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On the relevance of mitochondrial fusions for the accumulation of mitochondrial deletion mutants: A modelling study

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Summary

The molecular mechanisms underlying the aging process are still unclear, but the clonal accumulation of mitochondrial deletion mutants is one of the prime candidates. An important question for the mitochondrial theory of aging is to discover how defective organelles might be selected at the expense of wild-type mitochondria. We propose that mitochondrial fission and fusion events are of critical importance for resolving this apparent contradiction. We show that the occurrence of fusions removes the problems associated with the idea that smaller DNA molecules accumulate because they replicate in a shorter time – the *survival of the tiny* (SOT) hypothesis. Furthermore, stochastic simulations of mitochondrial replication, mutation and degradation show that two important experimental findings, namely the overall low mosaic pattern of oxidative phosphorylation (OXPHOS) impaired cells in old organisms and the distribution of deletion sizes, can be reproduced and explained by this hypothesis. Finally, we make predictions that can be tested experimentally to further verify our explanation for the age-related accumulation of mitochondrial deletion mutants.

Key words: aging; computer simulations; free radicals; mitochondrial fusion; mitochondrial mutants.

Introduction

The mitochondrial theory of aging sees the accumulation of defective mitochondria as the central mechanism responsible for the aging process. These cellular organelles originated early in evolution from purple bacteria (Schwartz & Dayhoff, 1978) and serve as endosymbionts in most modern eukaryotes. As a remnant from this time they still contain their own genetic material in the form of circular DNA (mtDNA). During evolution most genes needed by the mitochondrium have been transferred into the nucleus, leaving a small genome (c. 16.5 kbp in mammals) that only codes for 13 proteins in humans (Anderson *et al*., 1981). Mitochondria are involved in important processes like calcium storage, apoptosis and energy production via oxidative phosphorylation (OXPHOS). Their role as the main energy source for the cell together with certain properties of the mtDNA is crucial for their possible involvement in the aging process. Harman (1972) was the first to propose that reactive oxygen species, which arise as a bye-product of electron transport during OXPHOS might damage the mitochondrial DNA and thus impair the energy supply of the cell. Since all the genes necessary for growth and replication of the mitochondria are located within the nucleus, defective organelles could still propagate. Damage to the mtDNA would effectively turn the symbiont into a parasite. This idea has found many supporters (Miquel *et al*., 1980; Richter, 1988; Linnane *et al*., 1989) and leads to the view that defective mitochondria accumulate with age in cells and tissues, contributing to the gradual functional decline that characterizes aging.

Experimental support for this idea came from studies that found large mtDNA deletions in aging individuals (Linnane *et al*., 1990; Hattori *et al*., 1991; Yen *et al*., 1991). Deletion mutations accumulate preferentially in postmitotic tissues like heart, brain and skeletal muscle with high oxygen demand (Cortopassi *et al*., 1992; Lee *et al*., 1994; Brierley *et al*., 1998; Wanagat *et al*., 2001). Early studies were performed on tissue homogenates and although they found an age related increase of mutations, they concluded that the fraction of defective mtDNAs is well below 1% (Cortopassi *et al*., 1992; Randerath *et al*., 1996). However, it turned out that the underlying assumption that mitochondrial damage is distributed homogeneously within a tissue is wrong. The combination of PCR amplification of extended sequences together with single cell studies revealed that muscle tissue displays a mosaic pattern of mitochondrial damage. While even in old individuals most cells harbour little or no damaged mitochondria, there are a few cells that contain a large proportion of mitochondrial mutants (Khrapko *et al*., 1999; Cao *et al*., 2001; Gokey *et al*., 2004). These studies demonstrated that in affected cells the mitochondrial population was apparently taken over by a single mutant, which was different for different cells. This suggests that the cellular accumulation of defective mitochondria proceeds via clonal expansion of a single originating mutant. Finally, single cell studies showed that the age-related decline of mitochondrial

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respiratory function that had been observed in several species (Cortopassi & Arnheim, 1990; Müller-Höcker, 1990; Müller-Höcker *et al*., 1997) colocalizes with the occurrence of mitochondrial deletions. Two enzymatic abnormalities that occur in cells with defective organelles is a lack of cytochrome c oxidase activity (COX^-) and succinate dehydrogenase hyperactivity (SDH^{++}), a phenotype known as red ragged fibres.

But how might a single mutant mitochondrium out-compete the population of wild type (wt) mitochondria? Initially, it was suggested that the reduced genome size of the deletion mutant may provide a selection advantage (Hayashi *et al*., 1991; Wallace, 1992; Takai *et al*., 1999). A mutant that has lost 8kbp is only half the size of the wild type and might therefore replicate in half the time. This idea has fallen into disfavour because even though the mutant might have a replication advantage it should also suffer from a severe lack of ATP and a reduced proton gradient, both of which are important for fast mitochondrial growth. But most important for the dismissal of the faster replication idea is the fact that the time required for the replication of the mtDNA is only 1–2 h (Davis & Clayton, 1996). In contrast, the division time of mitochondria is of the order of 1–3 weeks (Huemer *et al*., 1971; Menzies & Gold, 1971; Korr *et al*., 1998). Consequently, it has been argued that it is very difficult to imagine how mtDNA replication could be the rate-limiting step in mitochondrial multiplication (de Grey, 1999; Elson *et al*., 2001).

