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Development of a Quantitative PCR (TaqMan) Assay for Relative Mitochondrial DNA Copy Number and the Common Mitochondrial DNA Deletion in the Rat

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Changes in mitochondrial DNA copy number and increases in mitochondrial DNA mutations, especially deletions, have been associated with exposure to mutagens and with aging. Common deletions that are the result of recombination between direct repeats in human and rat (4,977 and 4,834, bp, respectively) are known to increase in tissues of aged individuals. Previous studies have used long-distance PCR and Southern blot or quantitative PCR to determine the frequency of deleted mitochondrial DNA. A quantitative PCR (TaqMan) assay was developed to detect both mitochondrial DNA copy number and deletion frequency in the rat. This methodology allows not only the determination of changes in the amount of mitochondrial DNA deletion relative to total mitochondrial DNA but also to determine changes in total mitochondrial DNA relative to genomic DNA. As a validation of the assay in rat liver, the frequency of the common 4,834 bp deletion is shown to increase with age, while the relative mitochondrial DNA copy number rises at a young age (3–60 days), then decreases and holds fairly steady to 2 years of age. Environ. Mol. Mutagen. 44:313–320, 2004.

Key words: rat; mitochondrial DNA; quantitative PCR; mitochondrial deletion; aging

INTRODUCTION

Somatic mutations in mitochondrial DNA (mtDNA) are hypothesized to be a (or the) cause of aging and age-related disease [Harman, 1972; Linnane et al., 1989; Gadaleta et al., 1998]. According to the free radical theory of aging, mtDNA damage leads to free radical production that in turn causes further damage. Numerous studies have found increases in mitochondrial mutations in a variety of tissues from aged individuals. While point mutations of mtDNA have been shown to vary in both normal and malignant tissues and increase with age [Munscher et al., 1993; Yowe and Ames, 1998; Zhang et al., 1998; Fliss et al., 2000; Jones et al., 2001; Kirches et al., 2001; Penta et al., 2001; Lin et al., 2002; Khaidakov et al., 2003], most studies have focused on the induction of large deletions in mtDNA. In humans, a 13 bp direct repeat (at 8,470–8,482 and 13,447–13,459) in the mtDNA leads to the frequent occurrence of a 4,977 bp deletion by recombination between the repeats [Holt et al., 1989; Schon et al., 1989; Tang et al., 2000]. Heteroplasmic inheritance of this deletion causes Pearson syndrome or Kearns-Sayre syndrome [Holt et al., 1989, 1989; Schon et al., 1989; Rotig et al., 1991], but this deletion also occurs frequently during aging.

A similar common mtDNA deletion occurs in the rat due to a 16 bp repeat at 8,103–8,118 and 12,937–12,952; recombination between these repeats leads to a 4,834 bp deletion [Gadaleta et al., 1992; Edris et al., 1994]. This

Abbreviations: CD/DL, common mitochondrial deletion/mitochondrial D-loop; C_T, cycle threshold; ΔC_T, C_T experimental gene − C_T control gene; $\Delta\Delta C_T$, ΔC_T of unknown sample − ΔC_T of reference sample; DL/BA, mitochondrial D-Loop/β-actin.

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deletion also increases in aged rats [Gadaleta et al., 1992; Filburn et al., 1996; Seidman et al., 1997; Kang et al., 1998; Yowe and Ames, 1998; Nagley et al., 2001]. It can be increased by some drugs [Nagley et al., 2001; Suliman et al., 2002] or stress [Sakai et al., 1999] and decreased by dietary restriction in at least some tissues in rats [Kang et al., 1998]. Other mtDNA deletions also are seen in aged rats [Van Tuyle et al., 1996].

Quantitative PCR (qPCR) generally is used to detect changes in gene expression (RT-qPCR), but also can be employed to detect amplifications or deletions in genomic DNA. Quantitation studies have been performed either with Northern blots, Western blots, Southern blots, or quantitative PCR blots, all of which are tedious, technically demanding, and involve the use of radioisotopes. Furthermore, these PCR methods use an endpoint determination that may not be truly quantitative because of plateau effects.

