Biochemical and Biophysical Research Communications **291,** 8–16 (2002)
doi:10.1006/bbrc.2002.6394, available online at http://www.idealibrary.com on IDEAL®

Mitochondrial Genome Content Is Regulated during Nematode Development

William Y. Tsang and Bernard D. Lemire[1]
Canadian Institutes of Health Research Group in the Molecular Biology of Membrane Proteins, Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

Received January 7, 2002

Growth and development rely on the mitochondrial respiratory chain (MRC) as the major source of ATP. We measured the mitochondrial DNA (mtDNA) copy number of each of the *Caenorhabditis elegans* developmental stages. Embryos, L1, L2, and L3 larvae all have ~25,000 copies of maternally derived mtDNA. The copy number increases fivefold in L4 larvae and a further sixfold in adult hermaphrodites, but only twofold in adult males. The majority of mtDNA in adult worms is germline associated, and germline-deficient mutants show markedly reduced mtDNA contents. With sperm-deficient or oocyte-deficient mutants, we confirm that mtDNA amplification is primarily associated with oocyte production. When mtDNA replication is inhibited, a quantitative and homogeneous arrest as L3 larvae occurs. Thus, mtDNA amplification is a necessary component of normal development and its regulation may involve an energy-sensing decision or checkpoint that can be invoked when mitochondrial energy generation is impaired.

Key Words: **mitochondria; ethidium bromide; mitochondrial DNA; larval arrest; germline development; *glp-1; glp-4; fem-3; fem-1.***

The mitochondrial respiratory chain (MRC) is the major source of ATP for most cell types. It is composed of five multisubunit protein complexes containing subunits encoded by both the mitochondrial and the nuclear genomes. The human mitochondrial DNA (mtDNA), a small circular molecule that encodes 13 MRC subunits, is transcribed and translated independently of the nuclear genome. It is present in 10^3 to 10^4 copies per cell (1) and is maternally inherited, being transmitted through the egg cytoplasm (2). The *Caenorhabditis elegans* mtDNA, which encodes 12 MRC subunits, is similar in size and gene content to its human counterpart (3).

The mtDNA encoded MRC subunits are essential for the proper function of the MRC protein complexes. Expression of those subunits requires that the mitochondrial genome be replicated, transcribed, and translated (4). The newly synthesized subunits are subsequently assembled with nuclear encoded subunits that have been imported from the cytoplasm. Assembly of a functional MRC necessitates over 200 nuclear genes, making MRC biogenesis a complex proposition that requires ~1% of the *C. elegans* gene content (5).

The role of the mitochondrial genome in cellular energy metabolism and in organismal development is under active investigation (6–8). A large and heterogeneous group of disorders is associated with mutations in the human mtDNA. Depletion of the mitochondrial genome produces an autosomal recessive disorder resulting in myopathy or hepatopathy and early death (9). Ethidium bromide (EtBr), a DNA-intercalating dye, is a potent inhibitor of mtDNA transcription and replication in mammalian, avian, and yeast cells (10–12). The depletion (13) or elimination (11) of mtDNA with EtBr have been used to investigate the genetic function of the mitochondrial genome and to produce cells that are defective for mitochondrial respiration and ATP synthesis.

As a step toward a better understanding of the importance of the MRC in organismal development, we examined the genetic and developmental roles of mtDNA in *C. elegans*. We report that the *C. elegans* mtDNA copy numbers show two increases during normal hermaphrodite development: a fivefold copy number increase from the L3 to the L4 stage, and a further sixfold increase from the L4 stage to the adult. Adult males have fewer mitochondrial genomes, with approximately one-third the number of an adult hermaphrodite. Thus, mtDNA copy number is coordinated with the maturation steps of the life cycle. As revealed by germline-deficient mutants, it is the germline that con-

Abbreviations used: MRC, mitochondrial respiratory chain; mtDNA, mitochondrial DNA; EtBr, ethidium bromide; PCR, polymerase chain reaction.

[1] To whom correspondence and reprint requests should be addressed. Fax: (780) 492-0886. E-mail: bernard.lemire@ualberta.ca.

0006-291X/02 $35.00

TABLE 1

Life Span Is Extended by Ethidium Bromide

Strain	Life span (days)	Std. Dev. (days)	Number of animals
N2	11.9	1.6	30
EtBr-N2	13.8*	2.0	26

* $P < 0.01$ based on a two-tailed Student's t test to +/+.

tains the majority of organellar genomes and only a small component is linked to somatic development. With sperm- (*fem-3*) or oocyte-deficient (*fem-1*) mutants, we determined that oocytes contain substantially more mtDNA molecules than sperm. The oocytes have a copy number similar to that of embryos and early larvae suggesting that maternally-derived mtDNA transmitted to the oocyte is sufficient to support development through the early larval stages. Taken together, we conclude that the *glp-1, glp-4, fem-3,* and *fem-1* genes, which control germline development (14, 15) are upstream of the control of mtDNA copy number.

mtDNA amplification is a necessary component of the normal developmental program; when mtDNA replication is blocked, development is arrested. Embryos exposed to EtBr develop through the L1 and the L2 larval stages and arrest as L3-like larvae. The arrested animals are slightly smaller than normal L3 larvae and their gonad development does not proceed beyond the L2 stage. Surprisingly, the arrested animals have longer life spans than untreated animals. The L3 arrest phenotype is reversible upon drug removal. The transition from L3 to L4, which entails an increase in mtDNA copy number, is a particularly critical step in development that may involve an energy-sensing decision or checkpoint. When mitochondrial function is impaired, the checkpoint is invoked.

