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The Mutation Rate of the Human mtDNA Deletion mtDNA^{4977}

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Summary

The human mitochondrial mutation mtDNA^{4977} is a 4,977-bp deletion that originates between two 13-bp direct repeats. We grew 220 colonies of cells, each from a single human cell. For each colony, we counted the number of cells and amplified the DNA by PCR to test for the presence of a deletion. To estimate the mutation rate, we used a model that describes the relationship between the mutation rate and the probability that a colony of a given size will contain no mutants, taking into account such factors as possible mitochondrial turnover and mistyping due to PCR error. We estimate that the mutation rate for mtDNA^{4977} in cultured human cells is 5.95×10^{-8} per mitochondrial genome replication. This method can be applied to specific chromosomal, as well as mitochondrial, mutations.

Introduction

The human mitochondrial mutation mtDNA^{4977} is a 4,977-bp deletion that originates between two 13-bp direct repeats in normal mtDNA. This deletion is associated with myopathies and neuromuscular disorders such as progressive external ophthalmoplegia, Kearns-Sayre syndrome (KSS), and Pearson marrow/pancreas syndrome. Symptoms range from mild to severe, depending on the level to which the deleted molecules have accumulated. For a review of diseases associated with mutations in mtDNA, see DiMauro and Wallace (1993) and Wallace (1994).

The mtDNA^{4977} deletion has also been found at low levels in normal adults, and it appears to accumulate with age, primarily in nonmitotic tissues (Cortopassi and Arnheim 1990; Corral-Debrinski et al. 1991; Hattori et al. 1991; Yen et al. 1991; Cortopassi et al. 1992; Zhang et al. 1992). The level of accumulation is found to vary among different tissues and even within tissues. For example, studies on the brains of old, normal individuals have shown that the substantia nigra, caudate, and putamen can have higher levels of mtDNA^{4977}, by >300-fold, than the cerebellum (Corral-Debrinski et al. 1992; Soong et al. 1992).

The degree of accumulation of mtDNA^{4977}, whether at the high levels found in patients with severe symptoms or at the low levels found in normal older adults, is determined by the mutation rate, by selective factors that may affect deleted molecules, and by the initial level of deletions present at conception. Estimating the mutation rate is the first step in developing models that enable us to discuss quantitatively the roles played by each of these factors in the accumulation of deletions in both growing and stable populations of cells.

mtDNA deletions have been proposed to result from errors in DNA replication (Wallace 1994). In this paper, we describe an experiment that enables us to estimate the mutation rate for the mtDNA^{4977} deletion in cultured human cells. A number of cell colonies are grown, each arising from a single cell. For each colony, we can count the number of cells, and we can determine whether the colony contains at least one mutant molecule by subjecting the DNA to PCR amplification and testing for the presence of mutant sequences. We express the probability that the PCR product tests negative for the deletion mutation as a function of the number of cells in the colony, the mutation rate, the PCR efficiency, and the rate of PCR contamination. Mitochondria can turn over by being engulfed by lysosomes. In what follows, we refer to this turnover as "mitochondrial death." To make the model more general, we also include death rates for mutant and nonmutant genomes. Although, in our study of growing cells mitochondrial death is likely to be insignificant, death rates are significant in other applications of this model, such as nondividing cells.

Our procedure for estimating the mutation rate has its origin in the "P_0 method" of Luria and Delbrück (1943), which is based on the relationship between the mutation rate and the probability that a colony with a

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known number of cells contains no mutants. We found it necessary to modify considerably Luria and Delbrück's original method to account for the complex stochastic nature of mitochondrial replication and for the possibility of PCR errors.

Material and Methods

Cell Lines

The fibroblast strain GM03651C (25-year-old female), received from the National Institute of General Medical Science Human Genetic Mutant Cell Repository (Camden, NJ), was enucleated with cytochalasin B and fused to the mtDNA-less cell line $\rho^0$143B.206 (King and Attardi 1989). 51C.206.C11 is one of the resulting transmitochondrial cell lines that were selected for growth in the absence of uridine (Dulbecco's modified Eagle's medium supplemented with 5% dialyzed bovine fetal serum and 4.5 g of glucose per liter).

Quantification of mtDNA

The relative mtDNA content of cell line 51C.206.C11 was determined by slot blot analysis (Yoneda et al. 1992). For this purpose, total cellular DNA was treated with 0.4 M NaOH for 16 h at 37°C and then applied, in duplicate, to a Zetaprobe (BioRad) nylon membrane according to the manufacturer's (Schleicher and Schuell) recommendations. The blot was hybridized first to a probe containing 498 bp of the nuclear 28S rRNA gene. It was then stripped and rehybridized to a probe containing mtDNA sequences 41–2578. The ratio of the 28S signal to the mtDNA signal was calculated, using Phosphorimager data, for 51C.206.C11 and compared to the ratio obtained for triplicate samples of total cellular DNA from 143B.TK$^-$, the parent of the $\rho^0$143B.206 cell line, applied to the same blot. The mtDNA content of the 143B.TK$^-$ cells (9,100 molecules/cell) was derived from King and Attardi (1989). On the basis of this ratio, the average number of mitochondrial genomes per cell is estimated to be 7,600, a figure we use throughout this paper.