A further possibility that has been suggested is that deletion mutants have no selection advantage at all, but accumulate by selection-neutral random drift (Chinnery & Samuels, 1999; Elson *et al*., 2001). However, this scenario describes only a single specific case from a continuum of possibilities (negative to positive selection advantage). If the selection difference between mutant and wild-type deviates only slightly from zero the assumptions and predictions of this model no longer apply. And, considering the multiple consequences of a DNA deletion (smaller size of mtDNA, production of no or faulty proteins), it is difficult to see how this could result in a truly selection-neutral mutation. Other explanations have therefore tried to identify a selection advantage for mitochondrial mutants (since a disadvantage would obviously lead to its elimination). The *survival of the slowest* (SOS) hypothesis proposed by de Grey (1997) realizes that the fate of a mitochondrial mutant not only depends on its growth rate, but also on its rate of degradation. A mutant can gain a selection advantage either through an increased replication rate or through a decreased rate of degradation. The idea of the SOS hypothesis is that mitochondria are selected for degradation by lysosomes depending on their amount of membrane damage. The more damage, the faster the degradation rate would be. de Grey argues that mutant mitochondria have an impaired respiration chain and generate less of the reactive perhydroxyl radical, which is responsible for most of the membrane damage. Computer simulations show that such a mechanism can indeed provide the required selection advantage (Kowald & Kirkwood, 1999; Kowald & Kirkwood, 2000). Mitochondrial mutants with impaired respiration also suffer from a chronic ATP shortage and the SOS hypothesis is an elegant idea to explain how mutants can have a selection advantage under those conditions.

However, the above arguments depend heavily on the idea that mitochondria are individual entities, competing with each other inside the cell. The existence of mitochondrial fusions is therefore of crucial importance for this assumption. Mitochondria form dynamic networks, ranging from long tubular forms to single spherical organelles. Microscopic techniques indicate that these changes in number and morphology are due to frequent mitochondrial fission and fusion events (Bereiter-Hahn & Vöth, 1994). The molecular mechanism has been studied during the last years and several essential proteins have been identified (Yoon & McNiven, 2001; Bossy-Wetzel *et al*., 2003). Mitochondrial fusion seems to occur in yeast, plants and HeLa cells (Nunnari *et al*., 1997; Takai *et al*., 1997; Takai *et al*., 1999; Arimura *et al*., 2004; Karbowski *et al*., 2004) while our own work demonstrates mitochondrial fusions in human endothelial cells (Jendrach *et al*., 2005). It is clear that mitochondria of many tissue types can and do undergo fusion under certain conditions. Whether mitochondrial fusions occur in skeletal muscle cells under physiological conditions (which is particularly relevant for theories of aging) is currently unclear. However, given the large number of cell types where mitochondrial fusion has been observed, the aim of this article is to use computer simulation to study the possible consequences of frequent mitochondrial fusions for the accumulation of defective mtDNAs.

If fusion does occur, all the mitochondria of a cell will effectively form one large compartment with constant mixing of mtDNA as well as matrix and membrane components. Under these conditions, mtDNA deletions no longer lead to energy and proton gradient deficiencies within the cell since there is a common pool. Most importantly, however, the competing entities are no longer mitochondria, but individual mtDNA molecules. For the outcome of this competition only the mtDNA replication times are important, while the apparent mitochondrial growth rate (which is much longer) is irrelevant. In the following we therefore describe a model for the accumulation of defective mitochondria based on the idea that deletion mutants replicate faster than wild-type (*survival of the tiny*).

Results

The description and implementation of the model are described under Experimental Procedures (and see Figs 1 and 2)

High mutation rate

Figure 3 displays the results for simulations using a mutation rate of $6 * 10^{-7}$ day^{-1} kbp^{-1}. Part A of the figure is effectively a contour plot showing the accumulation of mtDNA mutants over time. The solid line, for instance, indicates the fraction of cells that contain at least one defective mitochondrial genome (> 0% mutants) at a given time. Under the mutation rate used in these simulations, all cells contained one or more mutants after 400 days. Since each simulation run computes the fate of the

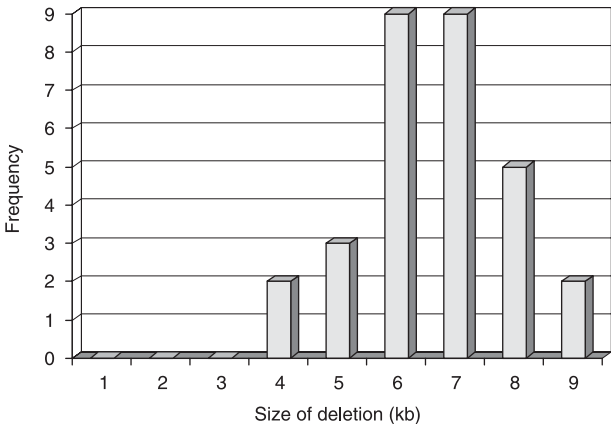

Fig. 1 Distribution of mitochondrial deletion sizes calculated from Cao *et al*. (2001). The authors determined the exact size of 30 mitochondrial deletion mutants in 38 months old rats. Mitochondrial deletion mutants in respiration deficient muscle fibres were amplified by PCR and sequenced, revealing deletion sizes from 4.4 kbp to 9.7 kbp. The histogram shows a conspicuous absence of small deletions below 4 kbp. Very large deletions are also rare.

mtDNA population of a single cell over time, the 'fraction of cells' is identical to the fraction of simulation runs performed. The other contour lines represent higher levels of defective mtDNAs. The point marked by the arrow shows that after approx. 200 days, 60% of the cells had a mtDNA population consisting of at least 20% defective genomes.

The other parameters used for this simulation are summarized in Table 1 and are as follows. No boost factor has been used (bF = 1), i.e. defective mtDNA did not increase oxidative stress. Parameters 'k' and 'c', necessary for the calculation of the replication rate, were set to 5000 and 1000, respectively. Together with the 10 day half-life of mitochondrial DNA, this resulted in a mtDNA population size of approx. 5500 (data not shown). This is in good agreement with studies of skeletal and heart muscle that find values between 3000 and 7000 mtDNA molecules per cell (Miller *et al*., 2003).

