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ACCUMULATION OF DELETIONS IN MtDNA DURING TISSUE AGING: ANALYSIS BY LONG PCR

Pascal REYNIER* and Yves MALTHIERY

Unité 38 de l'INSERM, Laboratoire de Biochimie médicale, Faculté de Médecine,
27 Boulevard Jean Moulin, 13385 Marseille cedex 5, FRANCE

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Multiple deletions of mtDNA have not only been implicated in aging, but also in a wide variety of pathological conditions. The enzyme system used in long-PCR makes it possible to synthesize the entire mitochondrial genome (16.5 kb), exposing the multiple deletions in mtDNAs implicated in and, at least partially, responsible for these pathologies. But it is not the number or type of anomalous mtDNA that is crucial, rather it is their frequency relative to the number of intact copies of the mitochondrial genome. Our work exposes the necessity of quantitating the number of normal mitochondrial DNAs. The accuracy of the technique and the small sample size required permit one to detect multiple deletions, located in a specific organ, and simultaneously measure the fraction of intact molecules. This fraction can then be correlated with mitochondrial dysfunction to serve both as an indicator of tissue aging and a monitor of an impending myopathy.

Mutations in mitochondrial DNA, as in any DNA molecule, can alter expression. However their environment leaves mtDNA molecules particularly vulnerable. Their rate of mutation is 10 to 20 times higher than that of nuclear DNA (1). These mutations occur during the lifetime of an individual and can bring on a pathological condition. generally, their number increases and they accumulate with age (2-4). The percentage of mtDNA affected defines the degree of heteroplasmy. This, in turn, indicates the extent to which intact and mutated mtDNAs coexist. A mutation in the heteroplasmic state in mitochondria can have phenotypic consequences if and when a certain threshold is surpassed. This threshold varies depending upon the tissues involved and their aerobic requirements; it obviously varies with age, the mitochondrial dysfunction appearing at lower degrees of heteroplasmy, i.e. lower thresholds, in older people(3).

Deletions and duplications are often found both in the process of mitochondrial dysfunction, and the aging process (5-7). These deletions are always present in the heteroplasmic state, as their consequences are too deleterious and the information carried on the mtDNA molecule too important for cellular survival. Biochemical dosages of respiratory chain complex activity indicate deficiency of the information in mitochondrial genomes. This enzymatic activity is noticeably reduced during aging (8), which can be linked to the accumulation of mtDNA

* To whom correspondence should be addressed. FAX: (33) 91 79 65 11.

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anomalies as age increases. Until now, these deletions were detected mainly by Southern blotting, which can only monitor the more common alleles. The first analyses made it possible to view mtDNA molecules with a major deletion. This led to the notion of a 'common deletion' of 5 kb (9), as well as one of a 7.5 kb deletion (10). Until very recently, the PCR analysis was limited by the (small) size of the DNA fragments which could be amplified. The analysis of these genomes was, thus, oriented toward the search for well-characterized deletions (11, 12); the rate of heteroplasmy was, then, assumed to be equal to the frequency of the analyzable deletion(s) in the mtDNA extracted from the tissue sample.

Multiple deletions have for many years been suspected or known to exist in certain pathologies of mitochondrial origin (13-17). Studies on this subject mainly consisted of punctual analyses of various presumed deletions, namely 'common deletions' as if these were a necessary and reliable indicator of all mtDNA deletions. The availability of more reliable polymerases (18) and the advent of mixtures of different polymerases has made possible in vitro synthesis of DNA segments measuring from 10 to 16.5 kb (size of the mitochondrial genome) (19-21). We have directed our attention to the study of large, multiple deletions in mtDNA associated with a variety of pathological conditions, especially myopathies, polymyalgia rheumatica and cardiomyopathies, as well as normal aging. For this we use enzyme combinations that amplify the whole mtDNA molecule. During our analyses, multiple deletions are found to occur very frequently, even though no one deletion is predominant or significantly represented. We have, therefore, developed a series of complementary methods that detect the complete spectrum of multiple deletions and allow to qantify the undeleted genomes.