Cloning Single Cells

One hundred microliters of growing cells, diluted to contain an average of slightly less than one cell per hundred microliters, was inoculated into the wells of a 96-well microtiter dish. Examination of the wells soon after plating identified those wells with no growing cells. These were not studied further.

Cells were observed under a phase contrast microscope and counted using a grid. When a well was seen to have between 50 and 4,500 cells, the media was removed, and the cells were washed and then lysed within the wells. The lysis buffer contained 1 μg *Escherichia coli* tRNA (Sigma R4251) as carrier, 0.2 mg/ml proteinase K, 1% SDS, 10 mM Tris pH 8.0, 0.5 M NaCl, and 3 mM EDTA. After incubation at 37°C, the contents of each well were phenol-chloroform extracted. DNA was precipitated with ethanol, washed with 70% ethanol, and brought up in 25 μl of water. The DNA was then used in the PCR reaction.

PCR Conditions

PCR amplification of mtDNA^{4977} molecules was carried out with previously described primers and reaction conditions (Cortopassi and Arnheim 1990; Soong et al. 1992), with the following modifications. In order to detect a single molecule of mtDNA^{4977}, methods developed for single sperm typing were used (Leeflang et al. 1994). In the first round of amplification, primers MT1A (Soong et al. 1992) and MT4 (Cortopassi and Arnheim 1990) were used for 30 cycles. One microliter was taken and then amplified in a second round for an additional 30 cycles by using MT1A and MT2 (Soong et al. 1992). The deletion-specific PCR product is 299 bp in length, and it was detected by ethidium bromide staining after 2% agarose gel electrophoresis. Three kinds of PCR controls were used. In some PCR reactions, water but no sample was added. Other control samples came from wells that received the original growth media but no cells; these samples were processed identically to those that received cells. Finally, fresh media controls were also processed identically to those that contained cells.

Sensitivity of mtDNA^{4977} Detection

mtDNA^{4977} PCR product was obtained by amplification using primers MT1A and MT4 on brain DNA from an elderly individual. PCR product was purified and run on a 2% agarose gel next to a lane containing a known amount of pBR322 DNA digested with *Msp*I. The intensities of staining with ethidium bromide were compared and used to calculate the amount of mtDNA^{4977} PCR product. A single molecule, on average, of the purified mtDNA^{4977} PCR product was added to 50 ng of cellular DNA (~7,500 diploid genomes) from a 15-wk-old infant who had repeatedly tested negative for the deleted mtDNA genomes using the 60-cycle protocol described in the previous section. On the assumption that each infant brain cell contained 5,000 mitochondrial genomes, each sample tested contained $5{,}000 \times 7{,}500 = 37.5$ million normal genomes and an average of one molecule of mtDNA^{4977} PCR product. These samples were subjected to the two-stage 60-cycle PCR protocol for mtDNA^{4977} deletions.

Results

The mtDNA^{4977} mutation rate was estimated using cells carrying mtDNA from a 25-year-old woman. The original cell line was obtained by fusion of fibroblasts

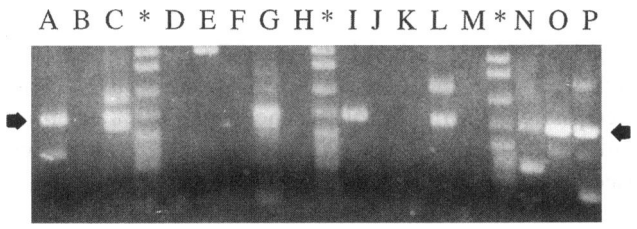

Figure 1 Representative data from the PCR analysis for mtDNA^{4977} in colonies derived from single cells. The 299-bp PCR product (arrows) is found in lanes A, G, I, L, and N–P, showing examples of category (ii) and (iii) results (for category definitions, see Results). Lanes B, D, F, H, J, K, and M gave no mtDNA^{4977} PCR product (category [i]). A mtDNA^{4977} band is seen in lane C. Explanations for this single example of a positive PCR reaction from a total of 173 negative controls are discussed in the Results section. Asterisk (*) denotes molecular weight marker, *Msp*I digest of pBR322.

from the donor to a transformed human cell line from which mtDNA had been eliminated, followed by selection for tumor cells carrying the donor's mitochondria (King and Attardi 1989). This approach made it possible to estimate the mutation rate of mitochondria without a long history of growth in culture. We are making the reasonable assumption that the mitochondrial mutation rate is unaltered by this experiment manipulation.