The diagram shown in Fig. 3(A) summarizes the total number of defective genomes that accumulate with time, but does not differentiate among different deletion sizes. For this purpose the histogram of deletion sizes at the end of the simulation (day 1127) is shown in Fig. 3(B). The histogram has been calculated from the mitochondrial deletion mutants that were present at the end of the 1000 simulation runs. The distribution is skewed with a maximum at around 8 kbp. Interestingly, deletions below 4 kbp are very rare, as observed experimentally (Fig. 1).

Why are there no small deletions, although one of the assumptions underlying the mutation process is that small deletions occur more often than large ones? A histogram of deletion sizes can be calculated for every time point of the simulation, and the results are very instructive. Figure 4 shows the deletion size distribution for four different, equally spaced, time points. As can be seen, early on (see day 281) many small deletions

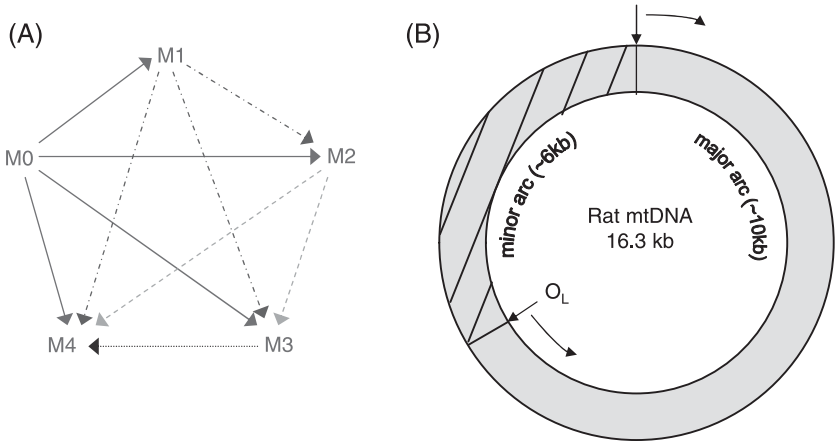

Fig. 2 (A) Overview of the mutations allowed by the model. Different mutants are labelled by numbers and the larger the number, the larger the deletion that this mutant carries. Arrows indicate possible mutations, showing that small ($M0 \rightarrow M_1$) or large ($M0 \rightarrow M_4$) pieces of DNA can be lost in a single mutation event. Mutants can suffer further mutations leading to even smaller mutants ($M1 \rightarrow M_4$ but not $M1 \rightarrow M_0$). (B) Schematic drawing of the rat mtDNA showing O_H, the origin of the heavy strand synthesis and O_L the origin of the light strand synthesis. The locations of the origins define the minor and major arc with the replication of the strands proceeding in the direction indicated by the arrows.

Table 1 Parameters and standard values used for the simulations

Name	Value	Description
baseRate	$6 * 10^{-7}$ day^{-1} kbp^{-1}	Mutation rate per day and kbp. Order of magnitude is in accordance with (Shenkar *et al*., 1996).
bF	1	Factor by which the mutation rate is increased if 100% of the mitochondrial population are deletion mutants.
K	5000	If the number of mitochondria is equal to 'k', the synthesis rate of new organelles is half maximal. This is related to the number of mtDNAs per cell which ranges from 700 in sperm (Diez-Sanchez *et al*., 2002), 7000 in heart muscle (Miller *et al*., 2003) to 25 000 in liver (Berdannier & Everts, 2001).
C	1000	Maximum number of mitochondria that can be synthesized per day.
degRate	ln(2)/10 day^{-1}	Degradation rate for all mtDNAs. Corresponding to a half-life of 10 days (Huemer *et al*., 1971; Menzies & Gold, 1971; Korr *et al*., 1998).

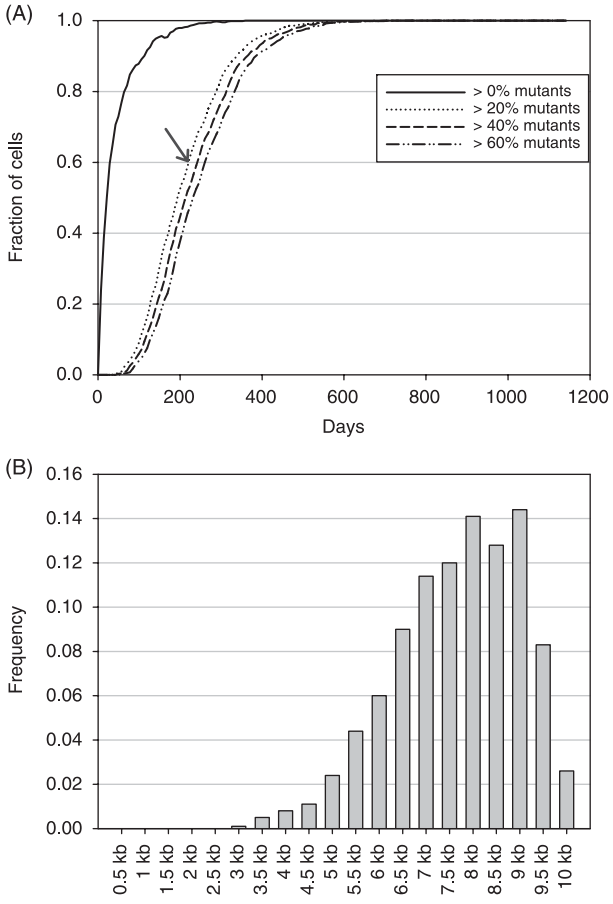

Fig. 3 Simulation results for a high mitochondrial mutation rate ($6 * 10^{-7}$ day^{-1} kbp^{-1}). (A) Summary of the accumulation pattern of defective mitochondria obtained by following the fate of 1000 cells. The curves show the fraction of cells that contain more than 0, 20, 40 or 60% defective mitochondria, i.e. after 200 days roughly 60% of the cells had more then 20% of defective organelles (marked by arrow). (B) Distribution of deletion sizes after 1100 days. The diagram is based on the results of 1000 simulation runs. For further simulation details see text.

down to 0.5 kbp are present. But as time progresses, the shape of the distribution changes: small deletions disappear and larger deletions emerge. It appears that the assumption included in the simulation of the mutation process, that mutants can mutate again, explains the disappearance of mtDNAs with small deletions.

