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High Direct Estimate of the Mutation Rate in the Mitochondrial Genome of *Caenorhabditis elegans*

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Mutations in the mitochondrial genome have been implicated in numerous human genetic disorders and offer important data for phylogenetic, forensic, and population genetic studies. Using a long-term series of *Caenorhabditis elegans* mutation accumulation lines, we performed a wide-scale screen for mutations in the mitochondrial genome that revealed a mutation rate that is two orders of magnitude higher than previous indirect estimates, a highly biased mutational spectrum, multiple mutations affecting coding function, as well as mutational hotspots at homopolymeric nucleotide stretches.

Understanding the onset of mitochondrial disease and effective evolutionary analysis require accurate estimates of the rate and pattern of mitochondrial mutation, both of which have been the focus of recent controversy. Phylogenetic estimates of the substitution rate in the control region and protein-coding sequences of the human mitochondrial genome range from 0.02 to 0.26 per site per 10^6 years (My) (*1*, *2*). By contrast, pedigree analyses of the human control region and protein-coding sequences suggest that the substitution rate is much higher: ~2.5 per site per My (*3*, *4*). This discrepancy may be a consequence of mutational hotspots in the control region and/or the mitochondrial disease state of the individuals included in the pedigree analyses (*5*, *6*). Because the rate and pattern of mitochondrial substitution observed over phylogenetic time are a function of both the baseline mutational spectrum and its subsequent modification by natural selection, they likely provide a highly biased view of the rate and pattern of mutation. Unfortunately, almost all of our current estimates are based on indirect arguments and observations that may be biased by the consequences of selection (*7*, *8*).

A direct estimate of the mitochondrial mutation rate and pattern was accomplished by sequencing 10,428 base pairs (bp) of the mitochondrial genomes of 74 *Caenorhabditis elegans* mutation accumulation (MA) lines maintained for an average of 214 generations by single-progeny descent (*9–12*). Each MA line was propagated in a benign environment across generations by a single, random worm. This resulted in an effective population size of each MA line equal to one; hence, the efficiency of natural selection was reduced to a minimum, ensuring that all mutations, except those with extreme effects, accumulated over time in a neutral manner. These lines are known to have undergone a substantial decline in productivity, survival to maturity, generation time, and fitness as a consequence of deleterious-mutation accumulation (*12*).

Among the 74 MA lines, we analyzed 771,672 bp and observed 26 mutations for a total mutation rate equal to 1.6×10^{-7} per site per generation ($\pm 3.1 \times 10^{-8}$), or based on an average generation time of 4 days, 14.3 per site per My (±2.8). Sixteen of these mutations were base substitutions (Table 1), yielding a direct estimate of the mitochondrial mutation rate for base substitutions equal to 9.7×10^{-8} per site per generation ($\pm 2.4 \times 10^{-8}$), or 8.9 per site per My (±2.2). This observed rate is two orders of magnitude higher than the phylogenetic estimates discussed above and exceeds rates derived from pedigree analyses (*1–4*). The 16 base substitutions occurred across unique sites in 15 different MA lines, suggesting that the observed rate of base substitution is a product neither of hotspots nor of lines predisposed to mitochondrial mutation. Furthermore, the observed numbers of lines with 0, 1, and 2 mutations are nearly identical to those expected on the basis of a Poisson distribution (*13*).

Animal mitochondrial DNA (mtDNA) evolution is characterized by a strong bias toward transition (G↔A or T↔C) substitutions, but it has been unclear whether this phenomenon is a consequence of selection or of the baseline mutational spectrum (*14*, *15*). Comparison of two natural isolates of *C. elegans*, N2 (England) and RC301 (Germany), revealed 27 transitions and 2 transversions in mtDNA (*16*). Similarly,

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among the MA lines, 13 transitions and 3 transversions were observed. Therefore, despite the near absence of selection in the MA lines a strong bias toward transitions remains, suggesting that the transition bias observed in phylogenetic comparisons is largely a reflection of the baseline mutation pattern (*17*).

