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Mitochondrial Respiratory Chain Deficiency in *Caenorhabditis elegans* Results in Developmental Arrest and Increased Life Span*

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The growth and development of *Caenorhabditis elegans* are energy-dependent and rely on the mitochondrial respiratory chain (MRC) as the major source of ATP. The MRC is composed of ~70 nuclear and 12 mitochondrial gene products. Complexes I and V are multisubunit proteins of the MRC. The *nuo-1* gene encodes the NADH- and FMN-binding subunit of complex I, the NADH-ubiquinone oxidoreductase. The *atp-2* gene encodes the active-site subunit of complex V, the ATP synthase. The *nuo-1(ua1)* and *atp-2(ua2)* mutations are both lethal. They result in developmental arrest at the third larval stage (L3), arrest of gonad development at the second larval stage (L2), and impaired mobility, pharyngeal pumping, and defecation. Surprisingly, the *nuo-1* and *atp-2* mutations significantly lengthen the life spans of the arrested animals. When MRC biogenesis is blocked by chloramphenicol or doxycycline (inhibitors of mitochondrial translation), a quantitative and homogeneous developmental arrest as L3 larvae also results. The common phenotype induced by the mutations and drugs suggests that the L3-to-L4 transition may involve an energy-sensing developmental checkpoint. Since ~200 gene products are needed for MRC assembly and mtDNA replication, transcription, and translation, we predict that L3 arrest will be characteristic of mutations in these genes.

The human mitochondrial respiratory chain (MRC)[1] is composed of >80 subunits, but requires >100 additional genes for its assembly (1). These additional genes encode the import apparatus for transporting polypeptides into the organelle, chaperones, and other assembly factors, as well as the machinery needed to replicate, transcribe, and translate the 13 MRC subunits encoded by mtDNA. The MRC generates the majority of cellular ATP. Thus, the loss of one member of this large class of genes can compromise the entire pathway of energy production. The consequences of such events are diverse and debilitating (2–4). The metabolism and structure of the *Caenorhabditis elegans* MRC closely parallel its mammalian counterpart (5). Moreover, the nematode mtDNA is similar in size and gene content to the human mtDNA (6).

The MRC is organized into five multisubunit proteins. Complexes I–IV form the electron transport chain, which couples the oxidation of NADH and succinate to the reduction of oxygen and the formation of a proton gradient. Complex I (NADH-ubiquinone oxidoreductase) is an elaborate enzyme that catalyzes the transfer of electrons from NADH to ubiquinone. Bovine complex I consists of ~43 subunits, an FMN cofactor, and up to eight iron-sulfur clusters (7, 8). The FMN- and NADH-binding sites, as well as one iron-sulfur cluster, are considered to reside in the 51-kDa subunit. One predicted *C. elegans* gene product (C09H10.3) bears strong sequence resemblance (75% identical amino acids) to the human gene. We have designated the gene encoding the *C. elegans* NADH-ubiquinone oxidoreductase 51-kDa subunit as *nuo-1*. Complex I deficiency is one of the most frequently encountered mitochondrial defects (9–11). Mutations in the *NDUFV1* gene, which encodes the human 51-kDa subunit of complex I, result in myoclonic epilepsy and leukodystrophy (12).

Complex V (the ATP synthase) uses the proton gradient to catalyze the synthesis of ATP from ADP and inorganic phosphate (13). A selective deficiency of the human mitochondrial ATP synthase caused by an uncharacterized nuclear mutation results in heart failure (14). The ATP synthase is composed of a membrane-intrinsic proton channel (F_0) and a peripheral catalytic domain (F_1). The F_1 domain comprises five types of subunits forming an $\alpha_3\beta_3\gamma\delta\epsilon$ complex. Three catalytic nucleotide-binding sites are associated primarily with the β-subunits (15). The *C. elegans* gene product C34E10.6 shares 80% identical amino acids with the human β-subunit gene. We have designated this gene as *atp-2*.

The contribution of the mitochondrial genome to cellular energy metabolism and organismal development is an area of active research (16–18). In humans, mtDNA mutations produce a heterogeneous group of disorders (19). Depletion of mtDNA, which also impairs MRC biogenesis, produces an autosomal recessive disorder resulting in myopathy or hepatopathy and early death (19). Mice heteroplasmic for a large deletion in their mtDNA demonstrate mitochondrial dysfunction in various tissues and die of renal failure (20).

In this work, we present the isolation and characterization of two lethal mutations in the nuclear *nuo-1* and *atp-2* genes, encoding subunits of complexes I and V, respectively. The mutants are phenotypically similar, being developmentally impaired and arresting in the third larval stage. Gonad development is even more severely affected, arresting in the second

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[1] The abbreviations used are: MRC, mitochondrial respiratory chain; L, larval stage; PCR, polymerase chain reaction; kb, kilobase pair(s); RNAi, RNA interference.

larval stage. Despite the developmental arrest, mutant animals have extended life spans. Mutant development to L3 larvae requires the contributions of maternally inherited protein and mRNA. The mutations also interfere with the proper expression of the dauer developmental pathway.

Chloramphenicol and doxycycline are specific inhibitors of mitochondrial protein synthesis and have been used to inhibit the synthesis of essential mtDNA-encoded subunits of the MRC (21, 22). The phenotypes of the *nuo-1* and *atp-2* mutants can be mimicked when the expression of the mitochondrial genome is prevented with these inhibitors. The common larval arrest induced by the mutations and drugs suggests that the L3-to-L4 transition may involve an energy-sensing developmental checkpoint. Our results suggest that an intact MRC is a prerequisite for normal development in the nematode. Assembly of a functional MRC requires ~200 genes or 1% of the genome content, and the *nuo-1* and *atp-2* mutations define the first two essential members of this large class of genes (23).