Low mutation rate

The above simulations using a mutation rate of $6 * 10^{-7}$ day^{-1} kbp^{-1} gave a deletion size distribution that closely resembles experimental observations. However, it was much too high since it resulted in all cells having at least 60% defective mitochondrial genomes around day 600 (Fig. 3A). This is incompatible with experimental findings that, even in old individuals, tissues show a mosaic pattern of cells with and without defective mtDNA.

In a next set of simulations the mutation rate was therefore reduced 20 fold to $3 * 10^{-8}$ day^{-1} kbp^{-1}, while all other parameters were kept constant. Now defective genomes accumulate much slower and after 1100 days only approx. 50% of all cells harbour defective genomes while the remainder contain only wild type mtDNA (Fig. 5A). This is in agreement with the observed mosaic pattern.

However, now the distribution of deletion sizes has changed considerably and contains a large fraction of small and very small deletions (Fig. 5B), no longer corresponding to the experimental distribution. What is the reason for this discrepancy? In the earlier simulations, mutants with small deletions disappeared because they suffered further deletion mutations. These second generation mutants had a replication advantage because of their smaller size and could thus replace the first generation mutants. But by reducing the mutation rate 20 fold, the production of second generation mutants is not frequent enough to drive this process. The result is the persistence of many mutants with small deletions until the end of the simulation.

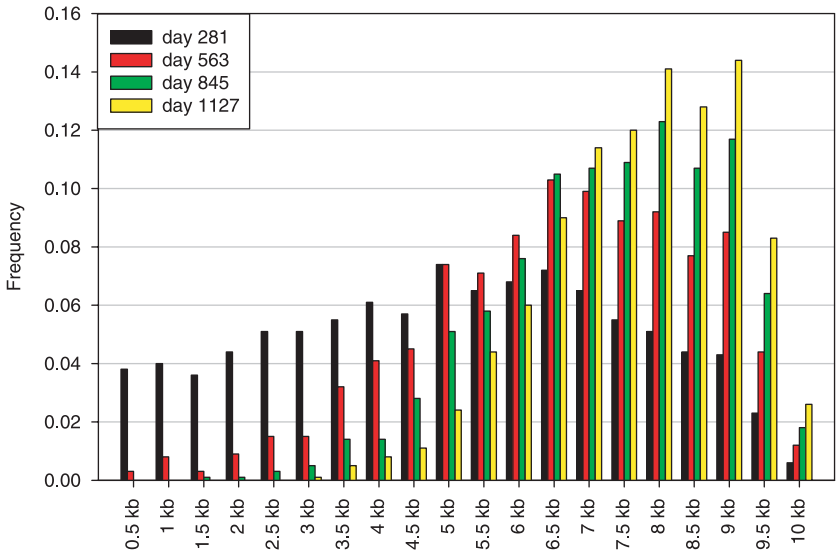

Fig. 4 Distribution of mitochondrial deletion sizes at various time points during the life of a rat. The simulation details are identical to Fig. 3, but this time the deletion size distribution is shown for four equally spaced time points. Early in life (day 281) the distribution contains a large fraction of small deletions down to 0.5 kbp. As time progresses, these small deletions disappear until finally (day 1127) the distribution shown in Fig. 3B has been reached.

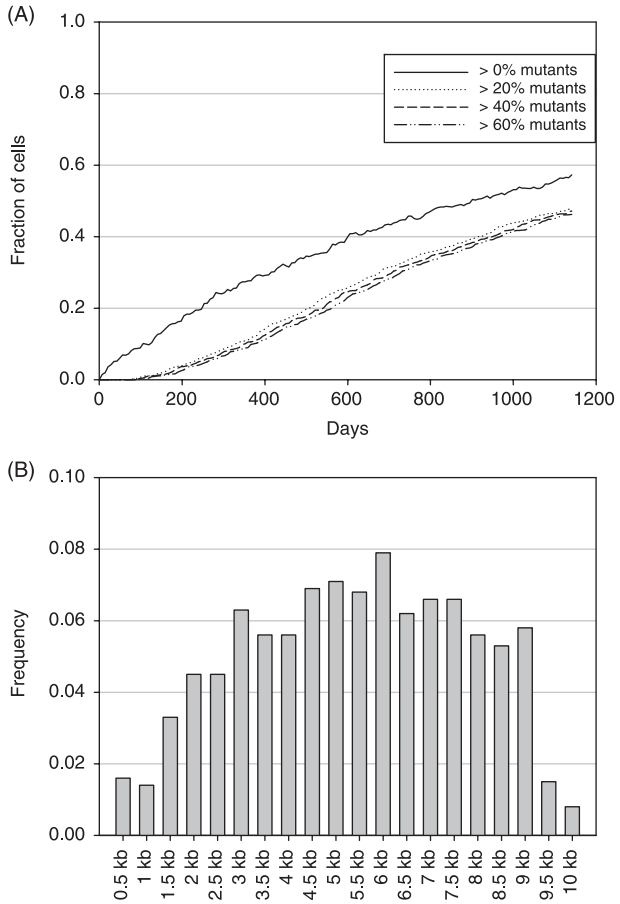

Fig. 5 Simulation results for a low mitochondrial mutation rate ($3 * 10^{-8}$ day^{-1} kbp^{-1}). (A) Summary of the accumulation pattern of defective mitochondria obtained by following the fate of 1000 cells. The mutation rate is 20 times lower than in Fig. 3 and as a consequence the fraction of cells harbouring defective organelles is, even in old animals, quite low.
(B) Distribution of deletion sizes after 1100 days. Although only the mutation rate has changed the distribution is very different from the one shown in Fig. 3B. This time many small deletions are present after 1100 days.