Animal mitochondrial genomes also share a universal strand-specific base compositional bias (*18*). This is reflected in the high frequency of thymine (T = 46%) and low frequency of cytosine (C = 9.3%) in the coding strand of *C. elegans* mtDNA (*9*). One hypothesis suggests that the skewed base composition is maintained by an underlying directional mutational bias (*19*, *20*). A stationary base composition maintained by directional mutation, coupled with a strong transition bias, predicts that an equal number of C→T and T→C transitions should be observed in the MA lines. However, we observed eight T→C mutations and only one C→T mutation, significantly rejecting the hypothesis that the high level of T and low level of C is maintained by directional mutation ($P < 0.05$) (Table 1). Our observations are more consistent with the view that the probability of mutation is proportional to the frequency of the base changing and that selection, rather than the mutational spectrum, is responsible for the universal base compositional bias.

A nonrandom distribution of substitutions with respect to coding function is expected in animal mtDNA evolution. Substitution patterns in the mitochondrial protein-coding genes among natural isolates of *C. elegans* display the typical bias toward synonymous sites (*21*). For example, between N2 and RC301, 26 synonymous substitutions and 3 replacements are observed. By contrast, in the MA lines 9 of the 15 mutations in nonhomopolymeric regions of mitochondrial protein-coding genes alter the amino acid encoded. The significant decrease in the proportion of synonymous mutations ($P < 0.001$) in the MA lines suggests a dominant role for purifying natural selection in the evolution of the mtDNA protein-coding genes in natural populations (*22*). For six of the nine mutations that change the amino acid encoded in the *C. elegans* MA lines, we have determined the same positions in *Caenorhabditis briggsae* (*23*). Five of these six amino acid residues are conserved between the two *Caenorhabditis* species. Seven of the nine positions are conserved between *C. elegans* and the distantly related nematode *Ascaris suum* (Table 1).

In addition to the 16 base substitutions, there are 10 insertion-deletion (indel) mutations among the MA lines (Table 2), 8 of which are associated with homopolymeric nucleotide runs and 1 that occurs in a short repetitive sequence. Five indels occur in a single stretch of 11 adenine (A) residues between the ATPase6 and tRNA^{Lys} genes. The indel mutation rate at this site is 3.2×10^{-4} per generation ($\pm 1.4 \times 10^{-4}$), or 0.029 per year (± 0.013). These observations suggest that simple repetitive sequences and specifically homopolymeric nucleotide runs are hotspots for indel mutations in *C. elegans* mtDNA. It is important to note that the mitochondrial genome of *C. elegans* contains numerous homopolymeric stretches that are both coding and noncoding. As expected in the relative absence of selection, coding homopolymers suffer the same mutational mechanisms as those in noncoding regions (see below). Similar indel mutations in coding homopolymers of the human mitochondrial genome have been associated with disease (*24*).

Four of the indels observed in the MA lines are predicted to drastically alter coding function (*25*). Two of the indels, a single-base deletion in a stretch of eight T residues in the nicotinamide adenine dinucleotide, reduced (NADH) dehydrogenase subunit 1 (ND1) gene in MA96 and a single-base insertion in a run of seven A residues in the NADH dehydrogenase subunit 4 (ND4) gene of MA100, result in premature stop codons. The predicted mutant ND1 and ND4 polypeptides are severely truncated and are missing many highly conserved residues. Although we cannot rule out the presence of wild-type copies in the mutant lines, the mutant versions of ND1 and ND4 were the only sequences detected [Web fig. 1 (*26*)] (*27*).

A third function-disrupting indel involves a 416-bp deletion in the mitochondrial genome of MA80, eliminating the entire tRNA^{Phe} gene, more than one-half of the tRNA^{Gln} gene, and the first 317 bp of the gene for cytochrome b. In wild-type sequences, the region deleted in MA80 is flanked by two homopolymeric A stretches (six and seven As, respectively). A single homopolymer (five As) remains at the site of the deleted sequences in MA80. Polymerase chain reaction (PCR)–based assays with primers flanking the deletion suggest that the mutant form is predominant in this line. However, by using a primer within the deletion, wild-type genomes can be detected in MA80 [Web fig. 2, A and B (*26*)].

