Increased frequency of deletions in the mitochondrial genome with age of *Caenorhabditis elegans*

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ABSTRACT

We have developed a long-extension-PCR strategy which amplifies approximately half of the mitochondrial genome (6.3 kb) of *Caenorhabditis elegans* using an individual worm as target. We analyzed three strains over their life span to assess the number of detectable deletions in the mitochondrial genome. Two of these strains are wild-type for life span while the third is mutant in the *age-1* gene, approximately doubling its maximum life span. At the mean life span in wild-type strains, there was a significant difference between the frequency of deletions detected in the mitochondrial genome compared with the mean number of deletions in young animals. In addition, deletions in the mitochondrial genome occur at a significantly lower rate in *age-1* mutants as compared with wild type. We cloned and identified the breakpoints of two deletions and found that one of the deletions had a direct repeat of 8 bp at the breakpoint. This is the largest single study (over 900 individual animals) characterizing the frequency of deletions in the mitochondrial genome as a function of age yet carried out.

INTRODUCTION

The molecular processes specifying senescence have yet to be elucidated. The free-radical theory of aging, as originally proposed by Harman (1,2), has recently gained much support from the observation that *Drosophila melanogaster* transgenic for catalase and Cu/Zn superoxide dismutase shows a 30% increase in the mean and maximum life span (3). A corollary of the free-radical theory, the mitochondrial DNA damage theory of aging (4), proposes that damage to the mitochondrial DNA results in an increase in the production of reactive-oxygen species (ROS) over the life span of the organism thereby causing macromolecular damage resulting in senescence.

Several studies support the mitochondrial DNA damage theory of aging and show that mitochondrial genomic deletions (dmtDNA) increase with age in a number of organisms (5–8). It is not yet clear how such deletions occur (9), nor what frequency of deletions would be required to cause significant alterations in cellular function. Quantitation of some dmtDNAs from a number

of tissues of aged humans has been performed and 0.1–12% deletions relative to wild-type have been found (5,10).

Caenorhabditis elegans is a small free-living nematode which has many advantages for investigating fundamental processes underlying senescence (11,12). The *age-1* mutation is a single-gene defect (12) which extends the mean and maximum life span by 65 and 110%, respectively (13,14). *age-1* mutants are generally stress-resistant and have been shown to have increased levels of Cu/Zn superoxide dismutase and catalase at older ages relative to wild type (15,16). Further, Age strains are more thermotolerant (17) and are hyper-resistant to paraquat (16, Melov *et al.*, unpublished data)—a superoxide anion generator, and hydrogen peroxide (15), which could result from a global up-regulation of ROS defenses. We previously reported that dmtDNA are found in *C.elegans* and that potential stem-loop structures are found at the breakpoints of most dmtDNAs and may be causally involved in generating such deletions (18). However, we did not address dmtDNA as a function of age.

We have examined over 900 individual animals in three distinct isogenic strains for an increase in dmtDNA over the life span of *C.elegans*. In these studies we assess the number of detectable dmtDNAs in the whole worm at a number of time points throughout life. This contrasts with other studies examining an individual dmtDNA as a function of age where the number of individuals examined has been small and genetically polymorphic. Further, we have developed a long-extension-PCR to examine ~50% of the mitochondrial genome in these aging populations. We wished to determine if dmtDNAs increased as a function of advancing age in *C.elegans* and whether there were differences between *age-1* mutants and wild-type in the rate of accumulation of dmtDNAs.

MATERIALS AND METHODS

Nematode strains

The following strains were used to facilitate the manipulation of large numbers of worms.

TJ1060 [*spe-9(hc88ts)* I *fer-15(b26ts)* II]

TJ1061 [*spe-9(hc88ts)* I *emb-27(g48ts)* II]

TJ1062 [*spe-9(hc88ts)* I *fer-15(b26ts)* *age-1(hx542)* II]

All strains are infertile at 25°C from the combined action of *spe-9* and either *fer-15* or *emb-27* (19). TJ1062 also contains a mutation in *age-1* which increases mean life span by ~65%.