Several methods have been developed using real-time PCR (either TaqMan or molecular beacons) to monitor either mtDNA copy number or the amount of the common deletion in human cells [Heid et al., 1996; Steuerwald et al., 2000; Gahan et al., 2001; Lim et al., 2001; Reynier et al., 2001; Rodriguez-Santiago et al., 2001; Szuhai et al., 2001; He et al., 2002; Miller et al., 2003]. Rodriguez-Santiago et al. [2001] determined mtDNA copy number in the brains of Alzheimer patients using qPCR of the mitochondrial *ND2* gene vs. the nuclear 18S gene. Wong and Bai [2002] determined copy number in patients with mitochondrial disease. Reynier et al. [2001] quantified absolute mtDNA copy number in oocytes. Gahan et al. [2001] used molecular beacons of the mitochondrial cytochrome b and nuclear *CCR5* genes to measure mtDNA copy number in human monocytes and in the fat of AIDS patients. Szuhai et al. [2001] also used molecular beacons for the mitochondrial tRNA *LYS* gene and the nuclear globin gene to determine heteroplasmy in MERRF syndrome patients using a $\Delta\Delta C_T$ method. Heid et al. [1996] and Lim et al. [2001] used the mtDNA and nuclear β-actin gene in a TaqMan relative curve method to quantitate mtDNA copy number, while Steuerwald et al. [2000] quantitated mtDNA copy number in oocytes using a standard curve method and SYBR green. He et al. [2002] used TaqMan probes against the commonly deleted *ND4* gene and the rarely deleted *ND1* gene to determine mtDNA copy number. Miller et al. [2003] determined the ratio of mtDNA (*CYTB* gene) vs. a nuclear gene (β-globin) as compared to plasmid standards. Lastly, a number of groups are now using real-time PCR to quantitate human mtDNA in patients treated with antiviral drugs for HIV infection [Cossarizza et al., 2003; Gourlain et al., 2003; Miura et al., 2003; Pace et al., 2003; Lopez et al., 2004].

Two groups have reported TaqMan methods to measure the human common deletion. Koch et al. [2001] used a nondeleted region probe and a probe specific for the deletion to quantitate the deletion in human keratinocytes, while Meissner et al. [2000] used similar probes to detect the deletion in human blood.

We wished to develop a fast and inexpensive method to measure changes in mtDNA copy number and the frequency of the common mtDNA deletion in rats. This report details the optimization of such a method quantating rat mtDNA as compared to a nuclear gene (β-actin) as well as quantitating the amount of the common deletion relative to the number of copies of the D-loop region. The reproducibility of the method is demonstrated and the method is used to determine the changes in relative mtDNA copy number and the frequency of the rat common deletion in 3-day-old to 2-year-old animals. This assay has also been used to show increases in mtDNA copy number and deletion frequency in sialodacryoadenitis virus-infected rats and also that diet can affect the frequency of mtDNA deletions after chemotherapy [Branda et al., 2002a, 2002b].

MATERIALS AND METHODS

Animals and DNA Extraction

A control female Fisher 344 rat (#6776) was obtained from Charles River Canada (St.-Constant, Quebec, Canada) and fed ad libitum a standard cereal-based rat chow (LM-485 Harlan Teklad, Madison, WI). For the aging experiments, Sprague-Dawley rats from newborn to 26 months of age (3-day-old, 10-day-old, 30-day-old, 45-day-old, 60-day-old, 120-day-old, 240-day-old, 365-day-old, and 735-day-old) were utilized. All time points used three animals except the 735-day point, where only two animals could be procured (others died in transit). The animals younger than 3 months were a gift from Dr. Deborah Damon, who obtained the mothers from Harlan Teklad (Madison, WI). The older animals were also obtained from Harlan Teklad at the indicated age, housed 1 week, and then euthanized. They were also fed the standard chow as above. The Sprague-Dawley rats that were old enough for gender to be determined (30-day-old) were all male. The rats were sacrificed and the liver tissue removed and stored at −80°C until processing. Total hepatic DNA was isolated using the Qiagen DNeasy Tissue kit (Valencia, CA).