DNA isolated from the descendants of 220 single cells were analyzed by PCR for the presence of mtDNA^{4977}. Among these 220 colonies, the number of cells ranged from 56 to 4,236, with most having between 1,000 and 3,000. The PCR assay results fell into three categories:

(i) In 118 colonies, no mtDNA^{4977} product was detected. Among these, 91 colonies had no PCR products, and 27 had bands of other sizes.
(ii) In 45 colonies, only the mtDNA^{4977}–specific PCR product was found.
(iii) In 57 colonies, in addition to the diagnostic 299-bp mtDNA^{4977} band, one or more bands, either larger or smaller than the observed mtDNA^{4977} PCR product, were detected.

Examples of the PCR products are shown in figure 1. Among a total of 49 wells that were inoculated, but which were not found to contain any cells, one was positive and may have resulted from either PCR product contamination, mitochondrial DNA from dead cells contained in the media, or cells present in a well but not observed under the microscope. Among 94 fresh media controls, no PCR products were detected. Thirty water-blank PCR reactions were also negative.

Only the presence or absence of the mtDNA^{4977}–specific band was considered in our data analysis, regardless of whether or not other bands were seen. There are several possible explanations for bands of other sizes. They could represent other deletions known to be present in human cells (Zhang et al. 1992; Wallace 1994) or PCR artifacts resulting from the large number of PCR cycles required to insure that even a single target molecule was detected.

The validity of our assay depends on our ability to detect a single deleted mitochondrial genome among the much larger number of undeleted genomes that are present in every colony of cells. The largest number of cells present in a colony was 4,236. Our estimate of the number of mitochondrial genomes in a 51C.206.C11 cell is 7,600. Thus, no colony would be expected to have more than ~32 million mitochondrial genomes. To test our sensitivity, we added on average one molecule of mtDNA^{4977} PCR product to 37.5 million mitochondrial genomes. After 60 PCR cycles on 50 samples, 26 were positive and 24 were negative for the mtDNA^{4977} product. Control samples that contained only the 37.5 million infant mtDNA genomes or no DNA were negative. We conclude that our PCR deletion assay is sensitive to the level of 1 deletion to 37.5 million undeleted mtDNA genomes, a sensitivity greater than that required (1 in 32 million; see above). However, we must also estimate the efficiency with which we can detect a single deleted molecule.

In order to estimate this efficiency, we have to describe the process by which deleted molecules are detected. Suppose that $k > 0$ deleted molecules are present and that they are amplified in the PCR reaction. The amount of PCR product produced by the ith deleted molecule will be denoted by Z_i. Different molecules amplify independently of one another, and the Z_i values have identical distributions. The reaction will be negative if $Z_1 + \ldots + Z_k \leq T$, where T is the threshold of detection of an ethidium stained band. No matter how the Z_i values are distributed, it is the case that for $k > 0$,

$$0 \leq \Pr(Z_1 + \ldots + Z_k \leq T) \leq (1 - \rho)^k ,$$

where $\rho = \Pr(Z_1 > T)$. The quantity ρ is the chance that a single deleted molecule is detected.

The PCR sensitivity experiment is designed to determine the ability to detect a single deleted molecule per tube. If we assume a Poisson distribution with mean 1 for the number of deleted mitochondria in each tube, then it follows from the last inequalities that the probability ρ_0 that a tube tests negative for the deletion satisfies

$$e^{-1} \leq p_0 \leq e^{-\rho} .$$

In our dilution experiment, 24 of 50 tubes tested negative. A conservative estimate of ρ follows by solving the equation $e^{-\rho} = 0.48$, giving the value $\rho = 0.73$. We use this estimate for ρ in what follows.

Table 1

Definitions of Symbols

Symbol	Definition
λ	Probability that a nonmutant sequence, on replication, produces one mutant and one nonmutant sequence
ω_1	Probability that a nonmutant dies before replicating
ω_2	Probability that a mutant dies before replicating
ρ	Probability that a mutant sequence is amplified to a detectable level by PCR
γ	Probability that PCR contamination, or a descendent of a mutant sequence in the initial cell, produces a signal

A Statistical Model of Deletions

Definitions of symbols used throughout the remaining discussion are presented in table 1. A statistical method for estimating a mutation rate, based on the detection of mutant molecules in cell colonies, must be based on two intuitively obvious facts: first, larger colonies are more likely to contain mutant molecules than smaller colonies; and second, the larger the mutation rate, the larger will be the proportion of colonies that contain mutants.

In their development of methods for fluctuation analysis, Luria and Delbrück (1943) described the P_0 method for estimating mutation rates in nuclear DNA. This method is based on a simple expression for the probability that a bacterial colony consisting of c cells grown from a single nonmutant would contain no mutants. If λ represents the probability that a replication of a nonmutant genome produces a mutant genome, then, since the c bacteria are the result of $c - 1$ independent replications, the probability that none are mutants is $(1 - \lambda)^{c-1} \approx e^{-\lambda c}$. For this result to be valid, it must be assumed that no deaths occur, and that no errors are made in detecting the presence of a mutant. For a complete discussion of the biological assumptions underlying the method, see Kendal and Frost (1988).