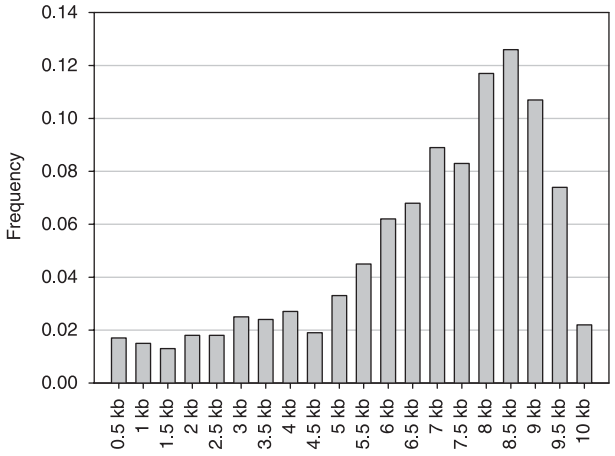

Fig. 6 Distribution of deletion sizes after 1100 days for a low mitochondrial mutation rate ($3 * 10^{-8}$ day^{-1} kbp^{-1}) and with boost factor = 100. In this simulation it is assumed that the presence of defective mitochondria leads to increased oxidative stress and an increased mutation rate. This mechanism leads to the disappearance of the small deletions seen in Fig. 5B.

Table 2 Distribution of the number of different mutants per cell for 5 different time points calculated from 1000 different simulation runs. Initially the only existing genotype is the wild-type, but with time mutant mtDNA molecules are appearing

Time (days)	1 genotype	2 genotypes	3 genotypes	4 genotypes
0	1	0	0	0
281	0.76	0.169	0.051	0.015
563	0.65	0.186	0.074	0.038
845	0.595	0.182	0.091	0.051
1127	0.565	0.224	0.082	0.054

Increased oxidative stress

The problem that emerged in the previous simulations is that either the deletion size distribution (high mutation rate), or the overall frequency of defective mtDNA (low mutation rate) is compatible with experimental data, but not both at the same time. What would be needed is a process that increases the mutation rate in cells harbouring mitochondrial mutants (to displace first generation mutants), but keeps the mutation rate low in cells that contain only wild-type genomes. The last assumption that went into the modelling of the mutation process can achieve exactly this outcome. There it was proposed that the presence of defective mtDNA leads to increased oxidative stress which in turn leads to an elevated mutation rate.

However, in the previous simulations the corresponding boost factor parameter, 'bF', was set to one, which disabled this process. Figure 6 shows the effects on the deletion size histogram of combining a low mutation rate of $3 * 10^{-8}$ day^{-1} kbp^{-1} with a boost factor of 100. Compared to the same simulation without boost factor (Fig. 5B), the distribution has changed as expected. A large fraction of small deletions has disappeared and the distribution now resembles again that of the high mutation scenario. The fraction of cells containing mitochondrial mutants is not affected by the parameter 'bF' and is thus identical to that seen in Fig. 5(A).

An important feature of the accumulation of defective mtDNAs is the clonal character found experimentally. In most affected cells only a single mutant genotypes exists apart from the wild-type. Table 2 shows the fraction of different genotypes within a cell for five different time points during the simulation displayed in Fig. 6. At the start of the simulation only one genotype is present, the wild-type. But as the simulation progresses, cells with more than one genotype appear. At the end of the simulation approx. 22% of the cells contain two genotypes (mostly wild-type plus one mutant) and approx. 8% contain three. In the majority of the cases with multiple genotypes, one genotypes exists in large excess over the others (data not shown).

Discussion

Aging is a complex biological process that affects all the cells, tissues and organ systems of an organism. At the molecular level,

diverse mechanisms seem to operate, ranging from telomere shortening, protein aggregation and oxidation to nonenzymatic glycosylation, cross-linking and the accumulation of defective mitochondria. Evolutionary theory strongly suggests that aging is caused by multiple sources of damage accumulation, operating in parallel (Kirkwood & Franceschi, 1992). Under such conditions it is important to understand the network of reactions that contribute to the aging phenotype. However, the initiation of such a systems biological approach has to include an understanding of the individual network components.

Damage to mitochondria with all its consequences is an important part of the overall picture, but the biochemical mechanisms that allow a mutant mitochondrion to supersede wild-type organelles and take over the cellular population of mitochondria are currently still unclear. It turns out to be of crucial importance for our proposition that mitochondria undergo frequent fusions and fissions *in vivo*. The SOS hypothesis (de Grey, 1997) can only work if the level of fusions is very low or nonexistent, while the idea that smaller mtDNA molecules (deletion mutants) have a replication advantage is only realistic when fusions occur. Previously we studied a situation where mitochondria do not undergo fusions (Kowald & Kirkwood, 1999; Kowald & Kirkwood, 2000) as representing one extreme in the possible range of fusion events. The jury is still out as to whether fusions occur under physiological conditions in post-mitotic cell types like neurons and skeletal muscle, but mounting evidence justifies a closer inspection of the selection consequences for small mtDNA deletion molecules. We therefore decided in this study to look at the other end of possible fusion rates, namely the case where mitochondria fuse and separate so frequently that they effectively form a single mitochondrial compartment.