Growth and maintenance of nematodes have been described (20). Mass cultures of each strain of worms were aged in synchrony on large NGM (nutrient growth media) plates as described (19). For experiment 1, survival analysis was carried out by transferring 25 animals at day 3 from the mass culture onto small NGM plates and scoring daily for death by touch provoked movement. In experiments 2–4, this assay was performed in triplicate. All strains were maintained at 25°C during the experiments.

Worm lysis

At each time point individual worms were picked at random from the aging population (by transferring the first worm seen in the microscope field) to a tube for PCR analysis (Table 1). Worms were picked from TJ1062 (experiment 4) at older ages of 17, 18, 20, 22 and 25 days of age because of its longer life span. For worm lysis, individual worms were placed into 10 µl of lysis buffer [50 mM Tris, pH 8.3, 250 µg/ml BSA, 1 mM MgCl₂ (Idaho technologies), 60 µg/ml of proteinase K (Stratagene)]. The worm

was then frozen at –70°C for at least 10 min and subsequently lysed by incubating at 60°C for 1 h and then at 95°C for 15 min. Lysed-worm preparations were stored at –20°C until used; 1 µl of the lysate was used in each PCR.

PCR and analysis of PCR products

PCR buffer was modified from the low magnesium buffer from Idaho technologies, and contained 50 mM Tris pH 8.3, 500 µg/ml BSA, 1.8 mM MgCl₂, 0.5% Ficoll 400, 1 mM Tartrazine. 0.1 U of Hot tub DNA polymerase (Amersham) was used per 10 µl PCR reaction. PCR was carried out in 10 µ micro-capillary tubes (Idaho technologies) in a 1605 Air thermal cycler (Idaho Technologies). The profile for long-extension PCR was an initial 2 min denaturation at 94°C, followed by 35 cycles of 0 s denaturation at 94°C, 0 s annealing at 55°C, and 5 min extension at 68°C, ending with a 10 min final extension at 68°C. The following primers were used for long-extension PCR.

Primer 9a (5'–3') TCG CTT TTA TTA CTC TAT ATG AGC G (nucleotides 1818–1842 mtDNA)

Primer 9b (5'–3') TCA GTT ACC AAA ACC ACC GAT T [nucleotides 8111–8090 mtDNA, (21)]

Table 1. Frequency of detectable dmtDNAs as a function of age

Age (days)	Experiment 1 TJ1061 (wild type)			Experiment 2 TJ1060 (wild type)			Experiment 3 TJ1060 (wild type)			Experiment 4 TJ1062 (<i>age-1</i> mutant)			
	<i>n</i>	dmtDN	<i>P</i> ₃	<i>n</i>	dmtDNA	<i>P</i> ₃	<i>n</i>	dmtDNA	<i>P</i> ₃	<i>n</i>	dmtDNA	<i>P</i> ₃	<i>P</i> _{wt}
3	36	7		17	0		19	4		18	3		NS
4	ND			16	0	NS	19	1	NS	19	9	NS	**
5	36	3	NS	ND			19	23	***	19	5	NS	***
6	36	7	NS	17	16	**	19	21	***	19	7	NS	***
7	19	14	*	19	3	NS	18	14	NS	19	10	NS	NS
8	36	33	**	ND			18	10	NS	19	3	NS	NS
9	19	25	**	18	19	***	19	42	***	19	17	**	***
10	17	14	**	16	8	*	19	25	***	19	7	NS	***
11							19	9	NS	18	23	***	**
12							19	34	**	19	20	***	NS
13							16	22	**	19	11	NS	*
14							19	21	***	19	12	NS	NS
15							18	22	***	19	14	**	*
16							18	22	*	18	9	*	NS
17										19	14	*	
18										19	14	NS	
20										19	10	*	
22										19	12	*	
25										18	5	NS	

n = number of individuals assessed on that day.

dmtDNA = number of dmtDNAs detected by Southern blotting.