In order to adapt Luria and Delbrück's result to our conditions, we must take three factors into account. First, we start with a single cell containing many mitochondrial genomes, rather than a single bacterial genome. Second, mitochondria can turn over by being engulfed by lysosomes. Results of Flory and Vinograd (1973) suggest that this turnover may be low in growing cell colonies. Third, PCR errors may result in the misclassification of some colonies as containing mutants when in fact they do not, and vice versa. We construct a mathematical model to compute the probability that a colony of a given size gives a negative signal for the deleted sequence.

The mitochondrial genomes in a human cell are distributed among mitochondria, with an average of between 4 and 10 genomes per mitochondrion. In our model, we assume that replications and deaths of mitochondrial genomes are stochastically independent from one genome to another. As a consequence of this assumption, we treat each mitochondrion as containing a single genome. In fact, deaths of genomes within a mitochondrion, considered as stochastic events, are highly dependent, since all die at the same time. In addition, an ideal stochastic model would probably include dependencies in the replication times of genomes within a mitochondrion and in both replication and deaths of genomes in distinct mitochondria within the same cell. The effect of ignoring such dependencies among genomes on the estimate of the mutation rate is assumed to be negligible.

Let ω_1 and ω_2 be the probabilities that a nonmutant and mutant, respectively, die before replication. Let λ be the probability that a copy of a nonmutant sequence is a mutant. We further assume that a mutant always gives rise to mutants upon replication. Let ρ be the probability that a single mutant sequence is amplified to a detectable level by PCR. We assume that DNA fragments amplify independently of each other.

We model mitochondrial replication as a two-type branching process, the two types being nonmutant and mutant. We begin by specifying a unit of time. This might be the length of time between replications of a cell, which we refer to as a *cell generation,* or the length of time between mitochondrial replications, which we refer to as a *mitochondrial generation.* For any given colony, the number of cell generations is equal to the number of replications of the initial cell, and the number of mitochondrial generations is equal to the number of replications of the mitochondria in the initial cell. In general, the mitochondrial generation time is somewhat shorter than the cell generation time, since mitochondria can die, while cells are assumed not to. A probability distribution for the number of copies of a given sequence after one unit of time is specified. We assume that, after one time unit, all sequences, other than the original sequence, are first generation offspring of the original one. For this assumption to be reasonable, the length of a time unit should not be much longer than the mitochondrial generation time.

Let $\phi_n^{(1)}(s_1, s_2)$ represent the probability generating function (pgf) for the joint distribution of the number of nonmutants and mutants in the clone of a single nonmutant sequence after n generations. Let $\phi_n^{(2)}(s_1, s_2)$ represent the corresponding pgf for the clone of a mutant. Let $f_N(s)$, $f_M(s)$ denote the pgf for the number of offspring of a nonmutant or mutant, respectively. As shown in the appendix, these pgf's determine the one generation pgf's $\phi_1^{(1)}(s_1, s_2)$ and $\phi_1^{(2)}(s_1, s_2)$, and the n generation pgf's follow from these by a standard recursion given in equation (A6) of the appendix.

The First Model

In our first model, we measure time in units of mitochondrial generations. We suppose that in each mitochondrial generation each mitochondrion either dies or survives and produces exactly one copy of itself (which may or may not be a mutant). The resulting branching process model has offspring pgf's, defined in (A1) and (A4) in the appendix, given by

$$f_N(s) = f_M(s) = s \,. \tag{1}$$

It follows from equations (A3) and (A5) in the appendix that the one-generation pgf's are

$$\phi_1^{(1)}(s_1, s_2) = \omega_1 + (1 - \omega_1)s_1((1 - \lambda)s_1 + \lambda s_2) \,.$$

$$\phi_1^{(2)}(s_1, s_2) = \omega_2 + (1 - \omega_2)s_2^2 \,.$$

To calculate the probability that the offspring of a nonmutant genome produce an undetectable amount of mutant PCR product, we must obtain a quantity to use as the number of mitochondrial generations, n. For the sake of simplicity, we assume this value is constant across mitochondria. We estimate this quantity as a function of k, the number of cells in a colony. During the time spanned by a single mitochondrial generation, the population of mitochondria increases on average by a factor of $2(1 - \omega_1)$, assuming the proportion of mutants is small. Therefore the number of mitochondrial generations n needed to produce a k-fold increase is approximately $n = \log k/\log(2[1 - \omega_1])$. In practice, we round $\log k/\log(2[1 - \omega_1])$ to the nearest integer.