MATERIALS AND METHODS

Biopsies: Samples of skeletal muscle are taken from the deltoid or quadriceps for histological analysis to establish diagnosis. Total DNA (genomic and mitochondrial DNA) are extracted following the technique described by Davis (22) using proteinase K on 50 mg of tissue. 25 to 30 µg of DNA are suspended in 50 µl of TE (10mM Tris, 1 mM EDTA, pH 8). Samples of myocardial tissue (a few mg) are obtained via a catheter from the left ventricle. The DNA, extracted by the same method, suspended in 20 µl of TE.

PCR using GoldStar DNA polymerase (Eurogentec, Liège): PCR is performed as described in reference 18. The 8.7 kb fragment is synthesized with primers D6 and R10, the 10.6 kb fragment with primers D5 and R10 and the 11.7 kb with primers D5 and R1.

LA-PCR with "Expand Long Template PCR System" (Boehringer, Mannheim): PCR is carried out with 100 ng of muscle DNA or 2 µl of myocardial DNA solution in a 50 µl final volume : 50mM Tris-HCl (pH 9.2 to 25°C), 14 mM $(NH_4)_2SO_4$, 2.25 mM $MgCl_2$, 50 pmole of each primer (Tab.I). 200 µM dNTP, 2.5 units of DNA polymerase mixture (Taq DNA and Pwo polymerases) according to the manufacturer's recommendation. The two primers used (R-Eag I & D-Eag I) overlap an Eag I site with a short overlapping sequence corresponding to the restriction site (T_m

Table I. Primers used for PCR amplifications of mitochondrial DNA

Primers	Sequence 5' -> 3'	position on L strand
D6	TCTAGAGCCCACTGTAAAG	8266-8284
R10	AGTGCATACCGCCAAAAGA	16149-16167
D5	TCATCGGCGTAAATCTAAC	9396-9414
R1	TAGGGTGATAGATTGGTCC	14284-14302
D-EagI	TAACGGCCGCGGTACCCTAACCGTG	13983-14107
R-EagI	CCGCGGCCGTTAAACATGTGTCACTG	13996-14121

much lower than T_m used in the reaction). The reaction is performed as follows: 1.5 minute at 94°C, 15 cycles of 20 secondes at 94°C, 10 minutes at 68°C followed by 15 cycles with an increase in synthesis time of 15 seconds per cycle, then 10 minutes at 68°C. Primers are added last according to the 'Hot Start' technique. The thermocycler used is an 'MJ Research Minicycler' and the reactions are in standard microtubes.

Electrophoreses: The reactions are analyzed on 0.5% agarose gels in TBE buffer (pH 8.3). 10 µl of the reaction products are placed on gels and migrated 90 minutes at 100V. The DNA is revealed with ethidium bromide. Some pictures are photographed by a computer assisted camera (Imager, Appligene) and densitometry analysis are performed with NIH Image 1.47 software.

RESULTS

The results reported here concern a group of 18 subjects of various ages affected by a myopathy and/or a rheumatic disorder of the polymyalgia rheumatica type in which mitochondrial dysfunction has been established by anatomo-pathological criteria (essentially the appearance of Ragged-Red-Fibers) and enzymatic criteria (activity of the I and IV complexes reduced by at least 50%)(23). Also included in this study were 5 cases of idiopathic dilated cardiomyopathies in which the mitochondrial function has not been studied, and 15 aged subjects (60-90 years old) showing no neuromuscular pathology.

A high-performance Taq polymerase (GoldStar) enables us to synthesize DNA fragments in the 1 to 12 kb range. DNAs corresponding to full, non-deleted sequences of mtDNA are always found in synthesis reactions, fragments of 8.7 kb (Fig. 1 & 2), fragments of 10.6 kb and 11.7 kb (Fig. 2). However, the relative quantities of DNA produced decreases with the size of the