All animal experiments were approved by the University of Vermont Institutional Animal Care and Use Committee and American Veterinary Medical Association guidelines for euthanasia were followed. The rats were euthanized by lethal injection of pentobarbitol followed by exsanguination. The University of Vermont Animal Care Facilities follow procedures that are in accordance with the Guide to the Care and Use of Laboratory Animals and are certified by the Association for Assessment and Accreditation of Laboratory Animal Care.

Oligonucleotide Primers and TaqMan Probe Design

Primers and probes for the rat D-loop and the rat mitochondrial deletion from the rat mitochondrial genome (Genbank accession X14848) and rat β-actin primers (Genbank V01217) were designed using Primer Express software (Applied Biosystems, Foster City, CA). Primers and probes were synthesized and HPLC-purified by the Oligo Factory (Applied Biosystems). The sequences of the primers and probes can be found in Table I.

Real-Time PCR

Mitochondrial deletion expression was quantified with a 5′ VIC reporter and a 3′ TAMRA quencher dye and D-loop expression with a 5′ 6-FAM

TABLE I. Sequences of the TaqMan Primers and Probes

Primer/probe	Sequence (5′–3′)
Mitochondrial D-loop	
Forward	GGTTCTTACTTCAGGGCCATCA
Reverse	GATTAGACCCGTTACCATCGAGAT
Probe	6FAM-TTGGTTCATCGTCCATACGTTCCCCTTA-TAMRA
Mitochondrial deletion	
Forward	AAGGACGAACCTGAGCCCTAATA
Reverse	CGAAGTAGATGATCCGTATGCTGTA
Probe	VIC-TCACTTTAATCGCCACATCCATAACTGCTGT-TAMRA
β-actin	
Forward	GGGATGTTTGCTCCAACCAA
Reverse	GCGCTTTTGACTCAAGGATTTAA
Probe	VIC-CGGTCGCCTTCACCGTTCCAGTT-TAMRA

TABLE II. Effects of Altering Primer Concentrations on C_T and ΔC_T

β-actin primer	D-loop primer	β-actin C_T	D-loop C_T	D-loop C_T − β-actin C_T (ΔC_T)
200 nM	100 nM	27.55	18.54	−9.01
200 nM	50 nM	27.09	19.03	−8.06
200 nM		27.45		
100 nM	200 nM	28.48	18.38	−10.1
50 nM	200 nM	29.95	18.45	−11.5
	200 nM		18.42	
Common deletion primer	D-loop primer	Common deletion C_T	D-loop C_T	Common deletion C_T − D-loop C_T (ΔC_T)
200 nM	200 nM	23.54	18.43	5.11
200 nM	100 nM	22.48	18.31	4.17
200 nM	50 nM	22.82	18.74	4.08
200 nM		23.32		
100 nM	200 nM	25.36	18.36	7.00
50 nM	200 nM	28.41	18.22	10.10

