Review

Techniques and Pitfalls in the Detection of Pathogenic Mitochondrial DNA Mutations

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Mutations in the mitochondrial DNA (mtDNA) are now recognized as major contributors to human pathologies and possibly to normal aging. A large number of rearrangements and point mutations in protein coding and tRNA genes have been identified in patients with mitochondrial disorders. In this review, we discuss genotype-phenotype correlations in mitochondrial diseases and common techniques used to identify pathogenic mtDNA mutations in human tissues. Although most of these approaches employ standard molecular biology tools, the co-existence of wild-type and mutated mtDNA (mtDNA heteroplasmy) in diseased tissues complicates both the detection and accurate determination of the size of the mutated fractions. To address these problems, novel approaches were developed and are discussed in this review. (*J Mol Diagn* 2003, 5:197–208)

The biogenesis of functional mitochondria results from the coordinate action of two genetic sources, nuclear and mitochondrial. The mitochondrial genome has become extremely specialized for the synthesis of components of the oxidative phosphorylation system, retaining, in the course of evolution, relatively few genes. More than 95% of all proteins located in the mitochondrial compartments are actually encoded by nuclear DNA and synthesized in cytoplasmic ribosomes. These include subunits of the respiratory complexes and factors that regulate mtDNA gene expression (eg, DNA and RNA polymerases, transcription factors, RNA processing enzymes).

Although almost all enzymes present in the mitochondria are encoded by the nuclear genome, the mitochondrion contains an average of 2 to 10 copies of its own DNA (mtDNA), a 16,569-bp circle of double-stranded DNA containing 37 genes specifying: 13 polypeptides, 22 transfer RNAs (tRNAs), and 2 ribosomal RNAs (1

Figure 1). All 13 polypeptides are components of the ATP generating oxidative phosphorylation system: seven are subunits of Complex I (ND1-ND6, ND4L), one is a subunit of Complex III (cyt *b*), three are subunits of Complex IV (COX I-COX III), and two are subunits of Complex V (ATPase 6 and ATPase 8). Each of these four complexes also contains subunits encoded by nuclear genes. All of the other proteins located within mitochondria, including all of the subunits of Complex II [succinate-ubiquinone oxidoreductase] are exclusively encoded by the nuclear genome.

In most healthy individuals, the wild-type form of mtDNA is present in all mitochondria (homoplasmy). In individuals bearing pathogenic mutated mtDNA, however, the mutated form is frequently found in a mixture with wild-type mtDNA (heteroplasmy). Because mtDNA is present in multiple copies within the cell, mutations are usually not immediately fatal to cells, as wild-type mtDNA can partially compensate for the defective or absent genes from the mutated mtDNA.² However, the mutated mtDNA population can accumulate and be passed on to progeny cells and to offspring. Similarly to nuclear DNA, mtDNA is subject to mutation. Although repair of mtDNA is about tenfold less efficient than repair of nuclear DNA, it does exist in mitochondria.^{3,4}

Fifteen years ago, the first connections were made between mtDNA mutations and human diseases.^{5,6} Since then, many neuromuscular diseases have been linked to specific mutations in the mtDNA (² Figure 1). Thus, the detection, quantitation, and characterization of mutations in mtDNA has become a critical step in the diagnosis of mitochondrial diseases. The genotype-phenotype correlations in mitochondrial diseases remain poorly understood. Although there is a clear association between specific mtDNA mutations with certain clinical phenotypes, there is a significant overlap and atypical

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Figure 1. Mitochondrial DNA morbidity map. The figure shows a representation of the human mtDNA with the location of the most common pathogenic point mutations and large deletions. A constantly updated list of these and many other mutations can be found in every issue of *Neuromuscular Disorders* (eg,⁴⁰). Also indicated is the location of specific protein coding, rRNA and tRNA genes (one-letter code). OH and OL, origins of replication of the heavy- (exterior circle) and light- (interior circle) strand; 12S and 16S, ribosomal RNA genes; ND1–6, genes encoding NADH-coenzyme Q oxidoreductase subunits 1–6; COX I–III, genes encoding cytochrome c oxidase subunits I–III; A6 and A8, subunits of ATP synthase; cyt b, cytochrome b.

presentations. Therefore, although we will describe mtDNA mutations in the context of their most common clinical manifestation, the reader should keep in mind that these associations are not absolute and testing for “atypical associations” is still an important diagnostic approach. Because of the unique features of mtDNA (eg, multiple copies and heteroplasmy), basic molecular biological techniques may yield results that can be misinterpreted. In this review we will describe techniques used in the detection of common mtDNA mutations and discuss approaches to avoid misinterpretation of the results.

Rearrangements of mtDNA

Phenotypes Associated with and Characteristics of Rearranged mtDNA

Deletions or duplications of mtDNA have been associated with a number of disorders, but most commonly with progressive external ophthalmoplegia (PEO), a paralysis of the extra-ocular muscles. PEO may be manifested either as a pure ocular myopathy, or as part of a more severe and fatal multi-system disorder termed Kearns-Sayre syndrome (KSS). However, while large-scale rearrangements of mtDNA are observed in almost all cases of KSS, they are observed in only half the cases of PEO.⁷ PEO and KSS patients are almost always sporadic, with no apparent genetic component. Even though KSS pa-

tients harbor partially deleted mtDNAs in the majority of their tissues, including, presumably, ova, it was surprising that a KSS mother with a large population of mutated mtDNAs in muscle had a normal child with no observable mtDNA deletions.⁸ Rearrangements of mtDNA have also been identified in patients with a sideroblastic anemia known as Pearson's syndrome⁹ and also in maternally inherited non-insulin-dependent diabetes and hearing loss.¹⁰

The size and location of the deletions do not correlate with specific clinical phenotypes. The deletions can range from approximately 2 kb to 10 kb and may encompass any mtDNA gene. It seems that the only regions that cannot be deleted are the origin of heavy-strand replication and the light-strand promoter, as they are required for the mutated mtDNA to be able to replicate. The most prevalent deletion in the patient population is a 4977-bp deletion (termed the “common deletion,”¹¹) encompassing several mitochondrial genes encoding structural proteins and tRNAs (from mtDNA positions 8470–8482 to 13447–13459). The presence of a long 13-bp direct repeat in the breakpoint suggests that homologous recombination (possibly combined with a replication slippage mechanism) was involved in the formation of the deletion (Figure 1,¹¹).