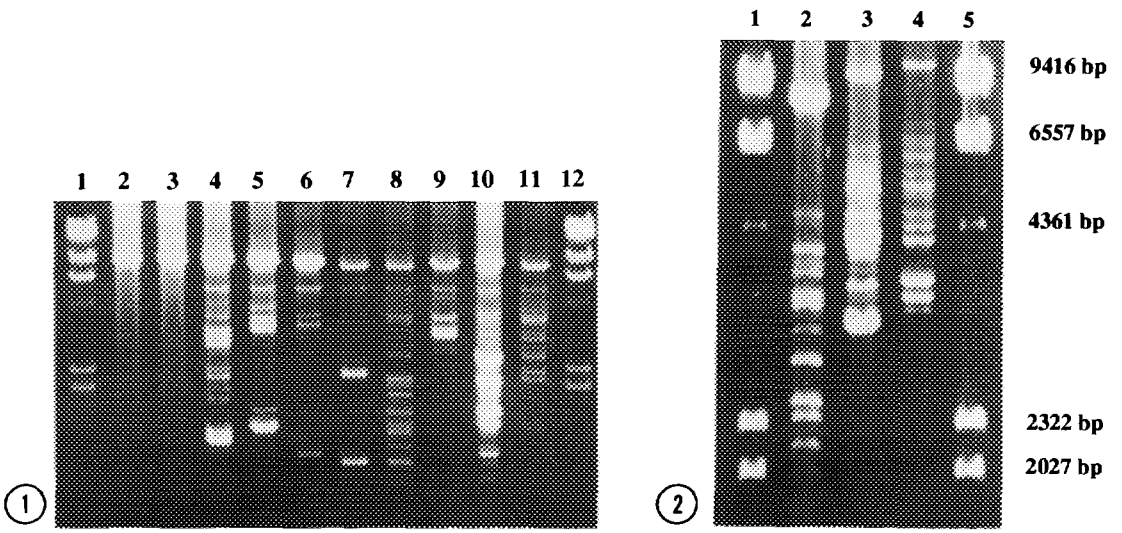

Fig. 1. Multiple deletions on mtDNA detected on 8.7 kb PCR products. PCR on skeletal muscle DNA from subjects affected with a mitochondrial disorder (myopathy or polymyalgia rheumatica) attested by an anatomo-pathological study (ages,respectively : 59, 62, 62, 73, 75 - lines 4 through 8), PCR on muscle DNA of subjects aged 88 and 91 (lines 9 and 10). PCR on myocardial DNA from a subject affected with a dilated cardiomyopathy aged 52 (line 11). Lines 2 and 3: analysis of mtDNA from young subjects (18 and 19 years). Lines 1,12 : marker of size (Lambda DNA cut by Hind III).

Fig. 2. PCR on an mtDNA with different primers. Synthesis of an 8.7 kb DNA with primers D6-R10 (line 2), of a 10.6 kb DNA with primers D5-R10 (line 3), of an 11.7 kb DNA with primers D5-R1. Molecular weight marker (DNA cut by Hind III (lines 1,5)).

synthesized fragment, and it seems that 12 kb is the limit for synthesis with this enzyme, at least under the experimental conditions used in the present study. Electrophoresis shows alleles which correspond to multiple deletions on mtDNAs (Fig.1). The number of alleles detected varies according to the tissue tested, from 4 to 15 and sometimes more bands being observed. These bands vary in size from 1 to 6.5 kb, corresponding to deletions of 7.5 and 2 kb, respectively. The patterns are individual and cannot be superimposed. Nevertheless, certain deletions seem to be detected frequently. Thus, a 3.5 kb band is present in most tests; it corresponds to a 5 kb deletion which might be the 'common deletion' already described. The intensities of synthesized bands vary greatly, showing that alleles carrying deletions are variably represented. Thus, the 3.5 kb band is of variable intensity, equal to the average of the other bands, but not indicative of the degree of heteroplasmy (Fig.1). The rate of synthesis is not directly related to size, although shorter alleles, corresponding to the larger deletions, are probably favored.

These amplifications are reproducible. Well-characterized bands appear with practically no smearing (Fig.1; rows 1 & 2). The pattern is reproducible, both with respect to quality and quantity, i.e. the relative intensity of the bands is reproducible. Specificity has yet been studied (18). This specificity is also confirmed by the use of 3 different pairs of primers (Fig. 2). The synthesis of a larger fragment, overlapping the 8.7 kb band, in the same DNA sample shows the same multiple deletion pattern, with bands displaced according to the primers used. It should be noted that starting at a certain size, the quantity synthesized depends upon the size of the fragment produced. Thus, when fragments between 8.7 kb and 11.7 kb are synthesized, band intensity decreases with increasing size, suggesting the preferential amplification of smaller DNA fragments. Therefore, although the technique indicates the presence of multiple deletions in the mtDNAs, it cannot be used to measure their relative abundance (the degree of heteroplasmy).

