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Mitochondrial Biogenesis and Dysfunction in *Caenorhabditis elegans*

by

William Yueyau Tsang 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Mitochondrial Biogenesis and Dysfunction in *Caenorhabditis elegans*" submitted by William Yueyau Tsang in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Abstract

The growth and development of an organism are energy-dependent processes and rely on the mitochondrial respiratory chain (MRC) as the major source of ATP. The MRC is composed of five complexes under the dual control of the nuclear and the mitochondrial genomes. A defective MRC has been implicated in a wide variety of multi-systemic and tissue-specific human diseases including diabetes, myopathies, neuromuscular and heart diseases.

We are developing the nematode, *Caenorhabditis elegans* as our model system for studying mitochondrial diseases. *C. elegans* has a simple anatomy with multiple tissue types, a sophisticated genetic system, and a mammalian-like MRC. The goal is to investigate the consequences of an impaired MRC and to understand the relationship between genotype and phenotype.

We have isolated two nuclear MRC mutations in the *nuo-1* and in the *atp-2* genes encoding the active site subunits of complexes I and V, respectively. Both mutations are homozygous lethal and result in developmental arrest at the third larval stage (L3). When the replication or expression of the mitochondrial genome is impaired with mitochondrial DNA (mtDNA) inhibitors, wild type animals arrest as L3 larvae and are phenotypically similar to the nuclear mutants. We believe that MRC dysfunction is lethal and produces a distinctive L3 arrest phenotype. Interestingly, the number of copies of mtDNA, an indirect indicator of energy needs, increases substantially from the L3 to the next larval stage, L4.

We have also isolated a large mtDNA deletion called *uaDf5* that removes multiple genes for MRC subunits. *uaDf5* animals are aphenotypic, and *uaDf5* mtDNA co-exists

with wild type mtDNA in a stable heteroplasmic state. The proportion of *uaDf5* mtDNA is below the threshold level of pathogenesis, which we believe is greater than 85% *uaDf5*.

We have identified the tissues in which *atp-2* gene function is dispensable for development beyond the L3 stage. By following the infrequent, spontaneous loss of a complementing wild type *atp-2* transgene during cell division, we have found that mosaic animals with a loss of the wild type *atp-2* gene in neuronal, pharyngeal, hypodermal, and intestinal cells can occasionally be tolerated. Thus, these tissues may rely less heavily on the MRC for energy production.

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ADP	adenosine diphosphate
ANT	adenine nucleotide translocase
ATP	adenosine triphosphate
atp-2(ua2)	*ua2* deletion in the *atp-2* gene
ATP6, 8	mtDNA-encoded subunits of ATP synthase
COI, II, III	mtDNA-encoded subunits of cytochrome *c* oxidase
Cyt *b*	mtDNA-encoded cytochrome *b* subunit
D-loop	displacement loop
e^-	electron
EMS	ethyl methane sulfonate
EtBr	ethidium bromide
FAD	flavin adenine dinucleotide-oxidized form
$FADH_2$	flavin adenine dinucleotide-reduced form
FMN	flavin adenine mononucleotide
GFP	green fluorescent protein
H^+	proton
H_20	water
IPTG	isopropylthiogalactoside
ISP	Rieske iron-sulfur protein
KSS	Kearns-Sayre syndrome
L1, L2, L3, L4	*C. elegans* larval stages
LHON	Leber hereditary optic neuropathy
MELAS	mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes
MERRF	myoclonus epilepsy with ragged-red fibers
MILS	maternally inherited Leigh's syndrome
MRC	mitochondrial respiratory chain
mtDNA	mitochondrial DNA
mtPTP	mitochondrial permeability transition pores
N2	*C. elegans* wild type strain
NAD^+	nicotinamide adenine dinucleotide-oxidized form
NADH	nicotinamide adenine dinucleotide-reduced form
ND1, 2, 3, 4, 4L, 5, 6	mtDNA-encoded subunits of NADH-ubiquinone oxidoreductase
nuo-1(ua1)	*ua1* deletion in the *nuo-1* gene
O_2	molecular oxygen
P_i	inorganic phosphate
Q	coenzyme Q or ubiquinone
rho^0	cells devoid of mtDNA
RNAi	RNA interference
ROS	reactive oxygen species
uaDf5	large-scale deletion in the mtDNA

Chapter 1

General Introduction

1.1 Introduction

This thesis describes the use of the nematode, *Caenorhabditis elegans*, as a model organism for studying and examining mitochondrial biogenesis, genetics, and dysfunction in a multicellular eucaryote. In particular, I will be concentrating on the genetic, phenotypic, and physiological consequences of mitochondrial dysfunction associated with mitochondrial respiratory chain (MRC) defects. The aim of this chapter is to give a comprehensive introduction to mitochondria, the mitochondrial respiratory chain, mitochondrial DNA (mtDNA), and human mitochondrial deficiencies and their phenotypes. I will also discuss and compare the advantages and disadvantages of a number of animal model systems for investigating mitochondrial function.

1.2 Mitochondria

Mitochondria are essential organelles in most eucaryotes. The first descriptions of mitochondria date back to the pre-1900s and report granule-like structures as seen with a light microscope. We now affirm that the ancestors of mitochondria are likely ancient bacteria that lived inside primitive eucaryotic cells (Gray *et al.*, 1999). The symbiotic relationship established between the two allowed the loss or transfer of genes from the bacterium to the nucleus of the eucaryote during the course of evolution (Gray *et al.*, 2001).

Mitochondria were identified and revealed by electron microscopy in the 1950s. They are sausage-like structures with dimensions of 3-4 μm in length and 0.5-1 μm in diameter (Figure 1.1). Mitochondria consist of a double membrane system consisting of an outer and an inner membrane, with the intermembrane space sandwiched between the

two (Addink *et al.*, 1972; Korman *et al.*, 1970; Palmer and Hall, 1972). The outer membrane contains porin proteins, which form pores and allow molecules of less than 10 kDa to diffuse freely across the outer membrane between the cytosol and the intermembrane space (Benz, 1994; Jap and Walian, 1990). Thus, the outer membrane is permeable to ions and most metabolites. The inner membrane encloses the matrix and is folded into invaginations called cristae (Daems and Wisse, 1966). These invaginations increase the membrane surface area, which is thought to be necessary for efficient respiration and energy production. In contrast to the outer membrane, the inner membrane is impermeable to most small molecules and ions, allowing only uncharged molecules of 100-150 Da to diffuse through passively (Ballarin *et al.*, 1996; Ballarin and Sorgato, 1996; Sorgato and Moran, 1993). Charged and large molecules can only cross the inner membrane with the aid of specific transport proteins (Palmieri *et al.*, 1996; Palmieri *et al.*, 2000). The controlled permeability compartmentalizes the external cytosol from the internal mitochondrial matrix and allows for the establishment and maintenance of an electrochemical gradient required for mitochondrial energy production. Both the outer and the inner membranes contain protein complexes that participate in the import of proteins into mitochondria (Neupert, 1997; Schatz, 1998). The matrix is the site of several important metabolic pathways including the Krebs cycle, the urea cycle, the oxidation of fatty acids, and the biosynthesis of heme and lipids (Scheffler, 2000). In yeast, worms, and humans, it is estimated that approximately 600-1500 genes, or a few percent of the genome, are devoted to mitochondrial function (Kumar *et al.*, 2002; Marcotte *et al.*, 2000; Taylor *et al.*, 2003).

Although the inner membrane and the outer membrane are considered to be two distinct membranes, they do come together at contact points to form the mitochondrial permeability transition pores (mtPTP) (Marzo *et al.*, 1998a; Petit *et al.*, 1996). These pores form the so-called "mega channels", which cause sudden increases in the inner membrane permeability to solutes of molecular mass up to 1500 Da. The mtPTP is composed of the outer membrane porin, the adenine nucleotide translocase (ANT) of the inner membrane, and other proteins involved in programmed cell death or apoptosis (Green and Reed, 1998; Marzo *et al.*, 1998b; Zoratti and Szabo, 1995). Prolonged or simultaneous opening of mtPTPs inactivates mitochondria and leads to cell death.

The classical sausage-shape structure revealed by electron microscopy (Figure 1.1) provides a static picture of the mitochondrion. We now know that mitochondria are not static; rather they form dynamic, fluid-like reticular or tubular networks capable of exhibiting an astonishing flexibility in terms of distribution and structure (Bereiter-Hahn, 1990). Mitochondria are quite mobile and can distribute themselves within a cell. Mitochondrial movement and distribution are the result of interactions between the mitochondrial outer membrane and various components of the cytoskeleton (Heggeness *et al.*, 1978; Starr, 2002; Yaffe, 1999). Movement is developmentally programmed (Barnett *et al.*, 1996; Mignotte *et al.*, 1987) and may serve to localize mitochondria to regions of high-energy demands. For instance, mitochondria are highly concentrated in the synapses of neurons (Morris and Hollenbeck, 1993; Morris and Hollenbeck, 1995) and in specific cytoplasmic regions of differentiated cardiac muscles and kidney tubules (Porter, 1973). In addition, mitochondria change their size, morphology, or number. In fact, mitochondria can undergo fission and fusion (Bereiter-Hahn and Voth, 1994).

Mitochondrial fission is thought to be an essential mechanism for mitochondrial multiplication and cell division. It is also required in non-dividing, fully differentiated cells as they grow in volume or in response to environmental changes. Fusion, on the other hand, may be necessary to "homogenize" mitochondrial proteins or genomes, thus mixing or exchanging mitochondrial contents between different mitochondria. Genetic studies in yeast, fruit flies, and mammals have led to the identification of several nuclear-encoded factors involved in mitochondrial fusion and fission (Beech *et al.*, 2000; Bleazard *et al.*, 1999; Hales and Fuller, 1997; Labrousse *et al.*, 1999; Nunnari *et al.*, 1997; Rapaport *et al.*, 1998; Smirnova *et al.*, 1998). Mutations in any one of these factors affect the equilibrium between these two processes, resulting in either excessive mitochondrial fragmentation (fusion defects) or interconnected mitochondria (fission defects).

1.3 Mitochondrial Respiratory Chain

Major milestones had been achieved in understanding the biochemistry and bioenergetics of mitochondria by the 1970s. Mitochondria are the sites of several important metabolic pathways including the Krebs cycle, the urea cycle, and the biosynthesis of heme, lipid, and amino acids (Scheffler, 2000). The primary function of mitochondria, however, is to generate adenosine triphosphate (ATP), the universal energy currency in a cell (Mitchell, 1987). The production of ATP is accomplished by the MRC, which accounts for the most abundant proteins found in the mitochondrial inner membrane. In bovine heart mitochondria, the MRC constitutes more than 50% of all inner membrane proteins.

The MRC is under the control of two separate genomes, the nuclear DNA and the mtDNA (Attardi and Schatz, 1988). The biogenesis of the MRC is complex. The genes encoding the MRC are transcribed, translated, and in the case of nuclear-encoded proteins, imported into mitochondria where they can be assembled and folded properly into functional multi-subunit complexes in the mitochondrial inner membrane. A typical, mammalian MRC is composed of five protein complexes (complex I to V) (Figure 1.2) (Hatefi, 1985; Mitchell, 1987). They are:

a) Complex I: NADH-ubiquinone oxidoreductase;

b) Complex II: succinate-ubiquinone oxidoreductase;

c) Complex III: ubiquinol-cytochrome c oxidoreductase or cytochrome bc_1 complex;

d) Complex IV: cytochrome *c* oxidase;

e) Complex V: ATP synthase or F_0F_1-ATPase.

Energy generation by the MRC occurs via a process called oxidative phosphorylation. Originally proposed by Peter Mitchell in the 1960s and now widely accepted, this process explains the coupling between the free energy of electron (e^-) transport and the utilization of this free energy in ATP production in the inner membrane of mitochondria (Mitchell and Moyle, 1967; Mitchell, 1976a; Mitchell, 1976b). A similar process of energy production also takes place in the thylakoid membrane of chloroplasts and in the plasma membrane of bacteria. In oxidative phosphorylation, electrons are transported along a sequential series of electron-transporting MRC complexes of increasing standard reduction potentials (Mitchell and Moyle, 1967; Mitchell, 1976a; Mitchell, 1976b). Each of these complexes contains a specific combination and arrangement of redox centers such as flavin adenine mononucleotide (FMN), flavin

adenine dinucleotide (FAD), cytochromes, iron-sulfur centers, and copper centers that act as electron carriers. The electrons are derived from reduced cofactors such as nicotinamide adenine dinucleotide (NADH-reduced form; NAD^+-oxidized form) and flavin adenine dinucleotide ($FADH_2$-reduced form; FAD-oxidized form), produced during the oxidation of nutrients such as glucose and fatty acids. Electrons enter the MRC at complex I when NADH is oxidized to NAD^+, or at complex II when succinate is oxidized to fumarate to form $FADH_2$. The electrons travel from complexes I or II to complexes III and IV, and ultimately to the final electron acceptor, molecular oxygen (O_2) to form water (H_2O). The passage of electrons between the MRC complexes releases energy that is stored in the form of a proton (H^+) gradient across the intact mitochondrial inner membrane, which is impermeable to protons and is osmotically sealed (Mitchell and Moyle, 1967; Mitchell, 1976a; Mitchell, 1976b). In particular, complexes I, III, and IV participate in the establishment of the proton gradient by coupling the movement of electrons with the active transport of protons from the matrix to the intermembrane space. Complex V uses this proton gradient to drive ATP synthesis from adenosine diphosphate (ADP) and inorganic phosphate (P_i) by allowing proton influx into the matrix (Mitchell and Moyle, 1967; Mitchell, 1976a; Mitchell, 1976b). The ATP is exported to the cytosol via the ANT in exchange for ADP (Stubbs *et al.*, 1978; Vignais, 1976). Two other important molecules have pivotal roles in the intermediate steps of electron transfer. Coenzyme Q, also called ubiquinone (Q), a lipid soluble compound, shuttles electrons between complexes I or II to complex III (Chazotte and Hackenbrock, 1989; Zhu *et al.*, 1982). Cytochrome *c* (cyt *c*), on the other hand, is a water-soluble, heme-containing

protein on the outer surface of the inner membrane that carries electrons from complex III to complex IV (Gupte and Hackenbrock, 1988a; Gupte and Hackenbrock, 1988b).

Each of the five complexes in the MRC is composed of a number of protein subunits, the majority of which come from the nuclear genome. Complex I is by far the largest of the five complexes (Table 1.1) (Saraste, 1999). Both the bovine and the human complex I contain ~43 subunits (Ragan, 1976; Skehel *et al.*, 1998; Smeitink and van den Heuvel, 1999; Smeitink *et al.*, 2001); 7 of them (ND1, ND2, ND3, ND4, ND4L, ND5, and ND6) are encoded by the mtDNA (Walker, 1992; Weiss *et al.*, 1991). The *Neurospora crassa* complex I is believed to have ~35 subunits; ~28 of them are highly conserved in humans (Videira, 1998; Videira and Duarte, 2002; Weiss *et al.*, 1991). In *Caenorhabditis elegans*, ~30 complex I subunits have counterparts in humans. Complex I can be subdivided into three domains, FP, IP, and HP. The FP and IP domains are peripheral and protrude into the mitochondrial matrix. These two highly conserved domains contain the catalytic site of the enzyme and carry one FMN and six iron-sulfur centers. The HP domain, on the other hand, consists primarily of hydrophobic subunits and serves to anchor the FP and the IP domains to the inner membrane (Walker, 1992). The main functions of complex I are to re-oxidize NADH to NAD^+ and thus recycle the cofactor for the next round of nutrient oxidation, to feed electrons into the MRC via coenzyme Q, and to generate a proton gradient (Yano, 2002).

Complex II is structurally the simplest of all the complexes (Table 1.1). It is composed of four nuclear-encoded subunits: a FAD-containing subunit, an iron-sulfur-containing subunit having three iron-sulfur centers, and two membrane-anchoring subunits (Lancaster, 2002; Lemire and Oyedotun, 2002). The FAD and the iron-sulfur

subunits form the catalytic domain of this enzyme and are highly conserved among eucaryotes. Complex II does not translocate protons; it oxidizes succinate to fumarate and shuttles the electrons to coenzyme Q (Hägerhäll, 1997).

The mammalian complex III typically contains ~11 subunits (Table 1.1). Only one subunit, Cyt *b* (cytochrome *b*), is encoded by the mitochondrial genome. The *Neurospora* complex appears to have up to 9 subunits, however only 7 of them have clear homologs in humans (Schulte and Weiss, 1995b). In *C. elegans*, 8 subunits have homologs in humans (Table 1.1). The key subunits of complex III are Cyt *b*, cyt c_1 (cytochrome c_1) and ISP (Rieske iron-sulfur protein). Together, these subunits form the functional core of the enzyme and deliver electrons from coenzyme Q to cytochrome *c* and contribute to the proton gradient (Saraste, 1999).

Like complex III, complex IV also functions to deliver protons across the inner membrane. It accepts electrons from cytochrome *c* and transfers them onto molecular oxygen, the final electron acceptor of the MRC (Saraste, 1999). The three major subunits, COI, which contains cytochromes *a* and a_3, and a copper center, COII, which carries a second copper center, and COIII, are mtDNA-encoded and form the catalytic core of this enzyme. In humans and in bovine, the enzyme is composed of ~13 subunits (Table 1.1) (Tsukihara *et al.*, 1996; Yoshikawa *et al.*, 1998). Comparisons of the primary protein sequences reveal that 9 of these subunits are conserved in *C. elegans* (Table 1.1).

The ATP synthase, or complex V, is the final complex in the MRC. It is comprised of at least 16 different subunits (Table 1.1) (Collinson *et al.*, 1994; Lutter *et al.*, 1993). In mammals, only two of these subunits, ATP6 and ATP8, are encoded by the mitochondrial genome. Complex V contains two domains. A membrane domain, called

F_0, contains a channel for proton transport. The catalytic domain, called F_1, extends into the matrix and is responsible for harnessing the proton gradient to drive ATP synthesis from ADP and P_i (Boyer, 1997; Boyer, 1999). The majority (~12) of the ATP synthase subunits are highly conserved in nematodes (Table 1.1). The α and the β subunits in the F_1 domain, which bind ADP and ATP, are the two most conserved proteins in complex V (Table 1.1).

1.4 Mitochondrial DNA

The mammalian mtDNA is a small, autonomous, double-stranded circular DNA molecule that resides in the matrix of mitochondria (Garesse and Vallejo, 2001). It has a size of ~16-kbp and encodes 13 MRC subunits (7 from complex I, 1 from complex III, 3 from complex IV, and 2 from complex V), two ribosomal RNAs (rRNAs; 12S-rRNA and 16S-rRNA), and all the transfer RNAs (tRNAs) necessary for mitochondrial protein synthesis (Figure 1.3). The mtDNA is extremely compact, containing no introns and only one non-coding region of ~1-kbp called the displacement loop (D-loop) (Clayton, 1982; Clayton, 1991; Clayton, 1992). The D-loop region is an important control region that contains one of the origins of replication and two promoters for mtDNA transcription. The maintenance of the mtDNA and the expression of mtDNA-encoded proteins require hundreds of nuclear-encoded proteins for replication, transcription, and translation. Interestingly, replication of the mitochondrial genome by the mtDNA polymerase γ is intimately linked to transcription. Initiation of mtDNA replication, which begins at the D-loop region, requires a short RNA primer generated by mitochondrial RNA polymerase-dependent transcription (Clayton, 1982; Clayton, 1991). Mitochondrial DNA

transcription and replication are controlled by at least three different mitochondrial transcription factors in mammals (Falkenberg *et al.*, 2002; Larsson *et al.*, 1997; Larsson *et al.*, 1998). Unlike nuclear DNA, the mitochondrial genome is normally present as thousands of copies per cell (Bogenhagen and Clayton, 1978; Piko and Taylor, 1987).

Mitochondria and mtDNA are generally believed to be inherited strictly from the maternal lineage (Berger and Yaffe, 1996; Dujon, 1981; Giles *et al.*, 1980; Hutchison *et al.*, 1974; Kaneda *et al.*, 1995). Paternal mtDNA transmission is rare but does occur under some circumstances in humans (Schwartz and Vissing, 2002), and in interspecific hybrids between closely related species of *Drosophila* (Kondo *et al.*, 1990) and mouse (Gyllensten *et al.*, 1991). The number of mitochondria and mtDNA copies are precisely maintained within a cell and vary within tissues (Bogenhagen and Clayton, 1974; Robin and Wong, 1988). The mechanisms that regulate organelle and mtDNA copy numbers are not understood (Marin-Garcia and Goldenthal, 2000), although a number of nuclear-encoded factors involved in mtDNA maintenance have been identified (Iyengar *et al.*, 1999; Iyengar *et al.*, 2002; Larsson *et al.*, 1998; Maier *et al.*, 2001). Mitochondrial division and mtDNA replication are not tightly coupled to the cell division cycle (Bogenhagen and Clayton, 1977; Clayton, 1982). They can respond to dietary intake (Brooks *et al.*, 1980), hormones (Nelson, 1990), exercise, aging, stress, hypoxia (Wallace, 1999), and environmental signals such as temperature (Labrousse *et al.*, 1999; Wu *et al.*, 1999). In normal somatic tissues, the number of mitochondria, the number of mtDNA copies, and the respiratory capacity of a cell correlate loosely with one another (Moraes, 2001; Williams, 1986). This correlation no longer holds true in transformed

cells (van den Bogert *et al.*, 1993) or in cells with mitochondrial dysfunction (Beziat *et al.*, 1993; Heddi *et al.*, 1999; Wiesner *et al.*, 1999).

A complete description of the changes in the numbers of mitochondria or mtDNA copies in any single eucaryotic species has not been reported. However, a relatively coherent picture can be assembled from studies involving a number of eucaryotic organisms. It is estimated that there are approximately 10^2-10^3 mitochondria and 10^3-10^4 copies of mtDNA in most mammalian somatic cells, with multiple copies of mtDNA (~2-10 copies) per mitochondrion (Nass, 1969; Satoh and Kuroiwa, 1991). Mitochondria and mtDNA copy number increase substantially during oogenesis. A mature oocyte contains several thousand mitochondria (Jansen and de Boer, 1998), and 10^5 copies of mtDNA (Michaels *et al.*, 1982; Piko and Taylor, 1987). These increases are necessary to supply the oocyte, and thus the developing embryo with sufficient mitochondria and mtDNA to support embryogenesis; the many cell divisions of early embryogenesis are not matched by mitochondrial division or mtDNA replication (Piko and Taylor, 1987). By the time the embryos reach ~100 cells, the number of mitochondria and the mtDNA copy number are reduced back to 10^2-10^3 and 10^3-10^4 per cell, respectively, and these numbers are in the same range as in somatic cells. In contrast, sperm mitochondria are actively destroyed and contribute very little, if at all, to the zygote (Hiraoka and Hirao, 1988). The mtDNA copy number of a sperm is about 10^2 (Hecht *et al.*, 1984). This down regulation is believed to be a mechanism for preventing paternal transmission of mtDNA (Larsson *et al.*, 1997).

1.5 Mitochondrial Dysfunction

Since mitochondria are such important organelles in a cell, it is not surprising that defective mitochondria can lead to serious pathogenic consequences. The field of mitochondrial medicine began in the late 1980s, whereas substantial progress had already been made in understanding the biochemistry and the bioenergetics of mitochondria. In 1988, the first mitochondrial diseases were characterized at a molecular level. Holt *et al.* reported large-scale mtDNA deletions in patients with mitochondrial myopathy (Holt *et al.*, 1988), and Wallace *et al.* characterized a point mutation in the ND4 gene in a family with Leber's heredity optic neuropathy (Wallace *et al.*, 1988). Since that time, the field of mitochondrial diseases has experienced a rapid growth, and to this date, hundreds of mutations affecting the MRC and the mtDNA have been identified (Larsson and Luft, 1999). Clinical manifestations of mitochondrial diseases are diverse and bewildering (Wallace, 1999). Mitochondria have also been implicated in the aging process and in neurodegenerative diseases such as Alzheimer (Hutchin and Cortopassi, 1995), Parkinson (Shoffner *et al.*, 1993; Shoffner, 1995), and Huntington diseases (Horton *et al.*, 1995), in cancer such as hereditary paragangliomas (Baysal *et al.*, 2000; Niemann and Muller, 2000), and in programmed cell death or apoptosis (Wang *et al.*, 2001).