Besides single mtDNA deletions, multiple species of partially deleted mtDNAs in the same patient have also been described.¹² Most of these patients suffer from autosomal-dominant form of PEO. The fundamental genetic defect in familial PEO most likely resides in a gene product affecting the tendency of the mtDNA to suffer deletions (reviewed in¹²). Multiple mtDNA deletions have been associated with mutations in the mitochondrial DNA polymerase (pol γ ,^{13,14} adenine nucleotide translocase (ANT1,¹⁵), and a putative mtDNA helicase (Twinkle,¹⁶). This genetic abnormality can also be observed in patients with a gastrointestinal pseudo-obstruction harboring mutations in the thymidine phosphorylase gene. (¹⁷)

De novo formation of mtDNA deletions may also play a role in the aging process. Accumulation of extremely low levels of partially deleted mtDNAs in the tissues of normal aged individuals, observable only with the polymerase chain reaction, was observed by several groups, with most effort being focused on the “common deletion” as a marker (reviewed in¹⁸). Partially deleted mtDNAs have been found to accumulate in ischemic heart disease and in other cardiomyopathies.^{19,20} The amount of mtDNAs harboring the “common deletion” present in aged muscle appears to be as much as 0.1% of total mtDNA, but other species of partially deleted mtDNAs¹⁸ also seem to accumulate during aging. Thus, it has been proposed that aged muscle and brain contains hundreds or even thousands of species of partially deleted mtDNAs, and that the total amount of partially deleted mtDNAs may, in some tissues, reach levels that could be physiologically significant in terms of the decline in oxidative metabolism in aging.

Although early studies in mtDNA-based diseases described patients that harbored wild-type and either duplicated or partially deleted mtDNA, more recent studies have shown that in many cases, the wild-type mtDNA

coexists with both partially deleted and duplicated mtDNAs.²¹ It has also been shown that cells which contained homoplasmic duplicated mtDNA displayed only a mild mutant phenotype.²²

Detection of Rearranged mtDNA

Southern Analysis

Most mtDNA rearrangements can be easily detected by Southern blot analysis. Total DNA to be used for these studies should be preferably purified from a non-dividing cell type such as muscle. Use of rapidly dividing cells such as hair or blood cells as a source of mtDNA may yield inconclusive results because heteroplasmy of rearranged mtDNA in these tissues is markedly shifted toward wild-type mtDNA.⁷ Total DNA prepared by standard procedures (ie, proteinase K/phenol/chloroform extraction) is suitable for mtDNA analyses.⁷ Although undigested circular mtDNA migrates abnormally, including an undigested mtDNA sample in the Southern analysis is helpful.

Restriction enzyme digestion using one of the few unique or nearly-unique restriction sites such as *PvuII*, *BamHI*, *PstI*, or *SnaBI*, followed by electrophoresis on a 0.7 to 0.8% agarose gel and Southern blot analysis can reveal an anomalous band if rearrangement is present.^{7,22} These four enzyme restriction sites are all found in different regions of the mtDNA genome, allowing a more precise characterization of the mtDNA molecule. Although normal older individuals could contain small levels of rearranged mtDNA molecules, the proportion of these mutated mtDNA species is likely to be under the limit of detection of the Southern blot technique. The presence of a "smear" beneath a wild-type mtDNA band may indicate a population of different partially deleted mtDNA forms, but this must not be immediately assumed. Instead, re-isolation of a fresh mtDNA sample is suggested to rule out damage of the original mtDNA sample as the cause of the smear. Traditionally, the probe used in Southern blot analysis is derived either from random primer radiolabeling of purified whole mtDNA or from radiolabeled PCR fragments amplified from the D-loop region, which has always been preserved in described partially deleted mtDNA molecules.²³ Alternatively, a whole mitochondrial genome PCR product can be used as template for probes.²⁴

As mentioned above, deletions and duplications of mtDNA are commonly present together in affected tissues. Studies of mtDNA bearing duplications can be conducted in much the same way as deletions alone, although one should approach this analysis with caution as the presence of a mixed population of mtDNA molecules will affect the usefulness of both the type of probe and the type of restriction endonuclease used.²²

Figure 2 illustrates the heteroplasmy of mtDNA from three cell lines with mtDNA rearrangements. Cell line $\Delta 10.5.2$ contains a heteroplasmic mtDNA deletion. Cell line $\Delta 16.10$ contains a mixture of partially deleted, wild-type, and partially duplicated mtDNA. Cell line $\Delta 16.10.40$ contains only partially deleted mtDNA. Cell line 143B

