle, bone) versus "more panmictic" tissues (blood, saliva, scraped buccal cells). The evaluation of overall heteroplasmy may therefore not be best accomplished by cloning of any particular tissue, unless there is particular interest in that tissue for research purposes. DNA sequencing of tissues such as blood, saliva, or buccal cells, besides identifying the homoplasmic baseline state, may detect cases of maternally inherited, pervasive heteroplasmy, where the minority component is at least 20% of the total population of cells. The homoplasmic mitochondrial DNA type of an individual establishes the baseline from which heteroplasmic variants may then be distinguished, regardless of the type of forensic sample. Interestingly, mtDNA forensic evidence most often includes hairs and bones, i.e., "clonal" samples, and these will usually be tested prior to the "panmictic", i.e., reference or known samples: blood, saliva, and buccal tissues.

Within the forensic community, sequence heteroplasmy is usually defined as the presence of distinguishable, different nucleotides at a single position on both strands of a sequencing product and in all overlapping PCR fragments containing that amplified site in a single sample [29,153]. In sequence heteroplasmy, most commonly transition pairs are observed simultaneously, such as A with G, or T with C. Pyrimidine heteroplasmy is more common than purine heteroplasmy. Transversion heteroplasmy is only rarely observed; this follows from the fact that transitions are more common mutations than transversions [27]. Notation for these observations can be given as the International Union of Applied and Pure Chemistry (IUPAC) designation, for example, when both a T and a C are observed, the notation is Y. Alternative notations for this pair have been given as T > C or T~C [153].

Heteroplasmy at two positions simultaneously, known as "triplasmy" [12], is expected at much lower frequencies, and, if suspected, should be evaluated to rule out the presence of a mixture of two or more mitochondrial DNAs from different sources (e.g., *see* [129]. Determination of the presence of a mixture can sometimes be accomplished by carefully inspecting all nucleotide positions in the sequence data to determine if mixed bases, no matter how minimal, are present in any of those locations. If numerous positions are observed to have two bases, then a mixture rather than heteroplasmy is the most likely explanation. However, for samples where the proportion of the minor type is less than 20%, the level at which sequencing detects mixed bases, the complete profile of the minor type may not be apparent in the sequenced product [127]. More research into the most appropriate handling of mixtures is clearly needed.

Also mentioned as a potential confounding artifact in forensic testing is the possibility of co-amplifying nuclear-origin (mitochondrial) pseudogenes with mtDNA, which then might be confused with heteroplasmy [119]. To date, no pseudogenes with homology to HV2 have been identified, but two pseudogenes with homology to HV1 have been described [152,173,174]. However, each of these has at least 20 nucleotide differences compared to the mtDNA HV1 sequences of modern humans. Therefore, heteroplasmy could not easily be confused with co-amplification of a pseudogene [28].

Length heteroplasmy is readily defined by the appearance of overlapping bases, or "out of sync" ripples that begin immediately 3' to the inserted or deleted base in the sequenced product and can progress to the end of the electropherogram. The level of difficulty in reading this sequence is correlated with the proportions of the variants; for example, a 90:10 ratio of C_8 to C_9 in HV2 will mean that the downstream base calls will be relatively easy, whereas a 50:50 mixture will be very difficult to evaluate and may require resequencing the PCR product with an additional primer that anneals 3' to or within the homopolymeric stretch on each strand. One form of notation for the heteroplasmic HV2 C-stretch is to report the dominant number of Cs as, for example, 309.1C (C_8) or 309.2 (C_9), and to note as well the presence of length heteroplasmy.

As previously mentioned, DNA sequencing captures the overall homoplasmic genotype of an individual and detects heteroplasmic variants when they are present at or above the level of about 20% of the sequencing product [167]. With this method, detection is dependent on the overall quality of sequence data and is enhanced by the absence of sequencing artifacts such as background "noise" above the baseline [108]. Several groups have used methods other than direct sequencing that detect lower levels of heteroplasmy; some of these methods grew out of a need to evaluate the mutation load of defective mtDNAs in patients with mitochondrial diseases. These methods include primer extension [53,85], temporal temperature gradient gel electrophoresis (TTGE) [20,31,169], real-time PCR [70,159], solid-phase minisequencing [91,144], restriction fragment analysis [54], and single-stranded conformation polymorphism analysis (SSCP) [133]. In addition, some applications that have evaluated heteroplasmy at more neutral sites, including the mtDNA control region, include sequence-specific oligonucleotide (SSO) typing [28], denaturing high-performance liquid chromatography (DHPLC/nuclear loci) [157], and denaturing gradient-gel electrophoresis (DGGE) [64,155]. These methods detect and sometimes quantitate heteroplasmy, even at levels as low as 1%, and can in theory detect any mismatched molecules within a sample (therefore being useful for detecting introduced or inadvertent mixtures or contaminants), but are not intended to capture complete mitochondrial DNA sequence data.