I will briefly describe a few human disorders commonly found in patients with mitochondrial dysfunction. For the purpose of my thesis, I will primarily focus on defects associated with the MRC to illustrate the diversity of clinical phenotypes and the tissue-specificity of some mutations (a more detailed listing is found in Table 1.2) (DiMauro and Andreu, 2000; Hirano and Vu, 2000; Sue and Schon, 2000; Wallace, 1999). It is important to bear in mind that in principle, any alternations in mitochondrial function and

homeostasis can lead to a mitochondrial disease. For instance, nuclear gene mutations affecting mitochondrial protein import (Koehler *et al.*, 1999), iron handling and transport (Allikmets *et al.*, 1999; Campuzano *et al.*, 1996), copper transport (Jaksch *et al.*, 2000; Papadopoulou *et al.*, 1999; Valnot *et al.*, 2000a), mitochondrial maintenance (Delettre *et al.*, 2002), deoxynucleotide pools (Nishino *et al.*, 1999), mtDNA copy number (Marin-Garcia and Goldenthal, 2000) and the ANT (Jordens *et al.*, 2002) have been identified in humans.

a) Kearns-Sayre syndrome (KSS)

This disorder, which normally develops before the age of 20, may cause blindness, eye muscle paralysis, severe heart problems, coordination problems, mental retardation, and a high incidence of diabetes. KSS is a result of insertion or deletion mutations in the mtDNA and is rarely inherited but rather occurs sporadically.

b) Leigh syndrome

This serious disease with an onset during infancy may cause brain abnormalities, vomiting, seizures, heart problems, epilepsy, and muscle weakness. Leigh syndrome is usually transmitted in a Mendelian fashion being most often due to nuclear mutations affecting complexes I, II, or IV, but may also result from mtDNA lesions (see below).

c) Maternally inherited Leigh syndrome (MILS)

The symptoms of this disease are similar to Leigh syndrome. MILS is maternally inherited and often occurs with mutations in a mitochondrial tRNA gene or in the ATP6 gene.

d) Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS)

MELAS patients suffer exercise intolerance, lactic acidosis, seizures, dementia, myopathy with ragged red fibers, and heart problems. Mutations in a mitochondrial tRNA gene, or in the mtDNA affecting complexes I, III, or IV are the main cause of this disease.

e) Myoclonus epilepsy with ragged-red fibers (MERRF)

MERRF usually appears before adolescence and causes epilepsy, loss of coordination, dementia, and myopathy with ragged-red fibers. MERRF patients arise sporadically or by maternal transmission of mutations in the mitochondrial tRNA genes.

f) Leber hereditary optic neuropathy (LHON)

Patients present with optic atrophy and sudden onset vision loss between the ages of 12 and 30 years. LHON is most commonly associated with point mutations in the mtDNA-encoded subunits of complex I (ND1, ND4, or ND6), or in cytochrome *b* of complex III.

g) Diabetes mellitus

Diabetes may be the most common mtDNA disease with approximately 1.5% of cases caused by mtDNA mutations. It results from impaired insulin secretion. Diabetes often occurs with mutations in the mtDNA.

A number of important observations can be made with regard to mitochondrial diseases. First, defects in the MRC can be attributed to mutations in either the nuclear or the mitochondrial genome. The majority of pathogenic mutations have been identified in the mitochondrial genome (DiMauro and Andreu, 2000). These include defects in the subunits of complexes I, III, IV, and V, and in rRNA and tRNA genes. Although most MRC subunits are encoded in the nuclear genome, only a few structural gene mutations

have been found, and these are restricted to complexes I and II. Several nuclear-encoded mutations that affect the assembly of complexes III or IV have also been documented (Table 1.2). The relatively few pathogenic nuclear-encoded mutations identified may reflect their rarity and/or their severity as well as the difficulty of identifying mutant genes in the much larger nuclear genome. Second, mitochondrial disorders can be genetically inherited, can occur sporadically, or can be induced by exposure to toxic chemicals (Barclay *et al.*, 2001; Feng *et al.*, 2001a; Ishii *et al.*, 1990; Nagley and Mattick, 1977; Ray and Butow, 1979; Spelbrink *et al.*, 1997). Diseases associated with nuclear DNA mutations most often follow the traditional Mendelian model of inheritance, whereas those associated with mtDNA lesions are inherited maternally (DiMauro and Andreu, 2000; Sue and Schon, 2000). Third, the gene mutations can be quite different and include deletions, insertions, inversions, and point mutations. Fourth, mitochondrial dysfunction can be caused directly by lesions in genes coding for the structural components of the MRC, or indirectly by lesions in genes controlling MRC biogenesis and assembly. Fifth, mitochondrial diseases can affect any cell or tissue in the body and at any age. Symptoms of mitochondrial diseases are often multi-systemic with prominent involvement of the heart, skeletal muscle, and the nervous system (Beal, 1995; Luft and Landau, 1995; Wallace, 1992). Sixth, there is currently no strict correlation between genotype and phenotype. One mutation can result in different diseases. For example, the same A3243G mutation in the $mtDNA^{leu}$ gene can result in MELAS without diabetes or diabetes without MELAS (DiMauro and Andreu, 2000). Conversely, the same disease can be caused by different mutations. LHON, for instance, can result from mutations in different mtDNA encoded complex I genes (DiMauro and Andreu, 2000). Finally, there

is currently no cure for mitochondrial diseases, although certain drugs and dietary supplements can significantly improve the symptoms (Beal, 2000).

How does a defective MRC contribute to the development of a mitochondrial disease? The MRC is the major source of energy for most cells and tissues. Tissues such as muscles and neurons are highly oxidative and rely heavily on the MRC for ATP production (Chinnery and Turnbull, 1997b). Furthermore, mitochondria are key regulators of signaling events leading to programmed cell death. Recent developments have demonstrated that mitochondria contain a number of factors that can mediate apoptosis (Marzo *et al.*, 1998b). Opening of the mtPTP leads to the release of these factors and triggers the activation of a cascade of specialized proteases called caspases that can lead to cellular degradation and destruction. In addition, the MRC is the major endogenous source of reactive oxygen species (ROS) such as the superoxide anion, hydrogen peroxide, and the hydroxyl radical (Halliwell, 1993; Papa and Skulachev, 1997). Superoxide anion is generated constantly at low levels during the normal transfer of electrons to molecular oxygen. It can then be converted by the mitochondrial manganese superoxide dismutase to hydrogen peroxide, which in turn can be detoxified to water by glutathione peroxidase. Excessive amounts of reduced metal centers can, by the Fenton reaction, convert hydrogen peroxide to hydroxyl radicals, the most reactive and damaging of all ROS. Once the levels of generated ROS surpass the detoxifying defense capability, mitochondrial and cellular lipids, proteins, and nucleic acids can be permanently damaged and lead to a progressive decline in mitochondrial function and energy production. Mitochondrial diseases can be caused by impaired mitochondrial

energy production, by oxidative damage inflicted by ROS, and/or by modulating the factors involved in apoptosis (Wallace, 1999).

How does mitochondrial dysfunction give rise to tissue-specific consequences? The tissue specificity observed in some mitochondrial defects can be attributed to a number of factors. First, the cells or tissues involved are those with highest demands or thresholds for MRC-generated ATP (Chinnery and Turnbull, 1997b; Larsson *et al.*, 1998). Tissues that are highly oxidative have higher thresholds for ATP requirements and are probably more sensitive to MRC defects. Second, some MRC genes have multiple isoforms, and the differential expression of these isoforms may predispose certain tissues to disease pathology (Grossman and Shoubridge, 1996; Poyton and McEwen, 1996). Third, mtDNA is present in multiple copies per cell and normally exists in a homoplasmic state where only one form of mtDNA is present. Occasionally, two or more forms of mtDNA co-exist within a cell in a state termed heteroplasmy (Chinnery *et al.*, 1999). Heteroplasmy can vary between different tissue types, increasing or decreasing as cells divide in a process called mitotic segregation (Grossman and Shoubridge, 1996). The severity of the disease is not only due to the nature of the mutation but also to the proportion of mutated mtDNA present in different cells or tissues (Chinnery *et al.*, 1999). In humans, the majority of mtDNA mutations that lead to a phenotype are pathogenic even in a heteroplasmic state (Chinnery, 2002). On the other hand, silent or non-deleterious mutations in the mtDNA can exist in a heteroplasmic or a homoplasmic state without any phenotypic consequences.

Three mechanisms help explain the fluctuation of mtDNA heteroplasmy during cell division. First, mtDNA replication is not tightly coupled to the cell division cycle

(Bogenhagen and Clayton, 1977; Clayton, 1982). This means that some mtDNAs may be replicated more often, whereas others not at all during mitosis. Second, mtDNAs can be randomly sampled and distributed among the daughter cells during cell divisions (Birky, 1983). Third, a replicative advantage for mtDNA molecules with large deletions may allow shorter, mutant mtDNA molecules to replicate more frequently than wild type mtDNA molecules; there is very little experimental evidence to support this idea (Schon *et al.*, 1997). A selection pressure involving nuclear DNA-encoded factors that maintain or regulate the mitochondrial genome has been postulated for the preferential replication of one form of mtDNA molecule over another (Battersby and Shoubridge, 2001; Le Goff *et al.*, 2002).

The level of heteroplasmy can also vary when mtDNAs are transmitted from the mother to her offspring (Chinnery *et al.*, 1997; Chinnery and Turnbull, 1997a). Even though thousands of mtDNA molecules are present in a cell, the segregation of mtDNA sequence variants can be unexpectedly rapid in some cases. In Holstein cows, a heteroplasmic sequence variant was observed to segregate rapidly, sometimes within a single generation (Ashley *et al.*, 1989; Hauswirth and Laipis, 1982; Koehler *et al.*, 1991; Laipis *et al.*, 1988). Rapid segregation of mtDNA genotypes has also been reported in humans carrying pathogenic or silent mtDNA mutations (Degoul *et al.*, 1997; Howell *et al.*, 1991; Howell *et al.*, 1994; Larsson *et al.*, 1992; Marchington *et al.*, 1997). These observations have resulted in the establishment of the genetic bottleneck hypothesis (Chinnery, 2002; Laipis *et al.*, 1988; Turnbull and Lightowlers, 1998), which states that only a fraction of mitochondria and/or mtDNA molecules is selectively sampled for amplification and transmission to the next generation. The mechanism for this

intergenerational genetic bottleneck remains obscure (Poulton *et al.*, 1998). The word bottleneck is a loose term that can refer to a process or a physical restriction in the number of mitochondria, mtDNA molecules, or both. It is generally believed that the bottleneck takes place during oogenesis prior to oocyte maturation (Marchington *et al.*, 1997; Marchington *et al.*, 1998). The size of the bottleneck may vary between different organisms (Ashley *et al.*, 1989; Jenuth *et al.*, 1996; Marchington *et al.*, 1998), and can be a low as a single mitochondrion or a few mtDNA molecules (Marchington *et al.*, 1998). Rapid segregation of mtDNA sequence variants is thought to be associated with inter-mitochondrial heteroplasmy where each mitochondrion contains either the wild type mitochondrial genome or the mutated form of the mtDNA, but not both.

Curiously, the segregation of mtDNA sequence variants can be unexpectedly slow in other situations. Stable and persistent intergenerational heteroplasmy has been observed in a number of systems including man (Howell *et al.*, 1992), mouse (Meirelles and Smith, 1997), *Drosophila* (Lecher *et al.*, 1994; Volz-Lingenhohl *et al.*, 1992), and cell culture (Yoneda *et al.*, 1992). Stable heteroplasmy may be attributed to a large bottleneck size and/or to intra-mitochondrial heteroplasmy where the two forms of mtDNA co-exist in every organelle. The faithful nucleoid model proposes that mtDNA molecules do not exist as independent entities within a mitochondrion but rather as groups forming polyploid nucleoids whose compositions do not change with replication (Jacobs *et al.*, 2000). In this scenario, the nucleoid is the segregating unit. The segregation of heteroplasmic mtDNA genotypes is expected to be slow if all the nucleoids are heteroplasmic.

1.6 Animal Models

With the growing number of nuclear and mtDNA lesions identified in humans, efforts have been increasingly aimed at elucidating the pathophysiological mechanisms of mitochondrial disorders. Since studies with human subjects are impractical or unethical, animal models are becoming invaluable for such investigations. In this section, I will briefly discuss a number of model systems currently being used for examining mitochondrial biogenesis and dysfunction.

Skeletal muscle biopsy samples (Morris *et al.*, 1995) and fetal tissues (Smeitink *et al.*, 2001) have been routinely used to study mitochondrial pathogenesis. While biopsy samples are considered to be the "golden standard" for the disease diagnostic process, cultured skin fibroblasts, chorionic villi, and amniocytes can be used for prenatal diagnosis. Mitochondrial fractions can be readily prepared for sensitive biochemical enzyme measurements (Morris *et al.*, 1995).

Intercellular transfer of mtDNA between cultured mammalian cells is a powerful system for the characterization of MRC mutants (King and Attardi, 1996). In this process, enucleated cells containing mitochondria and mtDNAs are transferred and fused with immortalized recipient cells devoid of mtDNA (rho^0) to form so-called cybrids. Nuclear DNA mutations can be similarly addressed by the fusion of a rho^0 cell, which contains the reference nucleus, with an enucleated patient cell still harboring the patient's mtDNA (Isobe *et al.*, 1997; Isobe *et al.*, 1998). The major advantage of this technique is that it allows for the examination of both nuclear and mtDNA mutations at the biochemical and genetic levels. However, cell cultures, cybrids, and biopsy samples are poor for tackling problems involving tissue specificity and genetic transmission. Moreover, cell cultures

may not accurately reproduce the *in vivo* situation when studying heteroplasmy and/or pathogenic mutations.

The yeast, *Saccharomyces cerevisiae* is an excellent model system for gaining insights into the functional basis of many human mitochondrial diseases (Attardi and Schatz, 1988). The major advantages of this organism are its amenability to genetic manipulations, its ability to grow aerobically and anaerobically, the uniformity of cell type, and the availability of a complete genome sequence (Goffeau *et al.*, 1996). However, yeast differs from humans in many respects. It is a unicellular, facultative anaerobic organism and can tolerate the loss of mtDNA or MRC function if provided with a fermentable carbon source such as glucose. It does not have a multi-subunit complex I as in mammals and does not possess any genes for complex I subunits in its mtDNA (Seo *et al.*, 1998; Seo *et al.*, 1999; Seo *et al.*, 2000). Furthermore, the yeast mtDNA is very different from its mammalian counterpart with respect to the gene content and size (Schmitt and Clayton, 1993). mtDNA heteroplasmy is unstable and segregation to homoplasmy is extremely rapid (Rickwood *et al.*, 1988). Also, yeast does not have differentiated or different tissue types (Rickwood *et al.*, 1988). Thus, yeast is probably not an adequate model system for investigating some aspects of mitochondrial diseases such as tissue-specificity.

The fungus, *Neurospora crassa*, has long been used as a model organism for studying the structure and bioenergetics of mitochondria, and in particular, complex I (Videira and Duarte, 2002). Mutations in complex I subunits have revealed that this enzyme is dispensable for vegetative growth, but is required during the sexual cycle for limited tissue differentiation. *N. crassa* expresses alternative mitochondrial reductases

and dehydrogenases (Schulte and Weiss, 1995a; Videira and Duarte, 2002). Thus, it is not suitable for studying some aspects of mitochondrial dysfunction.

The mouse has been developed as a system for studying mitochondrial dysfunction associated with nuclear gene mutations (Graham *et al.*, 1997; Huo and Scarpulla, 2001; Larsson *et al.*, 1998; Li *et al.*, 2000b). Tissue-specific nuclear gene knockouts can also be generated (Li *et al.*, 2000a; Silva *et al.*, 2000), and this allows for the investigation of tissue-specificity. Recently, defects associated with the mtDNA were introduced into mice by fusion of embryos with cybrids carrying a mutant form of mtDNA (Inoue *et al.*, 2000). The mouse is a sophisticated developmental system, containing multiple organs, such as the heart, the pancreas, and the brain. One major drawback of such a model is its relatively long life span, which makes it more cumbersome to maintain and more difficult to establish. The small litter size of the mouse makes it difficult to perform genetic screens. However, the complete genome sequence is now available, which should facilitate reverse genetic approaches.

The fruit fly, *Drosophila*, provides a sophisticated genetic and developmental system. The fly possesses multiple tissue types such as muscles and the central nervous system. Its genome sequence has recently been completed (*D. melanogaster* Sequencing Consortium, 2000). Previous mitochondrial investigations in this organism are extremely limited. Perhaps the best-studied example of a mutation affecting the MRC is a *Drosophila subobscura* mutant, which is heteroplasmic for a large mtDNA deletion that removes multiple MRC genes (Volz-Lingenhohl *et al.*, 1992). The heteroplasmy is stable and does not produce a phenotype or a decrease in ATP synthetic capacity despite the presence of 80% mutant mtDNA (Béziat *et al.*, 1997). Mutants with pathogenic levels of

heteroplasmy have not been isolated in this strain. Pathogenic mitochondrial mutations in a *Drosophila* model have only been identified recently, in nuclear genes involved in mtDNA replication and maintenance (Iyengar *et al.*, 1999; Iyengar *et al.*, 2002), and in the α subunit of the ATP synthase (Galloni and Edgar, 1999). The ATP synthase mutation has not been characterized extensively (Galloni and Edgar, 1999).

1.7 *Caenorhabditis elegans*

Caenorhabditis elegans is a small, free-living, multicellular eucaryote found in the soil. In the 1960's, Brenner selected it as a simple model for animal development and carried out a comprehensive study of the genetics and development of the nervous system (Brenner, 1974). We now understand more about the biology of *C. elegans* than of many other eucaryotes. It can be grown and propagated easily in the laboratory on an agar medium seeded with the bacterium, *Escherichia coli*, as the source of food. Its life cycle is complex but short, taking about three days to complete at 25°C (Figure 1.4) (Lewis and Fleming, 1995). A single self-fertilized adult hermaphrodite, which is about 1 mm in length, is capable of producing ~300 progeny that hatch, and develop through four larval stages (L1 to L4), which are separated by molts, before becoming adults (Figure 1.4) (Lewis and Fleming, 1995). When experiencing food deprivation or stress, young larvae can enter an alternative L3 stage called the dauer (Figure 1.4). An animal can remain dormant and survive for months in this stage, or it can exit dauer in response to favorable growth conditions (Riddle and Albert, 1997). *C. elegans* is anatomically simple (Figure 1.5) and increases in size throughout development; the most substantial increase happens during the L3-to-L4 transition (Lewis and Fleming, 1995). A newly hatched L1 larva has

558 somatic nuclei; this number increases to 959 in an adult hermaphrodite and 1,031 in an adult male (Wood, 1988). The number of germ cells also increases, from two in a newly hatched L1 to about 1,500-2,000 in an adult animal (Schedl, 1997). The hermaphrodite is protandrous: its germ cells differentiate into sperm in the mid-to-late L4 stage and oocytes in the adult stage. The matuaration of the somatic gonad and the hermaphrodite vulva occurs primarily at the L4 stage (Schedl, 1997). The nematode contains multiple tissue types such as neurons, muscles, intestine, pharynx, and the germline (Wood, 1988). Its transparent nature and its invariant cell lineage (Sulston and Horvitz, 1977; Sulston *et al.*, 1983) have allowed every cell to be followed precisely by microscopy.

The wealth of experimental technology and knowledge available in the nematode system may contribute significantly to the investigations of many fundamental biological processes that occur in nature. The availability of both the mtDNA sequence and the nuclear DNA sequence allows rapid investigation of gene function by both forward and reverse genetics. In 1998, the complete genome sequence of *C. elegans* became available, and the nematode became the first multicellular organism to enter the post-genomic era (*C. elegans* Sequencing Consortium, 1998). The construction of a physical map of the genome and its alignment to the genetic map facilitates the molecular analysis of mutant lesions. A number of human disease genes have homologues in the nematode, and studies in the nematode system have already yielded important insights into the function of some of these genes (Aboobaker and Blaxter, 2000; Culetto and Sattelle, 2000). Specific loss-of-function phenocopies can be produced by introducing double-stranded RNA into a worm in a phenomenon known as RNA interference, or RNAi (Fire *et al.*, 1998). The

RNAi phenomenon was originally discovered in the nematode and was subsequently found to be present in other eucaryotes. Many of the features of RNAi have been studied and described extensively in *C. elegans*. The powerful RNAi technique offers a quick and easy way to study the relationship between genotype and phenotype (Fire *et al.*, 1998). Finally, genomic DNA transformation of mutant animals by microinjection can provide valuable information linking genotype to phenotype (Mello and Fire, 1995).

Murfitt *et al.* performed the first detailed studies on mitochondria in the nematode in 1976 (Murfitt *et al.*, 1976). They developed conditions for bulk growth of nematodes and an isolation procedure for respiration-competent mitochondria. Their biochemical measurements on respiration rates and enzyme activities demonstrated that the structure, metabolism, and bioenergetics of the nematode MRC are very similar to their mammalian counterpart. Subsequently, it was shown that many intermediary metabolic pathways, including the Krebs cycle, were also present in *C. elegans* (O'Riordan and Burnell, 1989; Wadsworth and Riddle, 1989). In 1992, Okimoto *et al.* sequenced the nematode mtDNA, which turned out to be highly analogous in size and gene content to the human mtDNA (Okimoto *et al.*, 1992). The nematode mtDNA (~13-kbp) is slightly smaller than its human counterpart, and differs by lacking the ATP8 gene and by having a different gene organization and transcriptional pattern (the two rRNAs are indicated as s-rRNA and l-rRNA (Figure 1.6)). Most of the 12 MRC polypeptides encoded by the *C. elegans* mtDNA are conserved among eucaryotes (Table 1.1). A survey of the *C. elegans* nuclear genome reveals that the majority of the nuclear-encoded MRC genes is also present and is highly conserved in the nematode (Table 1.1). There appear to be more conserved

MRC subunits in the nematode than in the fungus, *N. crassa*, suggesting that *C. elegans* has a mammalian-like MRC.

C. elegans does have a few disadvantages. First, it is not an ideal system to carry out biochemical studies. The yields of isolated mitochondria are low and this may be partly attributed to the isolation procedure, which requires the disruption of the tough, external cuticle (Kayser *et al.*, 2001; Murfitt *et al.*, 1976). Second, it is technically difficult to isolate tissues for biochemical studies, and immortal cell lines and tissue culture remain unavailable (Christensen *et al.*, 2002). Third, methods for the introduction of foreign mtDNA molecules into worms have not been described so far. Thus, it is currently impossible to study the effects of specific, engineered mtDNA mutations in the nematode system.

There are several reports on mutations affecting MRC function in *C. elegans*. The nematode *dif-1*, *gas-1*, *mev-1*, *clk-1*, and *isp-1* mutants illustrate some of the possible phenotypes that MRC dysfunction may produce. *dif-1* embryos complete embryonic cell division but arrest prior to tissue differentiation (Ahringer, 1995). The product of the *dif-1* gene is a mitochondrial carrier protein whose function is unknown, but is thought to be involved in embryonic energy metabolism (Ahringer, 1995). The *gas-1* mutation was originally identified in a screen for anesthetic hypersensitivity (Kayser *et al.*, 1999). It is a missense mutation in the 49-kDa subunit of complex I (Table 1.1). Animals homozygous for the *gas-1* mutation are viable, have a reduced brood size, and are hypersensitive to oxygen and paraquat, a free radical generating compound (Hartman *et al.*, 2001). Curiously, the *mev-1* mutation shares many features with the *gas-1* mutation. The *mev-1* mutation, which was identified in a screen for hypersensitivity to paraquat

(Ishii *et al.*, 1990), is a missense mutation in the cytochrome b_{560} or *SDHC* subunit of complex II (Table 1.1) (Ishii *et al.*, 1998). *mev-1* homozygotes are viable with a reduced brood size and are sensitive to high oxygen concentrations (Ishii *et al.*, 1998). Both the *gas-1* and the *mev-1* mutations are thought to cause an increased production of ROS. Missense mutations in the *clk-1* and *isp-1* genes result in life span extension and a slowing of developmental timing and the pace of behaviors (Wong *et al.*, 1995; Feng *et al.*, 2001b). *clk-1* encodes a mitochondrial enzyme responsible for the final hydroxylation step in the biosynthesis of coenzyme Q (Ewbank *et al.*, 1997). *isp-1*, on the other hand, encodes the ISP subunit of complex III (Table 1.1).