Figure 2. Detection of partially deleted and partially duplicated mtDNA. Two cell lines harboring heteroplasmic mtDNA deletions ($\Delta 10.5.2$ and $\Delta 16.10$), one containing homoplasmic mtDNA deletions ($\Delta 16.10.40$) and a wild-type control (143B) were analyzed by Southern blot. The cartoons at the **right** illustrate the position of endonucleases recognition sites as well as the approximate location of the probes used (*). **A:** Illustrates our attempt to detect deletion dimers by partial digestion of the DNA with *PvuII* (P). *PvuII* linearizes both the wild-type and partially deleted molecules. Each set of DNA samples was either undigested or digested with 0.1 or 1 unit of *PvuII* for 30 minutes at room temperature. The probes shown adjacent to the mtDNA maps (*) were used for DNA detection. We did detect an intermediate form of mtDNA (indicated by **arrowhead**) that was larger than wild-type and was completely digested to one or more remaining bands. This band could be either a deletion dimer or a partial duplication, containing a wild-type + partially deleted segments. **B:** Southern blots designed to detect mtDNA molecules with partial duplications. This was accomplished by digesting the DNA with the *SnaBI* endonuclease (S), which has a single recognition site located within the deleted region (see mtDNA diagram). *SnaBI* digestion produced a fragment (**arrowhead**) in $\Delta 16.10$ that was larger than the wild type, and it was recognized by a probe corresponding to the deleted region. Taken together, the results showed that mtDNA from $\Delta 16.10.40$ was homoplasmic for a partial deletion, mtDNA from $\Delta 10.5.2$ was heteroplasmic for a partial deletion, and that mtDNA from $\Delta 16.10$ contained wild-type, partial deletion, and partial duplication forms of the genome.

contains only wild-type mtDNA. If a single cutter (eg, *PvuII*) is used as evidence of the identity of a rearrangement, it can correctly identify the single deletion, but it can be misleading when a partial duplication is present. If the restriction site lies within a duplicated region, Southern analysis will reveal a wild-type sized band and a smaller band (eg, Figure 2, Probe A, *PvuII* digest), incorrectly suggesting the presence of a wild-type, a large deletion and nothing else. However, a partial digestion can provide indications of partial duplications or deletion dimers (Figure 2A, arrow). Such partial digestions are relatively difficult to perform and interpret, and may not be a practical routine diagnostic tool. Moreover, the phenotypic consequences of a deletion dimer or monomer

Figure 3. Whole mitochondrial genome PCR from patients with mtDNA deletions and controls. Amplification products from muscle DNA from four controls (ages 3, 14, 67, and 91 years), two patients with multiple mtDNA deletions (mΔ#1 and mΔ#2) and five patients with single mtDNA deletions (Δ#1 to Δ#5) were analyzed by agarose gel electrophoresis (0.6%). **A:** Ethidium bromide staining of the long PCR amplifications. These amplifications were obtained with EXPAND from Roche Biochemicals (Indianapolis, IN) using the following oligonucleotide primers: mtDNA positions 10–40 and 16,496–16,465. The conditions were the ones recommended by Roche Biochemicals. **B:** Parallel analysis of the genomic DNA from the same muscle samples by Southern blot after *PvuII* digestion. The probe used in this Southern blot correspond to mtDNA positions 11,680–12,406.

are likely to be similar. Nevertheless, when the presence of a partial duplication is suspected, it is useful to differentiate a partial duplication from a deletion dimer.²²

The *SnaBI* digestion in Figure 2B indicates the presence of a duplication in sample Δ16.10 (upper band marked with arrow). Because probe B (which is within the deleted region) also detects this band, it cannot be a partially deleted or a deletion dimer molecule, and it is more likely to be a partial duplication.

PCR Analysis

One of the problems with Southern blot analysis is that mtDNA purified from cells with a mutant phenotype can have a population of rearranged mtDNA which is too small to be effectively analyzed using this method (eg, when studying aging). PCR amplification provides the solution to this problem, as the amplification of a shorter template is preferred over the amplification of a longer template. Applied to the identification of mtDNA deletions, this would allow preferential amplification of the under-represented partially deleted mtDNA. This procedure was first attempted using sets of primers amplifying large sections of the mtDNA genome.²⁵ These PCR products made from partially deleted mtDNA could then be electrophoretically compared to the wild-type products. The presence of the deletion could be noted by the appearance of smaller bands on a gel. This method is limited by the number of primers to be tested and the variability in the location of the deletions. This inefficient method is rapidly being supplanted by a technique which is much more powerful in detecting and identifying deletions.^{24,26} This technique makes use of the fact that the D-loop region is always preserved in partially deleted mtDNA molecules. Using PCR primers localized to this region, and a DNA polymerase preparation designed to amplify large regions of DNA, the entire mitochondrial genome may be amplified (²⁴ Figure 3, Table 1). The shorter, partially deleted mtDNA molecules will be preferentially amplified in PCR over the full-length wild-type mtDNA, allow-

Table 1. DNA Diagnostic Techniques for the Most Common Pathogenic mtDNA Mutations

Common clinical phenotype	nt Location	Gene location	Analysis test	Endonuclease digest	Primers	3' Mismatch sites	Gel condition	WT expected band	Mutant expected band	Ref.
LHON	G3460A	ND1	PCR/RFLP	<i>BsaHI</i>	3305–3329F 4261–4235B	n/a	1.2% Agarose	801/155	956	1
LHON	G11778A	ND4	PCR/RFLP	<i>BstUI</i>	11640–11662F 11805–11779B*	..ACTTC.. ..TGACG	12% PAGE	138/27	165	2
	T14484C	ND6	PCR/RFLP	<i>Sau3AI</i>	14455–14483F* 14578–14548B	..AACGA ..TTGGT..	12% PAGE	77/29/17	106/17	3
LHON/Dystonia	G14459A	ND6	PCR/RFLP	<i>BsoFI</i>	14150–14173F 14487–14458B*	..GAAGTC.. ..CTGCAG	12% PAGE	310/27	337	3
NARP/MILS	T8993G	ATP6	PCR/RFLP	<i>MspI</i>	8273–8305F 9950–9931B	n/a	1% Agarose	1004/657	704/657/300	4
MELAS/PEO	A3243G	tRNA ^{Leu} (UUR)	PCR/RFLP	<i>HaeIII</i>	3316–3134F 3353–3333B	n/a	12% PAGE	169/37/32	97/72/37/32	5,6
MERRF	A8344G	tRNA ^{Lys}	PCR/RFLP	<i>BanII</i>	8273–8305F 8372–8345B*	..CAATCC.. ..GTTGGG	12% PAGE	78/21	52/26/21	7
Deafness	A1555G	12S rRNA	PCR/RFLP	<i>BsmAI</i>	1092–1112F 3377–3357B	n/a	1.5% Agarose	1106/717/462	1586/717	8
KSS	Deletions	Multiple genes	Long PCR and/or Southern blot	For Southern: <i>PvuII</i> <i>PstI</i> <i>BamHI</i> <i>SnaBI</i>	For Long PCR: Forward nt10–40 Backward nt16496–16465	n/a	(Long PCR) 0.6% to (Southern) 0.8% Agarose	For Long PCR 16.5kb For Southern <i>PvuII</i> 16.6kb <i>PstI</i> 14.5kb & 2.1kb <i>BamHI</i> 16.6kb <i>SnaBI</i> 16.6kb	Variable lengths dependent on size reduction or increase	9 10 11 12

*, indicate primers with mismatches.