reporter and 3′ TAMRA-labeled quencher dye. PCR amplification was carried out in a 50 μL reaction consisting of 1 × TaqMan Universal Master Mix (Applied Biosystems) and varied concentrations of probe and varied concentrations of forward and reverse primers to optimize reaction conditions (Table II). Optimal reaction conditions were those concentrations that gave the maximum ΔR_n and minimum C_T. The final optimal reaction concentrations for the common mitochondrial deletion/D-loop assays (CD/DL; 50 μl reaction) consisted of 1 × TaqMan Universal mix, 200 nM each mitochondrial deletion forward and reverse primer, 100 nM each D-loop forward and reverse primer, 100 nM each mitochondrial deletion and D-loop probe, and ∼ 50 ng of sample DNA. For the D-Loop/β-actin (DL/BA) assays, final conditions (50 μl reaction) consisted of 1 × TaqMan Universal mix, 200 nM each β-actin forward and reverse primer, 50 nM each D-loop forward and reverse primer, and 100 nM each β-actin and D-loop probe, and ∼ 50 ng of sample DNA. The cycling conditions included an initial phase of 2 min at 50°C; followed by 10 min at 95°C, and 40 cycles of 15 sec at 95°C and 1 min at 60°C. Each sample was assayed in duplicate and fluorescence spectra were continuously monitored by the 7700 Sequence Detection System (Applied Biosystems) with sequence detection software version 1.6.3.

Analysis of Data

Data analysis was based on measurement of the cycle threshold (C_T), which is the PCR cycle number when the fluorescence measurement reaches a set value. Two types of experiments were performed. The first measured mtDNA copy number vs. nuclear DNA copy number by amplification of the mitochondrial D-Loop vs. the nuclear β-actin gene (DL/BA), while the second measured the amount of the mitochondrial deletion vs. the mitochondrial D-Loop (CD/DL). The difference in C_T values was used as the measure of relative abundance, i.e., in DL/BA experiments, C_T(DL) − C_T(BA) was used as the abundance of the mitochondrial genome, and in the MD/DL experiments, C_T(CD) − C_T(DL) was used as the abundance of the mitochondrial deletion. Descriptive statistics and plots were used to explore possible trends in ΔC_T values based on the day that the experiment was performed. Linear regression was used to characterize the strength of any trends to the extent that they were linear.

Analysis of variance (ANOVA) was used for comparisons of relative expression levels for different rats. Dunnett's [1955] method for multiple comparisons was used to compare results for older rats to those from the 3-day-old rats.

RESULTS

The objective was to develop an assay that could detect both changes in the amount of mtDNA in a cell (as compared to a known nuclear gene, which has two copies/diploid genome) and in the amount of the common mtDNA deletion as compared to the number of mitochondria. We chose to use a sequence in the rat β-actin gene as our

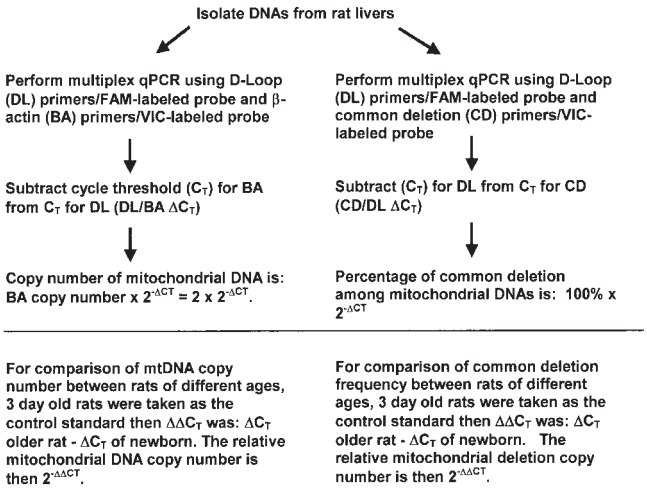

Fig. 1. Flow chart for the experiments and the calculation of results.

nuclear gene control, a sequence in the mitochondrial D-loop as the measure of the number of mitochondria, and a sequence that represented the new fusion sequence present only in deleted mtDNA as a measure for the common deletion. Figure 1 is a flow chart of the experimental design and result calculations.

TaqMan primers and probes were designed for the rat mitochondrial D-loop region, across the common deletion, and for the rat β-actin gene (Table I). PCR reactions were performed with these primers using liver DNA isolated from a single control F344 rat. The specificities of all three sets of primers were tested by demonstrating the correct size product on an agarose gel and by DNA sequencing, which revealed the correct sequence (data not shown).