MATERIALS AND METHODS

Strains. Worms were cultured as described (16). The following strains were used: N2, wild type (Bristol); CB1489, *him-8(e1489);* SS104, *glp-4(bn2);* JK1107, *glp-1(q224);* JK509, *glp-1(q231);* JK816, *fem-3(q20);* BA17, *fem-1(hc17);* and LB128, *atp-2(ua2) unc-32(e189)/qC1*[*dpy-19(e1259) glp-1(q339)*]. The Dpy Glp progeny of LB128 were used for the determination of the *glp-1(q339)* mtDNA copy number (Table 1). SS104 (*glp-4(bn2)*), JK1107 (*glp-1(q224)*) and JK509 (*glp-1(q231)*) are temperature-sensitive germline deficient strains. JK816 (*fem-3(q20)*) and BA17 (*fem-1(hc17)*) are temperature-sensitive oocyte-deficient and sperm-deficient strains, respectively.

Determination of mtDNA copy number. A quantitative PCR assay was set up that relates the initial number of template copies to the number of cycles at which exponential amplification of the PCR products first occurs (17). The standard curve was obtained as follows. N2 worm lysates were prepared by incubating worms in lysis buffer (50 mM KC1, 10 mM Tris–HCl, pH 8.3, 2.5 mM $MgCl_2$, 0.45% Nonidet P-40, 0.45% Tween 20, 0.01% gelatin, 100 μg/ml freshly added proteinase K) for 1 h at 50°C. This was followed by 10 min incubation at 95°C to inactivate the proteinase. PCR was performed to amplify a 1064-bp region of the mitochondrial genome, using the lysate as the source of template DNA and the mtDNA primers mtDNA1, 5′-CTTTTATTACTCTATATGAGCGTC-3′ and mtDNA5, 5′-GTAAATTCAACCATTCCACAAGG-3′. The PCR product was purified with the QIAquick PCR purification kit (Qiagen Inc., Mississauga, Ontario, Canada) and the concentration of the purified template was determined with a Sequoia-Turner 450 fluorometer. Increasing amounts of template ($10–10^8$ copies per reaction) were amplified in a DeltaCycler II (Ericomp, San Diego, CA), using a 10 μl reaction volume. PCR consisted of a single denaturation step (3 min at 92°C) followed by cycles of 15 sec denaturation at 92°C, 30 s annealing at 61°C, and 30 s elongation at 72°C. Depending on the initial amount of template DNA, tubes were removed at cycle numbers from 5 to 34 cycles. The entire contents of each reaction was subjected to 1% agarose gel electrophoresis, stained with EtBr, and the 1064-bp product was quantified with a Gel Doc 1000 system (Bio-Rad, Hercules, CA). Nonspecific products were not detected, except primer-dimers that were present in some samples (these were not quantified). The cycle number at which exponential amplification of PCR products first occurred was determined by plotting the logarithm of the fluorescence intensity versus the cycle number. The standard curve was constructed by plotting the cycle number versus the logarithm of the initial template copy number. The standard curve is linear between initial template copy numbers of 10^2 and 10^8. Quantitative PCR was performed for each initial template copy number at least 3 times and the results were reproducible. For sample analysis, 20 synchronized animals or oocytes (30 μl total volume) were lysed in a lysis buffer and the lysate was subjected to quantitative PCR (1 μl lysate per 10 μl reaction). Embryos were treated briefly with 20 mg/ml chitinase (Sigma-Aldrich, Oakville, Ontario, Canada) before lysis. To confirm the reliability and reproducibility of the PCR, we also established a standard curve for nuclear DNA copy number. Quantitative PCR was performed on lysates containing known numbers of worms from which the input nuclear DNA copy numbers can be calculated. In all cases, the input nuclear DNA copies and the measured copies agree closely, suggesting that nuclear DNA is quantitatively released during lysis.

Exposure to EtBr. EtBr was added at a final concentration of 125 μg/ml of agar to seeded NGM plates (16) and synchronized animals were placed directly onto the plates. All experiments were performed at room temperature (23°C) unless otherwise stated.

Measurement of life span. Gravid N2 hermaphrodites were allowed to lay eggs for 6 h in the presence or absence of EtBr at 25°C. Groups of five progeny animals were transferred to separate plates after hatching. Animals were monitored daily and were scored as dead when they no longer responded to light prodding on the head. Animals not exposed to EtBr were transferred daily during egg laying to keep them separate from their progeny.

Microscopy. Animals were mounted on 2% agarose pads and observed under a Zeiss Axioskop-2 research microscope (Carl Zeiss Canada Ltd., Calgary, Alberta) with a SPOT2 digital camera.

RESULTS

mtDNA Copy Number Varies with Developmental Stage

We determined the mtDNA copy number of each developmental stage of the wild type hermaphrodites using quantitative PCR. About 14 h after fertilization, an egg hatches as a first stage larva or L1 (16). Over the next 2 days, it will grow quickly through a series of four molts to become an adult. There are ~25,000 copies of mtDNA per embryo and that number remains essentially unchanged up to the L3 stage (Fig. 1). A

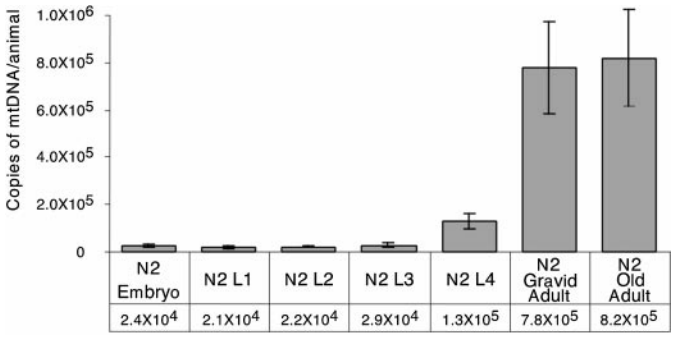

FIG. 1. The mtDNA copy numbers of the *C. elegans* developmental stages. Values represent the mtDNA copy numbers per animal averaged over 20 synchronized animals. At least three replicates were performed for each developmental stage.