The use of a mixture of two different DNA polymerases, one with no 3' -> 5' exonuclease activity (Taq DNA polymerase) and the other with a limited quantity of this exonuclease activity (Pwo DNA polymerase), makes it possible to synthesize larger DNA fragments (19-21). With partially overlapping primers we were able to amplify 16.5 kb, representing the total mtDNA molecule (Fig. 3). This synthesis is highly specific and without background noise. The quantity of material obtained is nevertheless lower than that obtained in a conventional PCR. We evaluate the amplification to be 10^4 to 10^5 times the input after 30 cycles of synthesis. Thus, using LA-PCR it is possible to demonstrate multiple deletions in mtDNA. Not only does the amplification of fragments of different lengths more accurately reflect their preponderance in the sample, than with the GoldStar polymerase (which tends to emphasize the smaller fragments), but it also can provide a quantitative estimate of the fraction of intact mtDNA molecules. Figure 4 shows the sizes of the whole mitochondrial genomes of 4 DNA samples extracted from affected muscles of patients. In column 2, the PCR products from DNA extracted from a cardiac muscle shows numerous deletions with a significant decrease in the intensity of the intact band at 16.5 kb. In column 3, DNA from a skeletal muscle sample is amplified. The intact band is severely reduced relative to deleted forms. In column 4, the PCR realized on a sample extracted from the healthy skeletal muscle of an older patient shows deleted forms without an obvious reduction in the intensity of the 16.5 kb. In column 5, a control PCR on the healthy skeletal muscle of a younger patient demonstrates no deleted forms 16.5 kb.

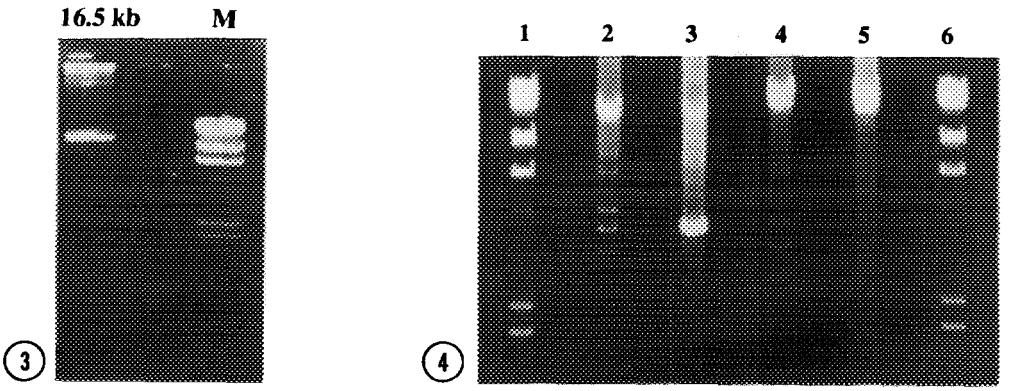

Fig. 3. Synthesis of the totality of mtDNA by long PCR with a mixture of Taq and Pwo polymerases. One-fifth of the PCR reaction is placed on the gel. M = Marker of size (Lambda DNA cut by Hind III).

Fig. 4. Long PCR of 4 different mtDNA preparations from muscular tissue. Two present multiple deletions (lane 2: cardiomyopathy; lane 3: skeletal muscle with a defect in the mitochondrial respiratory enzymes), a preparation of mtDNA from an older patient (lane 4) presents fewer deleted species, and one preparation of mtDNA from a young patient without deletions (lane 5). Lanes 1 and 6 correspond to a marker DNA (lambda cut by Hind III).