For qPCR, it is important that the efficiencies of the PCR reactions be close to 100% [i.e., the slope of the graph of C_T vs. log (DNA) is -3.33]. Figure 2 plots the results for the β-actin, D-loop, and common deletion PCR reactions. All the slopes are close to the optimal -3.33, with values of -3.346, -3.241, and -3.314 for the nuclear β-actin, mitochondrial D-loop, and mitochondrial common deletion reactions, respectively.

Several experiments were performed changing primer and probe concentrations to optimize the assay and to allow utilization of as little primer/probe per assay as possible for cost considerations. Table II shows the results of these experiments. Use of one primer at 50 nM while the other at 200 nM did decrease the C_T; however, use of 100 nM vs. 200 nM probes did not greatly affect the results.

TaqMan experiments can be quantitated comparatively in the same reaction to an endogenous control. To compare different experimental conditions, the ΔC_T of the experimental sample is subtracted from the ΔC_T of the control sample; the DNA concentration difference between the two experiments is given by $2^{-\Delta\Delta CT}$ (the $\Delta\Delta C_T$ method). We chose to use the $\Delta\Delta C_T$ method because it affords savings in reagents and time. However, in order to utilize this method,

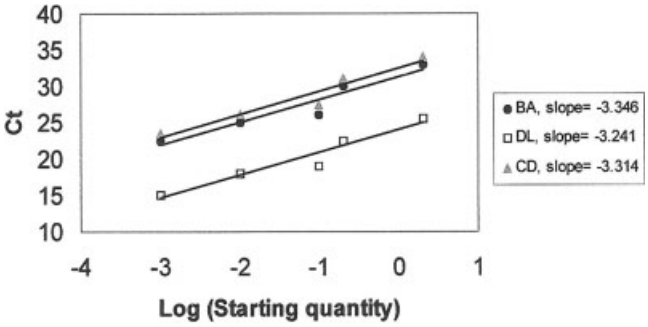

Fig. 2. Slopes of C_T vs. log (starting DNA). C_T was measured for five dilutions of input DNA for each primer and probe set separately [β-actin (BA), D-loop (DL), and common deletion (CD)]. C_T is plotted vs. the log of the input DNA. Slopes of the trend line for each probe were determined. A primer/probe set with 100% efficiency would have a slope of -3.33.

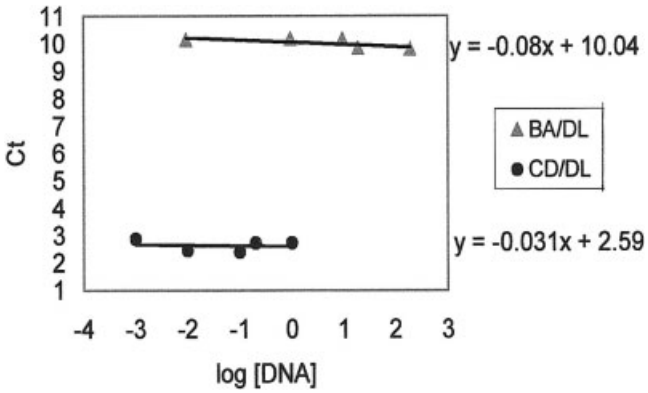

Fig. 3. Slopes of ΔC_T vs. input DNA for the multiplex PCR reactions. The multiplex reactions of β-actin with D-loop probe/primers to measure mtDNA copy number (DL/BA) and common deletion with D-loop probe/primers to measure the frequency of the common mitochondrial deletion (CD/DL) were performed on five dilutions of input DNA. The ΔC_T is plotted vs. the log of input DNA. Slopes should be < 0.1 for a good probe/primer set.

it is necessary to demonstrate that the ratio of the two C_Ts stays constant over a wide concentration range. In practical terms, this means that the absolute value of the slope of the ΔC_T vs. log amount of input DNA is less than 0.1. Figure 3 shows the plots for the D-Loop/β-actin (DL/BA) and common deletion/D-loop (CD/DL) results. For confirmation, experiments were performed with each PCR separately and in multiplex. These reactions gave similar results at a given concentration whether the PCR was a singleplex or multiplex (Table II).