ND = not done, NS = not significant.

*P*₃ = probability that the mean frequency of dmtDNA for that day is significantly different from that of the 3-day-old in that experiment, * < 0.05, ** < 0.005, *** < 0.0005.

*P*_{wt} shows the significance of the mean frequency of dmtDNA of experiment 3 compared with the mean frequency of dmtDNA of experiment 4 for each day. At days 4 and 11, TJ1062 has significantly more deletions than TJ1060, in all other comparisons, TJ1062 had fewer dmtDNAs than TJ1060.

The final concentration of primers 9a and 9b were 0.1 μ M and dNTPs (Pharmacia) were used at final concentrations of 200 μ M. Upon completion of the PCR, reaction products were analyzed by electrophoresis on horizontal 1% agarose slab gels containing ethidium bromide at 0.5 μ g/ml at 120 V in 0.5 $\times$ TBE buffer (22). Long-extension PCR amplified a 6294 base pair (bp) amplicon from the mitochondrial genome which represents 46% of the genome. Positive and negative controls were amplification of the 6.3 kb amplicon from 1 ng of genomic DNA derived from a mixed-stage population [primarily larval DNA prepared as in (20)] of wild-type (N2) and the absence of target DNA, respectively. Molecular weight markers were used to size reaction products and consisted of *Hind*III-restricted λ phage DNA (USB) and *Hae*III-restricted pBR322 DNA (Sigma).

Preparation of aged DNA and Southern blotting

Gels were photographed, denatured, neutralized and Southern blotted as described (22). The blot was then probed with radiolabeled 6.3 kb PCR product which had been isolated from an agarose gel using GeneClean™ (*Bio 101*). High-stringency washing (22) of the blot was then carried out, with subsequent exposure to Kodak XAR-5 film for 1–6 days at -80°C . In old worms, many bands detected by ethidium bromide were not mitochondrial in origin, and dmtDNA were identified by probing Southern blots of the gels with the 6.3 kb PCR product.

Genomic DNA from synchronous mass cultures of TJ1061 aged 3 or 10 days was prepared as described (20). Aliquots of the aged DNA (4 μ g) were digested using *S*all, *E*coRI or *H*indIII (NEB) overnight at 37°C in the recommended buffer. The resulting restricted DNA was electrophoresed on 1% TBE gels at 40 V in 1 $\times$ TBE buffer for 48 h. This gel was blotted and probed as described above.

Analysis of DNA sequence

Putative dmtDNAs were excised from the gel, and extracted by GeneClean™ followed by subcloning into the pCR II™ vector (Invitrogen) via a TA™ cloning kit (Invitrogen). Sequencing by a 373 sequenator (Applied Biosystems) was carried out (courtesy Dr J. Sikela) to confirm mitochondrial-genome origin and to establish deletion breakpoints. Sequence analysis was performed using Macvector™ (IBI) running on a Macintosh Ilci.

Statistical analysis

Deletions in the mitochondrial genome were scored by noting the number of small PCR products in each individual worm which hybridized with the 6.3 kb amplicon, and then summing across all worms all products at each time point (Table 1). Results from experiments 1 and 2 were scored blind and by separate researchers. To determine if there was a significant increase in the frequency of dmtDNAs with increasing age, a Wilcoxon non-parametric *t*-test was carried out to compare the number of dmtDNAs per worm at later ages to the number of dmtDNAs from worms at 3 days of age (Table 1, P_3). A Wilcoxon non-parametric *t*-test was carried out between experiments 3 and 4 to determine if there was a significant difference between the mean numbers of dmtDNAs on any individual day (P_{wt} , Table 1). Wilcoxon non-parametric *t*-tests were used because the data are not normally distributed. Survival statistics were performed using the Wilcoxon (Gehan) statistic on SPSS (SPSS Inc.). In

experiment 2, the variance at 3 days of age in mean dmtDNAs was zero, and we used the following formula to determine *t*-values for assessing significance of later ages when compared to 3 days of age:

$$\frac{\bar{x}}{\sqrt{\frac{s^2}{N}}} = t \text{ (Greg Carey, personal communication)}$$

