Detection of deletions in the mitochondrial genome of *Caenorhabditis elegans*

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ABSTRACT

We have examined an aging population of *Caenorhabditis elegans* via a PCR assay to determine if deletions in the mitochondrial genome occur in the nematode. We detected eight such deletions, identified the breakpoints of four of these, and discovered direct repeats of 4–8 base pairs at the site of all four deletions. Six of the eight repeats involved in the deletions are located in or immediately adjacent to tRNAs. Without a biochemical bias, the probability of direct repeats being present at all four breakpoints was 4×10^{-6} .

INTRODUCTION

The accumulation of damage to the mitochondrial genome with increasing chronological age has been hypothesized to cause aging (1–3) through the action of free-radicals produced by the respiratory chain (4,5). Such damage may not be repaired with high efficiency because the mitochondrial DNA (mtDNA) has a reduced repair capacity in comparison to the nuclear genome. This may explain the greater rate of accumulation of mutations in the mitochondrial genome when compared to the nuclear genome (6). A recent theoretical model predicts an upper threshold for such mutations above which the mitochondrial population would collapse causing a breakdown in ATP production and hence cell death (7). The ‘mitochondrial theory of aging’ is intuitively appealing but lacks evidence showing biological effects from the accumulation of deletions in the mtDNA.

Recent reports consistent with the mitochondrial theory of aging show an increase in the frequency of deletions in the mitochondrial genome (dmtDNAs) with advancing age. For example, dmtDNAs of several sizes have been demonstrated in an asymptomatic 69-year-old human (8). The excision sites or breakpoints of these dmtDNAs in this individual were associated with direct repeats of between 5 and 13 base pairs (bp) in size. Other studies have also demonstrated an increase in dmtDNAs with advancing age in both human and rat, albeit at frequencies of 0.1% and 0.02%, respectively, relative to the amount of wild-type mtDNA (9–11). DmtDNAs have also been detected at frequencies of 0.46–12% in specific regions of the aged human

brain (12,13). The physiological consequences of these levels of dmtDNA are at present unknown.

Severe physiological consequences of dmtDNAs are observed in several human mitochondrial myopathies. For example, Kearns Sayer (KSS) patients can have levels of dmtDNA approaching 100% in skeletal muscle (14). DmtDNAs of several sizes have been demonstrated in patients suffering from progressive external ophthalmoplegia (PEO) (15). dmtDNAs associated with PEO were identified by both Southern hybridization and the polymerase chain reaction (PCR) and sequence analysis revealed that small direct repeats were associated with the deletions (15). Apart from human and rat, there are no reports in other species of increased dmtDNA as a consequence of increasing age. We have examined small populations from a cohort of aging *Caenorhabditis elegans* to determine if we could detect deletions in the mitochondrial genome as a function of increasing age. We identified eight such deletions and determined the breakpoints of four of these. Short 4–8 base pair direct repeats were observed at all four junctions. In six of the eight potential breakpoints the location was in, or immediately adjacent to, genes coding for tRNAs.

MATERIALS AND METHODS

Nematode strains

All data presented in this report were derived from the N2 Bristol strain of *C.elegans* which has been well characterized both genetically and developmentally (16).

Worms were grown and maintained using methods outlined in Wood (17). Eggs were laid over an eight-hour period on NGM [Nutrient growth media (17)] plates, and then allowed to develop to young adults when they were transferred to small plastic petri dishes containing 3.5 ml survival media (S-basal with *E.coli* 10^9 per ml, 10 μ g cholesterol per ml) according to standard methods (18) at 20°C. The mean lifespan of N2 under these conditions is 17.9 ± 7.5 (SD) days with a maximum lifespan of 41 days (19).

Lysis of whole worms for individual mtDNA PCR

Ten worms were harvested at 7, 13, 18 and 25 days of age for use in the PCR. Individual worms were transferred from the

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liquid survival media to a small NGM plate and then into 20 μ l of lysis buffer [modified from (20)] consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl and 60 μ g ml⁻¹ of proteinase K (Stratagene). Individual worms in lysis buffer were then placed at -70°C for a minimum of 10 min, at 60°C for 1 h, then at 95°C for 15 min. Lysed worm preparations were stored at 2°C till needed. A 1.3 μ l aliquot of the lysate was then used for individual PCR.