roughly five-fold increase in copy number occurs with the transition from L3 to L4 (from ~25,000 to 130,000) and a further six-fold increase with the L4 larva to the adult transition (from 130,000 to ~800,000). The increases in mtDNA copy number per larva are not simply due to an increase in somatic cell number. For example, the hermaphrodite L1 larva has 558 somatic nuclei while the adult has 959 (18); this 1.7-fold change in the number of somatic nuclei is associated with an over 30-fold increase in mtDNA content. The replication of the organellar genome is not coupled to nuclear DNA replication, which is an active process during early development.

mtDNA Copy Number of Males

We measured the mtDNA copy numbers of males, produced from a strain with the *him-8* mutation. A wild type strain generates males at a frequency of 0.1–0.2% due to rare but spontaneous meiotic nondisjunction events; animals carrying a *him-8* mutation produce males at a high frequency (over 30%). The *him-8* mutation does not affect the mtDNA copy number; *him-8* L4 or adult hermaphrodite copy numbers are not significantly different from the wild type (Fig. 2). Interestingly, whereas *him-8* males and hermaphrodites have similar mtDNA contents at the L4 stage, adult males have about one-third the number of mtDNA copies (260,000) compared to adult hermaphrodites (800,000) (Fig. 2).

mtDNA Copy Number Is Affected in Germline-Deficient Strains

We postulated that the large increase in mtDNA copy number measured after the L3 stage is linked to reproduction. We measured the germline-related component of the mtDNA copy number increases in several germline proliferation deficient (*glp*) strains. The GLP-1 protein is related to the Notch family of transmembrane receptor proteins and controls germ cell mitotic division (19). Loss of function *glp-1* mutations result in the production of 4–8 germline cells compared to ~1,500 in wild type animals while the somatic gonad is morphologically normal (15). Wild type L4 larvae have 130,000 copies of mtDNA (Fig. 1) compared to the 67,000 copies in the *glp-1(q339*) mutant (Fig. 3). That number remains relatively unchanged as the *glp-1* animals mature (Fig. 3). At the permissive temperature of 15°C, the two temperature-sensitive *glp-1* mutants, *q224* and *q231*, have copy numbers similar to the wild type L4 (Fig. 3). The *glp-1* adult copy numbers increase, although they do not reach the levels of the wild type adult, suggesting that the temperature-sensitive alleles are hypomorphs even at 15°C (Fig. 3). At the restrictive temperature of 25°C, the mutants have only 60,000–70,000 copies as L4 larvae (Fig. 3) and even fewer copies as adults. The reason for the drop in mtDNA content of the adults is not known, but an active degradation of organellar genomes is suggested.

We also examined the effects of another germline proliferation deficient mutation, *glp-4* on mtDNA levels. The function of the *glp-4* gene product is not known at the molecular level. The *glp-4(bn2)* mutation limits the production of germline cells to ~12, while the somatic gonad retains a wild type morphology (14). The *glp-4(bn2)* mutant mtDNA copy number profile is similar to the *glp-1(q224)* and *glp-1(q231)* profiles (Fig. 3). The *glp-1* and *glp-4* results indicate that the increase in mtDNA copy number from the wild type L3 to the adult has two components. The first component is somatic (*glp-1* and *glp-4* independent) and occurs between the L3 and L4 stages. It accounts for a 3-fold increase (from ~25,000 in the wild type L3 to ~70,000 in germline-deficient L4 larvae). The second component is germline related, depends on *glp-1* and *glp-4* function, and occurs between the L3 and adult stages. It accounts for an over 10-fold increase (from ~70,000

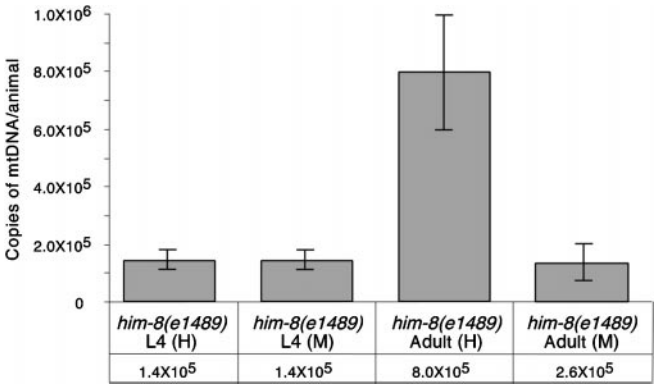

FIG. 2. The mtDNA copy numbers of a *him-8* mutant. Values represent the mtDNA copy numbers per animal averaged over 20 synchronized animals. At least three replicates were performed for each developmental stage. (H) and (M) indicate hermaphrodite and male, respectively.