The Second Model

In this model, we measure time in units of cell generations and describe the distribution of the number of mitochondrial replications that occur between cell divisions. As in the first model, nonmutant mitochondria die with probability ω_1, and mutants with probability ω_2. As before, mutants always have mutant offspring. The pgf of the number of offspring born to a surviving mutant is $f_M(s)$, with mean μ_2. Nonmutants mutate with probability λ, and the pgf of the number of offspring born to a surviving nonmutant is $f_N(s)$, with mean μ_1. The branching process that describes the numbers of nonmutants and mutants is another example of the model described in the appendix.

Each nonmutant mitochondrial genome present at the beginning of a particular cell generation produces on average a total of $(1 - \omega_1)(1 + \mu_1)$ copies (including itself, if it survives), whereas a mutant genome produces on average a total of $(1 - \omega_2)(1 + \mu_2)$. Since the number of mitochondria in a cell line should approximately double each cell generation, we assume that both these averages equal 2. That is,

$$\mu_i = \frac{2}{1 - \omega_i} - 1, \quad i = 1, 2 \,.$$

It remains to specify $f_N(s)$ and $f_M(s)$. For definiteness, we might assume that each distribution is geometric (having pgf $1/[1 + \mu_i - \mu_i s]$), or that each is Poisson (having pgf $\exp[-\mu_i(1 - s)]$).

Finally, we need a value of n to choose for the number of cell generations. In the absence of cell death, the number of cells doubles per division, so that a colony of size k results from $n = \log k/\log 2$ divisions. In practice, we round this to the nearest integer.

Statistical Methods

We now describe the calculation of the likelihood of the data under these models. We assume for now that ω_1, ω_2, and ρ are known. Let C be the number of colonies. For $i = 1, \ldots, C$, let k_i be the number of cells in the ith colony, and let $X_i = 1$ if the ith colony tests positive for the mutant sequence, and $X_i = 0$ if the ith colony tests negative. For the first model, we define $n_i = \log k_i/\log[2(1 - \omega_1)]$ rounded to the nearest integer, whereas for the second model, we define $n_i = \log k_i/\log 2$, rounded once more to the nearest integer.

Let m be the number of mitochondrial genomes in the cell that was the origin of the colony. We assume m is known, and for the sake of simplicity we assume m is the same for the initial cell in each colony. There are two sources of "contamination" we must consider. The first arises if a given PCR reaction is contaminated by an exogenous mutant sequence. The second arises if the original cell contains any mutant sequences. We denote the probability that either (or both) of these events occurs in a colony by γ. The probability P_0 that no mutants are detected in the ith colony is the probability p_0 that the descendants of the m initial cells do not produce a detectable amount of PCR product multiplied by the probability that the PCR is not contaminated. We show in the appendix that this probability satisfies the following inequality:

$$[\phi_{n_i}^{(1)}(1, 0)]^m(1 - \gamma) \leq P_0(k_i; \lambda, \gamma) \leq [\phi_{n_i}^{(1)}(1, 1 - \rho)]^m(1 - \gamma) \,.$$

We contend that because the PCR efficiency ρ is sufficiently close to 1, the two sides of this inequality have very similar values. In what follows, we use the lower bound for definiteness, and so set

$$P_0(k_i; \lambda, \gamma) = [\phi_{n_i}^{(1)}(1, 1 - \rho)]^m(1 - \gamma) \,. \tag{2}$$

The likelihood is the probability of observing the data $X_1, \ldots, X_C$, and it is defined below:

$$L(\lambda, \gamma) = \prod_{i=1}^{C} P_0(k_i; \lambda, \gamma)^{1-X_i}[1 - P_0(k_i; \lambda, \gamma)]^{X_i} . \quad (3)$$

The maximum likelihood estimators $\hat{\lambda}$ and $\hat{\gamma}$ of λ and γ are the values of λ and γ maximizing L (or log L). To get a better idea of the true value of λ, the maximum likelihood estimator (MLE) $\hat{\lambda}$ should be calculated across a range of reasonable values of ω_1, ω_2, and ρ. Alternatively, one could estimate ω_1, ω_2, and ρ along with λ and γ, if the data permit. We note that, while γ can be estimated from these data, the probabilities of the two different types of contamination cannot be estimated separately.

Estimates of the variances of the MLEs can be obtained by inverting the Fisher information matrix, whose entries are the expectations of the second partial derivatives of log $L(\lambda, \gamma)$. This is the matrix whose (r, s)th element is

$$\sum_{i=1}^{C} \frac{g_{ri}g_{si}}{P_0(k_i; \lambda, \gamma)[1 - P_0(k_i; \lambda, \gamma)]} ,$$

where

$$g_{1i} = \frac{\partial P_0(k_i; \lambda, \gamma)}{\partial \lambda} , \quad g_{2i} = \frac{\partial P_0(k_i; \lambda, \gamma)}{\partial \gamma} .$$

The partial derivatives of $P_0(k_i; \lambda, \gamma)$ are calculated with recursions similar to the one described in equation (A6) in the appendix.