RESULTS

In an earlier study we demonstrated that deletions in the mitochondrial genome (dmtDNAs) of *C. elegans* do occur (18) in a 4.3 kb region. Here we extend these earlier observations in three ways. First, we have examined a 6.3 kb region (approximately half of the mitochondrial genome) including part of the earlier region, by PCR. Secondly, we have conducted a systematic study of wild-type worms throughout their life to see if there is a continual increase in the number of dmtDNAs per worm with increasing age. Thirdly, we have asked whether the *age-1* mutant has a lower level of dmtDNAs than does wild-type.

Detection of deletions

We have assessed the frequency of dmtDNAs as a function of chronological age in four different experiments (Table 1). Primers 9a and 9b (Fig. 1) were used to amplify a 6.3 kb mitochondrial amplicon encoding five entire and two partial genes and five tRNAs. Single worms were picked from the aging cohort and lysed, releasing target DNA which was then assessed for the presence of dmtDNA. Three different genotypes were used in these studies and the frequency of dmtDNA was assessed up to 25 days of age, if the worms lived that long. A number of amplification products were detected and many of these were not mitochondrial in origin. We determined the number of true dmtDNAs by Southern blotting of the agarose gels containing putative amplicons of dmtDNA and probing with the 6.3 kb mitochondrial amplicon. In experiments 1 and 2 (Table 1) dmtDNAs were detected with high frequency and in an age-dependent manner. Populations of two wild-type strains were established and over the first week of life 16–36 individuals from each were assessed for the presence of dmtDNA (Fig. 2). A significant increase in the number of detectable deletion events was observed in TJ1061 at 7, 8, 9 and 10 days of age and TJ1060 at 6, 9 and 10 days of age over that seen in 3 day-old controls. An additional experiment on TJ1060 confirmed this increase in the frequency of dmtDNA with age (Table 1). In nine of 13 assays on worms 5 days old or older, we detected a significant increase in the frequency of detectable dmtDNAs. At a few ages the frequency of detected deletions was almost two per worm (Fig. 3C).

We found a number of different dmtDNAs occurring within the 6.3 kb region. More than 10 discrete deletions were detected, ranging in size from ~1.7 to 6 kb, with the smaller deletions being detected more frequently than the larger. Two putative dmtDNAs (SM1 and SM3) were cloned and the putative breakpoints of these deletions were determined by sequence analysis. A direct repeat of 8 bp was found at the putative breakpoints of SM1 but no direct repeat occurred at the putative breakpoint of SM3 (Fig. 4). However, examination of the sequence immediately 3' to the proposed upstream and downstream breakpoints showed 72% similarity (18/25 bp) between these regions (Fig. 4). Due to sequence differences between proposed upstream and down-

Figure 1. Map of the mitochondrial genome amplified by primers 9a and 9b. ND1: subunit 1 of NADH dehydrogenase; ATPase 6: subunit 6 of the F_0 ATPase; ND2: subunit 2 of NADH dehydrogenase; Cyt *b*: cytochrome *b*; COIII: subunit III cytochrome *c* oxidase; ND4: subunit 4 NADH dehydrogenase; COI: subunit 1 cytochrome *c* oxidase. Single letters refer to genes encoding tRNAs: lysine, leucine (UUR), serine, isoleucine, arginine, glutamine, phenylalanine, leucine (CUN) and threonine. The 109 bp region between ND4 and COI has the potential to form a stable hairpin structure (21).