Tully et al. presented a comprehensive examination of HV1 heteroplasmy by DGGE [155]. They noted that the level of sensitivity of heteroplasmy detection of this assay is 5%, and several samples with levels of less than 5% were also identified. In this study, three of 37 heteroplasmic positions that were detected by DGGE were revealed by sequencing; the proportions of these three variants were estimated by SYBR green labeling (Molecular Probes) to be 48%, 23%, and 16% of the total amount of DNA at those positions. Of the remaining variants, 30 were at/or estimated to be below 22% of the total, while four were in the range of 25–41%. This is somewhat consistent with observations that sequencing detects variants above 20% of the total, but as noted, four above this level were not detected. It was also interesting that although both strands of heteroplasmic samples were sequenced, the relative peak heights of the variants on light and heavy strands were not necessarily the same, a phenomenon that has been noted by others [127,167]. Repeat DGGE analysis of each blood sample gave reproducible results of both the proportions of heteroplasmy and each individual's overall dominant, homoplasmic genotype.

Interpretational guidelines for heteroplasmy in the application of forensic testing have been clearly stated in several peer-reviewed publications and are uniform [29, 74,153]. Because individuals may have heteroplasmy in some tissues and not in others, where a heteroplasmic questioned sample also has the mitochondrial DNA profile of the known sample — that is, the mitochondrial DNA sequences share a common nucleotide at the heteroplasmic position as well as all other sequenced nucleotide bases — the sample cannot be excluded as having come from the known individual. When both the questioned and known samples have an identical heteroplasmic position, the match is strengthened by some unknown factor that cannot currently be quantified but is related to the frequency of mutation at that site. A likelihood ratio (LR) calculation for this situation was described in detail for the case of Czar Nicholas, brother of Grand Duke Georgij, both of the Russian royal family. Skeletal remains of both individuals displayed sequence heteroplasmy at HV1 site 16169; the LR calculation allowed for the statement that the total DNA results were 1.3×10^8 times more likely if the remains were from the Romanov family than if they were an unrelated family (including the DNA results from the maternal lineage of the czarina) [84]. Situations such as this have led to the recommendation that cases be interpreted on an individual basis as needed [74].

A situation in which multiple samples in a case have the identical sequence heteroplasmy may not be rare; in 4 out of 13 forensic cases where the questioned sample displayed sequence heteroplasmy, the known sample or other questioned samples in the case had that identical site heteroplasmy [108]. Currently, the approach to searching a forensic database such as the SWGDAM mtDNA population database used by practitioners [112] for a profile with a sequence heteroplasmy is to designate the heteroplasmic base an ambiguous base. This allows the most conservative and inclusive statistical estimates of

upper bound frequencies of these types using the sampling equations found in the Holland and Parsons 1999 mtDNA review paper [74]. It is, however, not impossible to envision a time when the relative rarity of each mutational change can be estimated for use in a calculation to be applied in these "double heteroplasmy" cases, and this will be a welcome addition to the current applications.

Some authors have suggested that additional testing be undertaken when heteroplasmy is observed in some forensic samples in a case and not in others [29,74]. Even if such testing is not undertaken, or in some cases, cannot be undertaken due to limited evidence or known sample, the results from the original analysis must be considered valid because a particular heteroplasmy may be observed in only some samples and tissues from an individual. Of course, additional known hairs could be tested in order to look for the sequence heteroplasmy to attempt to strengthen the forensic failure to exclude, as in the situation with the Romanov family remains. Nevertheless, where doubt remains, repeat testing from the stages of DNA extraction, amplification, or sequencing is available in many cases to solidify the analytic conclusions.

III. CONCLUSIONS AND FUTURE DIRECTIONS

The scientific literature on heteroplasmy in humans has grown substantially since a 1995 observation suggested that heteroplasmy in normal individuals may not be a rare biological state [43]. In fact, we now know that limited sequence heteroplasmy is the norm, albeit more apparent in some tissues than others, and that this heteroplasmy is superimposed on a dominant homoplasmic state. Studies using the most sensitive methodologies for sequence heteroplasmy detection, such as DGGE, show that most control region heteroplasmic variants exist at low proportions compared to the wild-type variant [155]. In forensic settings where sequencing is the standard method of detection, this predominantly "minor component" phenomenon explains why the homoplasmic baseline type or wild-type is captured overwhelmingly, with only occasional observations of heteroplasmy. It also explains why it is rare to observe (1) complete single site changes between mother and offspring, and (2) a forensic case where any of the analyzed samples have a single nonheteroplasmic nucleotide difference between them. The latter phenomenon is related to the fact that randomly selected individuals from any population group will have on average 10.6 ± 4.9 mtDNA control region nucleotide differences between them [27]. The fact that it is rare to see single-site differences between samples (mother-offspring or within a single case) tells us that most novel mutations do not rise sufficiently in frequency to become replacement nucleotides, and most heteroplasmy does not result in single-site differences. Aside from the situation where all or most of an individual's tissues contain the novel variant in a heteroplasmic state because of maternal inheritance, this picture argues strongly for the homoplasmic type being preferentially maintained, with low-level heteroplasmic variants occasionally being generated and then removed via drift or selection. The former case, maternally inherited, pervasive heteroplasmy, seems to be the most advantageous type of heteroplasmy in forensic testing, as comparisons between different hairs, or between hair and blood, are likely to show heteroplasmy in both samples (and even between individuals in maternal lineage comparisons). In a case of this type, the forensic "match" is strengthened as in the case of the Romanovs. Interestingly, our dominant homoplasmy is maintained throughout life, despite ongoing exposure of mtDNA to mutagens, the continual destruction and replenishing of mtDNA in most tissues, and at least some evidence that heteroplasmy increases with age.