We modelled the mitochondrial mutation, replication and degradation process stochastically and asked under which conditions it is possible to reproduce important experimental findings, such as the time course of the fraction of cells containing mitochondrial mutants and the size distribution of mtDNA deletions. The stochastic approach is important to account for the probabilistic nature of mutations, which is likely to be responsible for the mosaic pattern of healthy and impaired cells seen in cross sections of tissues from old organisms. In some of the simulation runs, no mitochondrial mutants were present after 38 months, while in others a deletion event took place and the wild-type mtDNA was replaced by the mutant. A model composed of differential equations could not have captured such a pattern, since this approach corresponds to the experimental method of using tissue samples containing millions of cells. The result is in both cases an averaging effect, obscuring the true pattern of accumulation of mtDNA deletion mutants.

The results of the simulations show that it is possible to reproduce the experimental findings by considering only the three processes of mutation, replication and degradation. The model shows that the experimentally observed paucity of small deletions can be explained by the competition between small deletion mutants and mutants with larger deletions that occur less frequently but have a stronger selection advantage. However, to fulfil at the same time both constraints – correct deletion size distribution and low total fraction of cells with defective mtDNA – it is necessary to assume that the presence of deletion mutants increases oxidative stress and concomitantly the mutation rate (the so-called boost factor).

In the simulations a boost factor of 100 was used to adjust the shape of the deletion size histogram (Fig. 6) in the presence of a low overall mutation rate (Fig. 5A). Is such a high boost factor realistic? To our knowledge no experimental data exist regarding mitochondrial mutation rates under different levels of mtDNA mutants. However, a difference of two orders of magnitude for cells with zero vs. 100% mutated mtDNA doesn't seem unreasonable. Furthermore, a factor of 100 might be an overestimation, because the current model does not include apoptosis. If cells harbouring a high content of mtDNA mutants undergo frequent apoptosis, the frequency of cells with impaired mitochondria that is actually observed in cross sections of tissues will be much lower than the total number of affected cells that have appeared since birth. Thus apoptosis leads to an underestimation of the real mitochondrial mutation rate. But if the mutation rate is actually higher than assumed in Fig. 5, the boost factor needed to remove small deletions can be substantially lower than the value used in the current simulations.

At the end of the simulation shown in Fig. 6 78% of the cells contained either one or two genotypes while 8% harboured three different genotypes (Table 2). Most of the two genotype simulations represent a combination of wild-type and mutant, but in case where three genotypes coexist in a cell at least two different mutant mtDNAs are seen, a situation rarely observed experimentally. Closer inspection revealed that in those cases the frequency of one genotype often dominated over the others. Most likely this reflects a situation where a fresh mutation occurred very recently and is either about to overtake the dominant genotype or will itself disappear again. The fraction of cells with multiple genotypes observed in the simulations depends directly on the time that a new mutant needs to replace the existing genotype, which in turn depends on the boost factor used. By using a higher factor the frequency of small deletions would be even lower than in Fig. 6, while at the same time there would be less cells with multiple genotypes. This might indicate that a boost factor of even 100 may be too low.

In addition to the study on mitochondrial DNA deletions in rats (Cao *et al*., 2001), there has recently been a similar study in rhesus monkeys (Gokey *et al*., 2004). Among the similar observations, such as clonal expansion of a single mutant, a mosaic pattern of affected cells and high incidence of large deletions, there are also important differences. Two-thirds of the deletions had direct repeat sequences at their breakpoints and in one-third of the cases the light strand origin was deleted. It is not clear how the deletion mutants without a light strand origin could accumulate, but possibly they can make use of alternative origins or mtDNA replication might under certain circumstances proceed in a bidirectional mode (Bowmaker *et al*., 2003). However, the differences of the deletion process between rodents and primates means that different models

may be required for the two species. Since the mechanisms operating in primates are more complex (alternative origins, influence of repeats) and less well understood, our model aims to reproduce the experimental observations found in rats. But nevertheless we think that the results are relevant for primates, since in both species the major problem is to explain the problematic lack of small deletions.

Another interesting observation is that no deletions have been observed within the small arc of the rat mtDNA (Cao *et al*., 2001). If the break points are both within the small arc, O_H and O_L remain intact and therefore such mutants should accumulate. However, among the 30 deletion mutants investigated, none had a deletion within this area. A possible explanation coming from the present modelling study is the realization that because of the unidirectional mode of replication the sequences within the major arc count twice for the calculation of the replication time. A 6 kbp deletion within the minor arc (approximately the complete arc) is equivalent to a three kbp deletion within the major arc. Thus, although we did not explicitly model minor arc deletions within this study, we can predict that those deletions should be too rare to be observed among the 30 cases studied by Cao *et al*. (2001).

The simulation results and the idea that small mtDNA molecules have a replication advantage is also in agreement with experimental studies which demonstrate that mtDNA with large deletions repopulates human cells faster than wild-type mtDNA (Moraes *et al*., 1999; Diaz *et al*., 2002). Surprisingly, a similar replication advantage has been observed for mtDNA point mutations that cause the MELAS encephalomyopathy in humans (Yoneda *et al*., 1992). The replication advantage of these mutants cannot be explained by a reduced length of the mtDNA. Maybe, the changed sequence is able to attract molecular factors necessary to replicate more efficiently than the wild-type. This could also explain why Taylor *et al*. (2003) found an accumulation of point mutations in human gut crypt cells.

Interestingly, the model has very few free parameters (see Table 1). The degradation rate and the parameters 'k' and 'c' which control the steady state level of organelles are constrained by experiment or have no qualitative impact on the simulation results. This effectively only leaves the mutation rate and the boost factor to account for the good agreement of simulation and experimental results.