Figure 2. Ethidium bromide-stained agarose gels and autoradiograms of Southern blots of PCRs from individual worms from 3 and 9 day-old nematodes (experiment 1) showing PCR products with homology to the 6.3 kb amplicon. (A) An autoradiogram of individual-worm PCRs from 3 day-old TJ1061 probed with the 6.3 kb amplicon. (B) Autoradiogram of a Southern blot of PCRs from the same cohort in (A) aged to 9 days-old and probed with the 6.3 kb amplicon. (C) The ethidium bromide-stained agarose gel used to make the autoradiogram in (A). (D) The ethidium bromide-stained agarose used to make the autoradiogram shown in (B).

stream breakpoints, we can determine that the DNA from 1971 to 7589 bp was deleted, representing a deletion of 5620 bp. In SM1, a deletion of 5965 bp had occurred but due to the conservation of direct repeats, we are unable to determine which parts of each direct repeat had been excised during the deletion event. The putative upstream breakpoint in SM1 occurred in the coding region of ND1 and the putative downstream breakpoint occurred in the coding region of COI. The upstream breakpoint of SM3 also occurred in the coding region of ND1 and the downstream breakpoint occurred in the coding region of ND4.

Deletions in *age-1* mutants

In a final experiment we assessed dmtDNA in an aging cohort of TJ1062 which contains a mutation in *age-1* (Table 1, experiment 4) and compared this to a control non-Age strain. Assessments were conducted almost daily throughout the life of TJ1062, which is dramatically lengthened by the mutation in *age-1*, and the TJ1060 control strains. There was still an increase in the frequency of dmtDNA with chronological age (Fig. 3). Eight of 18 assessments on worms older than 3 days of age showed statistically significant increases from the frequency seen in 3 day-old worms (Table 1).

We were able to compare the frequency of dmtDNA in populations of *age-1* mutant and wild-type at 13 ages older than 3 days (Table 1, P_{wt}) and found that in 11 of 13 comparisons there were higher frequencies of deletions in the wild-type. Six of these comparisons reached statistical significance (days 5, 6, 9, 10, 13 and 15), while two comparisons showed higher levels of deletions in mutants than in the wild-type at a comparable age (Table 1 and Fig. 3). Overall then, there is a tendency for lower levels of dmtDNAs in *age-1* mutants, but the large amount of variability between days means that the comparisons are not uniformly significant. The overall mean frequency with which deletions were detected was 0.61 ± 0.46 for TJ1061 (experiment 1), 0.44 ± 0.47 for TJ1060 (experiment 2), 1.04 ± 0.59 for TJ1060 (experiment 3), and 0.57 ± 0.29 for TJ1062 (experiment 4) (Table 1).

We asked if dmtDNAs could be detected without amplification in a population of aging *C.elegans*. We therefore prepared total genomic DNA from young and old adults (3 and 10 days) from a synchronously-aging mass culture (TJ1061) and carried out restriction digests on aliquots of this DNA and analyzed it by Southern blotting using amplified mtDNA as probe. No difference between young and old adults could be detected, indicating that rearrangements of the mtDNA were lower than could be detected by Southern blotting (data not shown).

Detection of non-mitochondrial sequences

We observed a dramatic increase in the number of detectable products with increasing age in experiments 1–4 (e.g. a 56-fold increase between 3 and 6 days of age in experiment 2). When these bands were blotted and analyzed after hybridization with labeled 6.3 kb mtDNA, many were found to have no homology to this region of the mitochondrial genome of *C.elegans*. We believe that these do not represent amplification artifacts because they were never detected using young adult worms or larval DNA (used as the positive control). Seven of these products were sequenced and further analyzed.

Two of these products were completely homologous to the *E.coli* insertion sequence 1 element (*IS1*) over the 350 bp from which sequence was obtained (SM2 and SM5). We stripped and reprobbed the Southern blot from 5 days of age in experiment 1 with the *IS1* element and found all 36 worms had a PCR product of ~3 kb in size with homology to *IS1* (data not shown). *IS1* could not be detected by Southern blotting of PCR reactions amplified from *E.coli* which had been taken from the pre-spotted NGM plates and lysed in the same fashion as the worms (data not shown). Additionally, *IS1* could not be detected by Southern blotting of PCR of the positive control DNA. SM6 had homology to a Maize transposon, En-1 (74%). SM7 had homology to a number of cosmids in the physical map of *C.elegans* as well as the *C.elegans* transposon Tc2 and various mitochondrial genes from