EXPERIMENTAL PROCEDURES

Strains—Worms were cultured as described (24). The following strains or mutations were used: N2 (Bristol) wild-type strain; CB404, *unc-53(e404)*; DR2078, *mIn1[dpy-10(e128) mIs14]/bli-2(e768) unc-4(e120)*II *(mIs14* containing the *myo-2* and *pes-10* promoters and a gut enhancer fused individually to the green fluorescent protein coding sequence is integrated into *mIn1[dpy-10]*); SP127, *unc-4(e120)/mnC1[dpy-10(e128) unc-52(e444)]*II; MT1580, *dpy-10(e128) unc-53(n569)*II; SP471, *dpy-17(e164) unc-32(e189)*III; BC986, *sDp3*(III;f), *eT1*(III;V); and JK1122, *dpy-17(e164) unc-32(e189)/qC1 dpy-19(e1259) glp-1(q339)*III. The following strains were isolated or constructed: LB1, *nuo-1(ua1)* (the original unbalanced isolate); LB4, *nuo-1(ua1)/unc-53(e404)*; LB10, *nuo-1(ua1)/mnC1[dpy-10(e128) unc-52(e444)]*; LB21B, *nuo-1(ua1)/mIn1[dpy-10(e128) mIs14]*; LB100, *atp-2(ua2)* (the original unbalanced isolate); LB121, *dpy-17(e164)/atp-2(ua2) unc-32(e189)*; LB127, *atp-2(ua2) sDp3*(III;f); LB128, *atp-2(ua2) unc-32(e189)/qC1-[dpy-19(e1259) glp-1(q339)]*.

Isolation of Mutants and Genetic Analysis—Chemical mutagenesis was used to generate a library of deletion mutants that could be screened by PCR in a process called target-selected gene inactivation (25). A library of ethyl methanesulfonate-mutagenized N2 (synchronized and treated as L4 larvae, 50 mM for 4 h) was constructed by transferring ~1000 F_1 animals to each of 135 plates (26). After the plates had cleared, the worms were washed off and divided into three portions, two for freezing and one for DNA isolation (27). We visualized deletions using pairs of nested primers chosen to be ~3 kb apart, using duplicate or triplicate reactions with standard PCR conditions (27). The primers used were as follows. For *nuo-1*: NFP1, 5′-TGCCTGAAAAGCCATACTGTC-3′; NFP2, 5′-GTAAACGACACTCCGAAATTCC-3′; NFP3, 5′-CTCATTCATTCAGCGAGGTTG-3′; and NFP4, 5′-AGATCTTCTACGGACAAGGTG-3′. For *atp-2*: F1B1, 5′-CTGAATTCTGCACTGCAATCAC-3′; F1B2, 5′-ACGTTCAGCCTGAACTGAGAC-3′; F1B3, 5′-CCGATGTGTATTCTAGAGTTCG-3′; and F1B4, 5′-AGAACCAGAAGACATCTGTTCC-3′. One positive address was found for each gene, and the authenticity of the PCR product was determined by DNA sequencing. Three rounds of sibling selection were required to derive heterozygous clonal lines containing either the *nuo-1* or *atp-2* deletions. We took advantage of the fact that *nuo-1* maps at 3.10 map units on linkage group II, tightly linked to *unc-53*, which maps at 3.07 map units, to create LB4 (*nuo-1(ua1)/unc-53(e404)*) by outcrossing five times against CB404. The *nuo-1(ua1)* mutation was also balanced by crossing LB4 with SP127 to generate LB10 and by crossing LB10 with DR2078 to generate LB21B. For *atp-2*, we used the fact that *atp-2* maps at −2.00 map units on linkage group III, tightly linked to *dpy-17* (−2.16 map units) and *unc-32* (0.00 map units), to create LB121 (*dpy-17(e164)/atp-2(ua2) unc-32(e189)*) by outcrossing six times against SP471 and *dpy-17(e164)*. The *atp-2(ua2)* mutation was balanced by crossing LB121 with JK1122 to generate LB128. The *atp-2(ua2)* mutation was also outcrossed six times and balanced by crossing LB100 with BC986 and *unc-36(e251)* to generate LB127. cDNA clones for *nuo-1* and *atp-2* were obtained from Y. Kohara. The *nuo-1* and *atp-2* cDNA sequences were determined on yk389c4 and yk63b5, respectively.

Electrophoresis and Western Blot Analyses—100 synchronized L3 worms or 300 embryos laid over a 12-h period were picked, lysed, and subjected to 12% SDS-polyacrylamide gel electrophoresis. Proteins were transferred electrophoretically to a polyvinylidene difluoride membrane. The blot was treated with a rabbit polyclonal antiserum raised against *Saccharomyces cerevisiae* ATP2p (28). Detection was with a peroxidase-labeled goat anti-rabbit secondary antibody and the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Baie-d'Urfé, Québec, Canada).

Microscopy—Animals were mounted on 2% agarose pads and observed under a Zeiss Axioskop-2 research microscope with a SPOT-2 digital camera (Carl Zeiss Canada Ltd., Calgary, Canada).

Life Span Measurements—N2, LB21B, or LB128 gravid adults were allowed to lay eggs for 6 h at 25 °C. Once hatched, groups of five wild-type or homozygous *nuo-1* or *atp-2* progeny were transferred onto single nematode growth medium plates. Animals were monitored daily and were scored as dead when they no longer responded with movement to light prodding on the head. Wild-type animals were transferred daily during egg laying to keep them separate from their progeny.