The kinetics of the amplification of the deleted forms was followed by removing 5 µl of reaction product every second cycle. Figure 5a employs intact mtDNA extracted from healthy muscle, while figure 5b uses a DNA sample containing deleted forms. Figure 5c shows that the amplification kinetics are comparable for the intact and various deleted forms since the slopes of

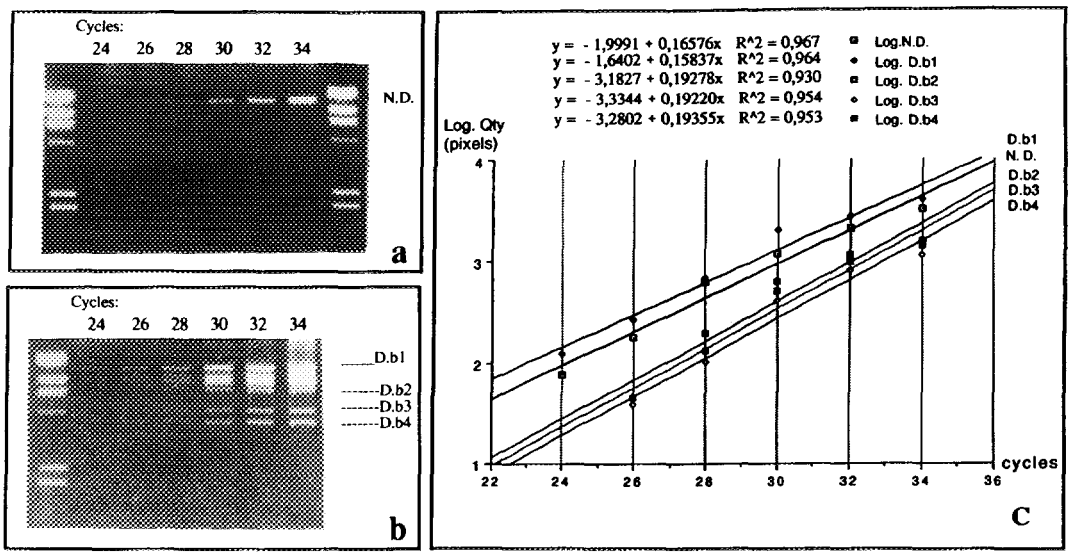

Fig. 5. Kinetics of mtDNA amplification by LA-PCR. PCR from an mtDNA extracted from muscular tissue, showing no deletions (a), and the same preparation as in lane 2 of Figure 5 showing multiple deletions (b). In (c) the optical densities (expressed in pixels) permit one to plot the kinetics of synthesis of each band (c). Extrapolating the exponential plot allows the calculation of the initial concentrations of mtDNA bands.
N.D corresponds to nondeleted (intact 16.5kb) mtDNA in Figure a. D.b1 is the intact band from Figure b; D.b2, D.b3, D.b4 correspond to the various mtDNAs with deletions in Figure b.