In order to demonstrate the reproducibility of the assay, the DL/BA and CD/DL assays were performed a total of 23 times over a period of 12.5 months on the same concentration of a single DNA sample from a 15-week-old rat (#6776). The mean ΔC_T ($\pm$ standard deviation) for the DL/BA value was -5.25 ± 0.93 while for mean CD/DL value was 8.13 ± 1.00. There was a significant linear trend in the ΔC_T values for the DL/BA measurement based on the day the experiment was done, with higher values (absolute

values) associated with experiments done on later dates. This linear trend explained roughly 23% of the total variability in the β-actin ΔC_T measurements. The trend is characterized as an average 0.0039 unit decrease in the ΔC_T values for each additional day after the first. For the CD/DL measurements, the trend in ΔC_T vs. day of the experiment had a more pronounced nonlinear trend. However, the linear trend still explains roughly 14% of the overall variability in the ΔC_T values. The linear trend shows an average 0.0033 unit increase in the ΔC_T values for each additional day. The amount of overall variability in the ΔC_T values was higher for the second half of the experiments as compared with the first half. For both measurements, the standard deviation of the results from the second half of the experiment was nearly twice as large as that of the first half. This could indicate some degradation or problems with uniform mixing in the later experiments. Furthermore, differential changes in the nuclear vs. mtDNA will have a significant effect on the DL/BA experiments. Measurements of the D-loop were made for both the DL/BA and CD/DL experiments. The correlation between the two different sets of measurements of D-loop was 0.903. This indicates good reproducibility on a single day and between the two types of assays, i.e., whether the D-loop was multiplexed with BA or CD, the same result was still obtained. The correlation between ΔC_T for DL/BA and ΔC_T for CD/DL is −0.409.

Finally, a series of livers from Sprague-Dawley rats of different ages (3 days to 23 months) was studied to determine changes in mtDNA copy number and changes in the frequency of the mtDNA deletion with age. Figures 4 and 5, respectively, show the results of these experiments based on $2^{-\Delta\Delta CT}$. From Figure 4, the mtDNA copy number appears to rise steeply at 30 days ($P = 0.003$ comparing the 30-day result to the 3-day result using Dunnett's procedure), then decreases slowly to 120 days, before holding fairly steady in old age. The frequency of the mitochondrial deletion rose until 60 days of age ($P = 0.007$ comparing the 60-day result to the 3-day result), then plateaued, before rising again until about 2 years of age ($P = 0.007$ comparing the 2-year result to the 3-day result).

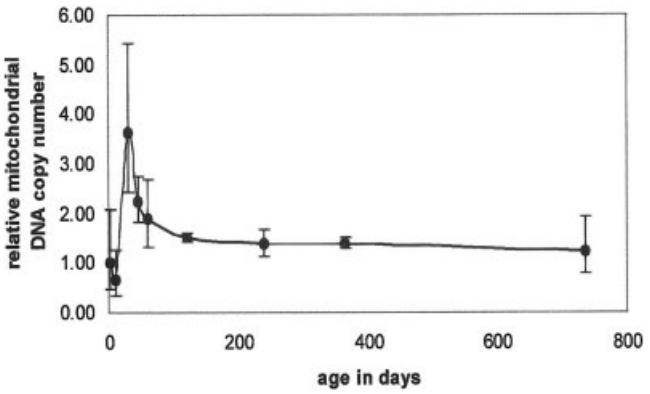

Fig. 4. Changes in mtDNA copy number with age. DNA was isolated from the liver of rats from newborn to 735-day-old (3-day-old, 10-day-old, 30-day-old, 45-day-old, 60-day-old, 120-day-old, 240-day-old, 365-day-old, and 735-day-old). All time points used three animals except the 735-day point, where only two animals could be procured (others died in transit). The 3-day-old DL/BA ΔC_T (mitochondrial copy number) was taken as the reference point for a $\Delta\Delta C_T$ to calculate $2^{-\Delta\Delta CT}$ (relative mtDNA copy number) that is plotted vs. the age (in days) of the rats.