Swimming, Pharyngeal Pumping, and Defecation—Animals were placed in drops of M9 buffer, and the simple rhythmic thrashing (swimming) of the animals was counted. The animals were scored for 1 min, and each animal was scored twice. Pharyngeal pumping and defecation were measured on seeded plates, and each animal was scored twice. Pumping was scored for 1 min. The time interval between two consecutive defecation muscle contractions was measured.

RNA Interference—For *nuo-1*, a 2.9-kb PCR product, amplified using N2 genomic DNA as a template with primers NFP2 and NFP4, was digested with *Sac*I and *Sac*II. The 0.8-kb *Sac*I/*Sac*II fragment was cloned into likewise digested pBluescript II SK and pBC-KS (both from PDI BioScience, Aurora, Ontario, Canada) to place the gene under the control of the T7 promoter in both the forward and reverse orientations. For *atp-2*, a 2.9-kb PCR product amplified with primers F1B2 and F1B4 was digested with *Xho*I and *Hin*dIII. The 1.1-kb *Xho*I/*Hin*dIII fragment was cloned into likewise digested pBluescript II SK and pBC-KS. The resulting constructs were transformed into a bacterial strain (BL21(DE3), PDI BioScience) expressing the T7 RNA polymerase gene from an inducible *lac* promoter and were grown either in the absence or presence of 1 mM isopropyl-β-D-thiogalactopyranoside overnight at 37 °C. As a control, empty pBluescript II SK and pBC-KS vectors were also transformed into BL21(DE3). N2 L4 hermaphrodites were fed with the bacteria, and their progeny were analyzed. All other *C. elegans* genes have a <60% nucleotide sequence identity over 200 nucleotides to the *atp-2* and *nuo-1* regions we used for our RNA interference (RNAi) studies, making it unlikely any cross-interference is responsible for the observed phenotypes (29).

Entry into and Exit from the Dauer Pathway—LB21B or LB127 was grown on single nematode growth medium plates. 7 days after the plates had cleared, SDS was added at a final concentration of 1% and gently stirred for 30 min. SDS-resistant animals were picked onto fresh plates at 15 °C to allow for recovery from dauer.

Exposure to Inhibitors—Chloramphenicol or 3-doxycycline was added to seeded nematode growth medium plates (24), and synchronized animals were placed directly onto the plates. All experiments were performed at room temperature (23 °C) unless otherwise stated.

RESULTS

Isolation of nuo-1 and atp-2 Mutants—We identified by sequence comparison two genes (designated *nuo-1* and *atp-2*) that encode the *C. elegans* homologs of critical mammalian MRC subunits. We employed target-selected mutagenesis to produce deletion mutations in both genes (25, 27). We prepared genomic DNA from an ethyl methanesulfonate-mutagenized library of animals and screened with nested oligonucleotide primers by PCR amplification of *nuo-1* and *atp-2* sequences.

For the *nuo-1* gene, we found one allele (designated *ua1*) in which a 1190-base pair sequence (nucleotides 26259–27448 in GenBank™ accession number Z50109) is replaced with a single A nucleotide (Fig. 1*A*). The deletion completely removes exons 1–3 as well as the beginning of exon 4. Exon 3 contains the sequences proposed to mediate NADH and FMN binding; thus, we believe that the *ua1* deletion is a null allele. In *Neurospora crassa,* loss of the 51-kDa subunit results in the complete loss of complex I activity (30). The intron/exon structure predicted by the GENEFINDER program was confirmed by sequence analysis of the yk389c4 cDNA (23). The protein is predicted to contain 479 amino acids and have a mass of 52,422 Da. The first 24 amino acids are predicted to be removed upon

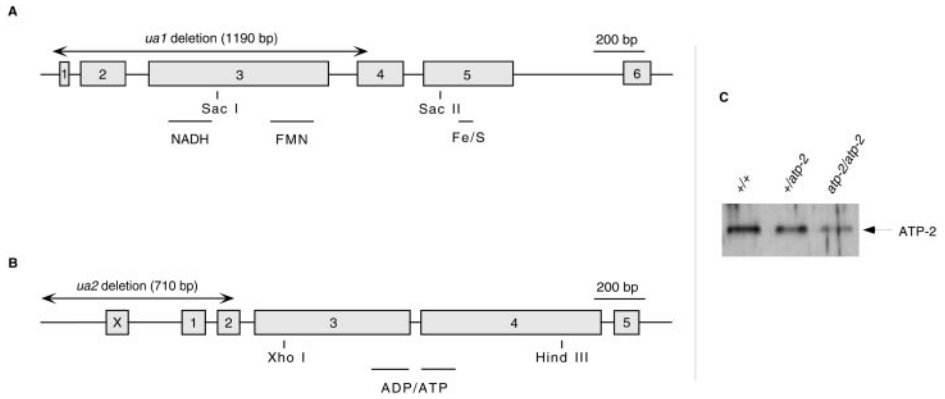

FIG. 1. *A*, the *nuo-1* gene structure. Exons are drawn as *rectangles*. Predicted cofactor- or substrate-binding regions and relevant restriction enzyme sites are indicated below the exons. *Fe/S*, iron-sulfur cluster. *B*, the *atp-2* gene structure. The exon labeled *X* is a computer prediction; it is not transcribed (see "Results"). Both the *ua1* and *ua2* deletions were confirmed by sequencing. *C*, a Western blot showing the amount of ATP-2 protein in 100 synchronized wild-type (+/+), heterozygous (+/*atp-2*), and homozygous (*atp-2*/*atp-2*) L3 animals (*arrow*). There is an ~2-fold difference in the ATP-2 level between +/+ and +/*atp-2* and a 4-fold difference between +/+ and *atp-2*/*atp-2*. *bp*, base pairs.

import into mitochondria, leaving a mature subunit of 50,076 Da (31).