1.8 Thesis Problem

In recent years, the development of animal models has become invaluable for studying and examining the pathogenic consequences and mechanisms associated with mitochondrial malfunction. However, there is currently no suitable model system that allows for the investigation of every aspect of mitochondrial biogenesis and dysfunction (DiMauro and Andreu, 2000; Taylor *et al.*, 1997). Although a considerable amount of knowledge has been gained over the years from studies carried out in yeast and in *Neurospora*, and more recently in mice, each of these systems presents its own disadvantages and limitations. Thus, there is a need to establish other animal models for tackling some of the problems raised in the field of mitochondrial biogenesis, genetics, and diseases. In my thesis, I describe the early development of the nematode, *C. elegans* as such a model organism. The conservation of metabolism, structure, and bioenergetics between nematode and human mitochondria, along with the many technological and

anatomical advantages offered by *C. elegans*, make it an excellent system for investigating mitochondrial function in a multicellular eucaryote.

Perhaps the major deficit in the field of mitochondrial medicine is an understanding of the relationship between genotype and phenotype (Grossman and Shoubridge, 1996; Lightowlers *et al.*, 1997; Wallace, 1993; Wallace, 1997; Wallace, 1999). How do MRC mutations give rise to a wide variety of disease phenotypes in humans? Are the processes the same in an anatomically simpler, multicellular organism like *C. elegans*? To address this question, a library of mutagenized animals was created and screened to identify lesions in a number of selected MRC genes (Jansen *et al.*, 1997; Tsang *et al.*, 2001). We have screened for deletion mutations in eight different nuclear MRC genes (Table 1.1) and in the mtDNA. Four authentic nuclear MRC mutations and two mtDNA mutations have been identified and confirmed by DNA sequencing. Chapters 2 and 4 describe the isolation and characterization of two nuclear MRC mutations and one mtDNA mutation, respectively. The phenotypes of these mutant animals were studied and compared. In addition, I have indirectly examined the consequences of mitochondrial dysfunction by inhibiting proper mtDNA expression with specific chemical inhibitors; these data are presented in chapters 2 and 5. How do these inhibitors affect normal nematode development? Are the phenotypes of these drug-treated animals similar to the nuclear and/or mitochondrial MRC mutants? Using these approaches, we have gained insight into the molecular mechanisms of mitochondrial dysfunction in the nematode system.

One peculiar aspect regarding mitochondrial diseases is the tissue-specificity associated with many human mitochondrial deficiencies (Beal, 1995; Luft and Landau,

1995; Wallace, 1992). In chapter 3, I have utilized a technique called mosaic analysis, which allows for an investigation into the function of a gene of interest in a cell or tissue-specific manner (Herman, 1995; Yochem *et al.*, 2000). Mosaic analysis allows for the investigation of genetic mosaics whereby some cells are genetically wild type while others are genetically mutant due to the spontaneous loss of a complementing transgene. I have used mosaic analysis to examine the tissue dependence of the *atp-2* gene.

Mitochondrial genetics is an area currently under active investigation (Moraes, 2001). In chapter 4, I have addressed questions regarding the fundamental principles of transmission, segregation, and maintenance of mtDNA in *C. elegans*. By screening a library of mutagenized worms, I have identified and cloned a mtDNA mutation. With the *uaDf5* mutation, I have been able to explore mtDNA inheritance and the processes involved in the maintenance of a heteroplasmic state.

The number of mitochondria and mtDNA genomes tend to correlate loosely with the energy demands of cells (Moraes, 2001; Williams, 1986). Surprisingly, there are currently no complete descriptions of changes in the number of mitochondria or mtDNA copy number in any eucaryotic species. Measurements of mitochondrial organelle number may be difficult to perform, since mitochondria are structurally dynamic in nature. Instead, in chapter 5, I have followed and measured the mtDNA content throughout development. I have determined how mtDNA copy numbers change during nematode development. In addition, I have recorded the mtDNA copy numbers of a number of mutant strains that are defective in germ line, oocyte, or sperm formation to assess the mtDNA copy numbers in different cell types.

Complex	Bovine	*Neurospora*	Human	*C. elegans*	P value[a]
I – FP	51kD	51kD	*NDUFV1*	C09H10.3 (*nuo-1*)*	$1.6x10^{-200}$
	24kD	24kD	*NDUFV2*	F53F4.10	$9.9x10^{-82}$
	9kD	?	*NDUFV3*	Y73C8C.3	0.26
I – IP	75kD	78kD	*NDUFS1*	Y45G12B.1	$3.3x10^{-225}$
	49kD	49kD	*NDUFS2*	T26A5.3 K09A9.5 (*gas-1*)	$6.0x10^{-165}$ $2.3x10^{-163}$
	30kD	31kD	*NDUFS3*	T10E9.7	$3.8x10^{-73}$
	23kD (TYKY)	23kD	*NDUFS8*	T20H4.5*	$4.8x10^{-66}$
	20kD (PSST)	19.3kD	*NDUFS7*	W10D5.2	$6.3x10^{-64}$
	18kD (AQDQ)	21kD	*NDUFS4*	ZK973.10	$1.6x10^{-35}$
	15kD	15kD	*NDUFS5*	Y54E10BL.5	$8.8x10^{-10}$
	13kD (A)	13kD	*NDUFS6*	F22D6.4	$1.8x10^{-20}$
	13kD (B)	29.9kD	*NDUFA5*	C33A12.1	$1.2x10^{-30}$
I – HP	ND1	ND1	ND1	ND1	$1.8x10^{-33}$
	ND2	ND2	ND2	ND2	$8.0x10^{-3}$
	ND3	ND3	ND3	ND3	$2.8x10^{-7}$
	ND4	ND4	ND4	ND4	$2.4x10^{-47}$
	ND4L	ND4L	ND4L	ND4L	>0.1
	ND5	ND5	ND5	ND5	$1.6x10^{-50}$
	ND6	ND6	ND6	ND6	>0.1
	B18	?	*NDUFB7*	D2030.4	$1.0x10^{-17}$
	SDAP (ACP)	9.5kD (ACP)	*NDUFAB1*	Y56A3A.19	$6.5x10^{-30}$
	B22	B22	*NDUFB9*	C16A3.5	$3.2x10^{-21}$
	SGDH	?	*NDUFB5*	C25H3.9	$9.8x10^{-11}$
	B14.5a	?	*NDUFA7*	F45H10.3	$4.8x10^{-11}$
	19kD (PGIV)	20.8kD	*NDUFA8*	Y54F10AM.5	$2.1x10^{-17}$
	MLRQ	?	*NDUFA4*	F13D12.6	0.48
	MWFE	9.8kD	*NDUFA1*	F19H8.1	0.14
	KFYI	?	*NDUFC1*	C25E10.4	0.15
	B17	?	*NDUFB6*	C09G5.1	0.37
	B12	?	*NDUFB3*	C18E9.4	$1.1x10^{-2}$
	B8	10.5kD	*NDUFA2*	Y63D3A.7	$1.1x10^{-18}$
	MNLL	?	*NDUFB1*	B0432.7	0.46
	B14.5b	?	*NDUFC2*	Y71H2AM.4	$4.2x10^{-5}$
	B9	9.5kD	*NDUFA3*	Y54E2A.8	0.35
	42kD	?	*NDUFA10*	K04G7.4A	$7.0x10^{-33}$
	AGGG	?	*NDUFB2*	F44G4.2	$9.1x10^{-8}$

	ASHI	?	*NDUFB8*	Y51H1A.3A/B	$2.2x10^{-13}$
	PDSW	12.3kD	*NDUFB10*	F59C6.5	$4.4x10^{-13}$
	B15	?	*NDUFB4*	F21A3.6	0.92
	B14	14.8kD	*NDUFA6*	Y57G11C.12	$6.7x10^{-12}$
	39kD	40kD	*NDUFA9*	Y53G8AL.2	$1.4x10^{-50}$
	B17.2	B17.2	B17.2	Y94H6A.8	$7.7x10^{-27}$
	B16.6	?	?	C34B2.8	$1.9x10^{-16}$
II	FP	FP	*SDHA*	C03G5.1* F48E8.3	$1.2x10^{-243}$ $2.8x10^{-12}$
	IP	IP	*SDHB*	F42A8.2*	$5.0x10^{-94}$
	CII-3 (QPS1)	?	*SDHC*	T07C4.7 (*mev-1*)*	$2.1x10^{-17}$
	CII-4 (QPS2)	?	*SDHD*	F33A8.5*	$1.1x10^{-9}$
III	I	I	*UQCRC1*	ZC410.2	$8.4x10^{-92}$
	II	II	*UQCRC2*	T10B10.2	$4.0x10^{-37}$
	III (Cyt *b*)	Cyt *b*	Cyt *b*	Cyt *b*	$1.8x10^{-81}$
	IV (cyt c_1)	cyt c_1	*CYC1*	C54G4.8	$1.0x10^{-63}$
	V (RISP)	RISP	*UCR1*	F42G8.12 (*isp-1*)	$4.2x10^{-58}$
	VI (14kD)	?	*UQCRB*	T02H6.11	$1.4x10^{-11}$
	VII (9.5kD)	VII	*UQCRQ*	F45H10.2 R07E4.3	$1.6x10^{-10}$ $1.6x10^{-10}$
	VIII (9.2kD)	VIII	*UQCRH*	T27E9.2	$4.7x10^{-11}$
	IX (8.0kD)	?	?	?	
	X (7.2kD)	?	*UQCR10*	F37C4.1	0.12
	XI (6.4kD)	?	*UQCR11*	Y54G2A.32	$7.0x10^{-2}$
IV	I (COI)	COI	COI	COI	$7.8x10^{-177}$
	II (COII)	COII	COII	COII	$3.9x10^{-40}$
	III (COIII)	COIII	COIII	COIII	$2.6x10^{-59}$
	IV	V	*COX4*	W09C5.8	$1.9x10^{-14}$
	Va	VI	*COX5A*	Y37D8A.14	$1.8x10^{-27}$
	Vb	IV	*COX5B*	F26E4.9	$1.6x10^{-12}$
	VIa	?	*COX6A1*	F54D8.2*	$5.7x10^{-15}$
	VIb	?	*COX6B*	Y71H2AM.5	$5.0x10^{-16}$
	VIc	?	*COX6C*	F29C4.2	$4.2x10^{-3}$
	VIIa	?	*COX7A*	ZC581.7	0.12
	VIIb	?	*COX7B*	K02B2.1	0.89
	VIIc	?	*COX7C*	F26E4.6	$9.4x10^{-8}$
	VIII	?	*COX8*	F14F11.2	0.12
V-F_0	a	a	ATP6	ATP6	$2.9x10^{-9}$
	b	b	*ATP5F1*	F02E8.1	$4.5x10^{-11}$
	c	c	*ATP5G3*	Y82E9BR.3	$2.0x10^{-21}$
	d	?	*ATP5H*	C06H2.1	$3.2x10^{-8}$

	e	?	*ATP5I*	R04F11.2	$2.0x10^{-5}$
	f	?	*ATP5J2*	R53.4	$1.0x10^{-8}$
	g	?	Subunit G	C53B7.4 K07H12.3	$6.7x10^{-12}$ $6.7x10^{-12}$
	F6	?	*ATP5J*	T05H4.12	$4.1x10^{-2}$
	A6L	8	ATP8	C03A7.3	0.23
	OSCP	ATP5	*ATP5O*	F27C1.7	$4.0x10^{-37}$
	IF_1	?	*ATPI*	B0546.1	$1.0x10^{-8}$
V-F_1	α	α	*ATP5A1*	H28O16.1	$5.9x10^{-226}$
	β	β	*ATP5B*	C34E10.6 (*atp-2*)*	$2.0x10^{-209}$
	γ	?	ATP5C1	Y69A2AR.18A Y69A2AR.18B	$1.1x10^{-82}$ $6.5x10^{-62}$
	δ	δ	*ATP5D*	F58F12.1	$1.9x10^{-30}$
	ε	ε	*ATP5E*	R05D3.6 ZC262.5	$1.5x10^{-5}$ $1.5x10^{-5}$

Table 1.1: Conservation of MRC subunits in eucaryotes

[a] Human protein sequences were blasted against the *C. elegans* protein sequences. The P value reflects the probability that the two sequences match with each other by chance. A P value of $<10^{-6}$ is defined as a "match" and implies a conserved homolog is present in *C. elegans*. A P value of $>10^{-6}$ may indicate a probable homologue in the nematode.

* These genes have been screened for deletion mutations.

Function	Gene	Mutation	Phenotype	Reference
Complex I structural	*NDUFV1*	Point; Deletion; Inversion	Myoclonic epilepsy; Leukodystrophy; Leigh syndrome	(Benit *et al.*, 2001; Schuelke *et al.*, 1999)
	NDUFS1	Point; Deletion	Leigh syndrome	(Benit *et al.*, 2001)
	NDUFS2	Point	Cardiomyopathy; Encephalomyopathy	(Loeffen *et al.*, 2001)
	NDUFS4	Duplication	Leigh syndrome	(van den Heuvel *et al.*, 1998)
	NDUFS7	Point	Leigh syndrome	(Triepels *et al.*, 1999)
	NDUFS8	Point	Leigh syndrome	(Loeffen *et al.*, 1998)
	ND1	Point; Inversion	LHON; MELAS; Exercise intolerance/weakness; Bilateral striatal necrosis	(Campos *et al.*, 1997; Cavelier *et al.*, 2001; Huoponen *et al.*, 1991; Musumeci *et al.*, 2000)
	ND4	Point	LHON; Dystonia; Exercise intolerance/weakness	(Andreu *et al.*, 1999b; Wallace *et al.*, 1988)
	ND5	Point	MELAS; Bilateral striatal necrosis; Leigh syndrome	(Santorelli *et al.*, 1997; Taylor *et al.*, 2002b)
	ND6	Point	LHON; Dystonia	(Johns *et al.*, 1992; Jun *et al.*, 1994; Valentino *et al.*, 2002)
Complex II structural	*SDHA*	Point	Leigh syndrome	(Bourgeron *et al.*, 1995; Parfait *et al.*, 2000)
	SDHB	Point	Hereditary paraganglioma	(Astuti *et al.*, 2001)
	SDHC	Point	Hereditary paraganglioma	(Niemann and Muller, 2000)
	SDHD	Point; Inversion	Hereditary paraganglioma	(Baysal *et al.*, 2000; Gimenez-Roqueplo *et al.*, 2001; Gimm *et al.*, 2000)
Complex III structural	Cyt *b*	Point; Deletion	MELAS; Exercise intolerance/weakness; Cardiomyopathy; Encephalomyopathy; Parkinson; Myoglobinuria	(Andreu *et al.*, 1999a; Andreu *et al.*, 2000; de Coo *et al.*, 1999; Schuelke *et al.*, 2002; Valnot *et al.*, 1999)

Complex IV structural	COI	Point; Deletion	Acquired sideroblastic anemia; Deafness/blindness/ ataxia; Motor neuron disease; Exercise intolerance/weakness; Myoglobinuria	(Bruno *et al.*, 1999; Comi *et al.*, 1998; Gattermann *et al.*, 1997; Karadimas *et al.*, 2000)
	COII	Point	Myopathy/ataxia/ dementia	(Clark *et al.*, 1999; Rahman *et al.*, 1999)
	COIII	Point; Deletion	MELAS; Exercise intolerance/weakness; Myoglobinuria; Encephalomyopathy	(Hanna *et al.*, 1998; Keightley *et al.*, 1996; Manfredi *et al.*, 1995)
Complex V structural	*ATP6*	Point	Bilateral striatal necrosis; MILS; Neuropathy/ataxia/ retinitis pigmentosa	(de Meirleir *et al.*, 1995; de Vries *et al.*, 1993; Thyagarajan *et al.*, 1995)
MRC biogenesis	rRNA in mtDNA	Point	Cardiomyopathy; Deafness; Diabetes	(Estivill *et al.*, 1998; Prezant *et al.*, 1993; Santorelli *et al.*, 1999; Yang *et al.*, 2002)
MRC biogenesis	tRNA in mtDNA	Point; Insertion	MELAS; MILS; MERRF; Diabetes; Myoglobinuria; Myopathy; Cardiomyopathy; Encephalomyopathy; Anemia; Deafness; Progressive external ophthalmoplegia; Alzheimer; Parkinson	(Bindoff *et al.*, 1993; Cavelier *et al.*, 2001; Goto *et al.*, 1990; Goto *et al.*, 1992; Hadjigeorgiou *et al.*, 1999; Kleinle *et al.*, 1998; Moraes *et al.*, 1993; Ogle *et al.*, 1997; Shoffner *et al.*, 1990; Shoffner *et al.*, 1993; Silvestri *et al.*, 1998; Taylor *et al.*, 2002a; Tiranti *et al.*, 1995; Vissing *et al.*, 1998)

MRC biogenesis	Multiple genes in mtDNA	Deletion	KSS; Progressive external ophthalmoplegia; Cardiomyopathy; Encephalopathy; Neuropathy; Huntington	(Campos *et al.*, 2000; Holt *et al.*, 1988; Horton *et al.*, 1995; Lestienne and Ponsot, 1988; McDonald *et al.*, 2002; Moraes *et al.*, 1989; Rotig *et al.*, 1991)
Complex III assembly	*BCS1L*	Point	Neonatal proximal tubulopathy; Encephalopathy	(de Lonlay *et al.*, 2001)
Complex IV assembly	*SURF1*	Point; Insertion; Deletion	Leigh syndrome	(Poyau *et al.*, 2000; Tiranti *et al.*, 1998; Tiranti *et al.*, 1999; Zhu *et al.*, 1998)
Complex IV assembly	*SCO1*	Point; Deletion	Neonatal hepatic failure; Encephalopathy	(Valnot *et al.*, 2000a)
Complex IV assembly	*SCO2*	Point	Infantile cardioencephalopathy	(Jaksch *et al.*, 2000; Papadopoulou *et al.*, 1999)
Complex IV assembly	*COX10*	Point	Encephalopathy	(Valnot *et al.*, 2000b)

Table 1.2: Human mitochondrial disease genes coding for structural, biogenesis, and assembly components of the MRC

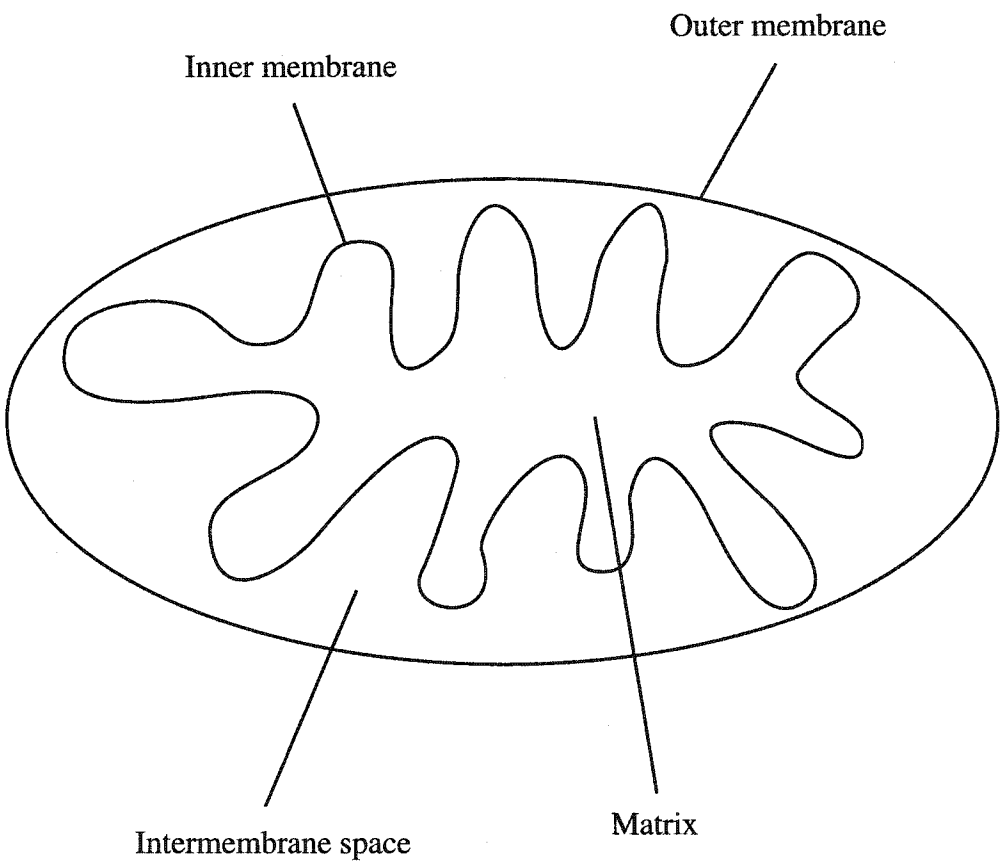


Figure 1.1: Structure of a mitochondrion

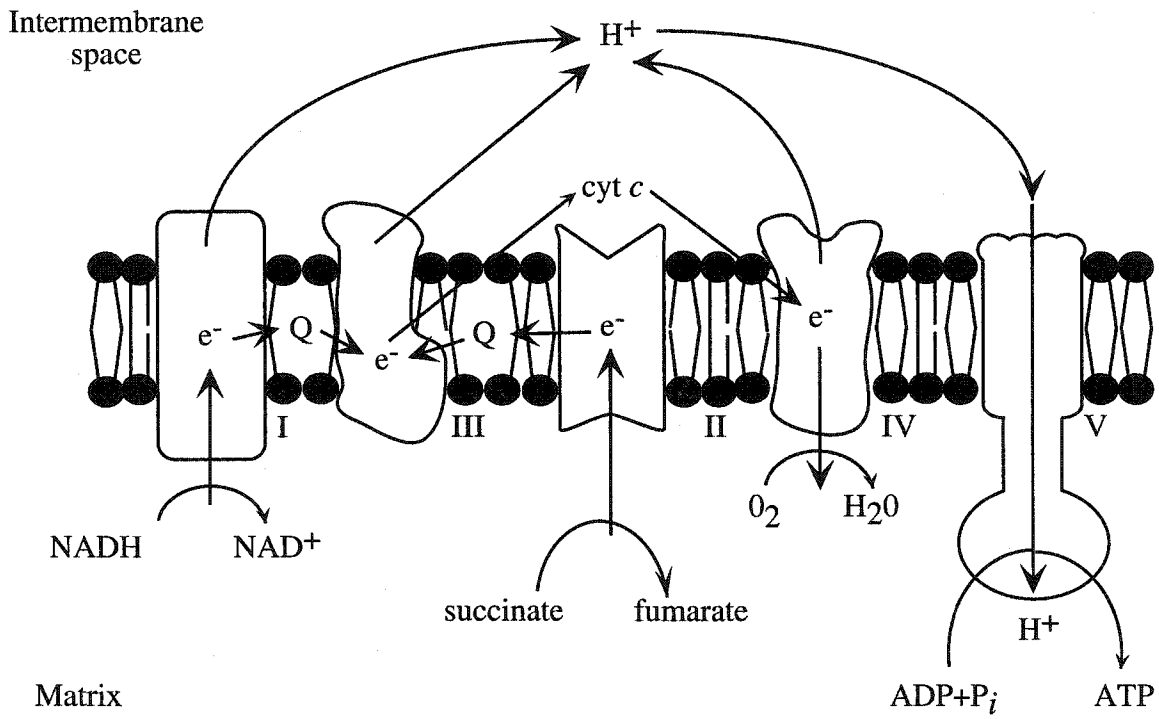


Figure 1.2: The mitochondrial respiratory chain

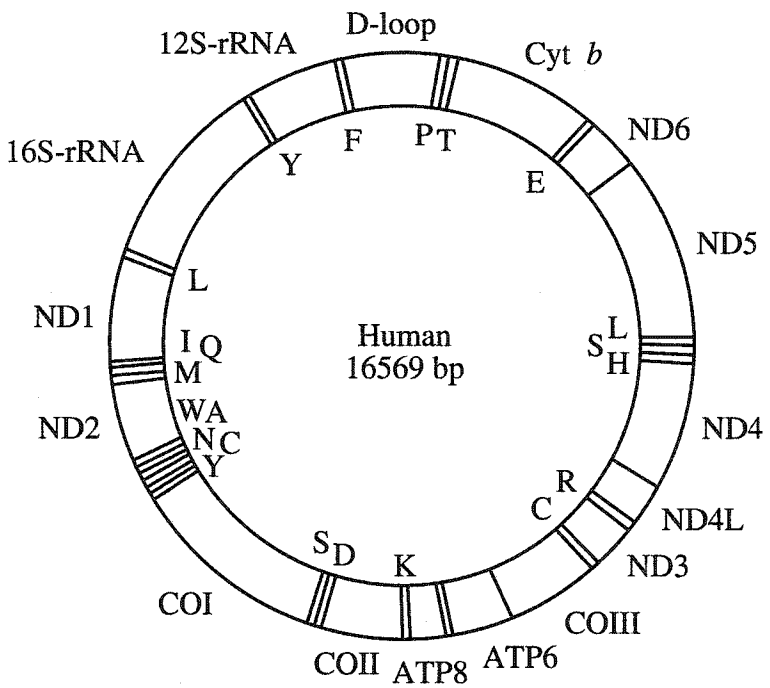


Figure 1.3: A circular map of the human mtDNA molecule
Each tRNA gene is identified by the one letter amino acid code.