3' mismatch sites illustrate the structure of the DNA duplex at the 3' end of the hybridizing oligonucleotide.

ing easier identification of the deleted region. Once the region surrounding the partially deleted mtDNA has been amplified, the breakpoints can be mapped using RFLP, and smaller PCR fragments encompassing the deletion breakpoint may be sequenced.

Point Mutations in mtDNA

Phenotypes Associated with and Characteristics of mtDNA Point Mutations

Given that the human mtDNA genome is very compact, with essentially all regions being part of a gene or involved in transcriptional regulation, almost any mutation could be deleterious. There is a growing list of pathogenic mtDNA point mutations that occur in both genes encoding proteins and in the mitochondrial tRNA genes causing a variety of diseases (Figure 1, Table 1).

Several point mutations in complex I genes have been described in patients with Leber's hereditary optic neuropathy (LHON) (Table 1). A G to A transition at position 11778 (G11778A) of mtDNA was found in several LHON pedigrees, accounting for approximately 50% of all cases.⁶ MtDNA mutations in the subunit 1 and 6 of complex I account for the rest.^{27,28} Neuropathy, ataxia, and retinitis pigmentosa (NARP) are associated with a missense mutation in the ATPase 6 gene, and was first described by Holt et al.²⁹ It was suggested that the "NARP" mutation impairs protons flow through complex V.^{30,31} The same mtDNA mutation causing NARP (G8993T) also can cause a severe infantile metabolic encephalomyopathy known as maternal inherited Leigh's syndrome (MILS).³⁰ Other mutations in the same gene have also been associated with NARP/LS.^{32,33}

Point mutations in the apocytochrome *b* gene have also been associated with human diseases. In most cases, the clinical phenotype is a pure myopathy.³⁴ However, an encephalopathy with Parkinsonian features has also been associated with cytochrome *b* mutation and increased free radical production.³⁵

Several metabolic syndromes have also been associated with mutations in mitochondrial tRNA genes, including mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS), and myoclonus epilepsy and ragged-red fibers (MERRF), and several other combinations of symptoms involving different tissues (Table 1). Among the more common mtDNA mutations in this group are an A to G transition at nucleotide 3243 in the tRNA^{Leu(UUR)} gene of the human mitochondrial genome has been found in the majority of patients with the clinical features of MELAS.³⁶ This same mutation also seems to account for a large number of PEO patients that do not have large-scale deletions.³⁷ Point mutations in the mitochondrial tRNA^{Lys} gene have been found in most patients with MERRF (A8344G,³⁸ and T8356C,³⁹). A constantly updated table in the journal "*Neuromuscular Disorders*" keeps abreast of the large number of pathogenic mtDNA mutations and associated phenotypes.⁴⁰

Detection of Pathogenic mtDNA Point Mutations

A number of established techniques, such as denaturing gradient gel electrophoresis (DGGE) or single-strand conformation polymorphism (SSCP) have been used to screen selected regions of mtDNA for pathogenic mutations. Frequently, this was performed to search for novel tRNA gene mutations in patients with evidence of mitochondrial protein synthesis defects.^{41,42} With the advances in DNA sequencing techniques, it is now relatively simple to sequence the mtDNA in its entirety. This latter approach has been used in the identification of pathogenic mtDNA mutations.^{43,44}

However, many neuromuscular diseases, which are caused by a single bp replacement, have a distinctive set of traits that can suggest the identity of the mutation. If the existence of deletions and/or duplications has been ruled out, and the disease symptoms fit a previously described phenotype/genotype correlation, a search may be performed with specific and simple tests for the presence of a few of the most common mutations. For example, the A to G transition at nucleotide 3243 (A3243G) is most often the cause of the MELAS syndrome or PEO. The most common mutation for a given disease should be tested for first, and, if negative, the presence of other possible mutations should be investigated (Table 1).

The tissue choice is an important consideration for the studies of mtDNA point mutations. In contrast to large rearrangements, point mutations of mtDNA are commonly observed in rapidly dividing tissues such as white blood cells. However, there are examples of patients with isolated myopathies, where the pathogenic mutation was restricted to muscle.^{34,45} As a rule of thumb, if there is multi-system involvement, and the underlying defect is a point mutation, it is very likely that such mutation will be detected in white blood cells or other rapidly dividing cell type (eg, mouth epithelial cells or hair roots). We have found hair roots to be an appropriate and convenient source of DNA for the study of mtDNA point mutations. Subjects can be instructed to insert 4 to 6 hairs (with roots) in a plastic tube with ethanol 50% and express-mail the tube to the laboratory. The mtDNA from hair roots can be exposed by the same procedure described for single cells in the section entitled "Single-Cell PCR." However, in such cases, the mutation should be already recognized (eg, in a family member), so that a single or a few PCR reactions would be required.⁴⁶ One should keep in mind that the amount of mutated mtDNA in rapidly dividing tissues is not always a good indicator of the amounts of mutated mtDNA in clinically affected tissues. Although Southern blot analysis can be used to detect point mutations, it is not normally used because it is more time-consuming and costly than PCR-based techniques and because it can be performed only if the point mutation in question creates or destroys a restriction site present in the mtDNA genome.