For the *atp-2* gene, we isolated one allele designated *ua2*, a 710-base pair deletion (nucleotides 947–1656 in GenBank™ accession number U10402) (Fig. 1*B*). The *atp-2* intron/exon structure was determined by sequence analysis of the yk63b5 cDNA. The first exon predicted by the GENEFINDER program (labeled *X* in Fig. 1*B*) is not present in the cDNA (23). Protein sequence corresponding to this first predicted exon is not present in any of the many ATP-2 homologs in the data base (data not shown). Translated exon 1 sequence aligns with the amino termini of other β-subunits and includes a possible initiator methionine at nucleotides 12–14. Furthermore, exon 1 (but not exon X) is transcribed as judged by the >25 expressed sequence tags in the GenBank™/EBI Data Bank. The *atp-2* gene, containing five exons, encodes a 538-amino acid protein with a predicted mass of 57,490 Da. The predicted mitochondrial targeting sequence is 72 amino acids long, leaving a mature subunit of 50,247 Da (31). The *ua2* deletion completely removes exon 1 plus 6 nucleotides of exon 2; 27 residues of the mitochondrial targeting sequence are deleted. Western blot analysis using a cross-reacting antiserum directed against the yeast ATP2p subunit did not detect a truncated *C. elegans* ATP-2, suggesting that the protein either is not expressed from the *ua2* allele or is unstable (Fig. 1*C*). The *atp-2(ua2)* phenotype (L3 arrest; see below) was not affected by placing the *ua2* allele in *trans* to a deficiency (*atp-2(ua2)*/*sDf121)*. This genetic evidence and the absence of detectable truncated protein led us to believe that the *ua2* allele is a null mutation.

Phenotypes of the nuo-1 and atp-2 Mutants—The complete *C. elegans* life cycle takes ~3 days at 25 °C (24). Following fertilization, the embryo undergoes cell proliferation, organogenesis, and morphogenesis, culminating in the hatching of the L1 larva after ~14 h (32). Over the next 2 days, development proceeds through three additional larval stages, L2, L3, and L4, until the adult emerges from the final molt. The gonad consists of four cells at hatching (33). During L2, the hermaphrodite gonad extends anteriorly and posteriorly, led by distal tip cells (34).

The *nuo-1(ua1)* and *atp-2(ua2)* mutations are recessive alleles that exhibit typical mendelian patterns of inheritance. Both resulted in the developmental arrest of homozygotes in the third larval stage. Gonad development was more severely affected, however, and was arrested in the second larval stage. Gonad development up to L2 proceeded normally (Fig. 2, *A*, *C*, and *E*). 6 h later in wild-type animals, the distal tip cells had migrated anteriorly and posteriorly, and the two gonad arms had extended considerably along the ventral surface (Fig. 2*B*). This migration of the distal tip cells did not proceed in *nuo-1* and *atp-2* animals, and the gonad primordium contained between 6 and 18 nuclei. This suggests that germ-line proliferation was also impaired (Fig. 2, *D* and *F*). In addition, mutant animals were somewhat smaller than the wild-type animals (Fig. 2, *D* and *F*). Over several days, the gonad arms partially extended, and the animals developed an L3-sized body. Surprisingly, the L3-arrested *nuo-1* and *atp-2* larvae had life spans significantly longer than those of the wild-type animals (Table I).

We reasoned that an impaired mitochondrial respiratory chain might lead to mobility or sensory defects due to the normally high rates of respiration in muscle and nerve cells. When placed in liquid medium, both the *nuo-1* and *atp-2* animals swam more slowly than wild-type animals (Table II). After being arrested for 2 days, their swimming rates were even further decreased. Pharyngeal pumping was irregular, and the rates were severely decreased in the mutants (Table II). The defecation cycle was also slowed (Table II). We also tested whether *nuo-1* animals could sense an osmotic gradient of sodium chloride (35). Mutant animals could sense and avoid the salt barrier (data not shown). Furthermore, the fractions of wild-type, *nuo-1*, or *atp-2* animals found away from a localized bacterial food source did not differ (data not shown). These observations indicate that the ability of the mutants to sense environmental stimuli was not abolished.

Maternal nuo-1 and atp-2 Expression Rescues Embryonic Arrest—RNAi with double-stranded RNA is a potent sequence-specific mechanism for eliciting mRNA degradation and inhibiting gene expression in *C. elegans* (36, 37). Simply feeding worms *Escherichia coli* expressing double-stranded RNA can result in interference (37, 38). We engineered two bacterial strains to express both sense and antisense RNAs, one by cloning a genomic *nuo-1* fragment encoding most of exons 3–5 and the other by cloning a genomic *atp-2* fragment encoding most of exons 3 and 4. Sense and antisense RNAs were produced from T7 promoters on two separate plasmids bearing different antibiotic markers. With a bacterial strain containing empty vectors as a control, the addition of isopropyl-β-D-thiogalactopyranoside to induce the expression of the T7 RNA polymerase had no effect on N2 worms (Fig. 3*A*). However, isopropyl-β-D-thiogalactopyranoside-induced transcription of the *nuo-1*- and *atp-2*-containing plasmids produced double-stranded RNA and led to the degradation of the maternal *nuo-1* and *atp-2* mRNAs; this resulted in ~10 and 65% embryonic arrest, respectively (Fig. 3*A*). Western blot analysis of the ar-