A goodness-of-fit test can also be constructed. The saturated model is the one in which the probability that a colony is mutant-free is estimated by the proportion of colonies that are mutant-free among those which have been assigned the same number of generations, n. Substituting these probabilities into the right side of (3) yields the saturated likelihood L_{SAT}. Suppose that there are s different values of n observed in the data. If our model is correct, the log likelihood ratio

$$\Lambda = 2[\log L_{SAT} - \log L(\hat{\lambda}, \hat{\gamma})] , \quad (4)$$

where $L(\lambda, \gamma)$ is defined by (3), has approximately a χ^2 distribution with $s - 2$ df.

Fitting the Models

We analyzed the data from the 220 colonies by using the first model described above. We assumed that there was no mitochondrial death, that the PCR detection efficiency ρ was 73%, and that the number m of mitochondrial genomes per cell was 7,600. The MLE of the mutation rate was $\hat{\lambda} = 5.95 \times 10^{-8}$ mutations per mitochondrial genome replication, with a standard error, estimated from the Fisher information matrix, of 1.14×10^{-8}. A 95% confidence interval for the deletion rate is therefore $(3.72 \times 10^{-8}, 8.18 \times 10^{-8})$. The MLE for the contamination rate is $\hat{\gamma} = 7.1\%$.

To examine the impact of the death rate and efficiency assumptions, we refit the data several times, varying the death rates between 0 and 0.2 and the efficiency between 0.6 and 1. The MLE of λ varied between 3.42×10^{-8} and 7.74×10^{-8}, with Fisher standard errors between 7.15×10^{-9} and 1.48×10^{-8}. The MLE of γ ranged from 7.1% to 11.1%. A more conservative confidence interval for λ is $(3.42 \times 10^{-8} - 2.96 \times 10^{-8}, 7.74 \times 10^{-8} + 2.96 \times 10^{-8}) = (4.60 \times 10^{-9}, 1.07 \times 10^{-7})$. It is reasonable to believe that the confidence level for this interval is greater than 95%.

To assess the effect of our assumption about the number m of mitochondrial genomes per cell (taken here to be 7,600), we repeated the analysis using several values of m. We noted that the resulting estimate of the mutation rate $\hat{\lambda}$ varied inversely with m in a nearly linear fashion; i.e., doubling m results in $\hat{\lambda}$ being reduced approximately by half.

The 220 colonies were grown in a series of batches, over a period of ~2 mo. Inspection of the data showed clearly that the colonies in the first two batches, 65 in number, tended to be much smaller than the remaining 155. The average size of the first 65 colonies was 1,147.4 cells and the average size of the last 155 colonies was 1,756.7 cells. For this reason, we repeated our analysis on these two groups of colonies separately.

For the last 155 colonies, the value of $\hat{\lambda}$ was 6.38×10^{-8}, and the value of $\hat{\gamma}$ was 0, assuming no mitochondrial death and a PCR detection efficiency ρ of 73%. For the first 65 colonies, assuming no mitochondrial death and a PCR detection efficiency of 73%, we obtained MLEs $\hat{\lambda} = 5.98 \times 10^{-8}$ and $\hat{\gamma} = 13.0\%$.

Estimates of the mutation rate are similar, whether based on the first 65 colonies, the last 155, or all 220, despite substantial differences in colony sizes and in estimated PCR contamination rates between the two groups of colonies. This indicates that the behavior of the estimator is rather insensitive to variations in experimental conditions.

We tested the adequacy of the model by using the goodness-of-fit test described above. For example, in analyzing the entire 220 colonies with a value of $\omega_1 = \omega_2 = 0$, each colony has a generation number between 6 and 12. The statistic Λ defined in (4) has $7 - 2 = 5$ df. The observed value of Λ was 4.70, showing that the model fits well. The same conclusion holds when the data are analyzed separately using the first 65 and the second 155 colonies.

We fit the second model to the data, as well. For the

geometric offspring distribution with $\omega_1 = \omega_2 = 0$, $\gamma = 0.73$, the MLE of λ is $\hat{\lambda} = 6.20 \times 10^{-8}$ with a standard error of 1.18×10^{-8}. This rate is per cell generation; however, when $\omega_1 = 0$, cell generations coincide with mitochondrial generations. The estimated contamination rate was 7.1%. These values are remarkably similar to the fitted values for model 1. Furthermore, the estimate of λ for model 2 exhibits the same robustness when ω_1, ω_2, and γ are varied as they did with model 1. The goodness-of-fit tests detect no serious departure from the model. The same broad conclusions apply when the offspring distributions were assumed to be Poisson. The overall conclusion is that estimates of the mutation rate are rather insensitive to the specific details assumed for the mitochondrial reproduction mechanism. Further discussion of statistical aspects of this model is given in the appendix.