PCR

PCR buffer was the standard Cetus buffer—10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂. One unit of AmplitaqTM was used per PCR reaction. PCR was carried out on a Perkin Elmer 9600 thermal cycler. The profile for long-extension PCR was an initial 3 min denaturation at 95°C, followed by 40 cycles of 15 sec denaturation at 94°C, 30 sec annealing at 62°C, and 4 min extension at 72°C, ending with a 10 min final extension at 72°C. Short-extension PCR used an identical profile except that the extension time at 72°C was 1 min. Short-extension PCR did not allow full extension of the 4.3 kb product and hence, preferentially amplified dmtDNA. A two phase hot-start with AmpliwaxTM beads (Perkin Elmer) was used standardly. The following primers were used for both short and long-extension PCR.

Primer 6a (5'-3')—GCA GAC GGA GTA TTT GGA AGG A
[nucleotides 6224–6245 mtDNA (21)]

Primer 6c (5'-3')—ATC ACA AAG GTC GAC ATA TCA A
[nucleotides 10534–10555 mtDNA (21)]

The final concentration of primers 6a and 6c were 0.4 μ M and dNTPs (Pharmacia) were used at final concentrations of 100 μ M. Upon completion of the PCR, reaction products were analyzed by electrophoresis on horizontal 1.2% agarose slab gels at 120 V in 0.5×TBE buffer. Long-extension PCR amplified a 4332 bp amplicon from the mitochondrial genome. Positive and negative controls were amplification of approximately 10⁵ copies of a cloned fragment of the mitochondrial genome which included the 4332 bp region bound by primers 6a and 6c (pBcl-3, a kind gift of Ronald Okimoto) or the absence of target DNA, respectively. Molecular weight markers were used to size reaction products and consisted of *Hind*III-restricted lambda phage DNA (USB) and *Hae*III-restricted pBR322 DNA (Sigma).

Analysis of DNA sequence

Putative dmtDNAs were excised from the gel, DNA extracted by GeneCleanTM (Bio101) and then subcloned into the pCRTM II vector (Stratagene) via a *T*ATM cloning kit (Stratagene) and sequenced by standard methodologies (22) to confirm mitochondrial genome origin and establish deletion breakpoints. Sequence analysis was carried out using MacvectorTM (IBI) running on a Macintosh IICI. The number of pairings of potential deletion endpoints and the probability of these pairings involving a repeat of at least 4 bp were determined using a program written in C and run on a UNIX workstation.

RESULTS

Primers 6a and 6c were used in the PCR to amplify a 4332 bp region of mtDNA from *C. elegans* (Figure 1). No products smaller than the 4.3 kb amplicon were seen in 20 long-extension PCRs using 10³–10⁸ molecules of cloned mtDNA (pBcl-3) as DNA target (data not shown). Furthermore, no products were

Figure 1. Map of region of mitochondrial genome amplified by primers 6a and 6c. This region contains the following genes: cytochrome oxidase subunit I (COI), cytochrome oxidase subunit II (COII), NADH dehydrogenase subunit 4 (ND4), the terminal third of cytochrome oxidase subunit III (COIII), the 5' region of the large-rRNA subunit (l-rRNA), and six mitochondrial tRNAs (T, C, M, D, G, H). The space between ND4 and COI is an intergenic region of 109 bp with the potential to form a stable hairpin structure (21).

Figure 2. Lanes 1–4 are reaction products from worms at 13 days of age. Note the presence of a dmtDNA at 436 bp in lane 1.

seen when pBcl-3 was examined by short-extension PCR. To determine whether dmtDNA could be detected *in vivo*, ten nematodes were harvested at 7, 13, 18 and 25 days of age from a synchronously aging cohort and analyzed by long- and short-extension PCR. In long-extension PCR, a 600 bp amplicon was observed in addition to the expected 4.3 kb amplicon (Figure 2). Sequence analysis of the 600 bp product showed no homology to the mitochondrial genome suggesting that the amplified DNA was nuclear in origin (data not shown) and was consistent with its absence of amplification in the pBcl-3 control. PCRs from individual animals were scored for the presence of products other than the 600 bp artifact and the 4.3 kb amplicon.