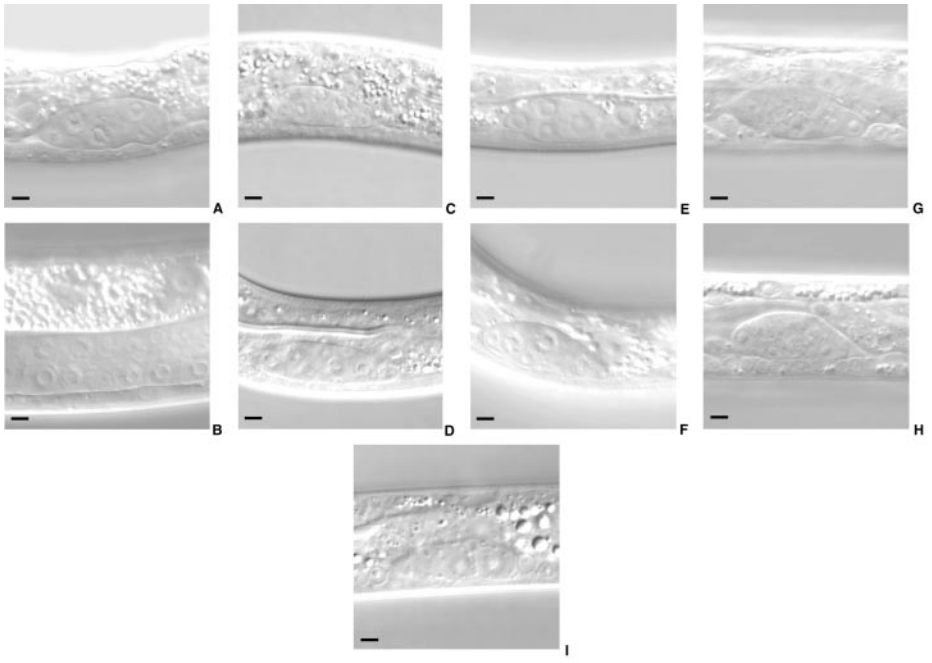

FIG. 2. **Nomarski photographs of developing gonads in control, *nuo-1*, *atp*-2, and drug-treated animals cultured at 25 °C.** *A*, N2, 27 h; *B*, N2, 33 h; *C*, *nuo-1*, 27 h; *D*, *nuo-1*, 33 h; *E*, *atp-2*, 27 h; *F*, *atp-2*, 33 h; *G*, chloramphenicol-treated N2, 27 h; *H*, chloramphenicol-treated N2, 33 h; *I*, doxycycline-treated N2, 150 h. *Bars* = 3 μm.

TABLE I
nuo-1 and atp-2 mutants have extended life spans

Genotype	Life span	Significance (p value)[a]
	mean days ± S.D.	
+/+	11.9 ± 2.7 (n = 27)	
nuo-1/nuo-1	14.8 ± 3.3 (n = 24)	<0.01
atp-2/atp-2	13.6 ± 2.6 (n = 31)	<0.05

[a] Based on a two-tailed Student's t test to +/+.

rested embryos confirmed that the double-stranded RNA reduced ATP-2 levels by ~2-fold (Fig. 3*B*). The majority (80–90%) of *nuo-1(RNAi)* and *atp-2(RNAi)* embryos arrested after gastrulation with very little tissue differentiation (Fig. 3*C*). A small fraction of the embryos (10–20%) arrested earlier, with the earliest arresting at the one-cell stage (Fig. 3*C*). The RNAi phenotype in each case was more severe than its mutant allele, suggesting that *nuo-1(+)* or *atp-2(+)* expression in the heterozygote hermaphrodite contributes to the survival of the corresponding homozygous mutant offspring. Thus, interference with maternal expression prevents or reduces the inheritance of *nuo-1*- or *atp-2*-derived protein by the embryo. We suggest that the maternal contribution of protein and mRNA allows the *nuo-1* and *atp-2* offspring to develop beyond the embryo stage before arresting. In agreement with this, we have detected full-length ATP-2 protein of maternal origin in the *atp-2* homozygotes; it was present at approximately one-quarter the level in the wild-type animals (Fig. 1*C*).

atp-2 (but Not nuo-1) Animals Are Dauer-defective—Entrance and exit from the dauer larval stage are developmental responses to chemosensory signals of food availability (39). Dauer larvae are resistant to treatment with 1% SDS (40). When starved LB127 animals were subjected to an SDS treatment, only *atp-2/atp-2 sDp3* animals were recovered, indicating that a wild-type *atp-2* gene, present on the sDp3 balancer, is required for dauer formation (Table III). To rule out the possibility that homozygous *atp-2* dauers are sensitive to the SDS treatment, we introduced either *daf-2(e1370)* or *daf-7(e1000)* alleles into the *atp-2* mutant background. Dauers were picked and subjected to genotype analysis by PCR. None of the >90 animals tested in this way had lost the sDp3 balancer (data not shown). Therefore, *atp-2* animals are dauer-defective.