Discussion

We have developed a method for estimating mutation rates in cultured cell colonies, using PCR detection, and applied it to estimate the mutation rate of the mitochondrial deletion mtDNA^{4977}. Our most conservative interval estimate of this rate is $(4.60 \times 10^{-9}, 1.07 \times 10^{-7})$. We note that, even if the PCR efficiency ρ were as low as 20%, the estimated mutation rate would be 1.33×10^{-7}, with an estimated standard error of 2.54×10^{-8}. Thus, the estimate of the mutation rate is rather insensitive to the estimate of ρ.

Our method is, in principle, applicable in a wide variety of settings, including chromosomal, as well as mtDNA, mutations. The sensitivity of the PCR assay would make possible estimates of chromosomal mutation rates for specific nucleotide substitutions, inversions, translocations, and duplications, as well as deletions. In addition, situations in which there are several mutant types can be accommodated, and mutation rates for each type can be estimated. Back mutations can be incorporated as well. The estimator appears to be rather insensitive to the specific stochastic details assumed for the genome replication mechanism and appears to be stable over a range of experimental conditions such as growth rates of colonies and rates of mistyping due to PCR errors. The validity of the approach in any given situation can be determined with a standard likelihood ratio goodness-of-fit test.

The degree of accumulation of mtDNA^{4977}, whether at the high levels found in patients with severe symptoms or at the low levels found in normal adults, is determined by the mutation rate, by selective factors that may favor deleted molecules, and by the initial level of deletions present at conception. With an estimate of the mutation rate of the mtDNA^{4977} deletion available, the magnitude of selection factors in the accumulation of deletions can be estimated as well. We are currently developing models for this purpose. Thus, one should be able to assess whether a selective advantage for deleted mitochondrial genomes is needed to explain the levels of mtDNA^{4977} observed in older humans. The number of deleted mitochondrial genomes in the oocyte that are required to produce a child with KSS can also be estimated once the values for the selective advantage and for the deletion rate are derived.

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Appendix

Branching Process Models

In this appendix, we outline the results for multitype branching processes that we exploit in the text. A convenient reference for this material is Harris (1963, chap. 2).

We model the evolution of the population of mitochondria as a two-type Galton-Watson branching process, the two types being nonmutant (type 1) and mutant (type 2). First we calculate the probability $P^{(j)}(m_1, m_2)$ that a parent of type j produces m_i offspring of type i, $(i = 1, 2)$ in a single generation. This is most easily done by calculating the joint pgf's $\phi_1^{(j)}$ given by

$$\phi_1^{(j)}(s_1, s_2) = \sum_{m_1,m_2} P^{(j)}(m_1, m_2) s_1^{m_1} s_2^{m_2}, \quad j = 1, 2 .$$

We suppose that a nonmutant dies with probability ω_1. If the nonmutant survives, it produces k *additional* offspring with probability q_k, $k = 0, 1, \ldots$. We define the pgf $f_N(s)$ of the distribution $\{q_k\}$ by

$$f_N(s) = \sum_{k \geq 0} q_k s^k . \tag{A1}$$

A surviving individual cannot mutate, but each new offspring undergoes mutation independently with probability λ. An offspring of a nonmutant is a nonmutant with probability $1 - \lambda$, and a mutant with probability λ.

Given that a type 1 individual survives and produces $K = k$ offspring, the total number of types 1 and 2 produced is $(N_1 + 1, N_2)$, where (N_1, N_2) is a multinomially distributed random vector with parameters k, $1 - \lambda$, and λ. It follows that

$$E(s_1^{N_1+1}s_2^{N_2} \mid K = k, \text{ parent survives}) = s_1[(1-\lambda)s_1 + \lambda s_2]^k, \quad k = 0, 1, \ldots . \tag{A2}$$

It follows that the unconditional pgf of the numbers of type 1 and 2 produced by a parent of type 1 is

$$\phi_1^{(1)}(s_1, s_2) = \omega_1 + (1-\omega_1)s_1 \sum_{k\geq 0} q_k((1-\lambda)s_1 + \lambda s_2)^k = \omega_1 + (1-\omega_1)s_1 f_N((1-\lambda)s_1 + \lambda s_2) . \tag{A3}$$

Mutants die with probability ω_2, and a surviving mutant produces k additional offspring with probability r_k, $k = 0, 1, \ldots$ having pgf

$$f_M(s) = \sum_{k\geq 0} r_k s^k . \tag{A4}$$

We assume that mutants remain mutants and that a surviving parent cannot change its type. An argument analogous to the one used to derive (A3) shows that the pgf $\phi_1^{(2)}(s_1, s_2)$ of the number of individuals of types 1 and 2 produced by a parent of type 2 is

$$\phi_1^{(2)}(s_1, s_2) = \omega_2 + (1-\omega_2)s_2 f_M(s_2) . \tag{A5}$$

For $n = 1, 2, \ldots$, let $P_n^{(j)}(m_1, m_2)$ denote the probability that the nth generation offspring of a single individual of type j consist of exactly m_1 nonmutants and m_2 mutants, and denote the corresponding pgf's by

$$\phi_n^{(j)}(s_1, s_2) = \sum_{m_1=0}^{\infty}\sum_{m_2=0}^{\infty} P_n^{(j)}(m_1, m_2)s_1^{m_1}s_2^{m_2} .$$