A fourth unusual mutation involves the insertion of one C in the aminoacyl acceptor stem region of the tRNA^{Asn} gene in MA35. No stem-disrupting mutations are observed in the tRNA genes among the natural isolates or between *C. elegans* and *C. briggsae*. PCR assays with primers specific to the wild-type and mutant forms suggest that MA35 is fixed for this change [Web fig. 2C (*26*)]. The most likely aminoacyl acceptor stem of this mutant tRNA is predicted to be less thermostable than the wild-type tRNA and may have an adverse effect on both translational efficiency and proper RNA processing in the mitochondrion of this MA line.

When assayed for the level of total fitness as in (*12*), the four lines with mutations expected to be most disruptive with respect to coding function (MA lines 35, 80, 96, and 100) experience a significantly ($P < 0.05$) lower intrinsic rate of increase (0.737 ± 0.164 day^{-1}) than nonmutant MA lines (1.087 ± 0.039 day^{-1}). Although the average selection coefficient against mitochondrial indel mutations is 0.32, further studies will be required to associate fitness changes with specific mitochondrial mutations. Significant fitness reductions in MA lines with amino acid replacements were not detectable at our level of experimental replication, but the conservation of these sites across considerable phylogenetic divergence suggests that they are deleterious enough to be eliminated in natural populations.

The mutation patterns observed in the MA lines of *C. elegans* are similar to those associated with human mitochondrial diseases, including the replacement of highly conserved amino acids, large deletions, and the high incidence of frameshift mutations at coding homopolymer stretches (*24*, *28*). The mitochondrial mutations isolated in this study

Table 1. Base substitutions in mtDNA of mutation accumulation lines. Positions of each mutation are as in the published sequence (*9*). ΔAA indicates a change in the amino acid encoded. For replacements in MA lines, the amino acids found in *C. briggsae* (*Cb*) and *A. suum* (*As*) are given. Sequences not determined in *Cb* are designated ND. Intergenic region is indicated by IG. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; L, Leu; M, Met; S, Ser; T, Thr; and V, Val.

Mutation	Line	Gene	ΔAA	*Cb*	*As*
531 C→T	32	ND6	A→V	A	A
1,453 T→C	91	12*S* rRNA			
2,068 T→C	55	ND1	Silent		
2,386 G→T	14	ND1	E→D	E	E
3,765 A→G	51	ND2	Silent		
5,011 G→A	80	Cyt. b	A→T	A	A
7,255 T→C	79	ND4	Silent		
7,756 T→C	63	IG			
8,355 T→C	55	COI	F→L	ND	F
8,393 T→C	28	COI	Silent		
8,718 T→A	98	COI	C→S	ND	C
9,254 T→C	71	COI	F→S	ND	F
10,042 A→G	81	COII	S→G	S	E
10,158 T→A	42	COII	Silent		
11,731 T→C	27	ND5	M→T	L	V
12,026 G→A	77	ND5	Silent		

Table 2. Indel mutations in mtDNA of MA lines. Positions of each mutation are as in the published sequence (*9*). Indels in homopolymers are numbered according to the first base in the homopolymeric stretch.

Mutation	MA line(s)	Gene(s)
1699 (+1C)	35	tRNA^{N}
2439 (−1T)	96	ND1
3235 (−1A)	2, 9, 14, 49	IG
3235 (+1A)	52	IG
4400 (−416bp)	80	tRNAs^{Q,F}, Cyt. b
6699 (+1A)	100	ND4
11918 (−TTA)	99	ND5

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can serve as models for future studies on the fitness effects of mitochondrial mutations and as models for investigating mitochondrial genetic disorders. Furthermore, the high rate and strongly biased pattern of mtDNA mutation detected here increase the probability of parallel mutations. The high potential for homoplasy must be considered when using mtDNA for evolutionary studies and when investigating the occurrence of recombination in mitochondrial genomes (*29*, *30*).