When starved LB21B animals were subjected to a detergent treatment, both +/*nuo-1* and *nuo-1/nuo-1* were recovered following the treatment (Table IV). Homozygous *nuo-1* dauers are easily identified because they do not contain any copies of the *mIn1* balancer and thus do not express green fluorescent protein. We did not recover SDS-resistant animals homozygous for the balancer; these animals did not form recognizable dauers upon starvation and were not resistant to the detergent treatment. The *nuo-1* dauers were morphologically similar to the heterozygote dauers. We observed that only heterozygous animals could recover from dauer and develop into L4 larvae when placed at 15 °C in the presence of food (Table IV). Thus, *nuo-1* homozygotes are competent to enter dauer, but cannot exit from it. The ability of *nuo-1* mutants to enter dauer demonstrates that not only are these animals physically the size of L3 larvae, but that they are also developmentally L3 larvae.

mtDNA Expression Is Necessary for Larval Development—A functional nematode MRC contains 12 subunits encoded by the mitochondrial genome. To mimic the effects of mutations in these mitochondrial subunits, we examined the effects of inhibiting mitochondrial translation by exposing N2 gravid adults to either chloramphenicol or doxycycline. In each case, the fraction of progeny animals that failed to develop to adults increased with increasing inhibitor concentration (Fig. 4). At 1.25 mg/ml chloramphenicol and 60 μg/ml doxycycline, >96% of the progeny developed through two larval stages and remained arrested as L3 larvae throughout their entire lives. Animals exposed to high concentrations of doxycycline (≥60 μg/ml) also arrested as L3 larvae, although they showed a considerable extension of the time needed to develop from L1 to L3 (>4 days for doxycycline as compared with 1–2 days for chloramphenicol). This suggests that doxycycline may have additional effects on worm metabolism unrelated to its effects on mitochondrial translation.

We monitored animal development in the presence and absence of doxycycline or chloramphenicol by differential interference contrast microscopy. Gonad development was severely impaired by both drugs. Up to 24 h after egg laying, chloramphenicol-treated animals were not readily distinguishable from control animals; both had typical L2-sized gonads (Fig. 2, *A* and *G*). 6 h later, untreated animals developed into L3 larvae, and their gonads showed substantial anterior and posterior elonga-

TABLE II
nuo-1 and atp-2 mutations impair swimming, pumping, and defecation

Genotype (stage)	Swimming[a]	Pharyngeal pumping[a]	Defecation[a]
	cycles/min	*contractions/min*	*s/cycle*
+/+ (adult)	112 ± 9 (n = 10)	258 ± 14 (n = 20)	56 ± 5 (n = 20)
+/+ (L3)	116 ± 7 (n = 20)	274 ± 17 (n = 20)	53 ± 8 (n = 20)
nuo-1/nuo-1 (L3; arrested 1 day)	81 ± 12 (n = 16)	97 ± 6 (n = 20)	187 ± 40 (n = 20)
nuo-1/nuo-1 (L3; arrested 2 days)	51 ± 19 (n = 6)	ND[b]	ND
atp-2/atp-2 (L3; arrested 1 day)	85 ± 7 (n = 20)	86 ± 10 (n = 20)	220 ± 39 (n = 20)
atp-2/atp-2 (L3; arrested 2 days)	51 ± 10 (n = 20)	ND	ND

[a] Values are means ± S.D.
[b] Not determined.

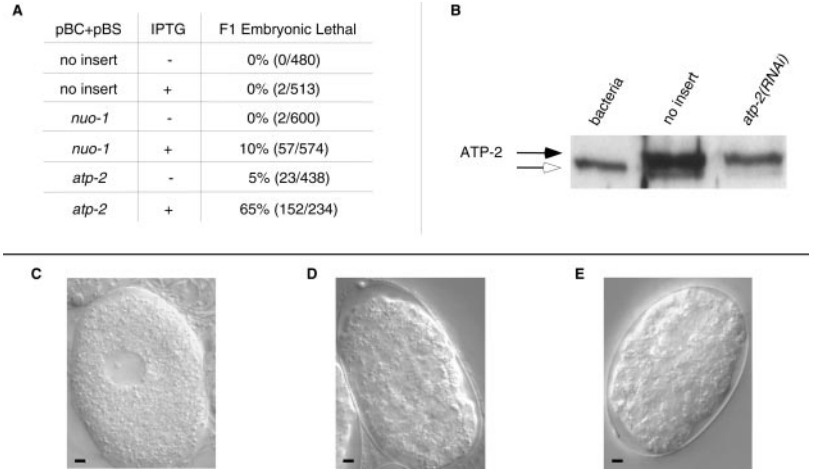

FIG. 3. **RNA interference and Nomarski photographs of arrested embryos.** *A*, embryonic lethality was scored at least 48 h after egg laying and expressed as a percentage of the total number of progeny. *B*, shown is a Western blot indicating the position of the full-length ATP-2 protein in control (*no insert*) and *atp-2(RNAi)* embryos (*closed arrow*). The first lane (*bacteria*) contains only BL21(DE3) bacteria. A lower band corresponding to the bacterial ATP-2 protein can be seen in all three lanes (*open arrow*). RNAi-mediated embryonic arrest occurred at the one-cell stage, *atp-2(RNAi)* (*C*), and at the comma stage, *atp-2(RNAi)* (*D*) and *nuo-1(RNAi)* (*E*). These embryos were photographed 7–9 h after egg laying. *Bars* = 3 μm. *IPTG*, isopropyl-β-D-thiogalactopyranoside.

TABLE III
atp-2 mutants are defective in entering into the dauer stage

	No. of animals scored	
	atp-2/atp-2 sDp3	*atp-2/atp-2*
Before 1% SDS treatment	38	54
After 1% SDS treatment	125	0
Recovery	123/125	NA[a]

[a] Not applicable.