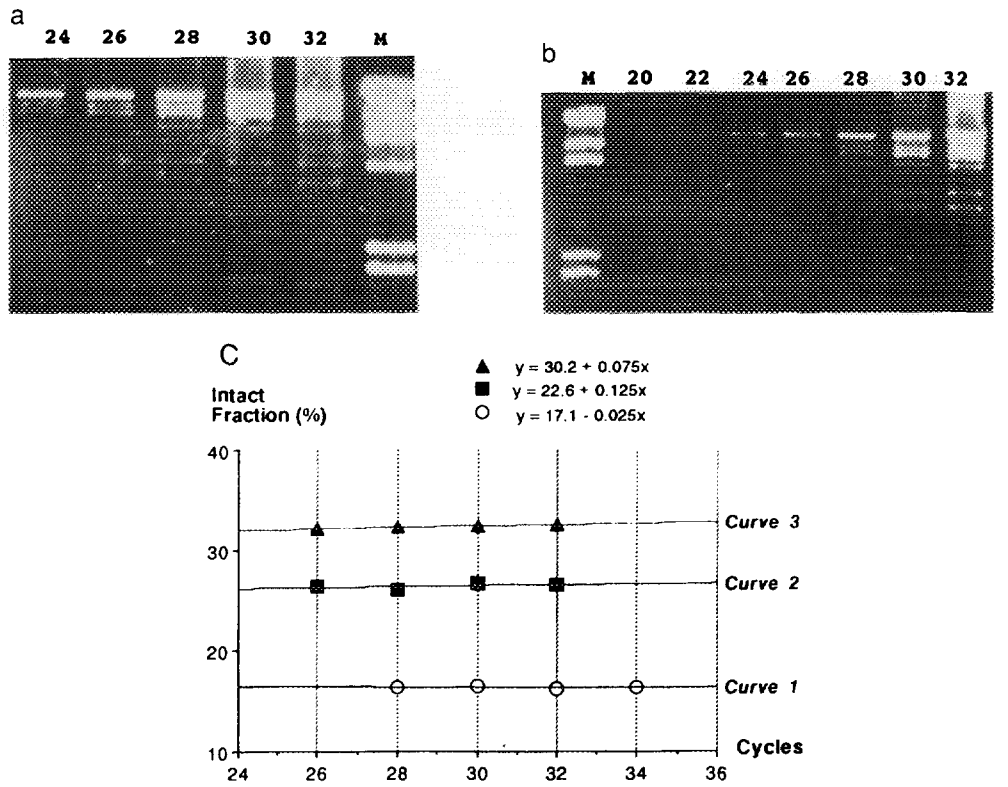

Fig. 6. Amplification of mtDNA plotted as the fraction of intact genome I : intact molecules / sum of deleted and non deleted species of mtDNA. In a, PCR of a mixture of 11 ng of DNA from 5a and 66 ng of DNA from 5b. In b, PCR of a mixture of 16.5 ng of DNA from 5a and 50 ng of DNA from 5b. In c, kinetics of amplification with I plotted as a function of the number of cycles of amplification. Curve 1: kinetics calculated from Figure 5b. Curve 2: kinetics calculated from Figure 6a. Curve 3: kinetics calculated from Figure 6b. A densitometer reading of the deleted species was statistically significant only at $\geq$26 cycles in these experiments.

the corresponding curves are similar. Plotting the fraction of intact mitochondrial genomes synthesized (calculated on a molar basis - Fig 6c, curve 1) as a function of the number of cycles of amplification gives an almost horizontal line which can be extrapolated to 17% on the ordinate.

If known quantities of intact mtDNA (from the sample amplified in Figure 5a) are added to the heteroplasmic sample of Figure 5b and the kinetics are measured (Figure 6), more accurate estimates of quantitative relation can be made. In figure 6a, 11 ng of total DNA from figure 5a were added to 66 ng of total DNA from figure 5b. In figure 6b, 16.5 ng of total DNA from figure 5a were added to 50 ng of total DNA from figure 5b. In Figure 6c, the fraction of intact molecules is plotted: intact / sum of all deleted and intact forms (after correcting band intensities as a function of their size) for the mixtures shown in Figures 6a (curve 2) and 6b (curve 3). In this case (Figure 4, lane 2 and Figure 5b), 6 deleted bands are detected and measured. Thus, Curve 2, provides an estimate of 23% for the fraction of intact molecules and curve 3 gives a value of 30% when projected upon the ordinate. When 14% of total DNA without deletions are added (11ng / 66ng+11ng), we observe an increase of 6% of intact DNA mesured with quantitative PCR (23%-17%). If we add 25% of the same DNA (16.5 / 50+16.5) instead of 14% we obtain a 13%

increase (30% - 17% = 13%), a result in good agreement with the expected theorical result of 13% (6% / 14% X 25% = 13%).

DISCUSSION

In the aging process, deletions accumulate in mitochondrial genomes. This observation is classic and has been well-documented (1). It was hoped that the appearance of particular mtDNA deletions could be held responsible for aging, for as these mtDNA anomalies accumulate with age mitochondrial function does diminish (3). The same deletion patterns in mtDNAs are found in analyzed cytopathies and in advanced conditions of aging, but never in subjects of the same age. These cytopathies show a premature tissue aging with multiple deletions in mtDNA identical to those found in very old individuals. These multiple anomalies in the mitochondrial genome probably correspond to a defect in the replication process of mtDNAs. Their origin is probably in the nucleus, linked to a dysfunction of the replication-repair machinery, which is entirely of nuclear origin.