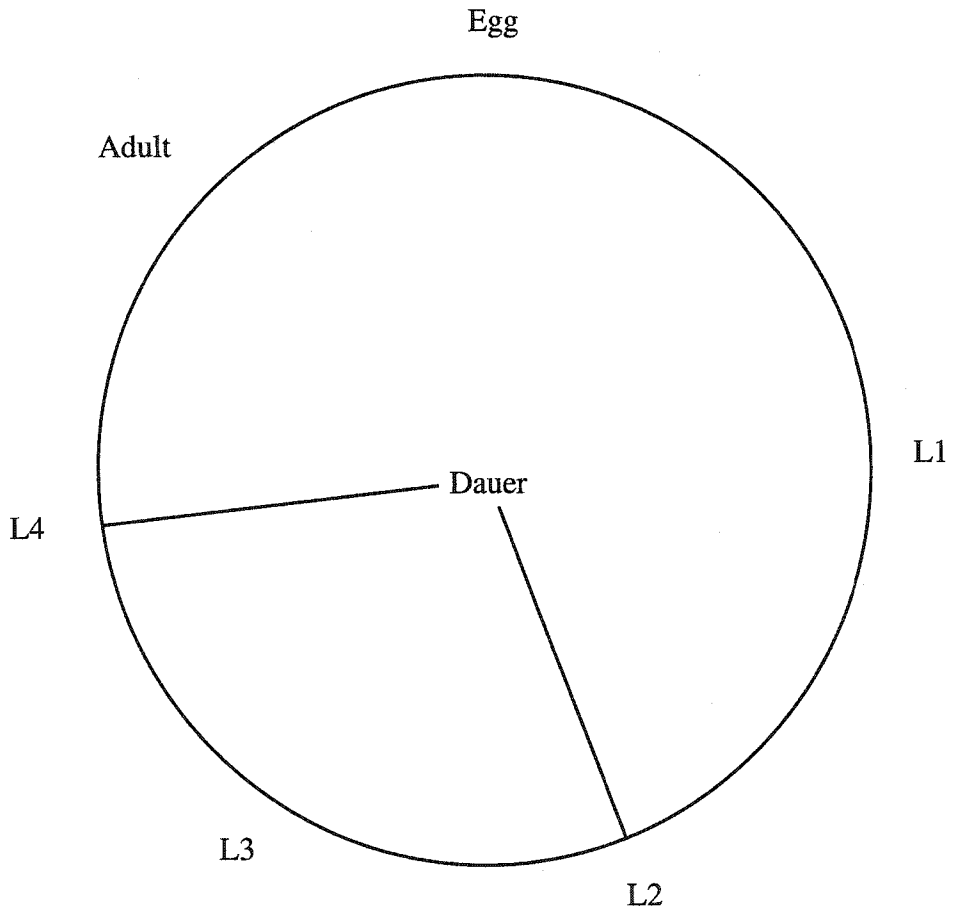
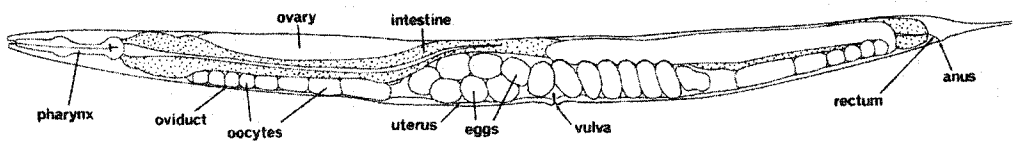
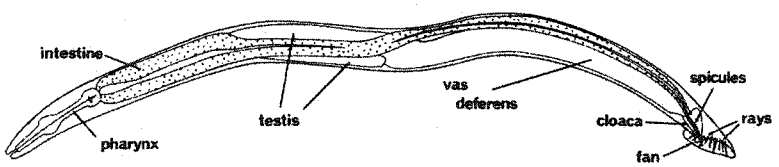


Figure 1.4: The life cycle of *C. elegans* (adapted from [Wood, 1988])



Hermaphrodite



Male

Figure 1.5: Photomicrographs showing major anatomical features of *C. elegans* adult hermaphrodite and male (reprinted from Developmental Biology, 56, Sulston and Horvitz, Post-embryonic cell lineages of the nematode, *Caenorhabditis elegans*, Pages 110-156, 1977, with permission from Elsevier)

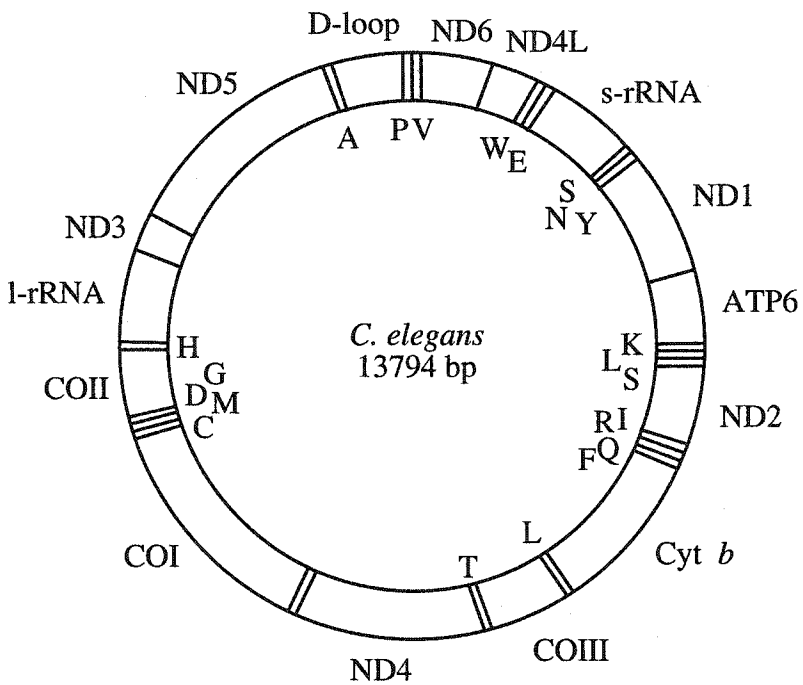


Figure 1.6: A circular map of the *C. elegans* mtDNA molecule
Each tRNA gene is identified by the one letter amino acid code.

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Chapter 2

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

A version of this chapter has been published. Tsang, W. Y., Sayles, L. C., Grad, L. I., Pilgrim, D. B., and Lemire, B. D. (2001) *J. Biol. Chem.* **276**, 32240-32246. Nomarski photographs of gonad development in *nuo-1* animals, the *nuo-1* life span, the *nuo-1* swimming rate, and the osmotic avoidance of *nuo-1* animals were performed by L. Sayles. L. Grad sequenced the cDNAs of *nuo-1* and *atp-2*. B. Lemire constructed the ethyl methane sulfonate mutagenized N2 library, isolated and sequenced the *nuo-1* deletion, and performed the dauer experiments on *nuo-1* worms. This work was made possible with the generous support and help of D. Pilgrim.

2.1 Introduction

The human mitochondrial respiratory chain (MRC) is composed of over 80 subunits but requires more than 100 additional genes for its assembly (Attardi and Schatz, 1988). 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 the mitochondrial DNA (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 (DiMauro and Andreu, 2000; Rubio-Gozalbo *et al.*, 2000; Sue and Schon, 2000). The metabolism and structure of the *Caenorhabditis elegans* MRC closely parallel its mammalian counterpart (Murfitt *et al.*, 1976). Moreover, the nematode mtDNA is similar in size and gene content to the human mtDNA (Okimoto *et al.*, 1992).

The MRC is organized into 5 multi-subunit 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. The bovine complex I consists of about 43 subunits, an FMN cofactor, and up to 8 iron-sulfur clusters (Ohnishi, 1998; Walker, 1992). The FMN and the 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 (Genbank Accession Number NP_496376) bears strong sequence resemblance (75% identical amino acids) to the human gene (Genbank Accession Number P49821) (Figure 2.1). 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 (Morris *et al.*, 1996; Robinson, 1998; Smeitink and van den Heuvel, 1999). Mutations in the *NDUFV1* gene, which encodes the human 51-kDa subunit of complex I, result in myoclonic epilepsy and leukodystrophy (Schuelke *et al.*, 1999).

Complex V, the ATP synthase, uses the proton gradient to catalyze the synthesis of ATP from ADP and inorganic phosphate (Boyer, 1997). A selective deficiency of the human mitochondrial ATP synthase caused by an uncharacterized nuclear mutation resulted in heart failure (Houstek *et al.*, 1999). The ATP synthase is composed of a membrane-intrinsic proton channel, the F_0, and a peripheral catalytic domain, the F_1. The F_1 domain comprises 5 types of subunits forming an a_3, $ß_3$, γ, δ, ε complex. Three catalytic nucleotide-binding sites are associated primarily with the ß-subunits (Abrahams *et al.*, 1994). The *C. elegans* gene product C34E10.6 (Genbank Accession Number NP_498111) shares 80% identical amino acids with the human ß subunit gene (Genbank Accession Number P06576) (Figure 2.2). We designate this gene as *atp-2*.

The contribution of the mitochondrial genome to cellular energy metabolism and to organismal development is an area of active research (Jenuth *et al.*, 1996; Larsson *et al.*, 1998; Wallace, 1999). In humans, mtDNA mutations produce a heterogeneous group of disorders (Zeviani and Antozzi, 1997). Depletion of mtDNA, which also impairs MRC biogenesis, produces an autosomal recessive disorder resulting in myopathy or hepatopathy and early death (Zeviani and Antozzi, 1997). Mice heteroplasmic for a large deletion in their mtDNA demonstrate mitochondrial dysfunction in various tissues and die of renal failure (Inoue *et al.*, 2000).

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 larval stage. Despite the developmental arrest, mutant animals have extended lifespans. 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 that have been used to inhibit the synthesis of essential mtDNA encoded subunits of the MRC (Nijtmans *et al.*, 1995; Piko and Chase, 1973). 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 by the 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 approximately 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 (*C. elegans* Sequencing Consortium, 1998).

2.2 Materials and Methods

Strains

Worms were cultured as described (Lewis and Fleming, 1995). The following strains or mutations were used: N2 (Bristol) wild-type strain; CB404, *unc-53(e404)*; DR2078 (a gift from M. Edgley), *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 (GFP) 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); JK1122, *dpy-17(e164) unc-32(e189)/qC1 dpy-19(e1259) glp-1(q339)*III; CB1370, *daf-2(e1370)*; DP44, *daf-7(e1372)*. *daf-2(e1370)* and *daf-7(e1372)* mutations are temperature-sensitive. Animals carrying a *daf-2* or a *daf-7* mutation grow normally at the permissive temperature of 15°C but arrest as dauers at the restrictive temperature of 25°C. 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) unc32(e189)*; LB127, *atp-2(ua2) sDp3*(III;f); LB128, *atp-2(ua2) unc32(e189)/qC1[dpy-19(e1259) glp-1(q339)]*; LB136, *daf-7(e1372)/daf-7(e1372) atp-2(ua2) unc-32(e189)*; LB139, *daf-2(e1370)/daf-2(e1370) atp-2(ua2) unc-32(e189).*

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 (Figure

2.3) (Jansen *et al.*, 1997). A library of ethyl methane sulfonate mutagenized N2 (synchronized and treated as L4s, 50 mM for 4 h) was constructed by transferring approximately 1,000 F_1's to each of 135 plates (Anderson, 1995). After the plates had cleared, the worms were washed off and divided into three portions, two for freezing and one for DNA isolation (Plasterk, 1995). We visualized deletions using pairs of nested primers chosen to be about 3-kbp apart, using duplicate or triplicate reactions with standard PCR conditions (Plasterk, 1995). The primers used were a) for *nuo-1*: NFP1, 5'-TGCCTGAAAAGCCATACTGTC-3', NFP2, 5'-GTAAACGACACTCCGAAATTCC-3', NFP3, 5'-CTCATTCATTCAGCGAGGTTG-3', and NFP4, 5'-AGATCTTCTACGG-ACAAGGTG-3' and b) for *atp-2*: F1B1, 5'-CTGAATTCTGCACTGCAATCAC-3', F1B2, 5'-ACGTTCAGCCTGAACTGAGAC-3', F1B3, 5'-CCGATGTG-TATTCTAGAGTTCG-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, 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 to 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) and *unc-32* (0.00), to create LB121, *dpy-17(e164)/atp-2(ua2) unc32(e189)* by outcrossing six times against SP471 and *dpy-17(e164)*. The *atp-2(ua2)* mutation was balanced by crossing LB121 to JK1122 to generate LB128. The *atp-2(ua2)*

mutation was also outcrossed six times and balanced by crossing LB100 to BC986 and *unc36(e251)* to generate LB127. LB136 and LB139 were generated by crossing LB128 to DP44 and CB1370, respectively. 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 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 the *Saccharomyces cerevisiae* ATP2p (Dibrov *et al.*, 1998). Detection was with a peroxidase-labeled goat anti-rabbit secondary antibody and the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech Inc., Baie d'Urfé, Québec).

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).

Lifespan measurements

N2, LB21B, or LB128 gravid adults were allowed to lay eggs for 6 h at 25 °C. Once hatched, groups of 5 wild type, homozygous *nuo-1*, or *atp-2* progeny were transferred onto single NGM 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, defecation, and food sensing

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. For food sensing, synchronized LB21 or LB127 animals were transferred to NGM plates and directly onto a localized food source. The number of phenotypically wild type or mutant animals found away from the food source was scored 3 h later.

RNA interference

For *nuo-1*, a 2.9-kbp PCR product, amplified using N2 genomic DNA as a template with NFP2 and NFP4 primers, was digested with *Sac* I and *Sac* II. The 0.8-kbp *Sac* I/*Sac* II fragment was cloned into likewise digested pBluescript II SK (PDI BioScience, Aurora, Ontario) and pBC KS (PDI BioScience, Aurora, Ontario) in order to place the gene under the control of the T7 promoter in both the forward and the reverse orientations. For *atp-2*, a 2.9-kbp PCR product amplified with F1B2 and F1B4 primers was digested with *Xho* I and *Hin*d III. The 1.1-kbp *Xho* I/*Hin*d III 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, Aurora, Ontario) expressing the T7 RNA polymerase gene from an inducible *lac* promoter and were grown either in the absence or the presence of 1mM IPTG 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 less than 60% nucleotide sequence identity over 200 nucleotides to the *atp-2* and *nuo-1* regions we used for our RNAi studies, making it unlikely any cross interference is responsible for the observed phenotypes (Bosher and Labouesse, 2000).

Entry into and exit from dauer

LB21B or LB127 were grown on single NGM plates. Seven 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. To test whether *atp-2(ua2)* animals carrying a dauer-constitutive mutation would enter dauer, the progeny of a LB136 or a LB139 *atp-2* heterozygote were grown at 25°C for 3-4 days and scored phenotypically and genotypically by PCR amplification using single worm lysates and the primers F1B2 and F1B4. The primer set distinguishes the wild type *atp-2* gene from the mutant *atp-2* gene. Single worm lysates were prepared by incubating single 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 *u*g/ml freshly added proteinase K) for 1 h at 50 °C, followed by 10 min incubation at 95 °C to inactivate the proteinase.

Exposure to inhibitors

Chloramphenicol or 3-doxycycline were added to seeded NGM plates (Lewis and Fleming, 1995) and synchronized animals were placed directly onto the plates. All experiments were performed at room temperature (~23 °C) unless otherwise stated.

2.3 Results

Isolation of *nuo-1* and *atp-2* mutants

By sequence comparison, we identified two genes, designated *nuo-1* and *atp-2*, which encode the *C. elegans* homologs of critical mammalian MRC subunits (Figures 2.1 and 2.2). We employed target-selected mutagenesis to produce deletion mutations in both genes (Jansen *et al.*, 1997; Plasterk, 1995). We prepared genomic DNA from an ethyl methanesulfonate mutagenized library of animals, and screened with nested oligonucleotide primers by polymerase chain reaction (PCR) amplification of *nuo-1* and *atp-2* sequences (Figure 2.3).

For the *nuo-1* gene, we found one allele (designated *ua1*) in which a 1,190 base pair sequence (nucleotides 26,259 to 27,448 in Genbank Accession Number Z50109) is replaced with a single A nucleotide (Figure 2.4A). 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 (Fecke *et al.*, 1994). The intron/exon structure predicted by the GENEFINDER program was confirmed by sequence analysis of the yk389c4 cDNA (*C. elegans* Sequencing Consortium, 1998). 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 import into mitochondria, leaving a mature subunit of 50,076 Da (Claros and Vincens, 1996).

For the *atp-2* gene, we isolated one allele designated *ua2*, a 710 base pair deletion (nucleotides 947 to 1656 as numbered in Genbank Accession Number U10402; Figure 2.4B). 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 Figure 2.4B) is not present in the cDNA (*C. elegans* Sequencing Consortium, 1998). Protein sequence corresponding to this first predicted exon is not present in any of the many ATP-2 homologs in the database (Figure 2.2). Translated exon 1 sequence aligns with the amino termini of other ß subunits and includes a possible initiator methionine at nucleotides 12 to 14. Furthermore, exon 1 but not X is transcribed as judged by the over 25 expressed sequence tags in Genbank. The *atp-2* gene, containing 5 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 (Claros and Vincens, 1996). 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 crossreacting antiserum directed against the yeast ATP-2 subunit does not detect a truncated ATP-2, suggesting the protein is either not expressed from the *ua2* allele or is unstable (Figure 2.4C). The *atp-2(ua2)* phenotype (L3 arrest, see below) is 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 lead us to believe the *ua2* allele is a null mutation.

nuo-1 and _atp-2_ phenotypes

The complete *C. elegans* life cycle takes about three days at 25 °C (Lewis and Fleming, 1995). Following fertilization, the embryo undergoes cell proliferation,

organogenesis, and morphogenesis culminating in the hatching of the L1 larva after about 14 hours (Wood, 1988). Over the next two days, development proceeds through three additional larval stages, L2, L3, and L4, until the adult emerges from the final molt. The gonad consists of 4 cells at hatching (Schedl, 1997). During the L2 stage, the hermaphrodite gonad extends anteriorly and posteriorly, led by distal tip cells (Sulston, 1988).

The *nuo-1(ua1)* and the *atp-2(ua2)* mutations are recessive alleles that exhibit typical Mendelian patterns of inheritance. Both result in the developmental arrest of homozygotes in the L3 larval stage. Gonad development is more severely affected, however, and is arrested in the L2 larval stage. Gonad development up to the L2 stage proceeds normally (Figure 2.5A, C, E). Six hours later in wild type animals, the distal tip cells have migrated anteriorly and posteriorly and the two gonad arms have extended considerably along the ventral surface (Figure 2.5B). This migration of the distal tip cells does not proceed in *nuo-1* and *atp-2* animals and the gonad primordium contains between 6 and 18 nuclei. This suggests that germline proliferation is also impaired (Figure 2.5D, F). In addition, mutant animals are somewhat smaller than the wild type (Figure 2.5D, F). Over several days, the gonad arms partially extend and the animals develop an L3-sized body. Surprisingly, the L3-arrested *nuo-1* and *atp-2* larvae have lifespans significantly longer than the wild type (Table 2.1).

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 the *atp-2* animals swim more slowly than wild-type animals (Table 2.2). After being arrested for two days, their

swimming rates are even further decreased. Pharyngeal pumping is irregular and the rates are severely decreased in the mutants (Table 2.2). The defecation cycle is also slowed (Table 2.2). We also tested whether *nuo-1* animals could sense an osmotic gradient of sodium chloride (Culotti and Russell, 1978). Mutant animals could sense and avoid the salt barrier. Furthermore, the fractions of wild type, *nuo-1*, or *atp-2* animals found away from a localized bacterial food source do not differ (Table 2.3). These observations indicate that the mutants' ability to sense environmental stimuli is not abolished.

Maternal *nuo-1* and *atp-2* expression rescues embryonic arrest

RNA interference (RNAi) with double-stranded (ds) RNA is a potent sequence-specific mechanism of eliciting mRNA degradation and inhibiting gene expression in *C. elegans* (Fire *et al.*, 1998; Tabara *et al.*, 1998). Simply feeding worms *Escherichia coli* expressing dsRNA can result in interference (Tabara *et al.*, 1998; Timmons and Fire, 1998). 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 to 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 2 separate plasmids bearing different antibiotic markers. With a bacterial strain containing empty vectors as controls, the addition of isopropylthiogalactoside (IPTG) to induce the expression of the T7 RNA polymerase had no effect on N2 worms (Figure 2.6A). However, IPTG-induced transcription of the *nuo-1* and *atp-2* containing plasmids produces dsRNA and leads to the degradation of the maternal *nuo-1* and *atp-2* mRNAs; this results in approximately 10% and 65% embryonic arrest, respectively (Figure 2.6A). Western blot analysis of the arrested embryos confirms that the dsRNA reduces ATP-2 levels by roughly 2-fold

(Figure 2.6B). The majority (80-90%) of *nuo-1(RNAi)* and *atp-2(RNAi)* embryos arrest after gastrulation with very little tissue differentiation (Figure 2.6C). A small fraction of the embryos (10-20%) arrest earlier, with the earliest arresting at the 1-cell stage (Figure 2.6C). An 1-cell stage arrest has been reported in the RNAi knockdown of other genes (Oka and Futai, 2000), although this phenotype is not commonly observed. The RNAi phenotype in each case is 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 allow 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 is present at approximately one quarter the level of wild type animals (Figure 2.4C).

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 (Troemel, 1999). Dauer larvae are resistant to treatment with 1% SDS (Tissenbaum *et al.*, 2000). When starved LB127 animals are subjected to an SDS treatment, only *atp-2/atp-2 sDp3* animals are recovered indicating that a wild-type *atp-2* gene is required for dauer formation (Table 2.4). 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(e1372)* alleles into the *atp-2* mutant background. Dauers were picked and subjected to genotype analysis by PCR. None of the over 90

animals tested in this way were homozygous for *atp-2* (Table 2.5). Therefore *atp-2* animals are dauer-defective.

When starved LB21B animals are subjected to a detergent treatment, both *nuo-1*/+ and *nuo-1/nuo-1* are recovered following the treatment (Table 2.6). Homozygous *nuo-1* dauers are easily identified because they do not contain any copies of the *mIn1* balancer and thus do not express GFP. We did not recover SDS-resistant animals homozygous for the balancer; these animals do not form recognizable dauers upon starvation nor are any resistant to the detergent treatment. The *nuo-1* dauers are 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 2.6). 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 gravid N2 adults to either chloramphenicol or doxycycline. In each case, the fraction of progeny animals that fails to develop to adults increases with increasing inhibitor concentration (Figure 2.7). 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 life. Animals exposed to high concentrations of doxycycline (≥60 μg/ml) also arrested at L3s,

although they showed a considerable extension of the time needed to develop from the L1 to the L3 stage (>4 days for doxycycline as compared to 1-2 days for chloramphenicol). This suggests that doxycycline may be having 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 is severely impaired by both drugs. Up to 24 hours after egg laying, chloramphenicol-treated animals are not readily distinguishable from control animals; both have typical L2-sized gonads (Figure 2.5A,G). 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 (Figure 2.5B). In contrast, chloramphenicol-treated animals are slightly smaller in size than control animals and their gonads remain unchanged from the 24-hour point (Figure 2.5H). With time, chloramphenicol-treated animals develop L3-sized bodies, but their gonads remain arrested at an L2-like stage. L2-sized gonads are also observed in doxycycline-treated animals, although the time required to reach the point of arrest is extended (Figure 2.5I). Thus, the effects of doxycycline or chloramphenicol on development are tissue-specific and may reflect high energy requirements in the gonad or a higher sensitivity of this tissue to these drugs.