TABLE IV
nuo-1 mutants are defective in exiting the dauer stage

	No. of animals scored		
	+/+	+/*nuo-1*	*nuo-1/nuo-1*
After 1% SDS treatment	0[a]	274	73
Recovery	NA[b]	253/253	0/73

[a] +/+ animals do not form recognizable dauers and are not resistant to 1% SDS treatment.
[b] Not applicable.

tion with a large increase in the number of germ cell nuclei (Fig. 2*B*). In contrast, chloramphenicol-treated animals were slightly smaller in size than control animals, and their gonads remained unchanged from the 24-h point (Fig. 2*H*). With time, chloramphenicol-treated animals developed L3-sized bodies, but their gonads remained arrested at an L2-like stage (data not shown). L2-sized gonads were also observed in doxycycline-treated animals, although the time required to reach the point of arrest was extended (Fig. 2*I*). Thus, the effects of doxycycline or chloramphenicol on development are tissue-specific and may

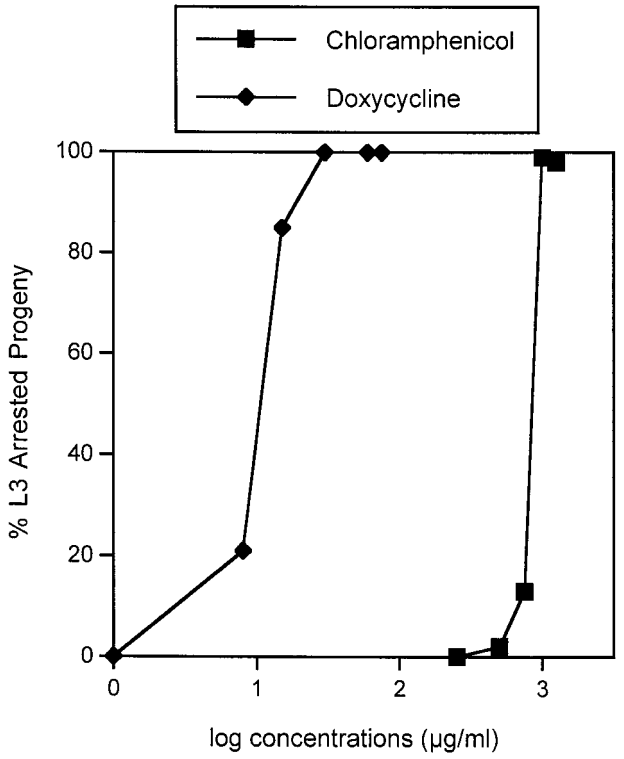

FIG. 4. **Exposure of N2 gravid adults to chloramphenicol and doxycycline.** The ratio of L3-arrested progeny to the total number of progeny scored (>89 for chloramphenicol and >120 for doxycycline) is expressed as a percentage. ■, chloramphenicol; ◆, doxycycline.

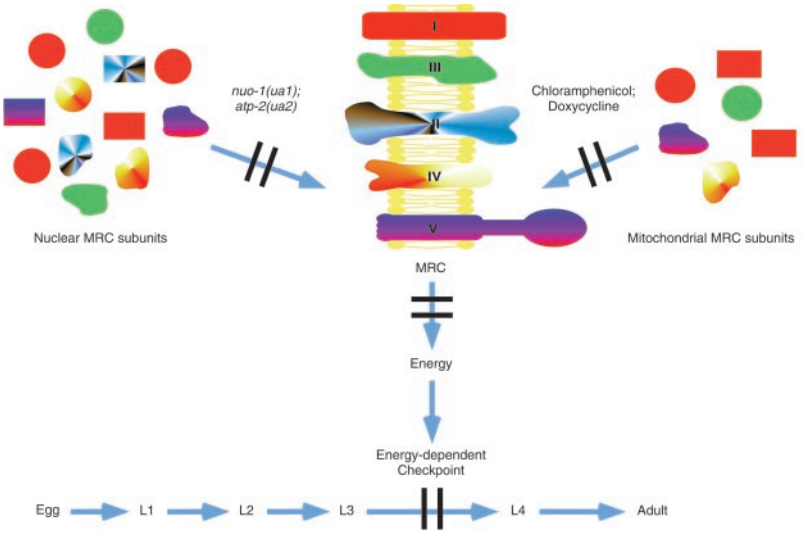

FIG. 5. **Model depicting roles of nuclear and mitochondrial genomes in *C. elegans* development.** Proper development requires energy production primarily by oxidative phosphorylation via the MRC. Both genomes contribute MRC protein subunits that assemble to form a functional MRC. Mutations or inhibitors affecting the production of these subunits lead to an impaired MRC with insufficient energy production. The transition from L3 to L4 is likely a large energy-demanding step in development and may involve an energy-sensing regulatory mechanism.

reflect high energy requirements in the gonad or a higher sensitivity of this tissue to these drugs.

DISCUSSION

A functional MRC is essential for nematode viability. Mutations in the *nuo-1* and *atp-2* genes, which encode MRC subunits of complexes I and V, respectively, both result in L3 arrest and lethality. Complexes I and V are physically and enzymatically distinct, yet participate in the common process of generating ATP via oxidative phosphorylation. The similar phenotypes of the *nuo-1* and *atp-2* mutants suggest that it is the loss of the energy-generating pathway that results in lethality characterized by L3 arrest (Fig. 5). Therefore, we propose that in addition to the nuclear encoded MRC subunits, the mitochondrial MRC subunits, the components of the mitochondrial import apparatus, the chaperones, and assembly factors, and the mitochondrial replication, transcription, and translation machinery will also be essential by virtue of their roles in MRC biogenesis and energy generation. We estimate there are ~200 genes or 1% of the genome involved in MRC biogenesis, and mutations in many of these may produce L3 arrest. In support of this generalization, we have generated L3-arrested animals by completely independent approaches. When mtDNA expression was impaired with doxycycline or chloramphenicol, wild-type animals also arrested as L3 larvae that phenotypically resembled the *atp-2* and *nuo-1* mutants. This was not unexpected since complexes I and III–V contain subunits encoded by the mitochondrial genome. The drug-arrested animals also had L2-sized gonads like the nuclear mutants. Thus, the *nuo-1* and *atp-2* mutations define the first two member genes of the large class of essential genes required for the assembly of a functional MRC in the nematode.