No dmtDNAs were detected in the ten worms assayed at 7 days of age. At later ages we detected 8 dmtDNAs in a total of 30 worms (2 dmtDNAs in 10 worms at 13 days of age, 3 dmtDNAs in 10 worms at 18 days of age, and 3 dmtDNAs in 10 worms at 25 days of age). An example of a dmtDNA can be seen at 436 bp in lane 1 in Figure 2, and several additional products can be seen at 13 and 18 days of age in Figure 3. Eight putative dmtDNAs were cloned and DNA sequence from the 5' and 3' end of the cloned dmtDNA was obtained. Comparison of the sequence from the cloned PCR products with that of the mitochondrial genome confirmed that all eight products were mitochondrial in origin. Sequencing revealed the breakpoints in four of these clones. We determined that these contained deletions of 3231, 3600, 3899, and 3898 bp (Figure 4). Direct repeats of 4–8 bp were found at the deletions site of all four dmtDNAs

Figure 3. dmtDNAs amplified by long-extension PCR from individual worms. The full-length PCR product can be seen at 4.3 kb and the artifact at 600 bp. Lane 1 shows PCR reaction products from an 18-day-old worm [* indicates the 731 bp product (b) in Figure 4]. Lane 2 shows 2 dmtDNAs from an 18-day-old worm [* indicates a 1100 bp product, (a) in Figure 4]. Lanes 3 & 4 show reaction products from individual worms aged 13 days of age [* indicates the 432 bp product in lane 3, (c) in Figure 4].

(4a) Size of deletion - 3231 bp. Long-extension PCR product of 1100 bp.

6280 9511
TTTACCGAATTCAT (GTCTTGTGTGGT.....GTAAGGTTTCAT) ACCCTTGA.

(4b) Size of deletion - 3600 bp. Long-extension PCR product of 731 bp.

6483 10083
ATTTAGAATATATAACTTGT (AATTATA.....GTTGATAATCGTTGT) GTTAT.

(4c) Size of deletion - 3899 bp. Long-extension PCR product of 432 bp.

6446 10345
TATTGATCTTACTAAT (ATAGCTATGAT.....TACTATAGAATAAT) TTAGC

(4d) Size of deletion - 3898 bp. Short-extension PCR product of 433 bp.

6463 10361
GCTATGATAGTTTA (ATTTAGAA.....TTAATAGTTTA) TATTAAAA

Figure 4. Nucleotide sequences flanking the breakpoints of cloned dmtDNAs. Size of deletion refers to the number of nucleotides deleted from the mtDNA. Deleted DNA is shown in parenthesis, direct repeats are shown by **bold face**. Additional sequence between the 5' and 3' ends of the deleted DNA is represented by ..|... Sequence numbering is taken from (21).

(Figure 4). One copy of the repeat had been deleted in each product.

We calculated the exact probability that a pair of arbitrarily chosen deletion endpoints share a direct repeat of at least 4 bp. To more closely resemble our observations, we required that each deletion remove at least 3000 bp, although the results were nearly identical without this restriction. The maximum possible deletion size was 4288 bp, the distance between the PCR primers. We counted 832913 pairwise combinations of potential deletion endpoints which could be identified with our PCR primers. This number includes the $(4289 - 3000) + (4289 - 3001) + (4289 - 3002) + \dots + 2 + 1 = 1289 \times 1290 / 2 = 831405$ combinations that are contained within the 4288 bp between the primers. It also includes the 1508 combinations in which the deletion overlaps a PCR primer, but the deletion endpoints occur in direct repeats that regenerate the primer sequence after the deletion event.

Figure 5. Potential stem loop structures in genes encoding the tRNA^{Thr} and tRNA^{His} (27). Direct repeats (4d in Figure 4) are boxed and occur in the corresponding position in the DHU loops of both tRNAs.

Figure 6. Potential stem loop structure in genes encoding the tRNA^{f-met} and tRNA^{Thr} (27, 28). Direct repeats are boxed and occur in deletions 4a and 4b (Figure 4) and are in corresponding positions of the anticodon loops.