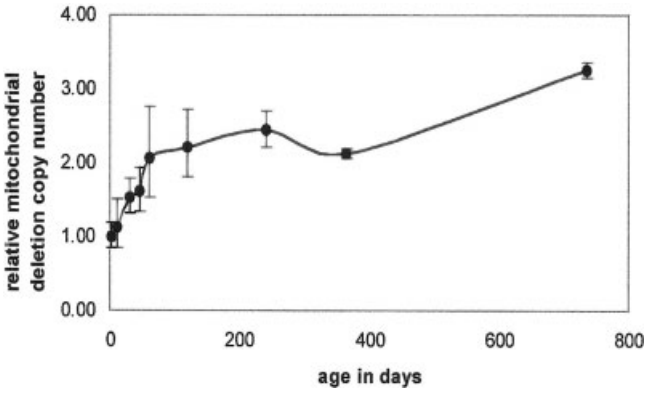

Fig. 5. Changes in the frequency of mitochondrial deletion with age. DNA was isolated from the liver of rats from newborn to 735-day-old as in Figure 4. The 3-day-old CD/DL ΔC_T (mitochondrial deletion frequency) was taken as the reference point for a $\Delta\Delta C_T$ in order to calculate $2^{-\Delta\Delta CT}$ (relative mitochondrial deletion copy number) that is plotted vs. the age (in days) of the rats.

DISCUSSION

Recently, several methods have become available to measure RNA or DNA quantitatively using fast and sensitive real-time (nonendpoint) nonradioactive methods. These involve PCR monitored continuously by fluorescence as the reaction proceeds. The most popular method uses the TaqMan system of Applied Biosystems (ABI). In TaqMan technology, a probe specific for the gene of interest is created with a fluorescent dye at the 5′ end and a quencher at the 3′ end. As the specific PCR for the gene of interest progresses, the 5′ exonuclease of the polymerase frees more and more fluorescent dye from the probe that is then quantified. The amount of mRNA or DNA for a specific gene in the experimental sample can be quantified absolutely using a standard curve made with solutions having a known number of copies, quantified relative to a known sample, or quantified comparatively in the same reaction to another gene (an endogenous control). In the first two cases, separate amplifications of an endogenous control are needed to control for PCR or reverse transcriptase efficiency and the amount of cDNA or DNA added to the reaction, while in the latter, the reactions for the control and experimental gene can be multiplexed. Our method simultaneously quantifies mtDNA and the common deletion rapidly and economically.

These experiments show that for control liver DNA, the mean DL/BA ΔC_T was −8.0, which indicates that (assuming 100% efficiency of both PCRs) the mitochondrial se-

TABLE III. Liver Mitochondrial Deletion Frequency in Rats

Deletion frequency in young rats	Deletion frequency in older rats	Reference
0.18% (3 months)	0.59% (23 months)	This study
0.0005% (7 months)	0.02% (27 months)	Gadaleta et al. [1992]
0.05% (6 months)	0.32% (24 months)	Kang et al. [1998]
0.001% (6 months)	0.5% (24 months)	Edris et al. [1994]
ND	1.7% (20 months)	Filser et al. [1997]
ND	1.88% (~24 months)	Yowe and Ames [1998]