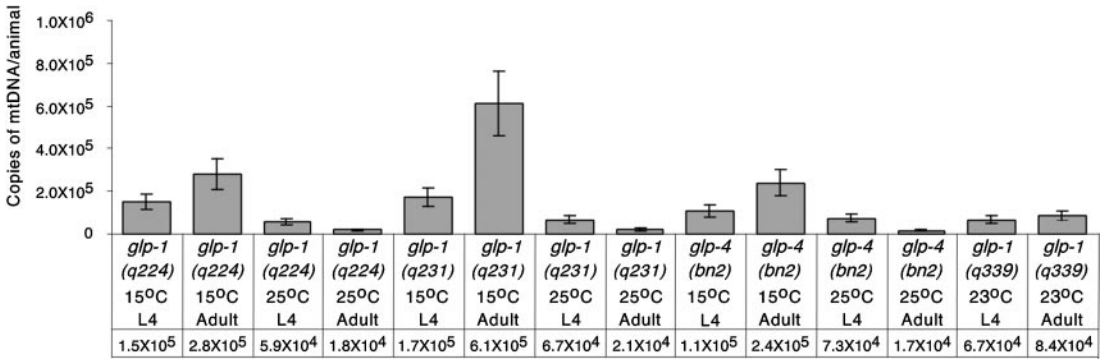

FIG. 3. The mtDNA copy numbers of *glp-1* and *glp-4* mutants. Values represent the mtDNA copy numbers per animal averaged over 20 synchronized animals. At least three replicates were performed for each developmental stage. The *glp-1(q339)* mutation is not temperature-sensitive.

copies measured in germline-deficient L4 larvae to ~800,000 in wild type adults).

mtDNA Copy Numbers of Sperm- and Oocyte-Deficient Strains

We also measured the mtDNA copy numbers of temperature-sensitive oocyte-deficient (*fem-3*) and sperm-deficient (*fem-1*) animals. The FEM proteins are required for spermatogenesis in both males and hermaphrodites (19). Loss of function *fem-1* mutations transform hermaphrodites into females with no sperm production (20). In contrast, gain of function *fem-3* mutations masculinize the hermaphrodite germ line, resulting in a vast excess of sperm produced and no oocytes (21). At the restrictive temperature of 25°C, *fem-3* animals only produce sperm, while *fem-1* animals only produce oocytes. Both strains are essentially wild type at the permissive temperature of 15°C and not surprisingly, the L4 and the adult mtDNA copy numbers of *fem-3* and of *fem-1* hermaphrodites at 15°C are similar to those of wild type (Fig. 4). At 25°C, *fem-3* and *fem-1* L4 larvae have copy numbers comparable to the wild type L4 (Fig. 4). However, we observed a large difference in mtDNA copy numbers between adult *fem-3* and *fem-1* hermaphrodites at 25°C. *fem-3* mtDNA copy numbers do not increase much between the L4 and adult stages (from 170,000 to 190,000; Fig. 4). With *fem-1* animals, the copy number increases approximately sixfold as it does in the wild type (from 140,000 to 800,000; Fig. 4). These results suggest that oocyte rather than sperm production accounts for the majority of the mtDNA copy number increase from L4 to adult. To confirm this, we measured the mtDNA copy number of *fem-1* oocytes from hermaphrodites raised at 25°C and found it to be roughly 18,000 copies per oocyte (Fig. 4).

mtDNA Expression Is Necessary for Larval Development

We examined the effects of preventing the amplification of mtDNA by inhibiting transcription and replication with EtBr. Single gravid N2 hermaphrodites were transferred onto plates containing increasing concentrations of EtBr and the development of their progeny was examined after 2–3 days. As the inhibitor concentration increases, the fraction of the brood that fails to develop to adulthood increases (Fig. 5). Above 83 μg/ml EtBr, all embryos hatched, developed through

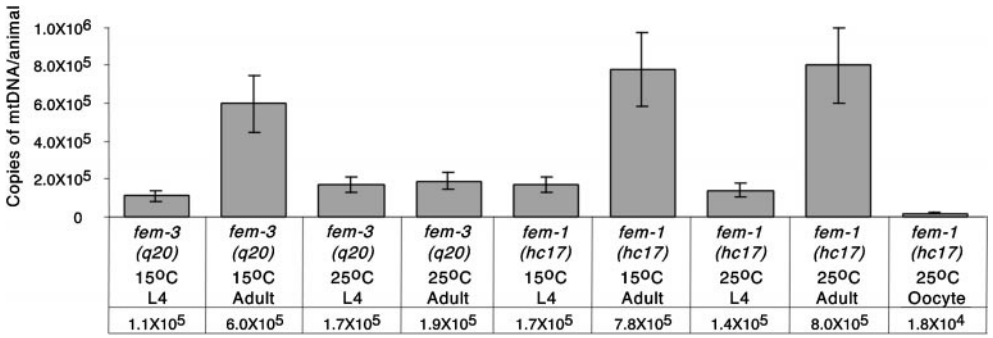

FIG. 4. The mtDNA copy numbers of *fem-3* and *fem-1* mutants. Values represent the mtDNA copy numbers per animal averaged over 20 synchronized animals. At least three replicates were performed for each developmental stage.

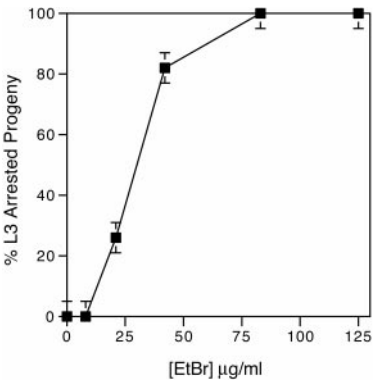

FIG. 5. Exposure of wild type hermaphrodites to EtBr. Gravid hermaphrodites were transferred to plates with increasing concentrations of EtBr. The ratio of L3 arrested progeny over the total number of progeny scored (>80) is expressed as a percentage.

two larval stages, and arrested as L3 larvae. Below 8 μg/ml, EtBr has no apparent effect on development. More importantly, at 125 μg/ml EtBr, >99% of the L3 progeny remain arrested throughout their entire lives. Similar results were obtained with *him-8* animals, which produce a high proportion of male offspring, indicating that the developmental arrest is not sex specific (not shown). It is important to note that the developmental arrest induced upon exposure to EtBr is reversible when the drug is removed, although the fraction of animals that recover decreases with the length of exposure (not shown). We also noted that animals arrested for shorter periods of time recovered more quickly than those arrested for longer periods. The reversibility of the L3 arrest phenotype indicates that EtBr is not acting by causing mutations in either the mitochondrial or the nuclear genomes since these would be expected to be heritable and irreversible. Furthermore, exposure to EtBr quantitatively produces L3 arrest; mutations by definition are rare events.