Figure 3. Survival curves (●) and mean numbers of dmtDNAs per worm (■) plotted as a function of increasing age. Error bars indicate the standard error of the mean. TJ1061: experiment 1, TJ1060: experiment 2, TJ1060: experiment 3, TJ1062: experiment 4. We determined the mean and maximum survival for the cohorts derived from each population by carrying out standard survivals (13). The mean survivals ($\pm$ SEM) were 10.0 ± 0.4 days for experiment 1, and 10.2 ± 0.3 days, 12.0 ± 0.3 days, 24.4 ± 0.8 days for experiments 2, 3 and 4, respectively. There was detectable heterogeneity between plates for the survival in experiment 4 ($P < 0.05$), and the value here represents the pooled life span data for all three plates. The life span of TJ1062 is 2-fold longer than the control performed at the same time and represents a statistically very significant extension.

Figure 4. Two dmtDNAs within the 6.3 kb amplicon with breakpoints shown. (A) Direct repeats are in bold and the breakpoints indicated by the brackets (). (B) Continuous sequence of the dmtDNA is shown in bold. Identical sequences between the two regions shown are indicated by |. DNA was deleted between 1970 and 7589 bp. Numbering taken from (21).

other species. The remaining two products had no identifiable homology to any sequence in GenBank.

Controls for PCR artifacts

As positive controls, primers 9a and 9b were used to amplify the 6.3 kb amplicon from 1 ng of target genomic DNA derived from L1 larvae. No amplification of any other products were detected in over 30 individual PCRs (data not shown). 'PCR homologous recombination' can occur between two separate sequences which are highly homologous in sequence content (23) in the PCR. To determine if the lysis procedure produced products other than the expected 6.3 kb product through PCR homologous recombination, we carried out serial dilutions of the target genomic DNA, and performed a lysis procedure on these dilutions, followed by the PCR. We detected no products other than the 6.3 kb amplicon on agarose gels (Fig. 5) indicating that PCR homologous recombination was not occurring. *Escherichia coli* (OP50) is the usual food source for *C.elegans* and we wished to rule out *E.coli* as a possible source of artifacts and non-mitochondrial PCR products. We assessed the ability of the PCR to amplify products from various dilutions of *E.coli* which had undergone the lysis

Figure 5. Titration of genomic DNA as target to determine the potential for artefact as a result of the PCR. Lane 1: molecular weight markers; lanes 2–5: PCR reactions varying amounts of target genomic DNA which had undergone the lysis procedure; lane 6: negative control (no target DNA); lane 7: positive control (1 ng of target genomic DNA, no lysis).

procedure. No amplified products were seen in a total of five such assessments (not shown).

DISCUSSION

The frequency of dmtDNAs was shown to increase with chronological age in four different populations of *C.elegans*. Comparison of two isogenic strains, TJ1060 (wild type) and TJ1062 (*age-1*) show that TJ1062 accumulates dmtDNAs at a lower rate than TJ1060. The observations reported here support similar studies in two other species where significant increases in the frequency of dmtDNAs were seen in elderly humans (24,25) and *Maccaca mulatta* (7). Thus, different eukaryotic species widely separated in evolutionary distance show an increase in the frequency of dmtDNA with increasing chronological age. Most importantly, our study shows that the frequency of dmtDNA can be modulated by genetic background.