quence is present in ~ 256 more copies than the β-actin sequence ($= 2^{-\Delta CT} = 2^8 = 256$) or at 512 copies/diploid nuclear genome, assuming the β-actin sequence is present in two copies. Liver has an average of 1,300 mitochondria per cell, with 8–10 copies of mtDNA/mitochondria, giving 13,000 mitochondrial DNA copies/cell [Loud, 1968]. However, calculating copy number from several published reports gives lower numbers. For example, Gadelata et al. [1992] report that rats have 2.30 μg mtDNA per mg of genomic DNA; this results in a copy number of ~ 860 in rat. Filser et al. [1997] reported 0.039% mtDNA, which converts to 146 copies/cell. Our calculated value is quite low compared to the value reported for liver but near those in Gadelata et al. [1992] and Filser et al. [1997]. The low values reported here for copy number could be a result of β-actin pseudogenes in the rat genome; our calculation assumes two copies/diploid nuclear genome but if there are four or six nuclear copies, then the result should be multiplied by two or three. Locus Link lists six actin-like genes in the rat. A Blast search, however, came up with a match to only the β-actin sequence. Further sequencing of the rat genome could reveal additional pseudogenes in the future. It must be pointed out that this comparative C_T method is not meant to give absolute quantitation but only relative quantitation between experimental conditions. Lastly, multinucleate liver cells accumulate in older livers and may result from exogenous treatment; these multinucleate cells will affect determinations of mitochondria/cell. As a result, the results in this study are expressed above as mitochondria per diploid genome; however, the figures of other researchers could include these multinucleate cells.

The results in Figure 4 show interesting changes in mtDNA copy number with age. At a young age (3–60 days), mtDNA copy number rises and then decreases; these changes in the mtDNA copy number could coincide with sexual maturity. The mtDNA copy number then holds fairly steady to 2 years of age (although a small increase cannot be ruled out). Others usually have found a slow rise with aging in mammals [Heerdt and Augenlicht, 1990; Barrientos et al., 1997; Lee et al., 1998; Wong and Bai, 2002]. For example, Lee et al. [1998] found an increase of mtDNA copy number with aging in human lung, and Barrientos et al. [1997] found a 1.6-fold increase in 80-year-olds vs. 20-year-olds. Heerdt and Augenlicht [1990] reported an increase in human mtDNA with development. Using hybridization to the *COIII* gene of mtDNA from liver, they found a level of ~ 3 at 22 weeks of gestation, ~ 5.5 at 32 weeks of gestation, and ~ 12 in two adults. Wong and Bai [2002] also report an increase in mtDNA from birth to 5 years of age in human muscle. However, Barazzoni et al. [2000] found that 27-month-old rats had only 50% of the mtDNA as 6-month-old rats. Based on in vitro studies, Tang et al. [2000] speculated that mtDNA copy number is inversely proportional to the size of the mtDNA, i.e., cells keep a constant mass of mtDNA, not a constant number of genomes.

In terms of an absolute value for the frequency of the mtDNA deletion, the ΔC_T for the CD/DL experiment of 9.11 indicates that the common deletion is 0.0018 (0.18%) of the total mitochondrial sequences for 3-month-old rats [$1/2^{\Delta Ct} = 1/2^{9.11} = 1/553$], while the ΔC_T for the 23-month-old rats of 7.41 indicates that the common deletion is 0.0059 (0.59%) of the total mitochondrial sequences at this older age [$1/2^{\Delta Ct} = 1/2^{7.41} = 1/170$]. The findings of other groups with regard to the frequency of mtDNA deletions are very variable, undoubtedly a result of the many experimental methods utilized as well as the tissues studied (Table III). The numbers found here are higher than most of those reported, although similar to Filser et al. [1997] and Edris et al. [1994]. In human liver, Wei et al. [1996] found a frequency of 0.00076% for 20- to 29-year-olds and a frequency of 0.076% in 70- to 79-year-olds, while Lee et al. [1994] found a frequency of 0.0076% in 70-year-olds. Our results (Fig. 5) clearly confirm the reported increase in deletions in older animals.

In conclusion, we have developed an assay to detect both relative mtDNA copy number and mtDNA deletion frequency in the rat. We have shown that the assay is robust and reproducible and have demonstrated the expected results with aging. This assay should be useful to study the effects of drugs, diet, or environmental changes on mtDNA.

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