For each pairwise combination of direct repeats having a length L , there are $L + 1$ possible deletion events that excise one copy of the repeat and generate an identical sequence (e.g. see Figure 4). Using the *C. elegans* mitochondrial genome we counted 38035 such possible events occurring within direct repeats of at least 4 bp. Then the probability that an arbitrary pairwise combination of deletion endpoints separated by at least 3000 bp involves a direct repeat of at least 4 bp is $38035 / 832913$, or approximately $1/22$. Thus, the probability that all four pairs of sequenced deletion endpoints involve repeats of at least 4 bp purely by chance is $(1/22)^4 = 4 \times 10^{-6}$. Therefore, the observation that all 4 sequenced dmtDNAs possess direct repeats at the site of deletion is not expected by chance.

Of the eight potential breakpoints shown in Figure 4, six occur in, or immediately adjacent to genes encoding tRNAs. In deletion 4d (Figure 4), the direct repeats at each end of the proposed deletion occur in corresponding positions of the DHU loop in the genes encoding tRNA^{Thr} and tRNA^{His} (Figure 5). The potential 4a breakpoint at 9511 bp and the potential 4b breakpoint at 6483 bp (Figure 4) occur in corresponding positions of the anticodon loops in the genes encoding tRNA^{f-met} and tRNA^{Thr} respectively (Figure 6). The breakpoint at 6446 bp in 4c (Figure 4) is 1 bp downstream of the termination codon of the COIII gene and 3 bp upstream of the start of the tRNA^{Thr} gene. The second breakpoint of 4c (10345 bp) is in a similar position relative to the first breakpoint, i.e. 1 bp downstream of the termination codon of the COII gene, and 2 bp before the start of the tRNA^{His} gene. The breakpoint at 6280 bp in deletion 4a in Figure 4, is in the coding region of COIII and the breakpoint at 10083 bp in deletion 4b is in the coding region of COII.

DISCUSSION

We have detected dmtDNAs in an aging cohort of *C.elegans*. This extends the range of species in which mitochondrial deletions have been observed. Although the numbers of dmtDNAs detected in 'young' (7 days of age) and 'old' (greater than 7 days of age) animals is not statistically different, it is intriguing that dmtDNAs occur in old animals but were not detected in young. dmtDNAs in rats and humans is detected primarily in post-mitotic tissues (e.g. the brain and skeletal muscle). Since all somatic cells of adult *C.elegans* are post-mitotic, an accumulation of damaged mitochondrial genomes may be occurring in one or more cell types. Individuals worms vary with respect to the numbers of dmtDNAs detected at any particular age. This variation occurs despite a common environment and genetic homogeneity and implies that the mechanism which gives rise to dmtDNA is stochastic.

Although the mechanism which causes dmtDNAs is not known, it has been suggested that direct repeats are implicated in their generation through recombination or slipped mispairing during replication of the mitochondrial genome (23, 24). For example, the so-called 'common' dmtDNA of KSS which deletes 4977 bp from the human mitochondrial genome is flanked by two direct repeats of 13 bp. We found that all four dmtDNA sequenced possessed direct repeats at the deletion point with one copy of the repeat being excised, presumably during the deletion event. Since the probability of four such events occurring by chance alone is 4×10^{-6} , this suggests a preference for deletions to occur at these repeats.

We detected only two dmtDNAs by short-extension PCR in the 40 animals assayed as compared to six dmtDNAs detected by long-extension PCR. We identified the breakpoints in three of the long-extension PCR dmtDNAs and in one short-extension dmtDNA. Short-extension times favour the detection of large deletions (9), and it may be that such large deletions are comparatively rare. Indeed, 11% of mitochondrial genomes of senescent mice contain deletions or insertions of approximately 400 bp (25) as assessed by electron microscopy, and such small deletions would not have been seen by short-extension PCR. From sequence analysis alone, it is not possible to determine the precise site of excision during a deletion event. However, all four deletion breakpoints that we analyzed, involved a direct repeat in or very near to a tRNA gene. This may implicate potential stem loop structures in the generation of dmtDNAs.

We are extending these studies to larger numbers of individuals at various chronological ages and in different genetic backgrounds to determine if dmtDNAs increase chronologically with age (Melov and Johnson, in preparation). We also hope to identify individual genes which influence the rate and formation of dmtDNAs in *C.elegans*. The availability of long-lived mutants (26) of *C.elegans* will enable us to test whether dmtDNA are causally related to lifespan, a biomarker of senescence, or causally unrelated to the determination of lifespan in these strains.

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