The mean life span of 26 EtBr-treated L3-arrested animals is 13.8 days, which is significantly longer ($P < 0.01$) than that of control animals not exposed to EtBr (Table 1). The control animals mature to adults and produce progeny, whereas the EtBr-treated animals remain as L3 larvae.

We monitored animal development in the presence and absence of EtBr by differential interference contrast microscopy. Gonad development is most severely impaired by EtBr. Up to 24 h after egg laying, EtBr-treated animals are not readily distinguishable from control animals; both have typical L2-sized gonads (Figs. 6A and 6B). Six hours later, untreated animals have developed into L3 larvae and their gonads show substantial anterior and posterior elongation with a large increase in the number of germ cell nuclei (Fig. 6D). In contrast, EtBr-treated animals are slightly smaller in size than control animals and their gonads remain unchanged from the 24-h point (Fig. 6C). With time, EtBr-treated animals develop an L3-sized body, although their gonads remain arrested at an L2-like stage. Thus, the effects of EtBr on development are tissue-specific and may reflect high energy requirements in the gonad or a higher sensitivity of this tissue to EtBr. The sensitivity of the gonad may be related to its high content of mtDNA at later developmental stages.

Exposure to EtBr at Different Stages of Development

Normal worm development is characterized by two increases in mtDNA copy number (Fig. 1). We exposed worms at different stages of development to EtBr to see whether the time of exposure might specifically block one or both of the increases in mtDNA copy number. When gravid hermaphrodites, embryos, or early L1 larvae are exposed to EtBr, the embryos hatch and the larvae develop to and arrest at the L3 stage. When late L1 or L2 larvae are exposed to EtBr, some animals arrest as L4 larvae while others continue development into adults (not shown). In contrast to the L3 arrested animals, the L4 arrested worms are not anatomically homogeneous, having heterogeneous gonad morphologies. Animals exposed to EtBr as L3 or older larvae also develop into adults (not shown). Adults developed in the presence of EtBr from the L2 stage or older exhibit one or more of the following defects: slow development, reduced body size, abnormal somatic gonad, abnormal or dead embryos, and reduced brood size (not shown). Their progeny arrest as embryos or as L3 larvae (not shown). Thus, later exposure to EtBr (late L1 and on) does not induce L3 arrest but rather results in a variety of developmental and reproductive consequences.

EtBr Affects mtDNA Copy Number

To verify that EtBr was indeed inhibiting mtDNA replication, we determined the mtDNA copy numbers of exposed animals. Exposure of embryos to EtBr results in arrest as L3 larvae, and prolonged exposure results in a continuous drop in mtDNA content (Fig. 7). As described above, a fraction of L2 worms exposed to EtBr arrest as L4 larvae. The mtDNA copy number of these L4 arrested animals remains at ~25,000 copies per animal (Fig. 7) and does not undergo the fivefold increase observed in untreated animals (Fig. 1). Thus, development from L3 to L4 can occur in the absence of the somatic mtDNA increase. L3 animals exposed to EtBr develop into adults despite reduced mtDNA copy numbers. Their mtDNA copy number remains unchanged as the animals develop from L3 to L4, increases approximately five-fold from L4 to adult, but returns to ~25,000 copies after the broods are laid (Fig. 7). The progeny have significantly reduced mtDNA complements and develop to and arrest as L3 larvae