The *nuo-1* and *atp-2* mutations are not the first respiratory chain mutations isolated in *C. elegans*, but they are the first null alleles. A *mev-1(kn1)* mutation is a missense mutation in the cytochrome *b* subunit of complex II (41). It does not completely abolish complex II activity and manifests itself in oxygen hypersensitivity and premature aging. The *gas-1(fc21)* mutation is a missense mutation in the 49-kDa subunit of complex I that confers anesthetic hypersensitivity (42) and impairs activity of the complex (43).

Why are the *nuo-1* and *atp-2* mutations not lethal during embryogenesis? The RNAi and Western blot experiments suggest that a complement of maternal protein and mRNA is inherited through the oocyte and that it is sufficient to support the energy requirements of early development. Through Western blot analysis, we showed that maternally contributed wild-type ATP-2 is present in the *atp-2* mutant, although at reduced levels (Fig. 1*C*). Using microarray analysis, significant levels of *nuo-1* and *atp-2* transcripts have been detected in and presumably inherited through oocytes (44). We believe the inherited *atp-2* mRNA and ATP-2 protein support the development of the mutant to L3 and its survival until senescence. These results imply that low ATP-2 levels are sufficient to support many basic cellular and organismal processes, but that higher levels are required for energy-intensive processes such as development and reproduction. Similarly, a maternal contribution of metabolic potential is seen in other organisms. *Tfam* knockout mice, which are deficient in mitochondrial transcription factor A, cannot replicate mitochondrial DNA, but embryos can still proceed through the implantation and gastrulation stages of development (17). Likewise, the loss of mouse cytochrome *c* causes embryonic lethality marked by a severe developmental delay and reduced size (45), yet the cytochrome *c*-deficient embryos do complete early developmental steps.

Recently, the genomic analysis of chromosome I by RNAi revealed that interference with genes encoding subunits of complexes I and III–V of the MRC produces embryonic lethality (46). Similarly, RNAi with the *atp-2* gene or with the *mev-1* gene, which encodes the cytochrome subunit of complex II, slows the pace of embryonic development and produces embryonic lethality (47). In fact, many genes of intermediary metabolism are essential for embryonic viability (46, 47).

Aerobic metabolism, as measured by oxygen consumption, peaks at L3 and L4 (48). Furthermore, it has been suggested that reproduction requires a considerable elevation of metabolic activity (48). Thus, we hypothesize that germ-line proliferation and organogenesis, which are associated with sexual maturation during L4, are energy-intensive processes and that the transition from L3 to L4 is associated with increased energy demands. The effects of the *nuo-1* and *atp-2* mutations on gonad development support the notion that a substantial energy requirement is necessary for gonad development, even early in life. We have observed significant increases in ATP-2 protein levels and in mtDNA copy number[2] associated with the transition from L3 to L4.

Animals with impaired mitochondrial energy metabolism due to the *nuo-1* or *atp-2* mutation or to exposure to chloramphenicol or doxycycline all appear to arrest at a common, precise point in development. The inhibitors and mutations have different mitochondrial targets, but all are expected to impair

[2] W. Y. Tsang and B. D. Lemire, submitted for publication.

energy generation by the MRC. By extension, all conditions that sufficiently impair MRC function are predicted to induce L3 arrest. We believe that the L3-to-L4 transition may involve an energy-sensing mechanism that invokes a developmental checkpoint in the mutants or the drug-treated animals when an energy deficit arises (Fig. 5). Recently, it was demonstrated that NAD is required for the genomic silencing that leads to increased longevity in response to calorie restriction (49). Since NAD is a central metabolic molecule, it is possible that by interfering with the MRC-mediated oxidation of NADH, the *atp-2* and *nuo-1* mutations result in altered transcription of genes that regulate development. Further work will be required to determine whether or not the connection between mitochondrial function and development is mediated through NAD.

Caloric restriction lengthens the life span of a variety of animals, including nematodes, perhaps by reducing energy production and decreasing metabolic rates (50). In *C. elegans*, the *eat* mutations produce defects in pharyngeal pumping that lead to a reduction in food uptake, a starved appearance, and an extended life span (50, 51). We suggest that the extended life spans of the *nuo-1* and *atp-2* mutants may be a consequence of caloric restriction due to reduced pharyngeal pumping rates. We have noticed that many older arrested animals have empty guts and appear starved (data not shown).

Human mitochondrial diseases are characterized by a diversity of phenotypes, many of which are tissue-specific in their effects (52, 53). The *nuo-1(ua1)* and *atp-2(ua2)* homozygous mutants arrested at the third larval stage, with their gonads showing an earlier L2 arrest. These mutants moved more slowly and were less energetic. Their rates of pharyngeal pumping were decreased, and the defecation cycle was extended. All of these features may be indicative of problems with muscular and/or neuronal function; both of these tissues are highly dependent on mitochondrial respiration. The *nuo-1* and *atp-2* mutations reproduce some of the features of mitochondrial diseases, suggesting that *C. elegans* may be a good model system for studying mitochondrial disorders (54, 55).

In conclusion, the loss of MRC function via mutations in key subunits of mitochondrial complex I or V or via the inhibition of mitochondrial translation is a lethal event that produces a specific developmental block. In the future, it should be possible to identify suppressor mutations in the putative checkpoint components that allow development to proceed past L3. Studies along these lines will provide insights into the molecular mechanisms underlying the arrest and may aid in the development of novel protocols for treating mitochondrial diseases.

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