PCR Restriction Fragment Length Polymorphism (RFLP)

Using the information that some point mutations in the mitochondrial genome cause the loss or appearance of a restriction site (Table 1), an endonuclease digest of a PCR amplification of the region surrounding a candidate mutation, can reveal the presence of a population of mutated mtDNA. For example, in most MELAS patients,³⁶ the change of mtDNA nucleotide 3243 from A to G causes the surrounding sequence to change from GAGCCC, which cannot be recognized by any known restriction enzyme, to GGGCCC which can be recognized by the enzyme *ApaI* or *HaeIII*. By amplifying a 238-bp region surrounding nucleotide 3243, and then digesting with *HaeIII* and separating the fragments on a 12% PAGE, mutated mtDNA would migrate as four fragments (97bp, 72bp, 37bp, and 32bp), whereas wild-type mtDNA would migrate as only three fragments (169bp, 37bp, and 32bp).⁴⁷ Some other mutations causing the addition or loss of restriction sites are listed in Table 1. This technique is the most commonly used diagnostic tool for detecting mtDNA point mutations. However, data derived from this method should be interpreted with caution because of the problems associated with PCR amplification of a heteroplasmic population of mtDNA. The section entitled "Quantitation of Heteroplasmy in mtDNA Bearing Point Mutations" addresses these problems.

PCR-Created RFLP

Although it is very convenient when a mutation creates or destroys a restriction endonuclease site, in most cases this does not happen. It is possible, however, to introduce a restriction site during the PCR. This analysis is performed by using a longer primer (at least 20 nucleotides long) which ends a few nucleotides 5' to the mutation site. The primer should be identical to wild-type mtDNA except for the end nearest the mutation, in which one or two mismatches will create a restriction endonuclease recognition site that includes the potentially mutated nucleotide. However, the last 3' nucleotide has to match the target sequence for the PCR to work. As the restriction site created by the primer near the mutation site will be very close to the end of the PCR fragment, the other primer should be chosen so that the resultant PCR fragment is a relatively small one (<300bp), and the restriction digestion should be separated on a polyacrylamide gel (Table 1).

An example is seen in the identification of the MERRF A8344G mutation. The region including and just downstream of the mutation site has the following sequence, GAACCA. Neither this sequence nor the sequence bearing the MERRF mutation (GAGCCA) is recognized by any known restriction endonuclease, but if a PCR primer changes the last A to a C, then the presence of the A8344G mutation will cause the resulting PCR fragments to change to GAACCA in the wild-type and GAGCCC in the MERRF mutation. The latter sequence can be di-

*Bsa*HI (G A/G C G T/C C) Digestion

SIL MUT	MUT	WT	UC	size (bp)
				-956
				-801
				-155

Figure 4. Pitfalls of PCR RFLP The PCR RFLP procedure applied to the G3460A LHON mutation. Showing an incorrect diagnosis of the presence of the G3460A LHON mutation (mut) using the PCR RFLP method. A silent mutation (SIL MUT) at nucleotide 3459 causes the destruction of a *Bsa*HI site, but does not cause a change in the final protein product. At the **top** is a diagram showing the nucleotide sequence of three possible PCR fragments (using primers indicated in Table 1) containing the 3460 LHON locus (mutated nucleotides indicated in **bold**). The corresponding amino acids are shown above the DNA sequence. At the **bottom** is a diagram of the expected electrophoretic pattern for a pure wild-type (WT), pure mutant, or pure silent mutation population of PCR fragments digested with *Bsa*HI. UC, undigested PCR fragment.

gested by *Ban*II, whose recognition sequence is G A/G G C T/C C (Table 1).

False-Positives/Negatives Using PCR/RFLP

It should be noted that, although such cases are rare, it is possible to obtain false-positive or false-negative results using these methods if there exists nearby in the mtDNA another mutation which can disrupt the creation of the new restriction site in question or destroy a site with a mutation which otherwise would go undetected.⁴⁸⁻⁵³ These secondary mutations are seen mainly in the coding regions of mitochondrial proteins and are usually silent mutations or missense mutations that do not affect the primary structure of the protein. For example, in Figure 4, a mutation associated with LHON, G3460A, is shown to produce a protein with the peptide sequence Asp-Thr, as opposed to Asp-Ala in the wild-type. This is due to the mutation of the DNA sequence from GACGCC to GACACC, which also destroys a *Bsa*HI site (recognition sequence G A/G C G T/C C). A mutation was found having the sequence GATGCC, which produces the wild-type peptide sequence of Asp-Ala, but would not be recognized by *Bsa*HI in a PCR/RFLP assay, creating the false impression that the G3460A mutation is present. It was therefore suggested that for diagnosis of mutations in coding regions, in identified cases, direct sequencing of the region surrounding the altered restriction site be

performed to rule out this possibility.⁴⁸ Similar problems also have been reported for the 3243 and 11778 mutations.^{49,50}

Quantitation of Heteroplasmy

Quantitation of Heteroplasmy of Rearranged mtDNA

As stated previously, mutated mtDNA commonly exists in a state of heteroplasmy with wild-type mtDNA. Quantitation of heteroplasmy in rearranged mtDNA can simply entail quantitation of bands representing wild-type and rearranged mtDNA on a Southern blot. The section entitled "Detection of Rearranged mtDNA" describes the procedure in detail. For an accurate determination, the probe used must hybridize with a non-deleted region only, thereby allowing a molar ratio determination between wild-type and rearranged mtDNA.