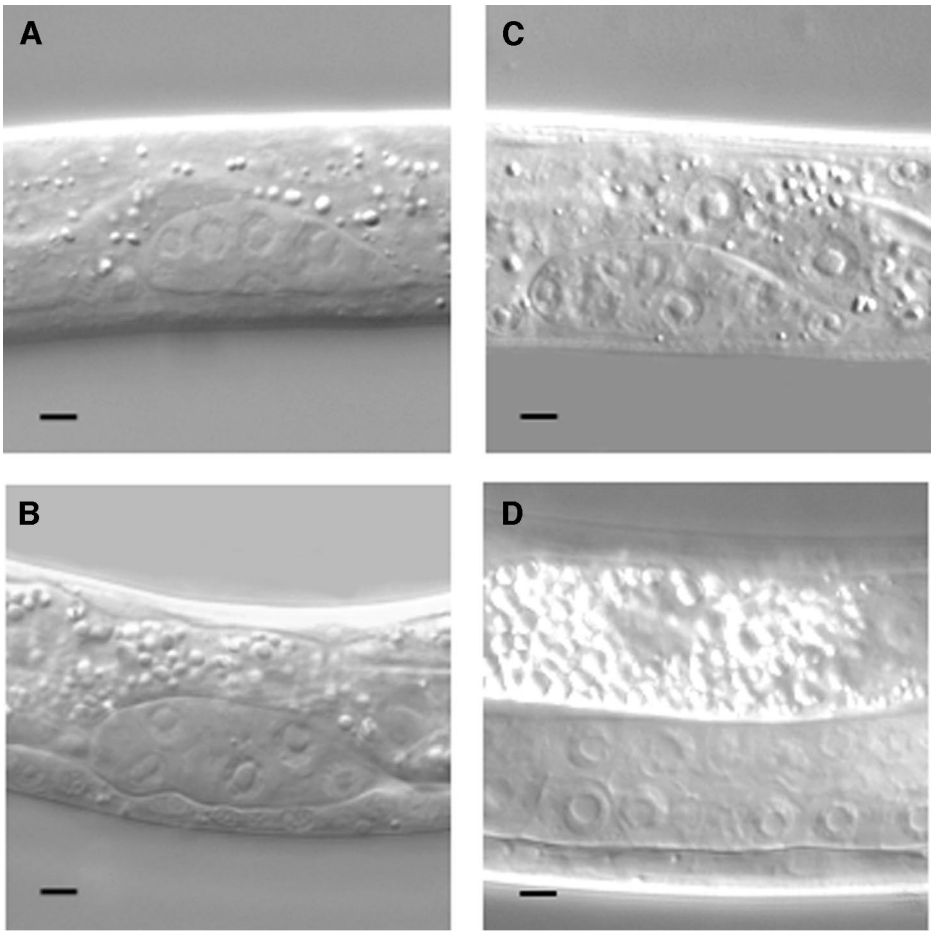

FIG. 6. Nomarski photographs of control and EtBr-treated N2 animals. (A) EtBr, at 27 h; (B) control, at 27 h; (C) EtBr, at 33 h; (D) control, at 33 h; bar, 3 μm. Worms were cultured at 25°C.

(Fig. 7). In untreated controls, the copy number exhibits a sixfold increase from L4 to adult and remains high in older adults after they have laid their broods (Fig. 1).

DISCUSSION

In this work, we focus on *C. elegans* development and its link to the control of mtDNA copy number. The mtDNA copy numbers of oocytes, embryos and of the first 3 larval stages (L1–L3) are similar. Since mtDNA is maternally inherited in *C. elegans* (Tsang and Lemire, submitted), these data suggest that mtDNA inherited through the oocyte is sufficient to support development through embryogenesis and the early larval stages. They also indicate that mtDNA synthesis is not a highly active process during early development and that it is not coupled to nuclear DNA replication. mtDNA synthesis appears to be similarly absent during the early development of *Xenopus laevis*, sea urchins, and *Drosophila* (13, 22, 23). When maternal mtDNA replication is inhibited, mtDNA-depleted larvae are produced; EtBr-treated hermaphrodites (exposed from the L3 stage of development) produce progeny with only one quarter the normal mtDNA complement (Fig. 7).

The maturation of the reproductive organs in L4 and adult hermaphrodites involves mtDNA amplification to supply the mtDNA needed for development and viability of the progeny. The mtDNA copy number increases about five-fold with the developmental transition from L3 to L4 and about sixfold from L4 to adult. The L4 stage is marked by a substantial increase in the body size of the worm, the end of somatic cell lineages, and a rapid proliferation of the germ cells. Thus, increases in mtDNA contents from L3 to L4 should support both somatic and germline development. Indeed, wild type L3 larvae that develop into adults in the presence of EtBr exhibit a wide variety of somatic (reduced body size, abnormal gonad development) and germline (abnormal embryos, reduced brood size) defects. In contrast, the increase in mtDNA from L4 to adults appears to be solely linked to germline development, being *glp-1* and *glp-4* dependent. The *glp-1* and *glp-4* mutants show the threefold somatic copy number

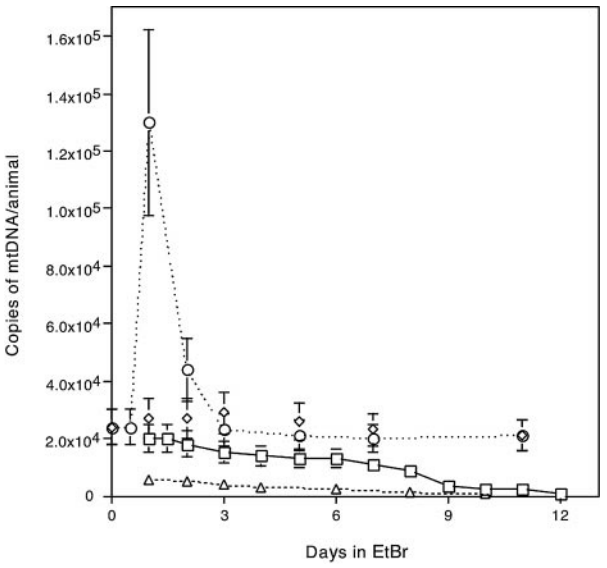

FIG. 7. The mtDNA copy numbers of EtBr-treated wild type animals. The mtDNA copy numbers per worm are averaged over 20 animals. The symbols used are: squares, L3 arrested animals developed from embryos; triangles, L3 arrested progeny of hermaphrodites exposed to EtBr from the L3 stage (32–36 h of age); diamonds, L4 arrested animals developed from L2 stage (26–30 h of age) animals exposed to EtBr; circles, adult hermaphrodites developed from L3 stage (32–36 h of age) animals exposed to EtBr. At least two replicates were performed for each time point.

increase from L3 to L4, but no further increase (or even a decrease) from L4 to adult.

Our data suggest the majority of the mtDNA copy number increase from L3 to adult is linked to reproductive function. The results with the *him-8, fem-3,* and *fem-1* mutants support this conclusion. *him-8* L4 animals carry 140,000 copies of mtDNA regardless of their sexes; this number increases substantially in adult hermaphrodites (800,000) but to a smaller extent in adult males (260,000). An adult *fem-1* hermaphrodite, which only makes oocytes at 25°C, has 800,000 copies of mtDNA. In contrast, an adult *fem-3* hermaphrodite, which only produces sperm at 25°C, has 190,000 copies of mtDNA, a value which is not much higher than that of the *fem-3* L4 larva (170,000 copies). Thus, oocyte production accounts for the majority of the mtDNA amplification measured in hermaphrodites. When we measured the mtDNA copy number of *fem-1* oocytes, it was found to be 18,000 copies per oocyte, confirming that oocytes have many copies of the mitochondrial genome. A *him-8* male undergoes an increase of ~120,000 copies of mtDNA during development from L4 to adult. If all of that increase is found in the ~3,000–4,000 sperm produced, this corresponds to 30–40 copies of mtDNA per sperm. The mammalian situation is similar; sperm have ~1,000-fold fewer copies of mtDNA than oocytes (24, 25). We estimate below that a germline nucleus is associated with an average of ~250 copies of mtDNA. This implies that an almost 100-fold mtDNA amplification occurs during oogenesis and that a reduction of mtDNA copy number occurs during spermatogenesis.