From the rich body of literature covered here, we have a good understanding of the mechanisms leading to heteroplasmy, its frequency in various tissues, and its characteristic appearance at specific locations in mtDNA. One forensic mtDNA laboratory reports that the overall frequency of sequence heteroplasmy in high-quality DNA sequence data, where a level of 20% heteroplasmy is detectable, is about 10% or less, depending on the tissue being examined. Given the additional observation that mitochondrial DNA forensic analyses result in a "failure to exclude" approximately 50% of the time [108], we can calculate that sequence heteroplasmy would be expected to play a role in forensic analysis and interpretation in about 5% of cases.

Of some interest to the forensic practitioner might be the implications of "brave new world" technologies, certain to increase as scientists tinker with reproductive biology and medical treatments. Although still controversial, the transfer of ooplasm from donor oocytes into human oocytes suspected to lack some required cytoplasmic factor has resulted in a number of healthy pregnancies [141]. The embryos, amniocytes, fetal placentas, and cord blood of these pregnancies showed heteroplasmy of both donor and recipient mtDNA types, something to consider in the next generation of forensic testing [22]. Similar results have been observed in nuclear transfer experiments to produce bovine offspring [149]; cloning may therefore impact nuclear-mitochondrial interactions and traits of economic importance, such as milk-fat yield and fertility [71,72,137]. As human cloning debates continue, we should look for discussions on the mitochondrial DNA heteroplasmy that may ultimately present as a result of new reproductive technologies.

In the medical community, *in vitro* experiments have been examining whether disease-related heteroplasmy could be therapeutically altered with a genetically engineered restriction endonuclease [139]. Chinnery [33] points out that recent work in mouse models of mitochondrial disease will delineate the many factors regulating mutation load, and this knowledge will lead to treatments for controlling heteroplasmy, perhaps raising the levels of wild-type normal molecules and decreasing mutant molecules. Lightowlers et al. [98] states that in patients with mitochondrial myopathy, their satellite cells, or muscle cell precursors, lack the mutant mtDNAs found in the muscle cells, and in theory could be stimulated to replace the defective tissues. Although decades away, it appears that gene therapy also has implications for the generation, maintenance, and eradication of heteroplasmy.

We have seen that maternally inherited and pervasive heteroplasmy may be advantageous in forensic science because of the likelihood of its appearance in multiple tissues from the same individual and consequent significance. However, the question remains whether sporadically observed heteroplasmy, with no evidence for or likelihood of matches to other samples in a case with the same phenomenon, is problematic when it arises in casework. We can consider several different scenarios involving a forensic casework sample, such as a shed hair with a single-site heteroplasmy being compared to a known sample that lacks this heteroplasmy. In the first scenario, the hair has the common 16093 C/T heteroplasmy while the known blood has either the CRS T or the substitution C; both samples have a background haplotype with additional rare substitutions that place the haplotype into a category containing one or only a few matches in the forensic database. Certainly there should be no doubt that this heteroplasmy, occurring at a known hot spot, has been well characterized in the literature, and that the conclusion would be a failure to exclude these two samples as being from the same maternal lineage. In the second scenario, the heteroplasmy in the hair is at a site previously undescribed as being a hot spot, the known has a nucleotide substitution with respect to the CRS at that position, and the background haplotype is "rare". Again, there seems to be no biological justification for doubting a common maternal lineage in this case; the rare haplotype and nucleotide substitution in the known would seem to be strengthening factors. In a third scenario, the heteroplasmy is at a hot spot on a common background (say, the 263G, 315.1C type). Again, knowing the site in question is a well-described hot spot means there is no doubt that this may be observed from time to time; we are actually more concerned that the common haplotype rather than the heteroplasmy may result in a false perception of the significance of the "match". Finally, in a fourth scenario, the hair heteroplasmy occurs at a site not previously described as hypermutable, on a common haplotype background. Here, more testing of different samples could be undertaken, if available, to attempt to clarify the situation. But as before, we are logically more concerned about the common haplotype than the heteroplasmy because we know that mutation, and therefore, heteroplasmy, can occur at virtually any site. In all four scenarios, there would be a scientifically supported failure to exclude. Obviously, in a scenario where a complete single site difference was observed between questioned and known samples, we would report an inconclusive test, and undertake more testing if possible.

The preceding four scenarios display a continuum of possibilities involving heteroplasmy that may arise in mtDNA forensic casework, some more challenging than others. Obviously, many other possible scenarios may come to mind. With the conservative interpretational guidelines that are currently being applied, which account for biological mechanisms in mitochondrial DNA, it is clear that two samples that share all nucleotide bases, even if heteroplasmy is present in one of the two samples, cannot be excluded as having come from the same maternal lineage [29]. This interpretation is clearly supported by all published and peer-reviewed scientific knowledge to date.

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