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10. Amplifications of mtDNA were performed in 50-μl reactions containing 67 mM tris-HCl (pH 8.8), 6.7 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 10 mM β-mercaptoethanol, 1 mM each of dGTP, dATP, dTTP, and dCTP, 0.5 μM of each primer, 10 to 100 ng of genomic DNA, and 2.5 U of *Thermus aquaticus* DNA polymerase (Perkin Elmer). Amplification was carried out by 35 cycles of denaturation at 94°C for 1 min, annealing at 52° to 55°C for 1 min, and extension at 72°C for 2 min. Primer sequences, positions spanned, and PCR product sizes are listed in Web table 1 (*26*). Primers were designed to the published sequence of N2 *C. elegans* mtDNA (*12*). PCR products were separated in a 2% SeaPlaque GTG (FMC Bioproducts) agarose gel. Bands were excised and purified with a QIAquick gel extraction kit (Qiagen). The purified PCR product was used as a template for cycle sequencing with dRhodamine dye terminators. PCR primers were used for sequencing reactions along with internal primers where necessary. Cycle sequencing was carried out by 25 cycles of denaturation at 96°C for 30 s, annealing at 50°C for 15 s, and extension at 60°C for 4 min. Unincorporated dye terminators were removed with Micro Bio-Spin Chromatography Columns (Bio-Rad). Sequences were determined with an ABI Prism 377 automated DNA sequencer (Perkin-Elmer). Sequences were aligned to the published N2 mtDNA sequence (*9*) with the ESEE computer alignment program [E. Cabot, Eyeball Sequence Editor (ESEE), Version 3.01 (1997)]. Two mutations were detected in the N2 progenitor of the MA lines, relative to the published sequence (5011 G→A, 8429 A→G). The first mutation is at a nonsynonymous site, replacing alanine with valine. The second is at a synonymous site. All substitution and indel mutations were confirmed by sequences from both strands of DNA.
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23. We have sequenced 8554 bp of mtDNA from the PB800 strain of *C. briggsae* as in (*10*). The PB800 strain is available from the CGC.
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27. For GeneScan analysis the region was amplified with the primer ND1-B(F) and the flourescently labeled primer ND1-D(R)-HEX. Amplifications were performed as in (*10*). Samples were separated on an ABI 377 Automated Sequencer (Perkin-Elmer) with TAMRA 500 internal size standards.
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Resetting of Circadian Time in Peripheral Tissues by Glucocorticoid Signaling

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In mammals, circadian oscillators reside not only in the suprachiasmatic nucleus of the brain, which harbors the central pacemaker, but also in most peripheral tissues. Here, we show that the glucocorticoid hormone analog dexamethasone induces circadian gene expression in cultured rat-1 fibroblasts and transiently changes the phase of circadian gene expression in liver, kidney, and heart. However, dexamethasone does not affect cyclic gene expression in neurons of the suprachiasmatic nucleus. This enabled us to establish an apparent phase-shift response curve specifically for peripheral clocks in intact animals. In contrast to the central clock, circadian oscillators in peripheral tissues appear to remain responsive to phase resetting throughout the day.

Daily rhythms in gene expression, physiology, and behavior persist under constant conditions and must, therefore, be driven by self-sustained biological oscillators called circadian clocks [for reviews, see (*1*, *2*)]. Circadian clocks can count time only approximately and must be adjusted every day by the photoperiod in order to be in harmony with the outside world. In mammals, light signals perceived by the retina are transmitted directly to the suprachiasmatic nucleus (SCN) via the retino-hypothalamic tract (*3*). The SCN, located in the ventral part of the hypothalamus, is thought to contain the master pacemaker, which synchronizes all overt rhythms in physiology and behavior (*4*).

In most systems, circadian oscillations rely on a negative feedback loop in gene expression that involves multiple clock genes. In *Drosophila*, the repertoire of essential clock genes includes *period* (*per*), *timeless* (*tim*), *clock* (*clk*), *cycle* (*cyc*), *doubletime* (*dbt*), *cryptochrome* (*cry*), and *vrille* (*vrl*) (*1*, *5*). During the past few years, one or more mammalian homologs to all of these genes have been uncovered. These include *Per1*, *Per2*, and *Per3*, *Tim*, *Clock*, *Bmal1*, *Tau*, *Cry1* and *Cry2*, and *E4bp4* (*1*, *5*, *6*).

Molecular oscillators may exist in most pe-

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