In *C. elegans,* a wild type L4 has ~250 germline nuclei (14). The difference of ~60,000 germline associated copies of mtDNA seen between a wild type L4 (130,000 copies; Fig. 1) and a *glp-1* or *glp-4* L4 at 25°C (~70,000 copies of mtDNA; Fig. 3) corresponds to an average of ~250 copies of mtDNA per germline nucleus. For comparison, somatic cells have a much lower complement of mtDNA. The L1 larva has ~560 somatic cell nuclei and ~25,000 copies of mtDNA for an average of ~45 mtDNA per cell. Similarly, the L4 larva has ~1,000 somatic cell nuclei and ~70,000 soma-associated copies of mtDNA (measured in germline deficient mutants) for an average of 70 mtDNA per cell. Both the somatic and the germline mtDNA copy number values are considerably lower than the 10^3 to 10^4 copies estimated to be present in most eukaryotic cells (26).

Prolonged exposure to EtBr can deplete mtDNA by inhibiting mtDNA replication. L3 arrested larvae contain only ~1,000 copies of mtDNA (down from an initial ~25,000 copies as embryos) after 10 days exposure (Fig. 7). This corresponds to 1–2 copies of mtDNA per somatic cell. Therefore, very few mitochondrial genomes are sufficient to support life, but more genomes are required for energy intensive processes such as development and reproduction.

Surprisingly, the L3 arrested animals generated with EtBr have extended life spans. The life-span extension may arise because of caloric restriction due to improper feeding behavior or impaired pharyngeal pumping (27). Mutations in two nuclear encoded MRC subunits significantly impair pharyngeal pumping rates and similarly extend life span (28).

We have proposed that an energy-related developmental checkpoint may control the L3 to L4 transition (28). This checkpoint appears to be related to energy status rather than directly to mtDNA copy number for the following reasons. First, although the transition from L3 to L4 coincides with a five-fold mtDNA copy number increase, this increase is not essential. L2 larvae exposed to EtBr develop to L4 larvae without an accompanying copy number increase; as L4, they still have ~25,000 copies of mtDNA (Fig. 7). Similarly, L3 animals exposed to EtBr develop into adults without the five-fold increase from L3 to L4. Their copy number increases from L4 to adult, but drops back down to ~25,000 copies after the broods are laid. Second, EtBr can quantitatively arrest development as L3 larvae. These larvae are anatomically homogeneous, suggesting they are all affected at a common developmental step or checkpoint. Third, doxycycline and chloramphenicol (two inhibitors of mitochondrial translation) can also cause L3 arrest, apparently at the same developmental step (28). Fourth, mutations affecting nu-

clear encoded MRC subunits of complexes I or V also quantitatively result in L3 arrested larvae (28). That EtBr, chloramphenicol and doxycycline, and nuclear MRC mutations all produce a common phenotype strongly supports an energy-related rather than a mtDNA copy number-related developmental checkpoint. These inhibitors and mutations affect different mitochondrial processes but all directly or indirectly impair energy generation by the MRC. By extension, other conditions that sufficiently impair MRC function will invoke the checkpoint-induced L3 arrest. As an example, the inhibition of genes of energy metabolism by systematic RNA interference frequently results in a larval arrest phenotype (29).

In conclusion, this study demonstrates a linkage between mtDNA copy number and development in three ways. First, mtDNA copy numbers increase only at specific stages of development. Second, a large portion of the mtDNA copy number increase during development is *glp-1*, *glp-4*, *fem-3*, and *fem-1*-dependent and appears to be specifically linked to germline development and maturation. Thus, these genes, which regulate germline development, are upstream of the control of mtDNA copy number. Third, when mtDNA replication is blocked, development is also blocked. These observations imply that mtDNA replication is regulated, apparently in response to the cellular or organismal energy status. We propose that an energy sensor is needed at the L3 to L4 transition for this function. We anticipate that mitochondrial dysfunction arising from any of several possible routes including mtDNA mutations will invoke the checkpoint and lead to L3 developmental arrest. Further efforts will be needed to understand the molecular mechanisms linking mtDNA copy number, MRC-mediated energy production, and *glp-1*, *glp-4*, *fem-1*, and *fem-3*-dependent development.

ACKNOWLEDGMENTS

We thank David Pilgrim for his advice and support and Catherine McPhalen for critical reading of the manuscript. W.Y.T. was supported by an Alberta Heritage Foundation for Medical Research Graduate Studentship and a Natural Sciences and Engineering Research Council Postgraduate Scholarship. The Canadian Institutes of Health Research Grant MT-15336 to B.D.L. supported this work. Some nematode strains used in this work were provided by the *Caenorhabditis* Genetics Center, which is funded in part by the NIH National Center for Research Resources (NCRR).