2.4 Discussion

A functional MRC is essential for nematode viability. Mutations in the *nuo-1* and the *atp-2* genes, which encode MRC subunits of complexes I and V, respectively, both result in L3 larval 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 the *atp-2* mutants suggest that it is the loss of the energy-generating pathway that results in lethality characterized by L3 larval arrest (Figure 2.8). 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 approximately 200 genes or 1% of the genome involved in MRC biogenesis and mutations in many of these may produce L3 larval arrest. In support of this generalization, we have generated L3 arrested animals by completely independent approaches. When mtDNA expression is impaired with doxycycline or chloramphenicol, wild-type animals also arrest as L3 larvae that phenotypically resemble the *atp-2* and *nuo-1* mutants. This was not unexpected since complexes I, III, IV, and V contain subunits encoded by the mitochondrial genome. The drug-arrested animals also have 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 the *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 (Ishii *et al.*, 1998). 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 (Kayser *et al.*, 1999) and impairs activity of the complex (Kayser *et al.*, 2001).

Why are the *nuo-1* and the *atp-2* mutations not lethal during embryogenesis? The RNAi and the 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 (Figure 2.4C). Using microarray analysis, significant levels of *nuo-1* and *atp-2* transcripts are detected in and presumably inherited through oocytes (Hill *et al.*, 2000). We believe the inherited *atp-2* mRNA and ATP-2 protein support the development of the mutant to the L3 stage 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 (Larsson *et al.*, 1998). Likewise, the loss of the mouse cytochrome *c* causes embryonic lethality marked by a severe developmental delay and reduced size (Li *et al.*, 2000), yet, the cytochrome *c* deficient embryos do complete early developmental steps.

The genomic analysis of chromosome I by RNAi revealed that interference with genes encoding subunits of complexes I, III, IV, and V of the MRC produces embryonic lethality (Fraser *et al.*, 2000). Similarly, RNAi with the *atp-2* gene or the *mev-1* gene,

which encodes the cytochrome subunit of complex II, slows the pace of embryonic development and produces embryonic lethality (Gonczy *et al.*, 2000). In fact, many genes of intermediary metabolism are essential for embryonic viability (Fraser *et al.*, 2000; Gonczy *et al.*, 2000).

Aerobic metabolism, as measured by oxygen consumption, peaks at the L3 and L4 larval stages (Vanfleteren and De Vreese, 1996). Furthermore, it is suggested that reproduction requires a considerable elevation of metabolic activity (Vanfleteren and De Vreese, 1996). Thus, we hypothesize that germ line proliferation and organogenesis, which are associated with sexual maturation during the L4 stage, 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 (Figure 2.9) and in mtDNA copy number (Tsang and Lemire, 2002) associated with the transition from L3 to L4.

Animals with impaired mitochondrial energy metabolism due to the *nuo-1* mutation, to the *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 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 (Figure 2.8). Interestingly,

nicotinamide adenine dinucleotide (NAD) is required for the genomic silencing that leads to increased longevity in response to calorie restriction (Lin *et al.*, 2000). Since NAD is a central metabolic molecule, it is possible that by interfering with the MRC-mediated oxidation of NADH, the *atp-2* and the *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 lifespan of a variety of animals, including nematodes, perhaps by reducing energy production and decreasing metabolic rates (Lakowski and Hekimi, 1998). 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 lifespan (Avery and Thomas, 1997; Lakowski and Hekimi, 1998). We suggest that the extended lifespans of *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.

Human mitochondrial diseases are characterized by a diversity of phenotypes, many of which are tissue-specific in their effects (Beal, 1995; Luft and Landau, 1995). The *nuo-1(ua1)* and the *atp-2(ua2)* homozygous mutants arrest at the L3 larval stage, with their gonads showing an earlier L2 arrest. These mutants move more slowly and are less energetic. Their rates of pharyngeal pumping are decreased and the defecation cycle is 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 (Aboobaker and Blaxter, 2000; Culetto and Sattelle, 2000).

In conclusion, the loss of MRC function via mutations in key subunits of mitochondrial complexes 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.

Genotype	Lifespan (days)	Significance (P value)[b]
+ / +	11.9 ± 2.7 (n=27)[a]	--
nuo-1 / nuo-1	14.8 ± 3.3 (n=24)	< 0.01
atp-2 / atp-2	13.6 ± 2.6 (n=31)	< 0.05

Table 2.1: *nuo-1* and *atp-2* mutants have extended lifespans

[a] Numbers are means ± SD (number of animals scored)

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

Genotype (stage)	Swimming (cycles/min)	Pharyngeal Pumping (contractions/min)	Defecation (sec/cycle)
+ / + (Adult)	112 ± 9[a] (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[b]
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[b]	ND[b]

Table 2.2: The *nuo-1* and *atp-2* mutations impair swimming, pumping, and defecation
[a] Numbers are means ± SD (number of animals scored)
[b] Not determined

	Number of animals scored				
Strain	Total	Adult[a]	L3; arrested 1 day	Adults found away from food source	L3s found away from food source
LB21	374	285	89	11 (4%)	4 (4%)
LB127	418	311	107	15 (5%)	4 (3%)

Table 2.3: The *nuo-1* and *atp-2* mutations do not impair the sensing of food stimuli
[a] Represents the total number of homozygous wild type and heterozygous animals

	Number 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]

Table 2.4: *atp-2* mutants are defective in entering into the dauer stage
[a] Not applicable

	Number of animals scored		
Stage	*daf-7 + / daf-7 +*	*daf-7 + / daf-7 atp-2*	*daf-7 atp-2 / daf-7 atp-2*
L3	0	0	24
dauer	27	48	0
L4 / adult	1	3	0
Total	28	51	24
	Number of animals scored		
Stage	*daf-2 + / daf-2 +*	*daf-2 + / daf-2 atp-2*	*daf-2 atp-2 / daf-2 atp-2*
L3	0	0	15
dauer	14	26	0
L4 / adult	1	4	0
Total	15	30	15

Table 2.5: *daf-7 atp-2* and *daf-2 atp-2* mutants are defective in entering into the dauer stage at 25°C

	Number of animals scored		
	+ / +	+ / *nuo-1*	*nuo-1* / *nuo-1*
After 1% SDS treatment	0[a]	274	73
Recovery	NA[b]	253 / 253	0 / 73

Table 2.6: *nuo-1* mutants are defective in exiting the dauer stage

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

[b] Not applicable

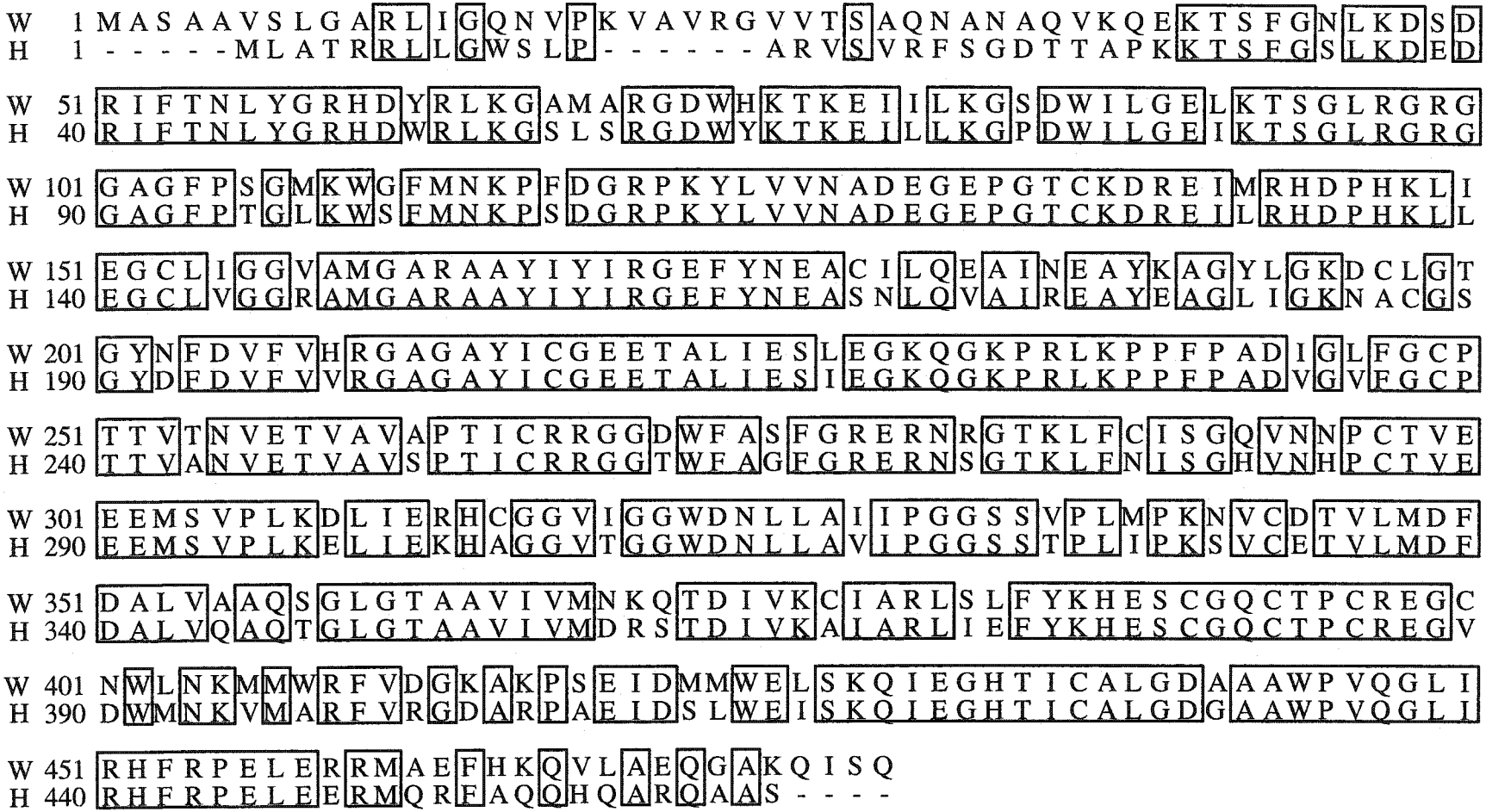


Figure 2.1: Alignment of the worm *nuo-1* (W) and the human *NDUFV1* protein sequences (H)
Alignment is generated using the PAM250 matrix with the program ClustalW 1.7 and edited with the program SeqVu 1.1. Gaps (hyphens) are used to optimze the alignment. Idential residues are boxed.

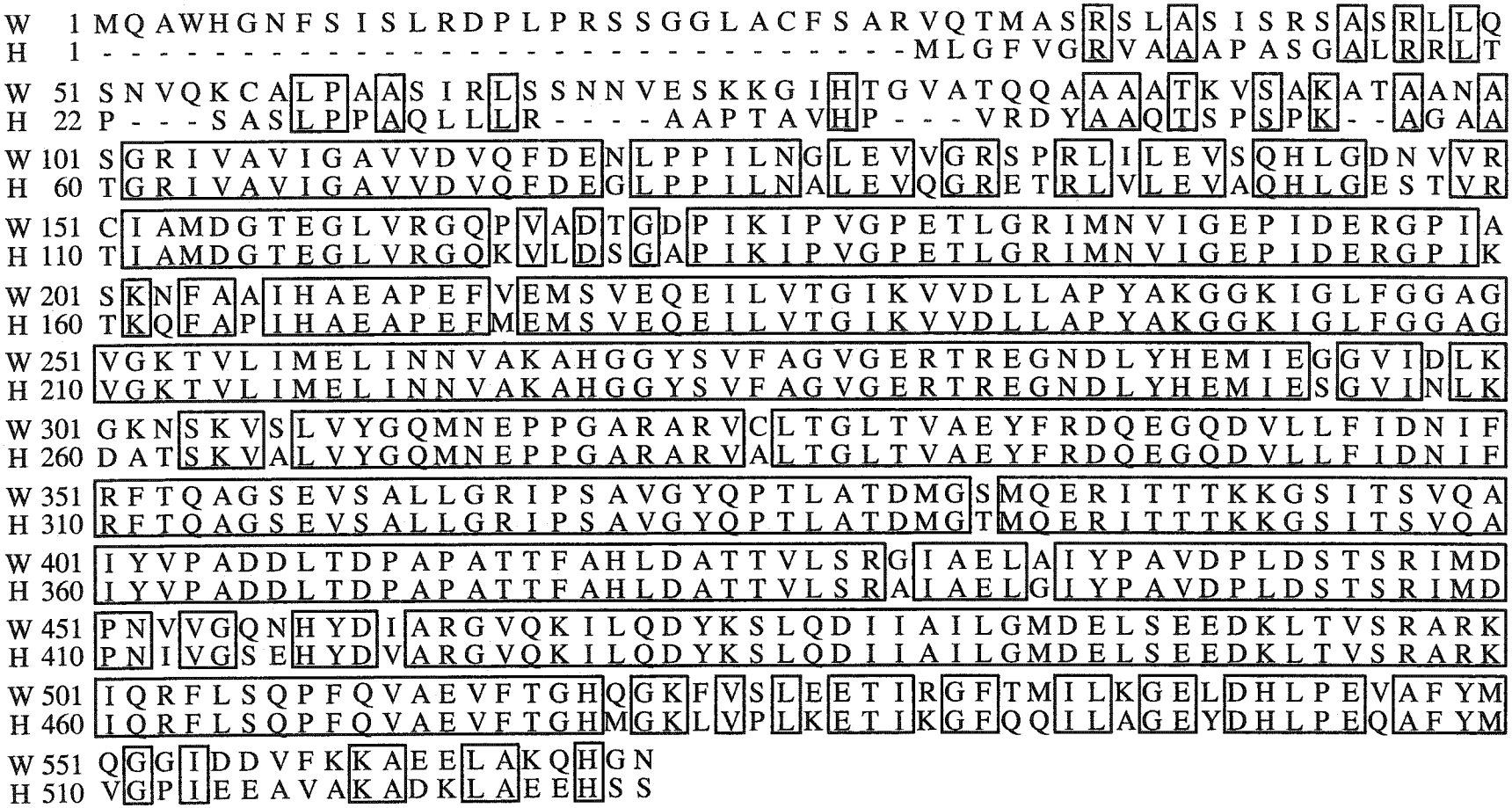


Figure 2.2: Alignment of the worm *atp-2* (W) and the human ß-subunit protein sequences (H)
Alignment is generated using the PAM250 matrix with the program ClustalW 1.7 and edited with the program SeqVu 1.1. Gaps (hyphens) are used to optimze the alignment. Idential residues are boxed.

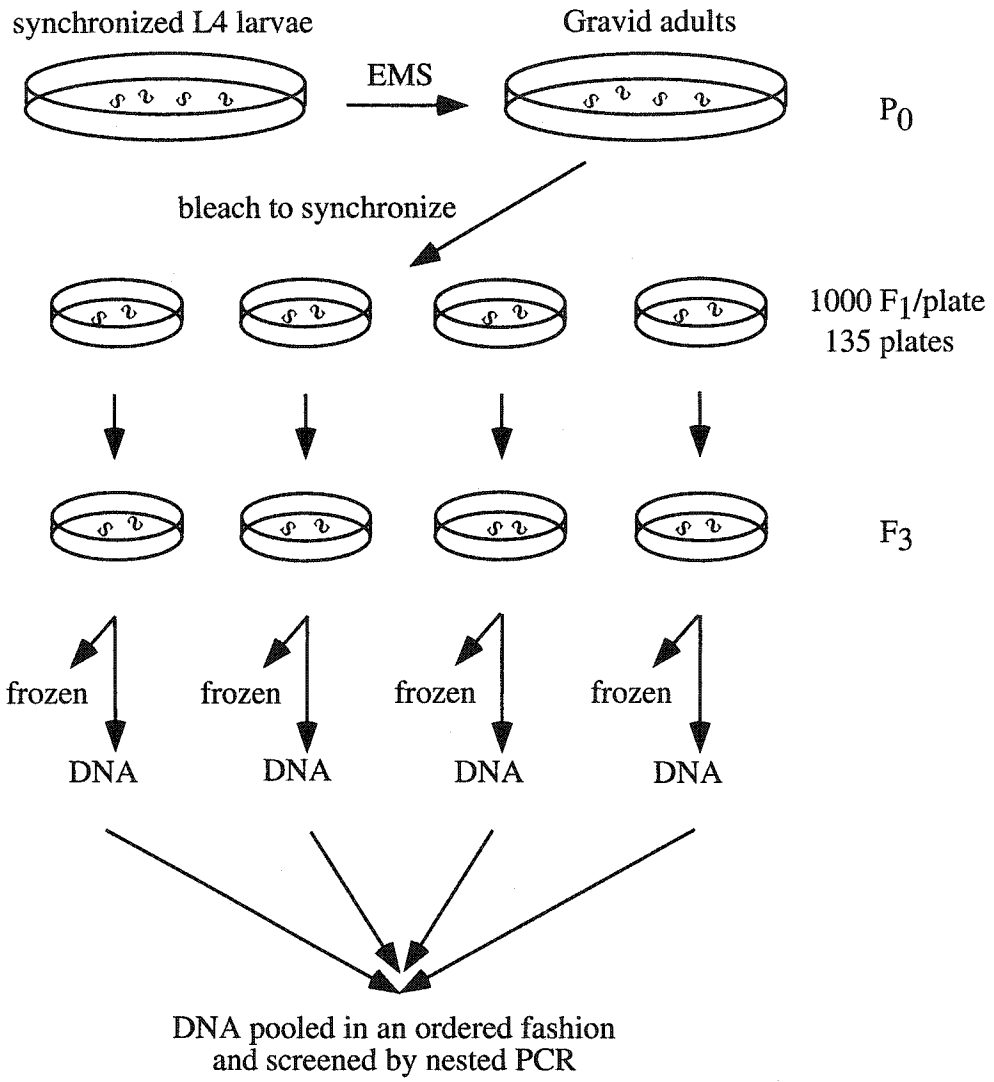


Figure 2.3: A library of ethyl methane sulfonate (EMS) mutagenized wild type animals

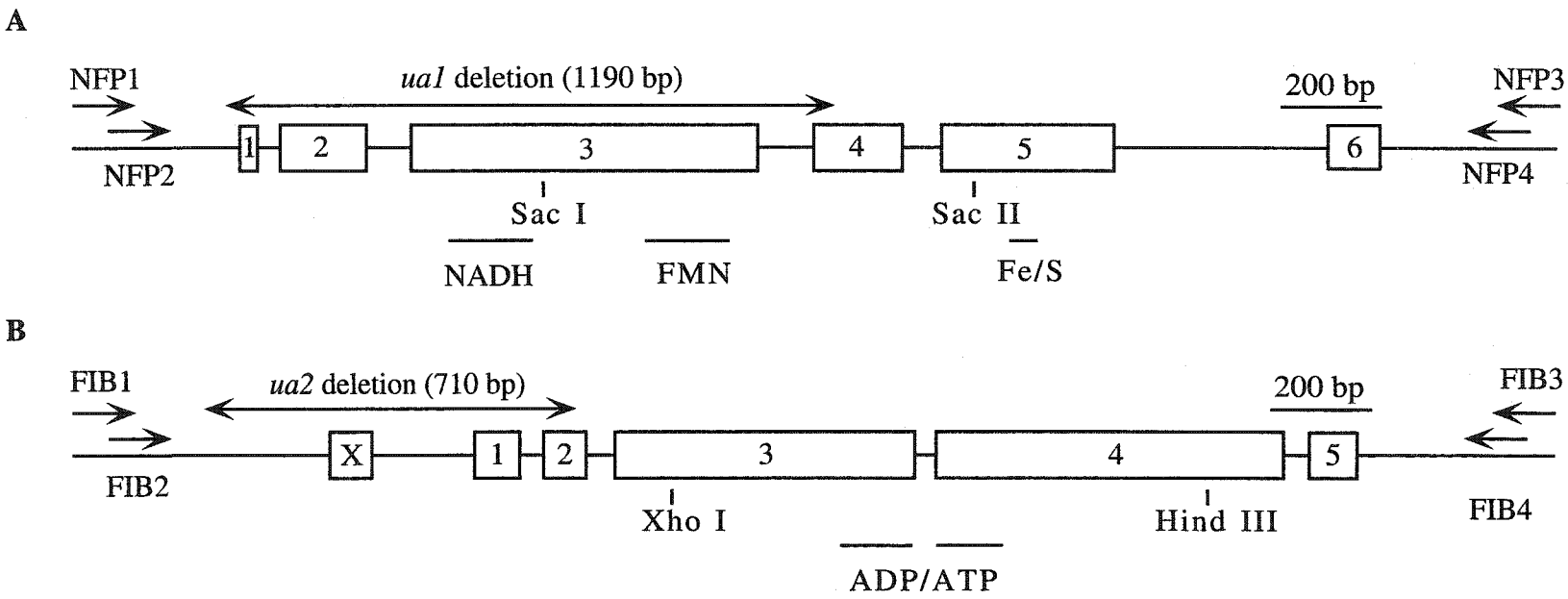
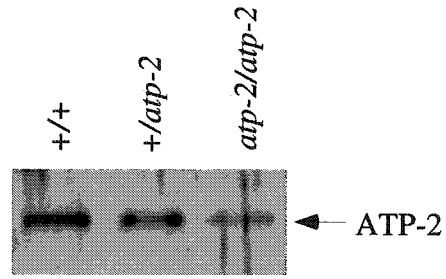


Figure 2.4: Gene structures and Western blot analysis
(**A**) The *nuo-1* gene structure. Exons are drawn as rectangles. Predicted cofactor or substrate binding regions and relevant restriction enzyme sites are indicated below the exons. Fe/S, iron-sulfur cluster. (**B**) The *atp-2* gene structure. The exon labeled 'X' is a computer prediction; it is not transcribed (see text). Both the *ua1* and *ua2* deletions were confirmed by sequencing. The locations of the primers (NFP and FIB) are indicated. (**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 roughly a two-fold difference in the ATP-2 level between +/+ and *+/atp-2* and a four-fold difference between +/+ and *atp-2/atp-2*.