If the amount of partially deleted mtDNA present in a sample is too small to visualize using a Southern blot, either because the total amount of cellular DNA is small, as is the case with the study of single cells,⁵⁴ or because the proportion of partially deleted mtDNA to wild-type mtDNA is very small,⁵⁵ then an alternative method should be used to estimate the amount of heteroplasmy in the cells. This is usually done by performing PCR using two different sets of primers, with one set amplifying the region surrounding the deletion and one set amplifying a control region in an area of the mtDNA genome that is not normally prone to any kind of rearrangements. First, the mtDNA is digested using restriction endonucleases that only recognize sites within the deleted area. This results in the amplification of only the partially deleted form of mtDNA at that region. The second amplification of a different non-affected mtDNA region serves as an internal control. The two products are separated on an agarose gel and the image of the bands is quantitated by densitometry. The ratio of the amount of mutated PCR product to the amount of total mtDNA PCR product is compared to a control standard in which similar PCR products are made from template DNA containing known mixtures of wild-type and partially deleted mtDNAs.⁵⁶

This procedure has also been simplified to require only three primers.⁵⁴ Figure 5 illustrates this procedure. The mtDNA heteroplasmy of transmitochondrial cybrids (see the section entitled "Cytoplasmic Hybrid (Cybrid) Construction" for better description of transmitochondrial cybrid production and uses) was examined by the 3-primer method. Primer 1 is common to both amplification products and hybridizes to a region outside the deletion. Of the other two, primer 2 hybridizes to the region inside the deletion, and primer 3 hybridizes to the region on the other side of the breakpoint from primer 1. In this procedure the DNA must remain undigested. To ensure the production of only two PCR fragments, the PCR program is designed to inhibit the production of large fragments, and primer 3 is chosen so that it hybridizes far enough away from primer 1 that the primer 1/primer 3 PCR prod-

Figure 5. Three-primer PCR for the quantitation of heteroplasmic mtDNA deletions. The use of one forward and two reverse primers has allowed the quantitation of the levels of mtDNA deletions in patients' tissues as well as in cell lines. **A:** Location of the oligonucleotide primers. **B:** Screening of several transmitochondrial cybrids (see section entitled "Cytoplasmic Hybrid (Cybrid) Construction" for description of transmitochondrial cybrids) by 3-primer PCR. Ethidium bromide treatment of one of the cybrids leads to a reduction in its mtDNA levels. After the drug is removed, cells repopulate with the residual mtDNA, a process that can lead to marked shift in mutated mtDNA distribution in resulting clones, as also determined by 3-primer PCR (**C**).

uct is never made from the wild-type. Thus, primer 3 will only participate in amplification of partially deleted mtDNA. Primers 1 and 2 will amplify the region representing wild-type mtDNA. Quantitation of the PCR products is performed as described above, with the actual percentage of mutated PCR product derived from comparison of the data obtained from a control standard curve. Real-time PCR can also be useful in determining the levels of mtDNA rearrangements.⁵⁷

Quantitation of Heteroplasmy in mtDNA Bearing Point Mutations

Quantitation of heteroplasmy in cells with point mutation brings up a problem that is not present in quantitation of heteroplasmy in rearranged mtDNA. In PCR amplification of rearranged mtDNA, the two fragments produced by the reactions described are different enough from each other as to be unique, even in the 3-primer PCR, and the products are easily distinguishable by size with no need for enzymatic modification of the fragments. In PCR analysis of point mutations, however, there is a chance that following the denaturation step, instead of hybridizing to primers, a DNA strand bearing wild-type sequence could anneal to another DNA strand bearing a mutated sequence. Since the single-stranded PCR products are identical with the exception of a single bp, the odds of annealing to either a wild-type or a mutated strand are equal. Binding to another wild-type DNA fragment would form a homoduplex, and binding one bearing a mutated sequence would form a heteroduplex. This by itself causes no problems, but when a PCR reaction containing this sort of heteroduplex is digested with a restriction endonuclease that can recognize the mutated sequence, a fragment bearing two mutated strands of DNA will be digested, whereas a fragment having two wild-type strands will not. However, a fragment having a strand of each will also not be digested because the restriction endonuclease cannot recognize the site. Direct quantita-

tion of heteroplasmy under these conditions will give results indicating a lower level of mutated DNA than is actually present, and the lower the actual amount of mutated DNA present, the higher the chance of forming a heteroduplex (see Figure 6A). This situation is reversed, of course, if the wild-type sequence bears the restriction site. Because of this problem, many different strategies have been devised to ascertain the actual amount of mutated mtDNA present in a tissue.

Quantitation of mtDNA point mutations heteroplasmy can be performed by Southern blot analysis. However, it is very costly and time consuming. Moreover, as mentioned before, it will only work for those mutations that disrupt or create a restriction site. Therefore, PCR-based techniques are more commonly used for these determinations.

Mutated Wild-Type Standard Curve

This technique accepts the error inherent in RFLP analysis of PCR products derived from heteroplasmic mtDNA by producing a standard curve against which experimental results can be compared. The standard curve can be made by amplifying multiple samples consisting of known percentages of pure wild-type and mutated templates.⁵⁸ The digested PCR products are then separated as described above and the percentages of wild-type and mutated products are quantitated. The quantitative value for the amount of the mutated product observed is used to calculate the observed percentage of mutated product present in the total sample. The percentage value thus obtained can then be plotted against the actual percentage of mutated mtDNA amplified. This curve can then be used to determine the percentage of wild-type mtDNA present in a patient sample. This method is straightforward, but assumes the availability of a diagnostic amount of pure forms of mtDNA. To get around this, it is possible to clone regions of the mtDNA genome from a mixed wild-type/mutant population into a plasmid vector. Purified plasmids can then mimic pure mutated or wild-type mtDNA. It should also be noted that for the greatest accuracy, the samples for this standard curve should be amplified at the same time as the test samples, and a standard curve should be prepared for every different PCR run.