The quantities $\phi_n^{(j)}$, $j = 1, 2$; $n = 1, 2, \ldots$ may be computed recursively, using the relations

$$\phi_n^{(1)}(s_1, s_2) = \phi_1^{(1)}[\phi_{n-1}^{(1)}(s_1, s_2), \phi_{n-1}^{(2)}(s_1, s_2)] \tag{A6}$$

and

$$\phi_n^{(2)}(s_1, s_2) = \phi_1^{(2)}[\phi_{n-1}^{(1)}(s_1, s_2), \phi_{n-1}^{(2)}(s_1, s_2)] ,$$

where $\phi_0^{(j)}(s_1, s_2) = s_j$, $j = 1, 2$. Proofs of these relations may be found in Harris (1963, p. 36).

In our application, we start with a cell containing m nonmutant mitochondria, and we must calculate the probability that the nth generation offspring of these individuals contain no detected mutants. We let Z_i be the amount of PCR product produced by the ith deleted molecule and T be the detection threshold of the PCR assay. We assume that different mutants amplify independently. The probability d_k that k deleted molecules do not produce a detectable amount of product is

$$d_k = \Pr(Z_1 + \ldots + Z_k \leq T) ,$$

with $d_0 = 1$. Since $Z_1 + \ldots + Z_k \leq T$ implies $Z_1 \leq T, \ldots, Z_k \leq T$, we see that

$$0 \leq d_k \leq (1-\rho)^k , \tag{A7}$$

where $\rho = \Pr(Z_1 > T)$ is the probability that any mutant amplifies to a detectable level. The probability that the nth generation offspring of the initial m nonmutant individuals test negative for the presence of the mutant is therefore

$$p_0 = \sum_{m_1=0}^{\infty}\sum_{m_2=0}^{\infty} P_{n,m}^{(1)}(m_1, m_2)d_{m_2} ,$$

where $P_{n,m}^{(1)}(m_1, m_2)$ is the probability that the m initial nonmutant molecules produce m_1 nonmutants and m_2 mutants after n generations.

From the inequalities in (A7), we see that

$$\phi_{n,m}^{(1)}(1, 0) \leq p_0 \leq \phi_{n,m}^{(1)}(1, 1-\rho) ,$$

where $\phi_{n,m}^{(1)}(s, t)$ is the pgf of the number of nonmutant and mutant molecules produced by the m initial nonmutant molecules. Since the offspring of different initial nonmutant molecules evolve independently, we have $\phi_{n,m}^{(1)}(s, t) = [\phi_n^{(1)}(s, t)]^m$, so that finally

$$[\phi_n^{(1)}(1, 0)]^m \leq p_0 \leq [\phi_n^{(1)}(1, 1-\rho)]^m . \tag{A8}$$

Either of these bounds can readily be computed recursively by setting $(s_1, s_2) = (1, 0)$ or $(1, 1-\rho)$ in equation (A6).

Statistical Aspects of the Analysis

We note first that it is not feasible to estimate the PCR efficiency, the death rate of mutants, and the mutation rate from the same experimental data set. Furthermore, the parameter ω_1 must be assumed known to calculate the number of generations. We conclude that models containing parameters in addition to λ and γ are intractable. Thus, it appears that in practice only two-parameter models can be fit to experimental data of the type that we have used here.

To address the nonidentifiability in more detail, define the function $L_{MAX}(\omega_1, \omega_2, \rho) = \sup_{\lambda,\gamma} L(\omega_1, \omega_2, \rho, \lambda, \gamma)$, where $L(\omega_1, \omega_2, \rho, \lambda, \gamma)$ is the likelihood function considered as a function of the five variables ω_1, ω_2, ρ, λ, and γ. We calculated values of the function $L_{MAX}(\omega_1, \omega_2, \rho)$ at several points and found that it is nearly constant on a two dimensional space in which ω_2 and ρ vary freely.

The assignment to each colony of an integral number

of generations could in principle limit the scope of the analysis. Essentially, we are rounding each colony size to the nearest power of some constant, which reduces the number of distinct colony sizes. For example, in the analysis of the 155 colonies described above, when a nonmutant death rate of 0 is assumed, each colony is assigned a number of generations between 9 and 12. It follows that, for each value of the parameters, each colony is assigned one of four probabilities of containing a mutant, and our likelihood function is a product of four binomial likelihoods. This would make it impossible to estimate more than three parameters without losing the ability to test the goodness-of-fit of the model. However, since the identifiability considerations described above appear to limit the model to two unknown parameters, the restriction to an integral number of generations does not appear to be important in this respect.

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