Our study deals with several types of pathologies. We have not found a deletion pattern specific to the various cytopathies studied. All these tissues show multiple deletion patterns, with a variable number of mtDNA alleles, i.e. no deletions specific to the pathology. The same type of mtDNA pattern is found in various cytopathies to various degrees. These multiple deletions recall those found in disorders associated with aging. So-called 'common deletions', those measuring 5 kb and 7.5 kb, do not constitute the major species being neither predominant, nor exclusive. The 5 kb deletion is often cited in the literature simply because it is the most sought after. The relative frequency of mtDNA molecules carrying the 'common deletion' has often been used as an indicator of mutant mtDNAs since this anomaly seemed representative. Our work demonstrates that such estimates of the rate of heteroplasmy are at best risky if not completely misleading. In fact, it is the observed differences in heteroplasmy using the 'Goldstar' enzyme that encouraged us to accurately measure the intact fraction using the long PCR protocol. The two enzymatic systems applied to the same mtDNAs yield consistent but distinct results. An effective Taq polymerase emphasizes the presence of the (supposed) minority deleted alleles. A mixture of polymerases favors the amplification of larger molecules, the seemingly lower degree of heteroplasmy is probably due to quantitative fidelity in the synthesis, more accurately reflecting the real molar ratio in the "defective" cells. Each PCR system has its particularities and advantages; for our purposes they are complementary. PCR with GoldStar DNA polymerase provides an overview of the multiple deletions in a potentially pathologic mtDNA. LA-PCR makes it possible to amplify and analyze mtDNA while minimizing the size of the tissue sample, e.g. myocardial biopsies whose analysis is critical to any assessment of disease severity. As we demonstrate, the former is qualitative, whereas the latter can be quantitative, accurately measuring the degree of heteroplasmy.

These performances probably depend on differencies in kinetics linked to different enzymatic processes. GoldStar DNA polymerase is a Taq polymerase having no 3' -> 5' exonuclease activity. In the mixture used for an LA-PCR, the 3' -> 5' exonuclease activity missing in the majority enzyme is compensated by the presence of this activity in the minority enzyme. The fidelity of the synthesis is, therefore, being obtained at the cost of a decrease in the quantity

of synthesized material. In both systems the syntheses are carried out at a very alkaline pH. This reduces the risk of depurination of the DNA matrix, which would halt synthesis (19).

An indication of the relative degree of heteroplasmy is provided in Figure 4, the fraction of intact mitochondrial genomes has decreased in the affected muscle tissue of patients with mitochondriological problems and can even be seen to decrease, albeit not as significantly, in muscle tissue from older patients. If one extrapolates curves of the 'long' amplification kinetics (Figures 5c and 6c) to the ordinate one realizes that the deleted forms comprise the majority of mitochondrial species in a pathological sample. LA-PCR permits a quantitative approach. Amplification kinetics of heteroplasmic mtDNA demonstrates a variation as a function of size (Fig 5) disfavoring the intact (16.5 kb) relative to shorter forms (4kb) given the difference in slope of amplification, but the bias is considerably less than that obtained with the GoldStar enzyme. Moreover, the amplification of the 16.5 kb form is strictly the same whether or not deleted forms are present and being coamplified with the intact mtDNA molecules (compare the D.b1 and N.D. curves in Figure 5c).

In the present analysis, the dilution kinetics presented in Figure 6 provide an accurate estimate of the intact fraction. It is a veritable competitive, quantitative PCR where the competitors are natural (the deleted forms).The curves represent, for one sample, three ratios of heteroplasmic and intact mtDNA plotted as a function of the cycle of amplification: first, the data already presented in 5b is shown in Figure 6c (curve 1). Curve 2 (from Figure 6a) and curve 3 (Figure 6b) represent molar fractions. The curves are going from 17% in the initial patient's tissue sample to 23% and 30% in the artificial mixtures. These results are consistent with the ratio of total DNA added (see results). Our estimate is preliminary and accurately describes an approach to quantitate the intact molar fraction, I, and establish the threshold by comparing mtDNA extracted from healthy and pathologic muscle samples derived from the same patient. Such quantification is approximate in the present publication, and will be more accurate when we have purified amplified mtDNA products and can artificially mix these DNA to establish standard dilution curves. The rate of heteroplasmy found here is consistent with those described as pathogenic in the case of point mutations in a transfer RNA (1) or in the case of multiple deletions (16, 17).

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