PCR "Last Cycle Hot"

As described above, the formation of heteroduplexes during the PCR reaction has a major effect of restriction fragment length polymorphism analysis. Heteroduplexes of PCR amplified strands are formed when the mtDNA region analyzed is heteroplasmic. At the end of the cycling procedure, many strands just re-hybridize to complementary ones instead of with oligonucleotide primers. In 1992 we devised a method to circumvent this problem⁵⁹ that is now widely used by investigators in the field (eg,⁶⁰). The method consists in performing one round of primer extension using radiolabeled α [³²P]dCTP on a

previously amplified fragment (Figure 6). By doing this, only fragments that participated in the single extension step would be radioactively labeled. The labeled duplexes would also remain as homoduplexes, as no denaturation step is performed after the extension with α [³²P]dCTP. All other duplexes would remain unlabeled. These unlabeled fragments would include previously formed homo- and heteroduplexes, which did not dissociate during the last denaturation step as well as newly formed homo- and heteroduplexes, which would not have participated in the single extension cycle. The labeled PCR reaction would be digested with the appropriate diagnostic endonuclease, separated on a polyacrylamide gel and subjected to autoradiography. The quantitation of the wild-type and mutant products seen on this autoradiogram can give a relatively accurate estimate of the percentage of wild-type and mutated mtDNA present in the sample tested as it does not detect heteroduplexes.

Single-Stranded Conformational Polymorphism (SSCP)

Although this technique is commonly used to discover new DNA mutations, it can also be used to quantitate mtDNA heteroplasmy. When Tanno et al⁶¹ compared this method against the other three methods described above, it was reported as the most accurate of the four methods, narrowly outperforming PCR "last cycle hot." This method involves amplifying the region surrounding a mutation site with one radioactively end-labeled primer and one unlabeled primer to create a short double-stranded PCR fragment. This PCR fragment is denatured and separated on a polyacrylamide gel containing glycerol. The gel allows the single-stranded fragments to renature, forming various secondary structures that persist through electrophoresis. Different single-stranded PCR fragments will form different sets of secondary structures and therefore create different electrophoretic banding patterns on the gel. The gel is then dried and subjected to autoradiography. To apply this basic concept to quantitation of heteroplasmy, conditions must be determined under which only two major products are formed, one from the wild-type PCR fragment and one from the mutant PCR fragment. These conditions include the length of the PCR fragment produced and the temperature at which the polyacrylamide gel is run. Once the conditions under which single wild-type and mutant bands can be formed have been determined, quantitation of the autoradiogram can reveal the amount of single-stranded PCR product derived from mutated mtDNA and the amount derived from wild-type mtDNA.

The major problem with SSCP as a way of quantitating heteroplasmy is the amount of labor needed to determine the optimal conditions for the study of each mutation. If the technique becomes more user-friendly, it may offer a viable alternative for heteroplasmy quantitation. Until then, the amount of preparatory work needed to determine these conditions makes the last cycle-labeled technique easier to use.

Figure 6. The use of "last-cycle hot" PCR for quantitation of heteroplasmy. Annealing of single-stranded DNA fragments amplified from wild-type (wt) and mutated (M) mtDNA causes the formation of heteroduplexes. These are created toward the end of the PCR cycling, as many molecules re-hybridize to full-length complementary strands and not to primers. Because heteroduplex sites cannot be digested by restriction endonucleases, they skew the results in favor of the undigested form, as heteroduplexes are also not digested by restriction endonucleases. This problem can be solved by "last cycle hot" PCR. A single round of annealing-extension is performed on the PCR product at the end of the PCR reaction. Because these duplexes are not denatured after extension, and because this last extension is the one that incorporates radioactive deoxynucleotides, the ^{32}P -labeled products will be exclusively homoduplexes. Therefore, the radioactive RFLP pattern will be a better representation of the actual levels of mtDNA heteroplasmy than the corresponding ethidium bromide staining.

Criteria for Pathogenicity

When a new mutation is found in the mtDNA of a patient with a mitochondrial defect, it is important not to assume pathogenicity until a firm link between the mutation and the cellular dysfunction has been established. Although some mutations in mitochondrial tRNA genes and in coding regions have been shown to cause diseases, the mtDNA evolves at a relatively high rate containing many polymorphic sites.²³ To avoid the mistake of incorrect assignment of the pathogenicity of a mutation, several criteria must be met to firmly link a mutation to the disease symptoms.

Presence of Heteroplasmy

Silent base substitutions present in the mtDNA are usually found in a state of homoplasmy.⁶² This is caused by the "bottleneck effect" of mtDNA inheritance,⁶³ which takes place during early oogenesis. Oogonia (oocyte precursors) bear only a small number of mtDNA molecules, and random genetic drift at this level causes a silent mutation to be brought to a state of homoplasmy.⁶³ It is not known why deleterious mutations in mtDNA are maintained, as one might think that the detrimental effects of the presence of the mutated mtDNA, acting according to the rules of population genetics, would cause the mutation to be selectively lost. Heteroplasmy, how-

Figure 7. Genotype-phenotype correlations at the single cell level. Three different patients with mtDNA mutations in tRNA genes were studied. Muscle fiber cross-section, obtained from frozen muscle, were stained histochemically for the activity of the enzyme cytochrome c oxidase (COX) or succinate dehydrogenase, which can detect ragged red fibers (RRF).⁶⁸ Single fiber segments were dissected from the activity-stained muscle sections with the aid of a micropipette.⁶⁸ The isolated fibers were transferred to an Eppendorf tube, treated for DNA release by alkaline lysis (30 minutes in 6 μL of 200 mmol/L KOH, 50 mmol/L DTT at 65°C, followed by neutralization with 6 μL of 900 mmol/L Tris-HCl pH 8.3, 200 mmol/L HCl). Two μL of the final solution is finally amplified by PCR. The amplified products were subjected to "last cycle hot" PCR (as described in Figure 6), digested with appropriate restriction endonucleases that can differentiate the pathogenic mutations from wild-type sequences, the products separated by PAGE (12% acrylamide) and analyzed by autoradiography.⁶⁸ Fibers with activity for cytochrome oxidase (COX) are marked as COX+ and COX-deficient fibers as COX-. Ragged-red fibers are marked as RRF. The correlation between the defective COX defect (or RRF) and the presence of high levels of the mutated mtDNA provides a convincing evidence for pathogenicity. The graphs on the right plot the data (obtained by "last cycle hot" RFLP) for a larger number of fibers, for each patient. Modified from *J Clin Invest* 1993, 92:2906-2915 with permission from the American Society for Clinical Investigation.⁶⁹

ever, is not a sufficient or required criteria for pathogenicity. Most patients with LHON have homoplasmic mtDNA mutations and heteroplasmy can be observed in association with non-pathogenic mutations, particularly in the D-loop region.⁶⁴