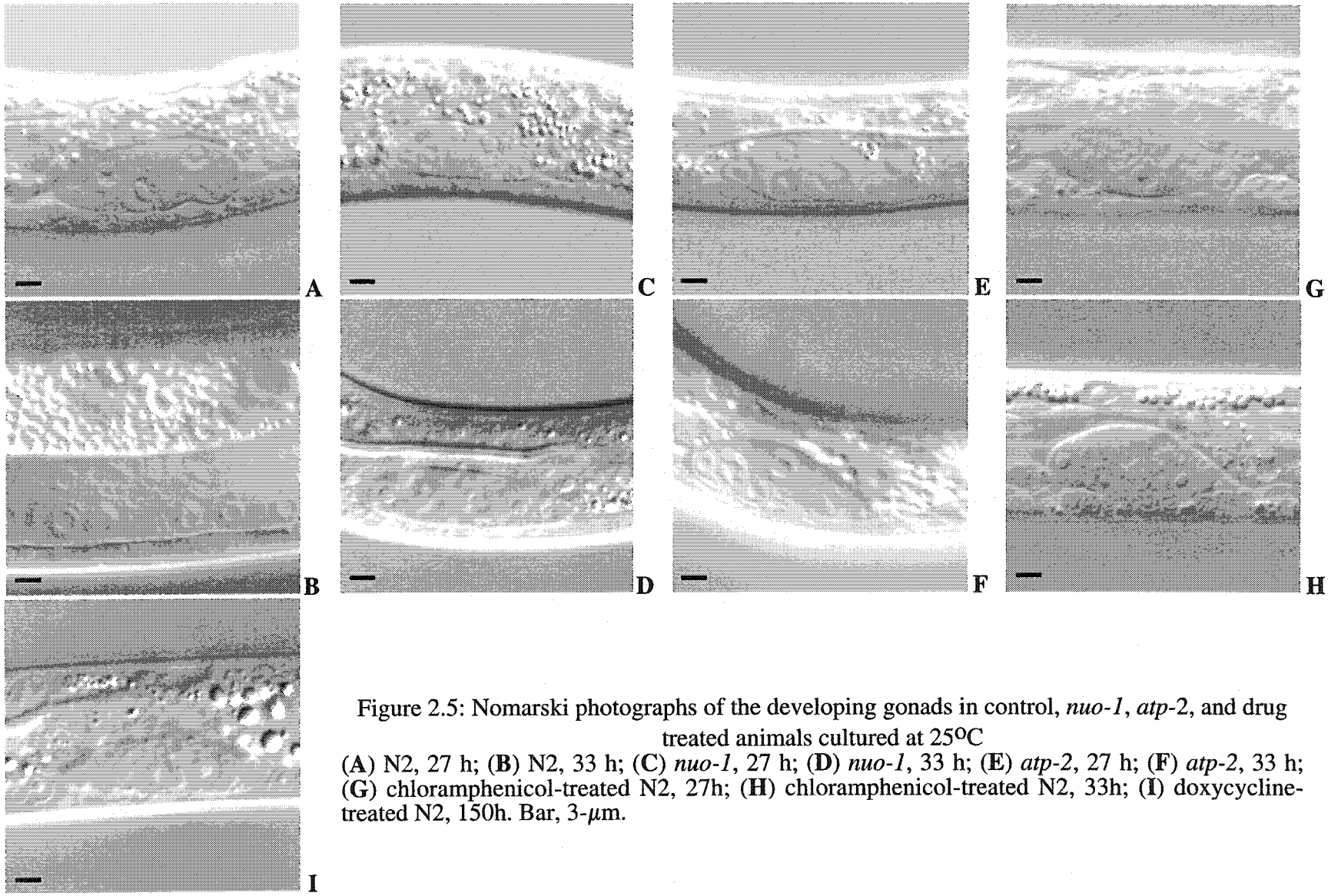
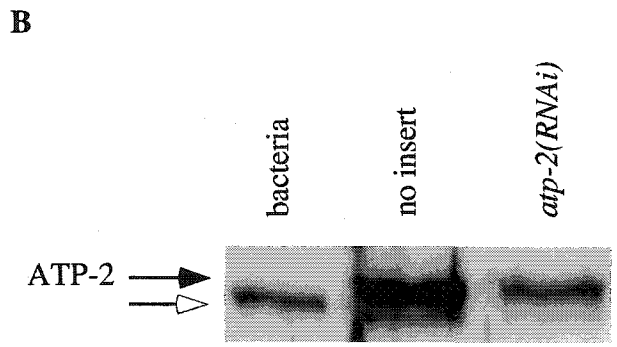


Figure 2.5: Nomarski photographs of the developing gonads in control, *nuo-1*, *atp*-2, and drug treated animals cultured at 25°C

(**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, 27h; (**H**) chloramphenicol-treated N2, 33h; (**I**) doxycycline-treated N2, 150h. Bar, 3-μm.

A

pBC+pBS	IPTG	F1 Embryonic Lethal
no insert	-	0% (0/480)
no insert	+	0% (2/513)
nuo-1	-	0% (2/600)
nuo-1	+	10% (57/574)
atp-2	-	5% (23/438)
atp-2	+	65% (152/234)



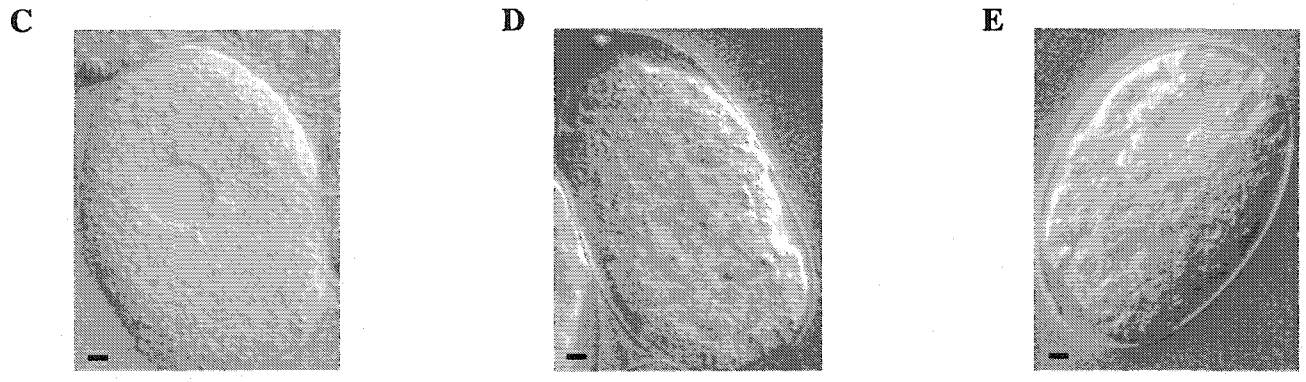


Figure 2.6: RNA interference and Nomarski photographs of arrested embryos
(**A**) Embryonic lethality was scored at least 48 hours after egg laying and expressed as a percentage of the total number of progeny. (**B**) A Western blot showing the position of the full-length ATP-2 protein in control (no insert) and in *atp-2(RNAi)* embryos (solid 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 3 lanes (open arrow). RNAi mediated embryonic arrest occurs at (**C**), the one-cell stage, *atp-2(RNAi)*; (**D**), the comma stage, *atp-2(RNAi)*; and (**E**), the comma stage, *nuo-1(RNAi)*. These embryos were photographed 7-9 h after egg laying. Bar, 3-μm.

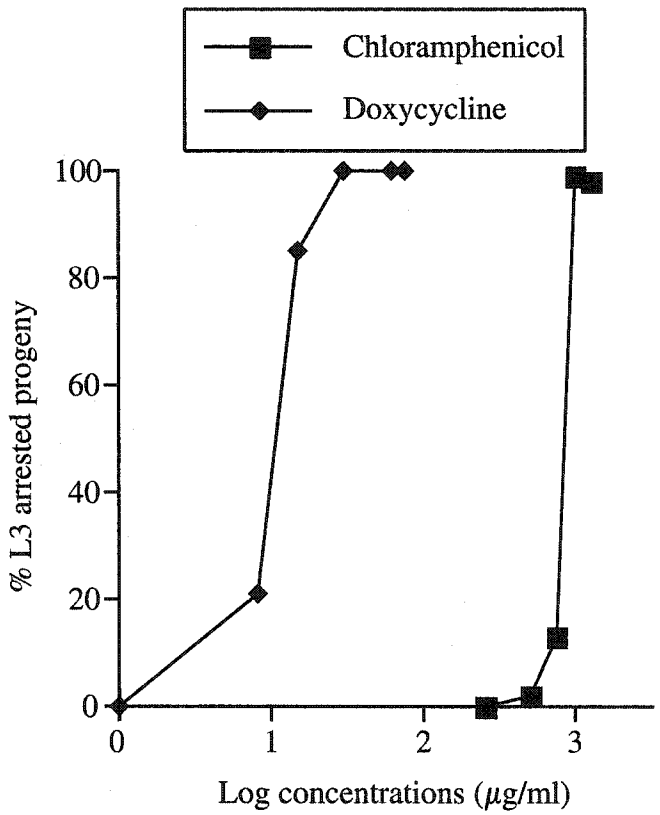


Figure 2.7: Exposure of N2 gravid adults to chloramphenicol and doxycycline The ratio of L3 arrested progeny over the total number of progeny scored (>89 for chloramphenicol; >120 for doxycycline) is expressed as a percentage. Chloramphenicol, squares; doxycycline, diamonds.

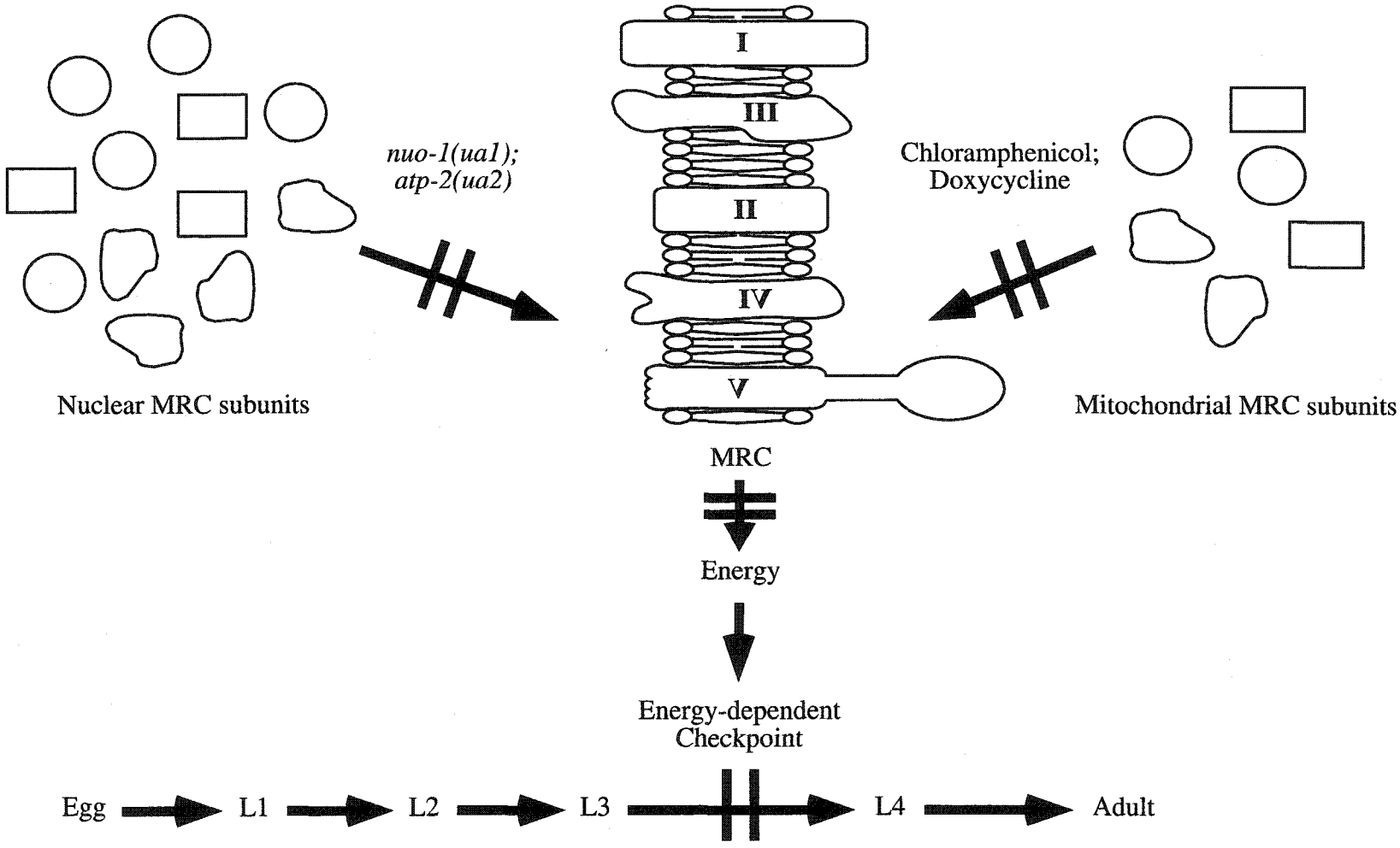


Figure 2.8: A model depicting the roles of the nuclear and the mitochondrial genomes in *C. elegans* development
Proper development requires energy production primarily by oxidative phosphorylation via the MRC. Both genomes contribute MRC protein subunits that assemble to form a functional MRC. Mutations 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.

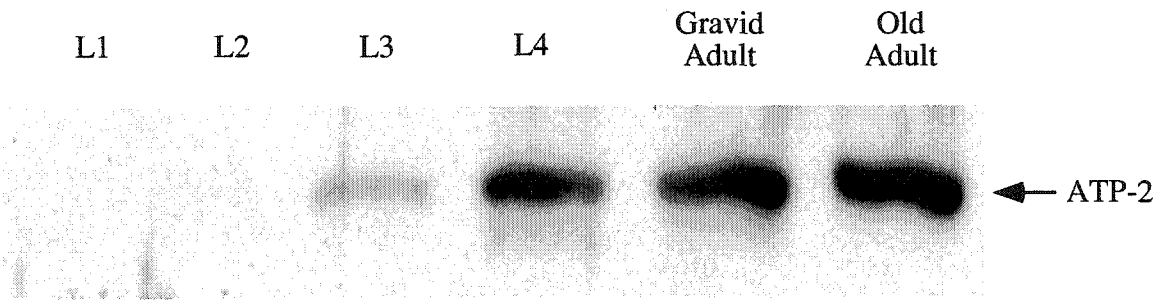


Figure 2.9: ATP-2 levels in N2 worms at different developmental stages
The blot was probed with a polyclonal antibody for *S. cerevisiae ATP2p*. 100 animals were loaded in each lane. There are significant increases in the ATP-2 level from L3 to L4 and from L4 to adults.

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Chapter 3

Mitochondrial ATP Synthase Controls Larval Development Cell Nonautonomously in *Caenorhabditis elegans*

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3.1 Introduction

The mitochondrial respiratory chain (MRC) is the major source of ATP for most cells. It is composed of four electron transporting protein complexes (I through IV) that generate a proton gradient across the mitochondrial inner membrane. The ATP synthase or complex V uses the energy of the proton gradient for ATP synthesis in a process called oxidative phosphorylation. The biogenesis of the MRC is complex, requiring the coordinate expression of genes from both the nucleus and the mitochondrial DNA (mtDNA). Defective mitochondrial energy production is often recognized as a contributor to a broad set of human diseases including myopathies, diabetes, neuromuscular and heart diseases (Beal, 1995; Luft and Landau, 1995; Wallace, 1992; Wallace, 1999).

An interesting aspect of mitochondrial diseases is that many are tissue-specific in their effects (DiMauro and Andreu, 2000; Sue and Schon, 2000). The tissue specificity observed in some mitochondrial energy deficiencies can be attributed to at least two factors. First, each tissue is thought to have different energy requirements. Tissues that are highly oxidative are more susceptible to MRC defects (Larsson *et al.*, 1998). Second, because it is present at thousands of copies per cell, wild type and mutated mtDNAs can co-exist in the same cell in a state called heteroplasmy (Wallace, 1992). Heteroplasmy can increase or decrease by mitotic segregation as cells divide (Grossman and Shoubridge, 1996). Disease severity can vary not only with the nature of the mutated mtDNA but also with the proportion of mutated mtDNA present. Since mtDNA is maternally inherited, mutant mtDNA levels in fertilized eggs can vary and lead to

incomplete penetrance among offspring (Chinnery *et al.*, 1997; Chinnery and Turnbull, 1997; Taylor *et al.*, 1997).

We have been studying the developmental, phenotypic, and genetic consequences of MRC defects in the model organism, *Caenorhabditis elegans* (Tsang *et al.*, 2001; Tsang and Lemire, 2002a; Tsang and Lemire, 2002b). One mutation we studied occurs in the *atp-2* gene encoding the active site or β-subunit of complex V. The *ua2* allele is a deletion that removes the first two exons of *atp-2* and is believed to be a null (Tsang *et al.*, 2001). Animals homozygous for *atp-2(ua2)* are inviable; they hatch and develop through two larval stages before arresting at the third larval stage (L3).

Is the *atp-2(ua2)* developmental arrest due to a tissue-specific energy deficit? We sought to address this question by analyzing the effects of mosaic expression of the *atp-2* gene in the nematode. *C. elegans* is a simple multicellular eukaryote with just under 1,000 somatic cells that develop into multiple tissue types including muscles, neurons, and hypodermis (Wood, 1988). The complete description of its invariant cell lineage makes *C. elegans* an ideal system for mosaic analysis (Sulston, 1988; Sulston and Horvitz, 1977; Sulston *et al.*, 1983). In mosaics, some cells are genotypically wild type while others are genotypically mutant due to the infrequent, spontaneous loss of a complementing transgenic array during cell division (Herman, 1995). The complementing transgenic array is marked with an independent gene whose activity can be easily detected. One such marker is the SUR5-GFP (green fluorescent protein) fusion protein in which a nuclear-targeted GFP is expressed under the control of the *sur-5* promoter (Yochem *et al.*, 1998; Yochem *et al.*, 2000). This marker had been successfully used for mosaic analysis (Hanna-Rose and Han, 2002; Yochem *et al.*, 1998) and has the

following features. First, it is expressed in almost every cell except the germ line (Yochem *et al.*, 1998). Second, it is expressed in a cell autonomous manner, meaning that only cells with the SUR5-GFP transgenic array will fluoresce (Yochem *et al.*, 1998). This is in contrast to cell non-autonomous expression in which a cell exhibits a phenotype (fluorescence) in the absence of the transgenic array because the array is present in neighboring cells. Third, the perdurance of the GFP is minimal after the array has been lost (Yochem *et al.*, 1998).

In this study, we found that the loss of the wild type *atp-2* gene in the ABa lineage, which gives rise to neuronal, pharyngeal and hypodermal cells, and/or in the E lineage, which generates all intestinal cells, could occasionally be tolerated with a fraction of these animals capable of developing beyond the L3 stage. These results suggest that all tissues require *atp-2* gene activity for optimal development. When *atp-2* gene function is lost in lineages that affect the body muscles, the gonad, or the vulva, L3 larval arrest occurs. However, in the L3, gonad and vulval development are at very early stages. The body muscles, which may rely heavily on the MRC for energy generation, constitute a large fraction of an animal's mass and may play a key role in developmental regulation. Development from the L3 stage to the next larval stage (L4) is a cell non-autonomous process, implying a consensus mechanism operates in the L3-to-L4 transition. Thus, *atp-2* may be necessary for the production or the regulation of a global, developmental signal required for the L3-to-L4 transition.

3.2 Materials and Methods

General methods and strains

General methods for culturing and handling of *C. elegans* were as described (Lewis and Fleming, 1995). The following strains were used: CB1489, *him-8(e1489)*; LB128, *atp-2(ua2) unc-32(e189) / qC1*[*dpy-19(e1259) glp-1(q339)*]III; VB91, *unc-4(e120)*II *dpy-17(e164) ncl-1(e1865) unc-36(e251)*III *svDp1[sDp3[atp-2(+)]-svEx12[unc-4(+)-sur-5::gfp]]* (obtained from S. Tuck); and LB146, *atp-2(ua2) unc-32(e189)*III *svDp1[sDp3[atp-2(+)]-svEx12[unc-4(+)-sur-5::gfp]]*. The *ua2* allele is a 710-base pair deletion in the *atp-2* gene. *svDp1* is an extrachromosomal transgenic array made by fusing the free duplication *sDp3*, which contains *atp-2(+)*, to an extrachromosomal array *svEx12*, which contains *unc-4(+)* and *sur-5::gfp*. *svDp1* complements *atp-2(ua2)* but not *unc-32(e189)*. *atp-2(+)* indicates that the wild type *atp-2* gene is present, whereas *atp-2(-)* indicates that it is absent.

Construction of strains for mosaic analysis

To generate LB146, LB128 hermaphrodites were crossed to CB1489 males to generate heterozygous F_1 *atp-2(ua2) unc-32(e189)/+* males. These F_1 males were then mated to VB91, and *gfp(+)* F_2 hermaphrodites carrying the *ua2* allele were picked onto single plates to allow for self-fertilization. F_3 *gfp(+) unc-32* hermaphrodites were selected and were designated as LB146. At every step, the presence of the wild type *atp-2* gene or the *ua2* allele was detected by PCR amplification using single worm lysates and the primers F1B2 and F1B4 as described previously (Tsang *et al.*, 2001). In this way, we confirmed that the wild type *atp-2* gene is absent in all *gfp(-)* L3 arrested LB146 animals, but is present in *gfp(+)* animals (Figure 3.1). We verified that the LB146 strain was

appropriate for mosaic analysis in two ways. First, we measured the germline transmission frequency of the complementing *svDp1* transgenic array and found it to be 61% (312 *gfp(+)* offspring out of a total of 509 observed). Arrays with germline transmission frequencies of 40-66% have been successfully used for mosaic analysis (Yochem *et al.*, 2000). Second, we measured the frequency of recombination involving *svDp1* and found it to be substantially lower than the frequency of finding genetic mosaics. We looked for very rare animals that were *gfp(-)* and *unc-32*, but did not arrest at L3. These animals were presumably recombinants and were found at a frequency ranging from 0 to 0.01% depending on the line (>10,000 animals observed per line). Only lines with a recombination frequency of no more than 0.003% were used for mosaic analysis. For comparison, the frequency of finding a genetic mosaic with an early loss of the array is about 0.8 to 4% (Yochem *et al.*, 2000).

Microscopy

Mosaic animals were identified as having incomplete or lower intensity fluorescence patterns as observed under a Leica MZ FLIII dissecting stereomicroscope equipped with a mercury vapor lamp (Leica Microscopy Systems Ltd., Willowdale, Ontario, Canada). Mosaic animals are mounted on 2% agarose pads in a M9 buffer containing 0.1% NaN_3 and photographed with a Zeiss Axioskop-2 research microscope (Carl Zeiss Canada Ltd., Calgary, Alberta, Canada) equipped with Nomarski and fluorescence optics and a SPOT2 digital camera.

Mosaic analysis

All animals were cultured at 25°C. Synchronized L1s were grown for about 2 days and mosaics were picked to separate plates. The mosaics were reassessed one day

later; animals were assumed to be arrested if they had not further developed past the L3 larval stage. When a cell was *gfp(-)*, it was assumed to have lost the *svDp1* array (and thus, the *atp-2(+)* gene). We identified the exact nature of mosaicism by examining a set of cells/nuclei within each lineage (Sulston and Horvitz, 1977; Sulston *et al.*, 1983) for the presence or absence of GFP. We assumed the minimum loss of *svDp1* consistent with the GFP pattern. The following cells/nuclei were scored: most anterior neurons (from the ABa lineage); hyp8, hyp9, hyp10, Pn.p cells, posterior neurons (from the ABp lineage); ASKL, ADLL, RID, ALA, m3L, m3VL (from the ABal lineage); ALM, BDU, m3R, m3VR (from the ABar lineage); excretory cell, rectal epithelium D, ASIL (from the ABpl lineage); DVA, ASKR, ADLR, ASIR, one body muscle (from the ABpr lineage); two distal tip cells, m3DL, m3DR, 28 body muscles (from the MS lineage); the terminal bulb of the pharynx (mostly from the MS lineage); all intestinal cells (from the E lineage); hyp11, 32 body muscles (from the C lineage); and 20 body muscles (from the D lineage). Cells derived from the P_4 lineage do not express the GFP reporter and cannot be scored. Thus, we are not able to distinguish between an array loss in D or in its parent, P_3 and this is designated as D(P_3).

3.3 Results

Mosaic analysis of *atp-2*

The complete *C. elegans* life cycle takes about three days at 25 °C (Lewis and Fleming, 1995). Following fertilization, the embryo undergoes cell proliferation, organogenesis, and morphogenesis culminating in the hatching of the L1 larva after about 14h (Wood, 1988). Over the next two days, development proceeds through three

additional larval stages, L2, L3, and L4, until the adult emerges from the final molt (Schedl, 1997). In early embryogenesis, the zygote (P_0) undergoes a series of cell divisions to generate six founder cells: AB, MS, E, C, D, and P_4 (Figure 3.2) (Sulston *et al.*, 1983). The sets of cells derived from each founder cell behave in characteristic and specific ways. Generally speaking, tissues are derived from more than one lineage. To identify the tissues or lineages in which the loss of the *atp-2(+)* gene is compatible with development beyond the L3 stage, we isolated *atp-2(ua2)* mosaic animals with spontaneous losses of the complementing transgenic array *svDp1*, which carries the *atp-2(+)* gene. The array is marked with SUR5-GFP; mosaic animals have less intense or incomplete GFP patterns. We focused on array losses that occurred early in embryogenesis (i.e. losses in the AB, ABa, ABp, P_1, P_2, EMS, E, MS, C, and D(P_3) lineages) to assess the effects of these large losses on L3 to L4 larval development.