Finally, the simulations also allow us to make two predictions that can be tested experimentally. According to the model, the mechanism which explains the lack of small deletions is the sequential replacement of mtDNA mutants with small deletions by mutants with large deletions. This process takes place over the lifespan of the rat (Fig. 4). Consequently, one prediction is that the deletion size distribution found in young animals should have a much larger fraction of small deletions. If it is possible to collect enough cells with defective mitochondria in young rats, this could be verified. The second prediction follows a similar line as regards deletions in the small arc. In old animals these deletions are not present because, in relation to the selection advantage they confer, they are equivalent to deletions in the large arc that are smaller than 3 kbp. In young animals, however, small arc deletions should be observable, occuring at the same frequency as large arc deletions of half the size. These predictions are, however, only valid if the removal of impaired cells by apoptosis is negligible.

In summary, we show that the existence or nonexistence of mitochondrial fusions is of crucial importance for explanations of how mutant mtDNAs can accumulate in cells. If there is fusion, *survival of the tiny* (SOT) is a feasible option to explain the accumulation of mitochondrial deletion mutants and gives simulation results that are in good agreement with experimental data.

Experimental procedures

Model description

The aim of the modelling exercise was to see if two major experimental findings can be reproduced by a model that assumes a replication advantage based on the reduced size of mtDNA deletion mutants. Firstly, it should reproduce the observation that even in aged organisms only a minority of cells harbours defective mitochondrial genomes, but, for that minority, the defective genome predominates. This mosaic pattern of damage distribution within tissues has been described by several authors (Khrapko *et al*., 1999; Cao *et al*., 2001; Gokey *et al*., 2004). Secondly, and most importantly, the model should be able to reproduce and explain the distribution of deletion sizes found by Cao *et al*. (2001) in old rats (Fig. 1). Using laser-capture microdissection the authors isolated 30 respiration deficient muscle fibres from 38 month old rats and obtained the exact size and position of the accompanying mtDNA deletions via PCR. All deletions were located within the major arc (see Fig. 2B) and Fig. 1 shows that the size distribution that completely lacks deletions that are smaller than 4 kbp and has only few deletions greater than 9 kbp.

We wanted to include only the most essential reactions in the model and therefore incorporated mutation, replication and degradation of mtDNA molecules.

Mutation

In this simulation study only deletion mutations affecting the large arc (Fig. 2B) of the rat genome are considered. It is assumed that deletions which include the heavy or light strand origin of replication (O_H and O_L) are no longer capable of replication and thus cannot accumulate. Deletions affecting exclusively the minor arc are also excluded, since they have not been observed (Cao *et al*., 2001) (see Discussion).

To further simplify and speed up the simulation, deletions are modelled with a granularity of 100 bp, i.e. deletions can be of 100, 200 or 300 bp, but not 250 bp. Consequently, the model contains 100 possible classes of mitochondrial genotypes, wild-type (M_0) and 99 different types of deletions ranging from 100 bp (M_1) to 9900 bp (M_{99}). Note that the name indicates the size of the deletion. Other assumptions that have been made regarding mitochondrial mutations are:

1. Deletion mutants can suffer from further deletion events ($M_x \rightarrow M_y$, $x < y$). Figure 2 A shows the resulting network of possible mutation reactions for five different types of mitochondria. However, since 100 different types were used in the simulations, the total number of mutation reactions is 4950.
2. Since no details are known of the biochemical mechanisms underlying the deletions, we assume that the mutation rate is proportional to the length of the mtDNA molecule and has the dimension per day per kbp. Thus larger mtDNAs have a higher probability of mutation than small mtDNAs.
3. Another important point is the outcome of a deletion event. What is the size distribution of the resulting deletion mutants? Without knowing the biochemical basis of the deletion process it is not possible to predict if all deletions will be of identical size or what type of distribution to expect. Under these circumstances we assume that the deletion is the result of two random cuts within the large arc. This leads to a distribution with a linearly increasing probability for the occurrence of small deletions. Basically, large deletions are very rare since only a few cut positions exist within the large arc that will result in a large deletion fragment.
4. Finally, we investigate the possibility that the presence of defective mtDNAs increases oxidative stress, and thus the mutation rate, within a cell. It is therefore assumed that the mutation rate is increased by a certain factor (the 'boost factor') if 100% of the mitochondrial population is defective. A linear relationship between the fraction of defective organelles and the increase in oxidative stress is assumed, so that a cell with X% mutants has its mutation rate increased by a boost factor * X/100.

Taking the above points into account the following equation can be constructed that calculates the mutation rates leading from one mutant type to another. The first term describes the effects of increasing oxidative stress caused by mitochondrial mutants (point 4). If there are only wild-type organelles (M_0), the term reduces to one, leaving the mutation rate unchanged, as calculated by the second term. But for a population consisting to 100% of mutants (M_1 to M_{99}) the first term evaluates to bF, the boost factor.

The second term incorporates the assumption that the mutation rate is per kbp and therefore the baseRate is multiplied by the length of the mtDNA of mutant M_i, which is given by $(16 - 0.1 * i)$. Furthermore, we have to take into account that small deletions are more likely to occur than large ones (point 3). There is a $1/100^2$ chance of placing two random cuts (limited to 100 bp intervals) such that a 10 kbp piece results, but there is a $100/100^2$ chance of placing the two cuts such that a 0.1 kbp piece results (i.e. a $(100 - k)/10\,000$ chance for a deletion size of $k * 0.1$ kbp). Since the total mutation rate is distributed among all possible resulting mutants $\left(\sum_{i=1}^{100} i/10\,000 \approx 1/2\right)$, the resulting equation is given by:

$$\text{mutRate}_{ik} = \left(\frac{(bF - 1) \cdot \sum_{i=1} M_i}{\sum_{i=0} M_i} + 1 \right) \cdot \frac{\text{baseRate} \cdot (16 - 0.1 \cdot i) \cdot (100 - k)}{5000}$$

Replication

It is assumed that replication time is proportional to the length of the mtDNA molecule. However, because of the special mode of replication of mtDNA, some regions of the mitochondrial genome count twice. The origins of replication of the heavy and light strands of the mammalian mtDNA molecule (O_H and O_L, respectively) are located at different positions on the molecule (Fig. 2B). The replication process proceeds asymmetrically via strand-displacement (Tapper & Clayton, 1981; Clayton, 2003). Leading strand synthesis starts at O_H and continues clockwise along the major arc until it reaches and exposes the origin of the lagging strand, O_L. Only now can lagging strand synthesis start counter-clockwise through the major arc, while leading strand synthesis continues through the minor arc until it has completed a full round of replication. However, the process is not finished until lagging strand synthesis has also completed the full circle of DNA.