Clinical/Biochemical Correlations and Family History

Although genotype-phenotype correlations are not fully understood in mitochondrial disorders, some clinical manifestations are sometimes typical of certain mtDNA mutations, for instance, stroke-like episodes in patients with the A3243G mutation in the tRNA^{Leu(UUR)} gene or myoclonus epilepsy in patients with the A8344G tRNA^{Lys} mutation. Mutations in mtDNA can be inherited, but may also be the product of a spontaneous event. If the mutation is maternally inherited, the presence of the mutation in other family members should be investigated. Individ-

Figure 8. The construction of cytoplasmic hybrids (cybrids) for the study of mtDNA mutations. Transmitochondrial cybrids can be obtained by fusing an enucleated mitochondrial donor with a cell line lacking mtDNA (p^0) with polyethylene glycol (PEG). The latter cell line bears a nuclear marker such as a thymidine kinase mutation, which allows for selection of cells that cannot incorporate the toxic thymidine analogue bromodeoxyuridine (BrdU). Cells lacking mtDNA (p^0 cell) are auxotrophic for uridine. Therefore, by screening the fusion product with a simultaneous nuclear (BrdU) and mitochondrial (uridine) selection, one can obtain transmitochondrial cybrids. TK, thymidine kinase; Ur, uridine; N, nucleus.

uals with higher percentages of the mutation in various tissues should also have a higher chance of showing disease symptoms. If the mutation is present in family members in high percentages, but cannot be linked to disease traits it may not be the cause of the disease. If blood is not easily available, hair roots can be easily tested by PCR. These can be mailed in tubes containing 50% ethanol.⁴⁶

It is important to keep in mind that mtDNA mutations require a threshold value for biochemical and clinical manifestations. In other words, the presence of mutated mtDNAs may be inconsequential if its levels are below a certain tissue-specific and mutation-specific threshold.² Nevertheless, the presence of a mitochondrial biochemical defect in tissues with high levels of the mutation are indicative of pathogenicity. The histopathology and histochemistry of affected tissues (eg, muscle biopsy) is also an important indicator of mitochondrial dysfunction, and gives crucial clues for the search for new mtDNA mutations. Biochemical and histochemical assays have been described in detail elsewhere^{65,66}

Single-Cell PCR

Deleterious mutations in the mtDNA can cause effects at the cellular level that can be visualized in various ways. Cells with defective mitochondrial function usually show a decrease in or lack of components of the oxidative phos-

phorylation pathway. Using cytochemical or immunocytochemical techniques,⁶⁷ the intracellular levels of these components can be ascertained. Single cells containing either normal or very low levels of components in the oxidative phosphorylation pathway, can be microdissected and subjected to single cell PCR, amplifying the region of mtDNA surrounding the mutation in normal or affected cells.⁶⁸ Using one of the PCR-based methods described above, the level of mtDNA heteroplasmy is determined for these cells. If the mutation is pathogenic, it would be expected that cells having diminished levels of specific mitochondrial components would also tend to have a higher concentration of mutated mtDNA molecules. If higher levels of mutated mtDNA consistently co-segregate with a detectable phenotype change, it is likely that the mutation is pathogenic. This approach has been used effectively for microdissected muscle fiber segments stained for cytochrome c oxidase activity⁶⁹ (Figure 7).

Cytoplasmic Hybrid (Cybrid) Construction

This procedure allows researchers to study the effects of the mtDNA mutation on cells that bear 0% to 100% mutated mtDNA. This entails enucleating fibroblasts from patients and fusing them to a cell line that lacks mtDNA (p^0 cells,⁷⁰). This allows the creation of immortal cytoplasmic hybrid (cybrid) lines bearing only mtDNA from the patient (Figure 8). Clones can then be screened for heteroplasmy. The level of mtDNA heteroplasmy can also be manipulated in these cells. Growth under conditions which cause mtDNA to fail to replicate, such as the presence of ethidium bromide,⁷¹ will block mtDNA replication until, optimally, a single copy of mtDNA is present per cell. The drug treatment is then stopped and the cells are allowed to repopulate their mtDNA levels. If the residual molecules were mostly mutated, on repopulation the cell clone will have a higher level (in some cases becoming homoplasmic) of the mutated mtDNA. A second screening is then conducted to find cells with desired levels of wild-type and mutated mtDNAs (see example in Figure 5). These cell lines are then subjected to various tests of mitochondrial function including, but not limited to: mitochondrial protein synthesis, oxygen consumption, and function of various components of the oxidative phosphorylation pathway. If it can be shown that defective mitochondrial function is found in cells bearing mutated mtDNA, then the mutation is likely to be a pathogenic cause of disease.

Conclusion

When the link between mutations in mitochondrial DNA and human diseases was discovered, it became imperative to optimize means to characterize these mutations. Over the last several years, many commonly used molecular biological techniques have been adapted to the task, and others were used for the first time. Most of these tests are now routinely used for diagnosis. However, with the continuing discovery of new mutations, it becomes

important to be able analyze these mutations in a manner which is time- and cost-effective. New advances in multiplex detection systems would greatly facilitate the diagnosis of pathogenic mtDNA mutations.

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