The results are summarized in Table 3.1. Animals with no array losses (class 1) are phenotypically wild type and develop into fertile adults. Animals that do not inherit the array (loss in the P_0 cell; class 32) arrest at the L3 stage and are phenotypically identical to *atp-2(ua2)* mutants. This indicates that the array does not confer a maternal effect distinguishable from the native *atp-2* gene. As expected, animals belonging to these two classes are the most common. Among the genetic mosaics, we found that animals with array losses in the AB (class 2), ABp (class 4), P_1 (class 5), EMS (class 6), P_2 (class 7), MS (class 8), C (class 10), and D(P_3) (class 11) precursor cells always arrested as L3 larvae. Consistent with these findings, losses of the array in more than one of the above-mentioned lineages also result in L3 developmental arrest (classes 15, 17-24, 27-31). Since all of the 81 body muscle nuclei in the L1 larva are derived from the

ABp (1), MS (28), C (32), and D (20) lineages, these data suggest that the loss of the *atp-2(+)* gene in the body muscle correlates closely with larval arrest. Losses in these same lineages affect all gonadal and vulval cells, but these tissues are in the very early stages of development in the arrested L3 larvae. The same losses also affect a subset of neuronal, hypodermal, and pharyngeal cells, but each of these tissues can be partially *atp-2(-)* as shown by the class 3 (ABa) mosaics that develop past L3. Therefore, body muscle, by virtue of its abundance, may have a special role in L3 to L4 development. However, it is unlikely that body muscles alone control development since the loss of the *atp-2* array in the single muscle cell derived from the ABp lineage leaves >98% of body muscle cells *atp-2(+)*. It seems improbable that class 4 (ABp) mosaics arrest because of that single *atp-2(-)* muscle cell.

In contrast, animals in which the *atp-2(+)* gene is lost in the ABa (class 3) or the E (class 9) lineages do not always arrest development as L3 larvae. Some of these mosaic animals had developed to the L4 larval stage and a few to adulthood at the time of observation (Figure 3.3). These data suggest that *atp-2(ua2)* animals can tolerate a loss of the *atp-2* activity in the neuronal, hypodermal, and pharyngeal cells of the ABa lineage (class 3). A complete loss of *atp-2* activity in the E lineage (class 9), which gives rise to all intestinal cells, can occasionally be tolerated. Not surprisingly, animals with partial losses in the E lineage (class 12) also often proceed past the L3 stage. The percentage of non-arresting animals for the class 12 mosaics is increased over the class 9 mosaics (~60% versus ~30%). Thus, at least in the E lineage, the probability of development beyond the L3 stage increases with the number of *atp-2(+)* intestinal cells. Thus, the lack of *atp-2* activity in any given cell is compatible with either L3 larval arrest or continued

development, suggesting that a threshold level of *atp-2* activity is involved in the developmental decision.

Interestingly, we also found a single L4 worm in which the *atp-2(+)* gene had been lost in both the ABa and the E lineages (class 25). Partial or complete losses of the wild type *atp-2* gene in the E lineage lead to L3 larval arrest when combined with losses in the C, $D(P_3)$, or ABp precursor cells (classes 13-14, 16, 26). This reinforces our earlier observation that *atp-2* function in the muscle cells of the C, $D(P_3)$, and ABp lineages is closely correlated with larval development.

We also sought to determine whether the presence of the complementing array in any single tissue type or lineage is sufficient to rescue the L3 arrest. For instance, the entire hypodermis is generated from the AB and the C lineages (Figure 3.2). Thus, mosaic animals with losses in both the EMS and P_3 lineages would lead to a maximal loss of *atp-2(+)* activity without affecting the hypodermal cells; the entire hypodermis is genotypically wild type. We observed such animals (class 21); they did not develop past L3. Similarly, L3 larval arrest was also observed when *atp-2(+)* was present in all neuronal lineages (class 16), in all intestinal cells (class 30), in the gonad cells (class 31), in the vulval lineages (class 24), in the pharyngeal lineages (class 19), in the germ line (class 29), and in all but one body muscle cell (classes 2 and 4). Thus, expression of *atp-2(+)* in any single lineage or tissue is not sufficient to support the L3 to L4 transition.

Cell non-autonomous function of *atp-2*

The function of the *atp-2* gene in larval development could either be cell autonomous or cell non-autonomous. We examined mosaic animals in greater detail by focusing on three different developmental processes: body size, gonad development, and

vulval development. In brief, there are dramatic morphological differences readily observed by Nomarski microscopy associated with the overall body size, the gonad, and the vulva during development from an L3 larva to an L4 or an adult animal (Figure 3.4). The somatic gonad is exclusively derived from the MS lineage (Figure 3.2) and its development does not depend on the germ line (Austin and Kimble, 1987). Vulval development requires a signal secreted from the anchor cell, which is derived from the MS lineage, to the vulval precursor cell from the ABp lineage (Felix and Sternberg, 1997).

Our data strongly suggest a cell non-autonomous function for *atp-2*. If *atp-2* gene activity were cell autonomous, we would expect that *atp-2(+)* tissues would adopt an L4 or an adult morphology while *atp-2(-)* tissues would remain at an L3 stage. For example, we would expect to observe L4 or adult-sized gonads in mosaics only carrying *atp-2(+)* in the MS lineage. Similarly, we would expect L4 or adult vulval morphologies in mosaics only carrying *atp-2(+)* in the ABp and MS lineages. In contrast, if *atp-2* function is cell non-autonomous, then each cell's or tissue's state of development will not necessarily correspond to its *atp-2* genotype. In this case, some *atp-2(+)* cells may remain at the L3 developmental stage and conversely some *atp-2(-)* cells may continue development past L3. We observed arrested gonad and vulval development in L3-sized larvae in class 3 and class 16 mosaics, which are *atp-2(+)* in the ABp and the MS lineages (Figure 3.5C, D, E, F). The phenotype of the two classes of mosaics is similar to that of the homozygous *atp-2(ua2)* mutant (Figure 3.5A). Conversely, we always observed L4- or adult-stage gonad and vulval development in L4- or adult-sized mosaic animals of classes 3, 9, and 12, and the phenotype of these three classes of mosaics is

similar to that of a wild type animal (Figure 3.5B). We have not observed any mosaics with a mixture of L3 and L4-like features. Thus, body size, gonad and vulval development are not independent processes determined by the respective tissue genotypes in *atp-2* mosaics.

3.4 Discussion

In this report, we have presented evidence that certain mosaic animals can tolerate partial or complete losses of *atp-2* gene function in neurons (ABa), the hypodermis (ABa), the pharynx (ABa), and the intestine (E) without eliciting developmental arrest. In contrast, *atp-2(+)* losses that affect even a fraction of body muscles invariably result in an L3 arrest phenotype. These data suggest that every tissue depends on *atp-2* function for optimal development. We speculate that body muscles rely more heavily on the MRC for energy production and thus, may be more sensitive to MRC defects than other tissues. This is consistent with our previous findings that muscular function is impaired in *atp-2(ua2)* mutants (Tsang *et al.*, 2001). Tissues derived from the ABa or E lineage, especially those that are highly oxidative such as neurons, may contain substantial amounts of maternally inherited *atp-2* transcript and/or protein in their mitochondria. This may help explain why they can occasionally tolerate the loss of wild type *atp-2* gene. Alternatively, these tissues may be capable of utilizing metabolites other than ATP for their energy needs that other tissues cannot. Since *atp-2* is involved in energy production, development beyond the L3 stage may require the synthesis of a metabolite in *atp-2(+)* cells. This metabolite may then be transported to and utilized by *atp-2(-)* cells. Metabolite and substrate transport across the inner membrane of mitochondria is

mediated by a number of mitochondrial carrier proteins, which are highly conserved among eucaryotes (Palmieri *et al.*, 2000). One characterized gene in the nematode, *dif-1*, encodes a mitochondrial carrier protein of unknown function and is expressed in a tissue-specific manner (Ahringer, 1995). However, it should be noted that *C. elegans* does not have a circulatory system that would transport a metabolite to all parts of the animal.

The L3 arrest phenotype is not likely determined solely by the status of the body muscles, since the presence of the complementing array in all but one body muscle nucleus does not allow normal development to occur (Table 1, class 4). Thus, *atp-2* function is apparently required in more than one tissue type. No single lineage appears to be sufficient to prevent larval arrest. It is possible that a specific set of cells arising from different lineages is important for the L3 to L4 transition. Alternatively, proper development may require a certain threshold number of *atp-2(+)* cells in each tissue or lineage. The latter hypothesis is supported by our data from the E lineage losses; partial losses in E lineage (Table 1, class 12) are less likely to arrest development than complete losses (Table 1, class 9).

The developmental processes we have studied appear to be controlled by a global mechanism; cells that are *atp-2(-)* can prevent distant *atp-2(+)* cells from developing past the L3 stage. Thus, all cells in the mosaic animals must somehow reach a consensus to continue development past the L3 stage or not. The consensus mechanism probably does not rely directly upon *atp-2* function but rather upon the production of a downstream signal, which may reflect the energy status of the signaling cell. Candidate signaling molecules include hormones or neuropeptides, both of which have important roles in the nematode (Chitwood, 1999; Gerisch *et al.*, 2001; Li *et al.*, 1999; Nelson *et al.*, 1998). The

magnitude of the signal may have to reach a certain minimum threshold in order for larval development to proceed past the L3 stage. If this is the case, then the number of cells contributing to signal production is an important parameter. In addition, some cells or tissues may contribute differentially to the magnitude of the signal. For instance, the body muscles may produce of a large proportion of the developmental signal.

A comparable signaling mechanism has been proposed for the cell non-autonomous function of the *daf-2* gene in dauer formation (Apfeld and Kenyon, 1998). DAF-2 is an insulin-like growth factor (IGF) receptor believed to respond to insulin-like ligands (Kimura *et al.*, 1997). Mutations in the *daf-2* gene reduce IGF signaling and lead to an increase in the formation of dauer larvae. Expression of *daf-2* in only a few neuronal cells is adequate to prevent dauer formation, suggesting that DAF-2 operates at a distance in addition to acting in those cells (Apfeld and Kenyon, 1998), and that a signaling molecule downstream of *daf-2* is received by cells throughout the animal and controls development. Downstream of *daf-2* are *daf-16*, which encodes a fork-head transcription factor, and *daf-9*, which encodes a *daf-16*-regulated cytochrome P_{450} hydroxylase involved in steroid biosynthesis (Jia *et al.*, 2002; Ogg *et al.*, 1997). The DAF-9 generated steroid molecule, which is a secondary signal emanating from *daf-2(+)* cells, is thought to be the ligand for the DAF-12 orphan nuclear hormone receptor; ligand binding is necessary for the inactivation of DAF-12's dauer-promoting activity (Antebi *et al.*, 2000). We hypothesize that a signaling mechanism analogous to the one in the *daf* system may mediate an energy-related signal in larval development. It is interesting to note that the *C. elegans sir-2.1* gene, which encodes a protein with 31% sequence identity to the yeast Sir2p, regulates lifespan determination and dauer formation (Tissenbaum and

Guarente, 2001). Sir2p is an NAD-dependent histone deacetylase whose function reflects cellular metabolism (Guarente and Kenyon, 2000); the cellular ratio of NADH/NAD can be profoundly affected by MRC defects such as the *atp-2(ua2)* mutation (Wallace, 1994). Alternatively, a regulatory protein that directly senses ATP levels may be involved (Dennis *et al.*, 2001).

In summary, our findings demonstrate two important points. First, *atp-2* gene function in body muscles is tightly correlated to nematode larval development from the L3 to the L4 stages, although it is essential in all tissues for optimal development. Second, *atp-2* function is cell non-autonomous suggesting that MRC defects interfere with the production of a global developmental signal. Deciphering the molecular pathway connecting MRC function and development will be necessary for understanding mitochondrial disorders.

Class	P_0						L3	L4	Adult	L4 + Adult
	AB		P_1							
	ABa	ABp	EMS		P_2					
			MS	E	C	$D(P_3)$				
1	+[a]	+	+	+	+	+				>10000
2	-[b]	-	+	+	+	+	32[d]			
3	-	+	+	+	+	+	2	2[e]		2[g]
4	+	-	+	+	+	+	7			
5	+	+	-	-	-	-	51			
6	+	+	-	-	+	+	11			
7	+	+	+	+	-	-	2			
8	+	+	-	+	+	+	2			
9	+	+	+	-	+	+	14	4	2[f]	6
10	+	+	+	+	-	+	3			
11	+	+	+	+	+	-	5			
12	+	+	+	P[c]	+	+	27	25	13	38
13	+	+	+	P	-	+	1			
14	+	+	+	P	+	-	1			
15	+	+	+	P	-	-	1			
16	+	+	+	-	+	-	4			
17	+	+	+	-	-	P	1			
18	+	+	+	-	P	-	1			
19	+	+	+	-	-	-	2			
20	+	+	-	+	-	-	1			
21	+	+	-	-	+	-	2			
22	+	+	-	-	-	+	2			
23	+	-	-	-	-	+	1			
24	-	+	-	-	-	+	1			
25	-	+	+	-	+	+		1		1
26	-	-	+	P	+	+	1			
27	-	-	-	+	+	+	1			
28	-	-	+	+	-	-	1			
29	-	-	-	-	+	+	3			
30	-	-	-	+	-	-	2			
31	-	-	+	-	-	-	2			
32	-	-	-	-	-	-	>10000			

Table 3.1: Phenotypes of *atp-2* genetic mosaics

[a] *atp-2(+)*
[b] *atp-2(-)*
[c] Partial losses of *atp-2(+)* in that particular lineage
[d] Number of animals arresting at the L3 stage
[e] Number of animals developing to the L4 stage
[f] Number of animals developing to the adult stage
[g] Number of animals developing to the L4 or adult stages

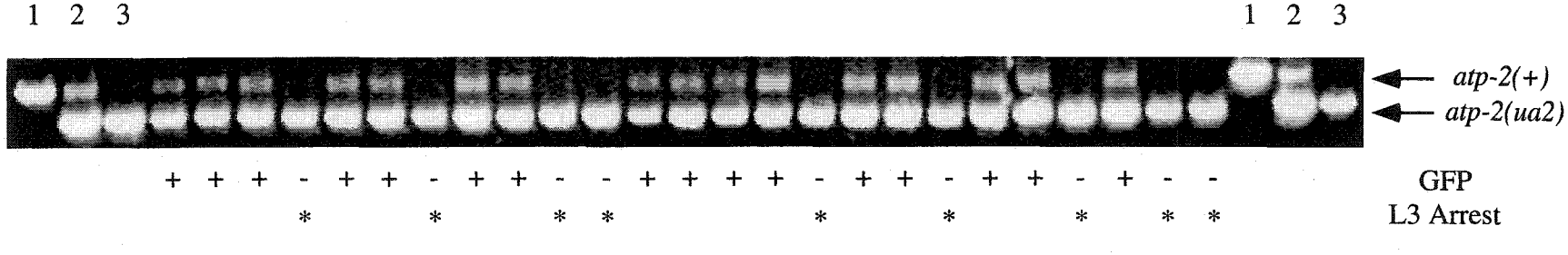

Figure 3.1: PCR analysis of LB146 *atp-2* genotypes
Individual worms were lysed, analyzed by PCR using the primers F1B2 and F1B4, and the products resolved by agarose gel electrophoresis. The *atp-2(+)* and the *atp-2(ua2)* alleles result in the production of 2.7 and 2.0-kb bands, respectively. Control lysates were prepared from (1) LB128 +/+, (2) LB128 *+/atp-2(ua2)*, and (3) LB128 *atp-2(ua2)/atp-2(ua2)* animals. LB146 animals are *gfp(+)* if they carry the transgene or *gfp(-)* if it has been lost. An asterisk (*) indicates an animal arrested as an L3 larva.

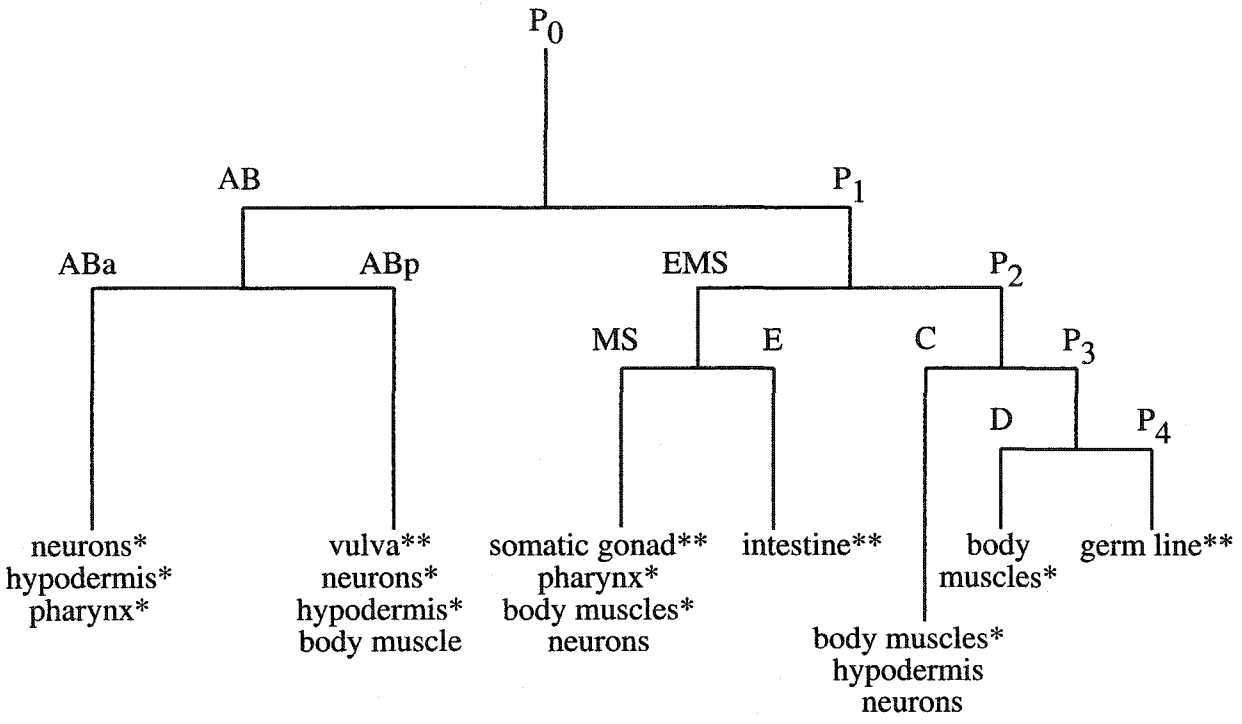

Figure 3.2: A diagram depicting the lineal origins of the major tissues
Tissues marked with two asterisks (**) are derived exclusively from that particular lineage. Tissues marked with only one asterisk (*) have significant contributions (>~10%) from that particular lineage. Other tissues have minor contributions (<~10%) from that particular lineage. Adapted from [Wood, 1988].

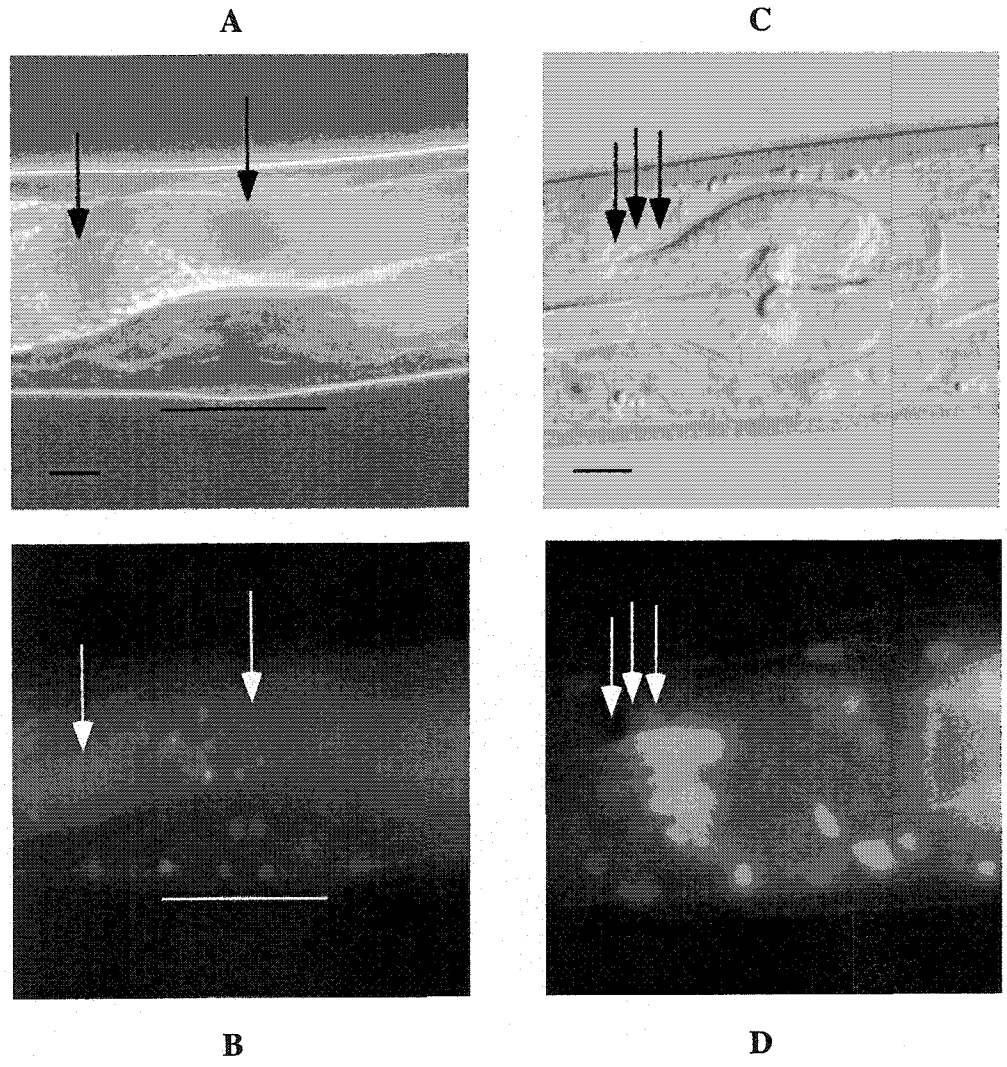

Figure 3.3: Nomarski (**A, C**) and fluorescence (**B, D**) images of two L4 mosaic animals
(**A, B**) A class 9 mosaic. This animal had lost *atp-2(+)* in the E lineage. Lost of GFP fluorescence in intestinal cells is evident (arrows). A L4 vulva can be seen (underlined). Bar: 10-μm. (**C, D**) A class 3 mosaic. This animal had lost *atp-2(+)* in the ABa lineage. Left, middle, and right arrows point to ASKL (derived from ABa), ADLL (derived from ABa), and ASIL (derived from ABp) cells, respectively. Note the absence of GFP in ASKL and ADLL and the presence of GFP in ASIL. Bar: 10-μm.