The time for the total replication process is therefore the time required for the synthesis of the full length molecule plus the time that the start of light strand synthesis lags behind the start of heavy strand synthesis. For the wild-type molecule this is the time required to synthesize 16 kbp +10 kbp = 26 kbp so that the major arc effectively counts twice. As a consequence a mutant with a complete deletion of the major arc replicates 26/6 = 4.33 times faster than wild-type and not 16/6 = 2.66 times, as might have been expected.

To construct a model that leads to a steady state population of mtDNA molecules we have to add negative feed back such that the total synthesis of mtDNA declines with increasing copy number in the cell. The following equation shows how this feed back enters into the calculation of the replication rate for mutant M_i. The first term is a Hill type product inhibition depending on the total number of genomes, a parameter which controls when the total synthesis rate has reached 50% of its maximum (k). The latter is an exponent determining how sharply the system reacts if the number of mtDNA departs from 'k'. The term includes a constant specifying the maximum number of mtDNA that can be produced per day (c).

$$\text{repRate}_i = \frac{c \cdot k^5}{\left(\sum_i M_i\right)^5 + k^5} \cdot \frac{\frac{26}{26 - i \cdot 0.2} \cdot M_i}{\sum_i \frac{26}{26 - i \cdot 0.2} \cdot M_i}$$

If there are no mtDNA molecules, the total number of newly synthesized genomes is 'c', if the number of mtDNAs is equal to 'k' the synthesis rate is reduced to 'c/2' and if the number of mtDNAs is very large the total synthesis rate approaches zero. We also have to specify how the total amount of synthesized mtDNAs is distributed between the different types (mutants). This is done by the second term that basically weights the different types according to their size advantage and number. If there is only wild-type (M0), the second term simplifies to 1, indicating that all synthesized genomes will be wild-type. If there is a mutant with a 10 kbp deletion (M100) together with

equal amounts of wild-type, a fraction 1/(1 + 26/6) = 0.1825 of the newly synthesized genomes will be wild-type and a fraction (26/6)/(1 + 26/6) = 0.8125 will be of type M100.

Degradation

If new mtDNAs are synthesized some genomes have to be degraded to maintain a stable population size. Unfortunately, little is known about the process of mitochondrial degradation. It is not known if they are selected for degradation according to the amount of membrane damage, as suggested by the SOS hypothesis (de Grey, 1997) or if they are selected randomly. However, here we are exclusively interested in the effects of different mtDNA sizes and in this context degradation of mitochondria means degradation of mtDNA molecules. For the purpose of this study we assume identical degradation rates, and therefore half-lifes, for all types of mtDNAs (M_0 to M_{99}). For all simulations shown, the half-life was set to 10 days (see also Table 1).

Implementation

The model outlined above was simulated stochastically to explicitly take into account random fluctuations and the discrete nature of the studied biological objects. Mutation events with their inherently small probabilities require a nondeterministic approach to describe adequately the finding that in old animals some cells do contain mitochondrial mutants and others do not. Gillespie developed exact stochastic simulation algorithms that directly calculate the change of the number of molecules of the participating species during the time course of a chemical reaction (Gillespie, 1977). Thus stochastic algorithms calculate explicitly the time until the next reaction takes place (e.g. A→B + C) and keep track of how the number of molecules in the system is changed by this reaction (A decreased by one, B and C increased by one). Such an approach also deals with the discrete nature of molecule numbers. This is of special importance for self-replicating entities (like mtDNAs) if the number of objects is either zero or one. One mutant mtDNA can multiply and take over the cell, while zero mtDNA molecules obviously cannot. Differential equations, however, assume that variables can attain continuous values, so that 0.001 mtDNAs is a valid simulation result. Because this value is different from zero it is theoretically possible that a population of mutants can recover from such low levels and replace the wild-type: an unrealistic situation which is avoided by stochastic simulations.

The free software package *Dizzy* was used for all simulations (Ramsey & Orrell, 2005). Dizzy is written in Java and implements several stochastic (Gillespie, Gibson-Bruck, Tau-Leap) and deterministic (ODE solvers) simulation algorithms. Models can be described in Systems Biology Markup Language (SBML) (Hucka *et al*., 2003) or using a proprietary model definition language (CMDL). Because of a special language construct it was possible to define the 4950 mutation reactions with just five lines of code in CMDL. To be comparable with the experimental conditions of Cao *et al*. (2001), Gillespie's direct method was used to calculate the fate of a mitochondrial population within a cell for 1100 days, corresponding to 38 months of a rat's life. On a modern workstation such a calculation took approx. 1 h. The simulation was then repeated 1000 times on a Linux cluster to have enough information for a statistical analysis of the accumulation process of defective mitochondria.

The CMDL script that was used for simulations as well as a Python program used for the analysis of the Dizzy output is available as supplementary material.

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Supplementary Material

The input file that describes the model and was used for simulations with the software package Dizzy, as well as a Python program used for the analysis of the Dizzy output, are available as supplementary material on the Blackwell Synergy website.