REFERENCES

1. Clayton, D. A. (1991) Replication and transcription of vertebrate mitochondrial DNA. *Annu. Rev. Cell Biol.* **7,** 453–478.
2. Wallace, D. C. (1993) Mitochondrial diseases: Genotype versus phenotype. *Trends Genet.* **9,** 128–133.
3. Okimoto, R., Macfarlane, J. L., Clary, D. O., and Wolstenholme, D. R. (1992) The mitochondrial genomes of two nematodes, *Caenorhabditis elegans* and *Ascaris suum*. *Genetics* **130,** 471–498.
4. Attardi, G., and Schatz, G. (1988) Biogenesis of mitochondria. *Annu. Rev. Cell Biol.* **4,** 289–333.
5. *C. elegans* Sequencing Consortium. (1998). Genome sequence of the nematode *C. elegans:* A platform for investigating biology. *Science* **282,** 2012–2018.
6. Jenuth, J. P., Peterson, A. C., Fu, K., and Shoubridge, E. A. (1996) Random genetic drift in the female germline explains the rapid segregation of mammalian mitochondrial DNA. *Nat. Genet.* **14,** 146–151.
7. Larsson, N. G., Wang, J. M., Wilhelmsson, H., Oldfors, A., Rustin, P., Lewandoski, M., Barsh, G. S., and Clayton, D. A. (1998) Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. *Nat. Genet.* **18,** 231–236.
8. Wallace, D. C. (1999) Mitochondrial diseases in man and mouse. *Science* **283,** 1482–1488.
9. Zeviani, M., and Antozzi, C. (1997) Mitochondrial disorders. *Mol. Hum. Reprod.* **3,** 133–148.
10. Hayakawa, T., Noda, M., Yasuda, K., Yorifuji, H., Taniguchi, S., Miwa, I., Sakura, H., Terauchi, Y., Hayashi, J., Sharp, G. W., Kanazawa, Y., Akanuma, Y., Yazaki, Y. ,and Kadowaki, T. (1998) Ethidium bromide-induced inhibition of mitochondrial gene transcription suppresses glucose-stimulated insulin release in the mouse pancreatic β-cell line βHC9. *J. Biol. Chem.* **273,** 20300–20307.
11. King, M. P., and Attardi, G. (1996) Mitochondria-mediated transformation of human ρ^{o} cells. *Methods Enzymol.* **264,** 313–334.
12. Morais, R. (1996) Isolation of avian mitochondrial DNA-less cells. *Methods Enzymol.* **264,** 296–304.
13. Pikó, L., and Chase, D. G. (1973) Role of the mitochondrial genome during early development in mice. Effects of ethidium bromide and chloramphenicol. *J. Cell Biol.* **58,** 357–378.
14. Beanan, M. J., and Strome, S. (1992) Characterization of a germ-line proliferation mutation in *C. elegans*. *Development* **116,** 755–766.
15. Austin, J., and Kimble, J. (1987) glp-1 is required in the germ line for regulation of the decision between mitosis and meiosis in *C. elegans*. *Cell* **51,** 589–599.
16. Lewis, J. A., and Fleming, J. T. (1995) Basic culture methods. *Methods Cell Biol.* **48,** 3–29.
17. Koekemoer, T. C., Downing, T. G., and Oelofsen, W. (1998) An alternative PCR assay for quantifying mitochondrial DNA in crude preparations. *Nucleic Acids Res.* **26,** 2829–2830.
18. Wood, W. B. (1988) *in* The Nematode *Caenorhabditis elegans* (Wood, W. B., Ed.), pp. 1–16, Cold Spring Harbor Laboratory Press, New York.
19. Schedl, T. (1997) *in C. elegans* II (Riddle, D. L., Blumenthal, T., Meyer, B. J., and Priess, J., Eds.), pp. 241–269, Cold Spring Harbor Laboratory Press, New York.
20. Nelson, G. A., Lew, K. K., and Ward, S. (1978) Intersex, a temperature-sensitive mutant of the nematode *Caenorhabditis elegans*. *Dev. Biol.* **66,** 386–409.
21. Barton, M. K., Schedl, T. B., and Kimble, J. (1987) Gain-of-function mutations of *fem-3*, a sex-determination gene in *Caenorhabditis elegans*. *Genetics* **115,** 107–119.
22. Chase, J. W., and Dawid, I. B. (1972) Biogenesis of mitochondria during *Xenopus laevis* development. *Dev. Biol.* **27,** 504–518.
23. Rubenstein, J. L., Brutlag, D., and Clayton, D. A. (1977) The mitochondrial DNA of *Drosophila melanogaster* exists in two distinct and stable superhelical forms. *Cell* **12,** 471–482.
24. Pikó, L., and Taylor, K. D. (1987) Amounts of mitochondrial DNA and abundance of some mitochondrial gene transcripts in early mouse embryos. *Dev. Biol.* **123,** 364–374.
25. Hecht, N. B., Liem, H., Kleene, K. C., Distel, R. J., and Ho, S. M. (1984) Maternal inheritance of the mouse mitochondrial genome

is not mediated by a loss or gross alteration of the paternal mitochondrial DNA or by methylation of the oocyte mitochondrial DNA. *Dev. Biol.* **102,** 452–461.

26. Shadel, G. S., and Clayton, D. A. (1997) Mitochondrial DNA maintenance in vertebrates. *Annu. Rev. Biochem.* **66,** 409–435.
27. Lakowski, B., and Hekimi, S. (1998) The genetics of caloric restriction in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* **95,** 13091–13096.
28. Tsang, W. Y., Sayles, L. C., Grad, L. I., Pilgrim, D. B., and Lemire, B. D. (2001) Mitochondrial respiratory chain deficiency in *Caenorhabditis elegans* results in developmental arrest and increased life span. *J. Biol. Chem.* **276,** 32240–32246.
29. Fraser, A. G., Kamath, R. S., Zipperlen, P., Martinez-Campos, M., Sohrmann, M., and Ahringer, J. (2000) Functional genomic analysis of *C. elegans* chromosome I by systematic RNA interference. *Nature* **408,** 325–330.