We sequenced the breakpoints of two of the larger dmtDNAs and identified a direct repeat associated with one breakpoint. There was significant homology in the other dmtDNA between regions upstream and downstream of the putative breakpoint. One dmtDNA we sequenced was similar to dmtDNAs we have previously identified (18) in that a direct repeat was found at the breakpoint. None of the dmtDNAs sequenced here were found in our earlier study, although many more dmtDNAs could be sequenced. Tandem repeats at the site of the deletion has also been found in other species (6,7,26) and the so-called 'common deletion' in humans has a 13 bp direct repeat (10,24,27–29). One proposed mechanism for the generation of dmtDNAs involves direct repeats in a 'slipped mispairing' (9) model of replication and our data supports such a model. We have previously suggested that potential stem-loop structures may be involved in generating dmtDNAs (18). We also have identified a deletion in which a direct repeat was not found at the breakpoint, and this too has precedent in humans (26).

The number of products amplified by the PCR primers increased with chronological age but many of these were not derived from the mitochondrial genome. We cannot explain this increase in non-mitochondrial PCR products as an artifact because we could not reproduce this phenomena using procedures that could be responsible for such artifacts. In Southern experiments we detected no changes between young and old mtDNA. Similar lack of alterations between young and old mtDNA analyzed by Southern blotting have been observed in *D.melanogaster* (30). Attempts to create artifact amplicons from total genomic DNA derived largely from larval populations were unsuccessful as were attempts to amplify PCR products not derived from the mitochondrial genome using *E.coli* as a target.

Analysis of three genetically distinct isogenic populations of *C.elegans* shows an increase in the mean number of deletions in the mitochondrial genome per worm with increasing chronological age. Comparison of two strains, TJ1060 (wild-type) and TJ1062 (*age-1*) which differ by a single gene, show that over the life span TJ1062 accumulates dmtDNAs at a lower rate than TJ1060. This suggests that *age-1* in addition to modulating life span and stress response, also has physiologic consequences on the frequency of detectable dmtDNAs.

The observation that dmtDNAs increase in the nematode *C.elegans* with increasing chronological age is an important corollary to the mitochondrial damage theory of aging. Here, we have not attempted to quantify the increase of any individual dmtDNA across life span, but rather, we addressed the frequency of detectable dmtDNAs which occur within 46% of the mitochondrial genome in populations of aging *C.elegans*. It has often been postulated that any one particular dmtDNA represents 'the tip of the iceberg' (31) and that there are many more dmtDNAs occurring which are undetected due to limitations of the assay. If the 6.3 kb we analyzed is representative of the rest of the genome, there may be more than 20 different species of dmtDNA created within an aging animal. We cannot say whether any one species of dmtDNAs is more prone to accumulate in a particular cell, and our results represent all dmtDNAs detected in whole *C.elegans*. We conclude that the frequency of dmtDNAs increases progressively in *C.elegans*, and that at the mean life span, an individual worm is likely to have accumulated at least one dmtDNA detectable by PCR, at least for the two strains with wild-type life span (TJ1060 and TJ1061). The animal to animal variability in levels of dmtDNA we observed may be due to elimination of cells with high levels of dmtDNAs through cell death. Conversely, such variability may be due to elimination of the dmtDNAs through an as yet unknown mechanism. *age-1* has been shown to be hyper-resistant to paraquat and hydrogen peroxide, as well as having higher levels of Cu/Zn SOD and catalase at older ages. It may be that through the action of *age-1*, ROS are reduced in the cell thereby preventing the accumulation of dmtDNAs. It has previously been hypothesized that damage to the mitochondrial genome will cause an increase in the production of ROS (34), and if ROS are causally implicated in generating dmtDNAs, such action would have the effect of generating further ROS, thereby causing a positive feedback loop.

This study used the largest population of animals of any species ever assessed for the frequency of dmtDNAs as a function of age. We also show for the first time that increased frequency of dmtDNAs with age can be seen in invertebrates. The comparison between a long-lived mutant, which appears to be generally stress-resistant, and wild type shows that dmtDNA are not

generated as frequently in the mutant. Thus, at the very least, dmtDNAs are an interesting biomarker of life span. By utilization of recently published techniques for long-extension PCR (37,38) capable of amplifying the entire mitochondrial genome (39), it should be possible to assess and quantify the complete 'spectrum' of dmtDNA which occur as a result of age.

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