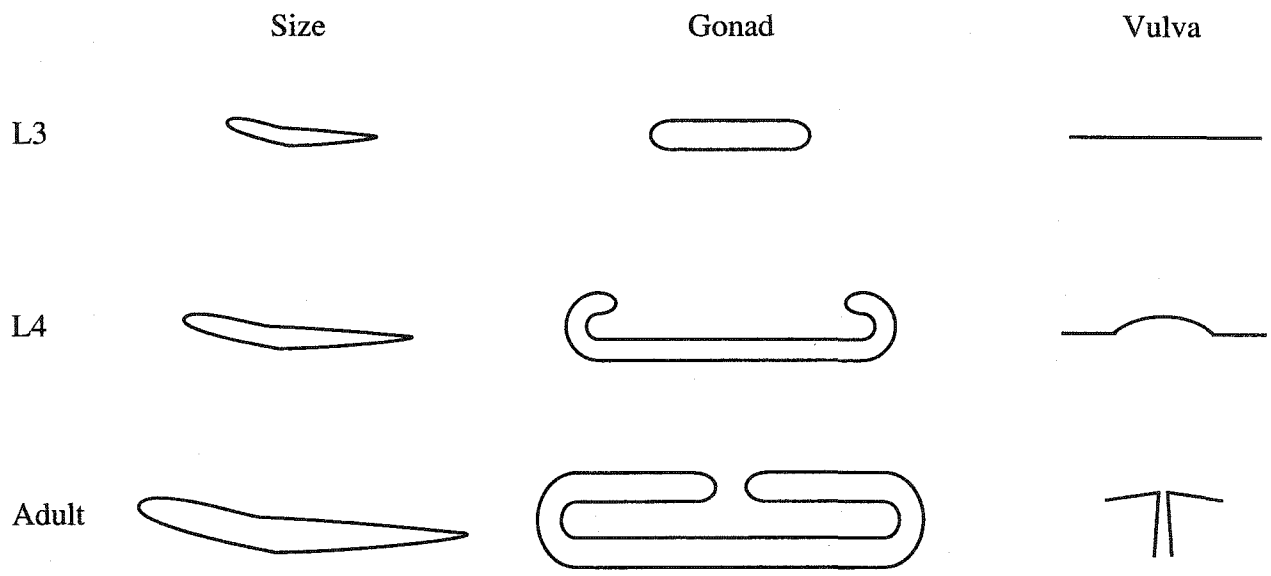

Figure 3.4: Morphological differences in the body size, the gonad, and the vulva associated with larval development from the L3 stage to the adult

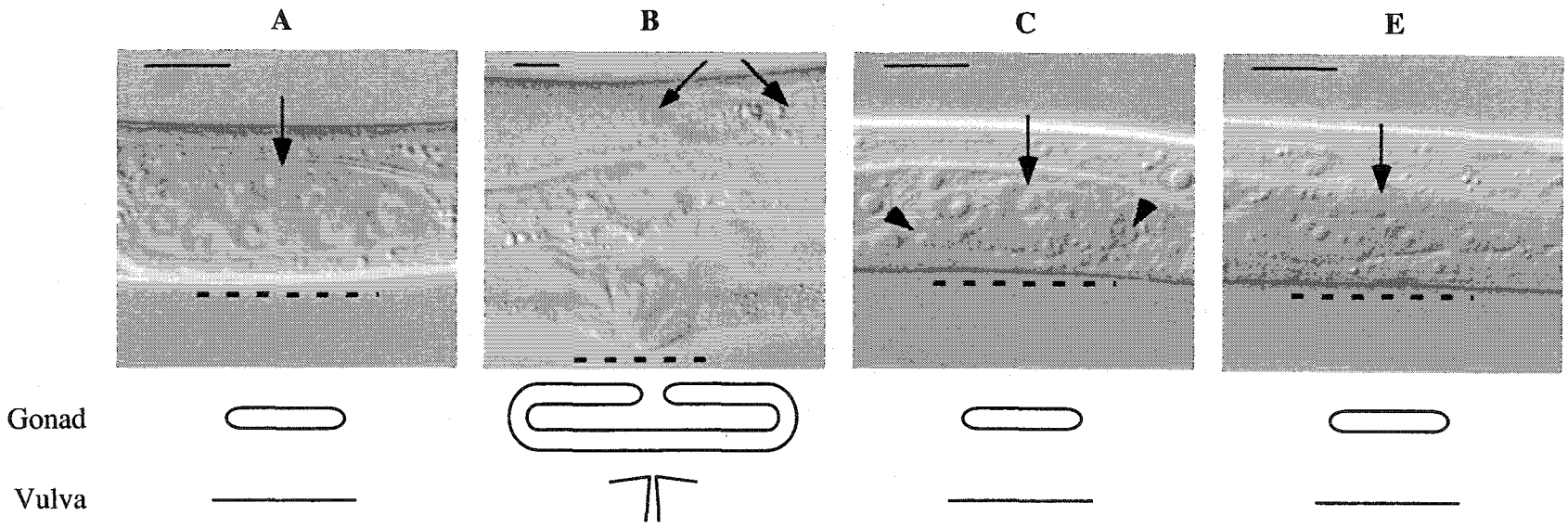

Figure 3.5: Nomarski (**A**, **B**, **C**, **E**) and fluorescence (**D**, **F**) images of selected mosaic animals

The positions of the gonads or gonad arms are indicated with arrows. The positions of the vulva or the Pn.p cells are underlined with a dashed line. (**A**) An L3 homozygous *atp-2(ua2)* animal with an arrested gonad and no vulval development. Bar: 12-μm. (**B**) An adult *atp-2(+)* animal with a reflexed, elongated gonad and a mature vulva. Bar: 10-μm. (**C, D**) A class 16 mosaic. This animal had lost *atp-2(+)* in the E and P_3 lineages; GFP fluorescence and thus *atp-2(+)* are present in the ABp (out of focus) and MS lineages (the two fluorescent distal tip cells are indicated by triangles). Bar: 10-μm. (**E, F**) A class 3 mosaic. This animal had lost *atp-2(+)* in the ABa lineage; *atp-2(+)* is present in the ABp (the fluorescent Pn.p cells are underlined) and MS lineages (out of focus). Bar: 12-μm. Both mosaic animals were arrested as L3 larvae and had a phenotype similar to an *atp-2(ua2)* mutant shown in (**A**). Pictorial gonad and vulva representations are drawn below the Nomarski pictures.

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Chapter 4

Stable Heteroplasmy but Differential Inheritance of a Large Mitochondrial DNA Deletion in Nematodes

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4.1 Introduction

The mitochondrial respiratory chain (MRC), composed of five highly conserved protein complexes, is the major source of ATP in most eucaryotes. The biogenesis of the MRC is a complex process relying on the coordinate expression of both nuclear and mitochondrial DNA (mtDNA) encoded genes (Attardi and Schatz, 1988). Defective mitochondrial energy production in humans gives rise to a broad set of diseases, many of them associated with defects in the mtDNA (DiMauro and Andreu, 2000). In humans, the mtDNA encodes 13 polypeptide subunits of the MRC, 22 tRNAs, and two rRNAs, all of which are required for the elaboration of a functional MRC (DiMauro and Andreu, 2000). The mtDNA of the nematode *Caenorhabditis elegans* encodes the same numbers of RNA species, but only 12 MRC subunits; it is missing the gene for subunit 8 of the ATP synthase (Okimoto *et al.*, 1992). In both species, the remainder of the MRC subunits and the components of the replication, transcription, and translation machineries are the products of nuclear genes that are imported into mitochondria.

The transmission of traits associated with the mtDNA displays a number of interesting genetic peculiarities (Grossman and Shoubridge, 1996). First, most cells contain hundreds to thousands of identical copies of mtDNA; this is the normal case and is known as homoplasmy. The presence of more than one form of the mtDNA constitutes a state known as heteroplasmy. Second, mammalian mtDNA is strictly transmitted along the maternal line. Third, a mutation in a single copy of mtDNA can propagate and lead to heteroplasmic states with widely differing proportions of mutant mtDNA. The genotypes of heteroplasmic individuals are likely constantly evolving due to mitotic segregation and selection and the proportion of mutant mtDNA in different tissues can vary markedly.

Fourth, it is generally believed that shorter, deletion-containing mtDNA molecules have a replicative advantage over wild type mtDNA molecules. Finally, increasing proportions of mutant mtDNA do not have significant effects on energy production until a threshold level in the range of 60% to 90% is reached. Tissues or organs with the highest energy demands such as skeletal muscle, brain, and heart are more seriously affected by impaired energy production (Wallace, 1992b).

We report the isolation and the genetic characterization of a large deletion in the *C. elegans* mtDNA. The *uaDf5* mutation affects four protein-coding and seven tRNA genes. It is maternally inherited and has been stably transmitted for over 100 generations. Animals carrying the *uaDf5* mtDNA are heteroplasmic with the average proportion of ~60% being maintained throughout development. Individual animals carry about 20% to 80% *uaDf5*, and no homoplasmic animals have ever been found. No deleterious consequences have been observed despite high levels of *uaDf5* mtDNA in some animals. Interestingly, the total mtDNA copy number of heteroplasmic animals is increased two-fold over wild type levels, suggesting that mtDNA amplification may be a genetic compensatory mechanism to minimize the effects of mtDNA mutations.

Our data suggest that the stable *uaDf5* heteroplasmy is maintained by at least two opposing forces, one driving *uaDf5* levels up and the other pushing it down. The proportions of *uaDf5* mtDNA in the offspring of single, self-fertilized hermaphrodites with intermediate levels of *uaDf5* mtDNA (50% or 65%) form normal (non-skewed) distributions centered on the parental mean. However, hermaphrodites with high proportions of *uaDf5* mtDNA (75% or 81%) have broods with skewed distributions; the mean content of the offspring is much lower than the *uaDf5* level of the parent

hermaphrodite. Conversely, hermaphrodites with low proportions of *uaDf5* mtDNA (24% or 29%) also have broods with skewed distributions of *uaDf5* but with the means being higher than the level of the parent. The biased inheritance of the *uaDf5* mtDNA observed at high and low levels of heteroplasmy prevents the appearance of homoplasmic wild type or mutant progeny.

4.2 Materials and Methods

Strains

Worms were cultured as described (Lewis and Fleming, 1995). The following strains were used: N2 (Bristol) wild type strain; CB164, *dpy-17(e164)*; CB1489, *him-8(e1489)*; CB251, *unc-36(e251)*; LB141, *uaDf5//*++M, the original *uaDf5* isolate; LB131, *dpy-17(e164)*III *him-8(e1489)*IV *uaDf5//*++M; LB138, *him-8(e1489)*IV *uaDf5//*++M. The 'M' refers to the mtDNA and the double slash in '*uaDf5//*++' indicates that the *uaDf5* and the wild type mtDNAs are not equimolar.

Isolation of mutants and genetic analysis

Chemical mutagenesis (ethyl methanesulfonate, 50 mM for 4 h) was used to generate a library of deletion mutants in an N2 background that could be screened by PCR in a process called target-selected gene inactivation (Figure 2.3) (Jansen *et al.*, 1997; Tsang *et al.*, 2001). We detected mtDNA deletions using pairs of nested primers chosen to be about 4-kbp apart using duplicate or triplicate reactions under standard PCR conditions (Plasterk, 1995; Tsang *et al.*, 2001). The 4-kbp region encompasses nucleotides 1,852 to 5,857 of the mtDNA (Okimoto *et al.*, 1992). The primers used were: mtDNA1, 5'-CTTTTATTACTCTATATGAGCGTC-3', mtDNA2, 5'-GGGAAGAAG-

ACAAAATCGTCTAG-3', mtDNA3, 5'-CGAATTTAAACCCGTCTATAACG-3', and mtDNA4, 5'-TATAGCAATATCCTTACCTCAAGC-3'. One positive address was found and the authenticity of the PCR product was determined by DNA sequencing. Three rounds of sibling selection were required to derive a clonal line. The *uaDf5* mutation was outcrossed six times by crossing LB141 to CB164 and CB1489 to generate LB131 and LB138. To evaluate the inheritance pattern of the *uaDf5* mtDNA, we performed two genetic crosses. Heteroplasmic LB131 males were mated to CB251 hermaphrodites and the genotypes of individual, phenotypically wild type outcross progeny were determined by PCR with primers mtDNA1 and mtDNA3. CB1489 males, which are homoplasmic for wild type mtDNA, were mated to LB131 hermaphrodites. Outcross progeny, which are phenotypically wild type, and their self progeny were analyzed by PCR.

Quantitative PCR

PCR was used for the detection and measurement of mtDNA levels. Two standard curves, one for the *uaDf5* allele and the other for wild type mtDNA were generated by relating the initial number of template copies to the number of cycles at which the product DNA had attained an arbitrary fluorescence intensity of 100 when resolved by agarose gel electrophoresis and stained with ethidium bromide (Figure 4.1); this occurs during the exponential amplification phase. A 1,036-bp region of the *uaDf5* mtDNA was amplified with the primers mtDNA1 and mtDNA3. The mtDNA1 and mtDNA5 (5'-GTAAATTCAACCATTCCACAAGG-3') primers amplify a 1,064-bp region of the wild type mtDNA. For the construction of standard curves, purified PCR products generated from either mtDNA1 and mtDNA5 (wild type template) or mtDNA1 and mtDNA3 (mutant template) were used as templates of known concentrations, determined with a

Sequoia-Turner 450 fluorometer. PCR was performed with a DeltaCycler II (Ericomp, San Diego, California) and consisted of a single denaturation step (3 min at 92 °C), followed by cycles of 15 sec denaturation at 92 °C, 30 sec annealing at 61 °C, and 30 seconds elongation at 72 °C. PCR products were subjected to 1% agarose gel electrophoresis, stained with ethidium bromide, and quantified with a Gel Doc 1000 system (BioRad, Hercules, California). Non-specific products (except primer-dimers in some samples) were not detected. Worm samples were lysed by incubation in a lysis buffer (50 mM KCl, 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 *u*g/ml freshly added proteinase K) for 1 h at 50 °C. For single worm lysis, 10 to 15 μl of the lysis buffer was used. For populations, 100 to 2,000 worms were lysed in 100 μl of lysis buffer. This was followed by 10 min incubation at 95 °C to inactivate the proteinase. One μl lysate was used per reaction and the copy numbers of the wild type and the *uaDf5* mtDNAs were determined from the standard curves. The percentage of *uaDf5* was determined as the ratio of the *uaDf5* copy number over the total copy number. The reproducibility and reliability of this method were confirmed by performing a number of control reactions with mixtures containing variable, known proportions of input wild type and mutant templates (Figure 4.2). Total mtDNA copy numbers were determined as described previously (Tsang and Lemire, 2002), using primers that recognize sequences present on both wild type and *uaDf5* genomes; mtDNA6, 5'-AGCACACGGTTATACATCTACAC-3' and mtDNA7, 5'-AAATCTAGTACCAACCATACCAG-3' amplify a 628-bp product (Figure 4.3). Twenty synchronized animals were lysed in 30 μl total volume and 1 μl was used as template for the total copy number reactions.

Swimming, pharyngeal pumping, defecation, brood size, and lifespan

Swimming, pharyngeal pumping, defecation, and lifespan were measured as described previously (Tsang *et al.*, 2001). For the determination of brood size, individual L4 hermaphrodites were transferred onto freshly seeded NGM plates every 6-14 h. The number of progeny on each plate was counted.

4.3 Results

Isolation of a mtDNA deletion

We employed target-selected mutagenesis to identify deletion mutations in the mitochondrial genome. We amplified a region of the mtDNA (nucleotides 1,852-5,857) that encodes 13 genes (Figure 4.4). This region was chosen because it does not encode replication or transcription control regions and is analogous to the common deletion found in patients with Kearns-Sayre syndrome (Moraes *et al.*, 1989; Okimoto *et al.*, 1992). We isolated and cloned one allele designated *uaDf5* that is a 3.1-kbp deletion (nucleotides 2,310-5,362; Figure 4.4). It removes a total of 11 genes, including the four MRC subunits, ND1, ATP6, ND2, and Cyt *b*, and seven tRNA genes, $tRNA^{lys}$, $tRNA^{leu:UUR}$, $tRNA^{ser:AGN}$, $tRNA^{phe}$, $tRNA^{gln}$, $tRNA^{arg}$, $tRNA^{ile}$. DNA sequencing revealed that the deletion occurred between 9-bp GTAATTGCT direct repeats at nucleotides 2309-2317 and 5362-5370; only one repeat is retained in the deleted molecule.

Maternal inheritance and maintenance of *uaDf5*

To confirm the mitochondrial location of the *uaDf5* deletion, the following genetic crosses were performed. When a heteroplasmic *uaDf5*-carrying hermaphrodite was mated to a homoplasmic wild type male, 100% of the outcross progeny carried the

uaDf5 genome (Figure 4.5). When the heteroplasmic F_1 hermaphrodite progeny were allowed to self-fertilize, all the F_2 progeny were heteroplasmic for the *uaDf5* mtDNA (Figure 4.5). Conversely, when a heteroplasmic *uaDf5*-carrying male was mated to a homoplasmic wild type hermaphrodite, none of the outcross progeny carried the *uaDf5* mtDNA (Figure 4.5). Thus, the inheritance of the *uaDf5* allele is non-Mendelian; it is exclusively transmitted through the maternal lineage. This is consistent with the *uaDf5* allele being an authentic mtDNA deletion rather than a portion of the mtDNA that has integrated into the nuclear genome (Wallace *et al.*, 1997).

The co-existence of wild type and *uaDf5* mtDNAs is stable. Since the initial isolation of the *uaDf5* mutation, the deletion has been outcrossed at least six times and maintained for over 100 generations. Over a period of about three years, we have randomly selected individual worms for mtDNA genotyping. In all cases, the animals were heteroplasmic with a heteroplasmy ranging from ~20% to ~80%. We have never detected any homoplasmic wild type or homoplasmic mutant animals arising from heteroplasmic *uaDf5* lines.

The complete *C. elegans* life cycle takes about three days at 25 °C. Following fertilization, the embryo undergoes cell proliferation, organogenesis, and morphogenesis culminating in the hatching of the L1 larva after about 14 hours. Over the next two days, development proceeds through three additional larval stages, L2, L3, and L4, until the adult emerges from the final molt. We measured the percentage of *uaDf5* during development in a synchronized population. The level of heteroplasmy was maintained at ~60% (Figure 4.6).

Random inheritance of *uaDf5*

Although every heteroplasmic animal contains two forms of mtDNA, the proportion of the two forms varies between individuals. We investigated the inheritance of the *uaDf5* allele by determining the proportion of *uaDf5* mtDNA in each individual from the brood of a single heteroplasmic LB138 hermaphrodite. The hermaphrodite, after having laid 195 embryos, was determined to have 50% *uaDf5*. Each embryo was allowed to mature to adulthood and hermaphrodites allowed to lay their broods before mtDNA contents were measured. The mean and the median of the *uaDf5* mtDNA proportions in the offspring were roughly equal to the *uaDf5* content of the parent. The *uaDf5* mtDNA contents of the offspring formed a normal (non-skewed) distribution ranging from ~20% to ~80% (Figure 4.7A and Table 4.1). The distribution of *uaDf5* contents was similar when male and female offspring were considered separately. Thus, the inheritance of mutant mtDNA is a random sex-independent process. A normal distribution of *uaDf5* contents was also observed in the partial brood of a single hermaphrodite with 65% uaDf5 (Figure 4.7B and Table 4.1). In this case, the average and the median of the *uaDf5* mtDNA proportions among the progeny were also similar to the content of the parent (Table 4.1). The distribution and its mean are shifted to the right as predicted for a parent containing a much higher proportion of *uaDf5*. The range of the distribution is narrower due to the smaller sample size. Despite the three to four-fold differences in *uaDf5* contents between individuals, none were obviously compromised. They are unaffected in terms of fertility, longevity, mobility, pharyngeal pumping rates, and defecation (Table 4.2). Many of these traits are affected in animals with respiratory chain mutations (Feng *et al.*, 2001; Kayser *et al.*, 1999; Tsang *et al.*, 2001; Wong *et al.*, 1995).

Biased inheritance of *uaDf5*

We reasoned that if *uaDf5* mtDNA were always inherited in a random fashion, then a hermaphrodite with a high proportion of *uaDf5* might produce offspring with a phenotype attributable to the mtDNA deletion. A fraction of the progeny might even be expected to be homoplasmic for the deletion. When a single hermaphrodite containing 75% *uaDf5* was allowed to lay a part of its brood, the proportions of *uaDf5* mtDNA of the progeny formed a skewed distribution with the mean and median values being much lower than that of the hermaphrodite (Figure 4.8A and Table 4.1). Had the inheritance of *uaDf5* mtDNA been random and formed a normal distribution, half of the progeny (18/36) would be expected to have greater than 75% *uaDf5*; only five such animals were found. A similar finding was observed in the partial brood of a single hermaphrodite carrying 81% *uaDf5* (Figure 4.8B and Table 4.1). In this case, only three progeny carried more than 81% *uaDf5* as compared to the 14 expected if the distribution had been normal. None of the individual progeny contained more than 83% *uaDf5*.

Similarly, we predicted that a fraction of the progeny from a hermaphrodite having a low content of *uaDf5* would be homoplasmic for the wild type mtDNA, having lost all the *uaDf5* molecules. When a single hermaphrodite containing 29% *uaDf5* was allowed to lay a part of its brood, the proportions of *uaDf5* mtDNA of the progeny formed a skewed distribution (Figure 4.9A and Table 4.1). Both the mean and median values of the distribution were much higher than the content of *uaDf5* in the parent hermaphrodite (Table 4.1). Had the inheritance of *uaDf5* mtDNA produced a normal distribution, half of the progeny (13/26) would be expected to have less than 29% *uaDf5*; only three such animals were found. A similar inheritance pattern was also observed in

the partial brood of a single hermaphrodite carrying 24% *uaDf5* (Figure 4.9B and Table 4.1). In this instance, only two progeny animals carried less than 24% *uaDf5* as compared to the 17 expected for a normal distribution. None of the individual progeny contained less than 19% *uaDf5*.

mtDNA copy number of *uaDf5* animals

The average mtDNA copy number in wild type adult hermaphrodites was determined to be roughly $8.2x10^5$ per animal (Figure 4.10) (Tsang and Lemire, 2002). The copy number of an *him-8* adult hermaphrodite ($8.0x10^5$) is not significantly different from the wild type, suggesting that the *him-8(e1489)* mutation does not affect the mtDNA copy number (Figure 4.10). Interestingly, an adult male has about one-third the number of mtDNA copies ($2.6x10^5$) as compared to an adult hermaphrodite ($8.0x10^5$) (Figure 4.10). We have attributed this difference in mtDNA content between the sexes to the particularly high mtDNA requirement of oogenesis (Tsang and Lemire, 2002). We determined the mtDNA copy number of the *uaDf5* strain, LB138, which also contains the *him-8(e1489)* mutation. LB138 adult hermaphrodites and males have $1.5x10^6$ and $5.0x10^5$ total copies of mtDNA per animal, respectively (Figure 4.10). Thus, for both sexes, *uaDf5* animals have approximately twice the number of mitochondrial genomes as wild type animals.

3.4 Discussion

In this work, we report the isolation of a *C. elegans* strain heteroplasmic for a large deletion in the mtDNA. Large deletions have been found in a number of organisms, including yeast (Dujon, 1981), mice (Inoue *et al.*, 2000), fruit flies (Petit *et al.*, 1998),

and humans (Holt *et al.*, 1988). Deletion mutations in the *C. elegans* mtDNA have also been reported (Denver *et al.*, 2000). In other *C. elegans* studies, it was determined that the frequency of mtDNA deletions increases with age (Melov *et al.*, 1995), However, the transmission, maintenance, inheritance, and proportions of the deleted species have not been reported previously in the nematode. Studies along these lines are essential to improve our understanding and knowledge of mitochondrial diseases associated with mtDNA defects.

Deletions in mtDNA are thought to arise by recombination at or slipped mispairing during replication of direct sequence repeats (Wallace, 1992a). The *uaDf5* deletion involves 9-bp direct repeats, with one copy retained in the mutated mtDNA. In each of four other *C. elegans* mtDNA deletions, direct repeats of 4-9 base pairs were also found, with one copy of the repeat deleted in each product (Melov *et al.*, 1994).

In obligate aerobes, mtDNA deletions are invariably heteroplasmic, presumably because homoplasmy is lethal (Grossman and Shoubridge, 1996; Petit *et al.*, 1998). It is believed that the nature of the mutation and the energy requirements of the affected tissues determine the maximal proportion of mutated mtDNA tolerated. Thus, individuals bearing mtDNA deletions do not necessarily exhibit a phenotype. In humans, mtDNA deletions can produce Kearns-Sayre syndrome, which is characterized by paralysis of the eye muscles, heart block, ataxia, hyperparathyroidism, and a high incidence of diabetes (Wallace, 1992a). Heteroplasmic mice with a greater than 85% mtDNA deletion content are susceptible to renal failure (Inoue *et al.*, 2000). In contrast, heteroplasmic *Drosophila subobscura* containing about 80% mtDNA deletion remain unaffected (Petit *et al.*, 1998). Similarly, the *uaDf5* mutant does not exhibit any developmental or reproductive defects

despite some individuals carrying ~80% *uaDf5* deletion. Perhaps simpler organisms can tolerate higher proportions of mtDNA mutations or the threshold level for the *uaDf5* mutation may exceed ~80%.

Worms carrying the *uaDf5* deletion exhibit a two-fold increase in mtDNA copy number (Figure 4.10). The increase may enhance the production of functional mtDNA transcripts and proteins and in part compensate for the mutation. An increase in mtDNA content was also observed in a heteroplasmic *Drosophila* mutant (Béziat *et al.*, 1997). In other studies, an increased expression of mitochondrial genes has been linked with deficiencies in mitochondrial energy metabolism (Heddi *et al.*, 1999; Murdock *et al.*, 1999; Wiesner *et al.*, 1999). It is interesting to note that adult *uaDf5* animals still contain similar numbers of copies of wild type mtDNA as their homoplasmic wild type counterparts but in addition, they contain *uaDf5* molecules.

Heteroplasmic fruit flies containing mixtures of *D. mauritiana* and *D. melanogaster* mtDNAs segregate homoplasmic lines for one of the two mtDNA forms (Niki *et al.*, 1989). Similarly, complete allele switching within one generation has been reported for a heteroplasmic D-loop sequence variant in Holstein cows (Koehler *et al.*, 1991). These inheritance patterns are most compatible with intermitochondrial heteroplasmy, with each mitochondrion containing either the wild type mitochondrial genome or the mutated form of the mtDNA. This is in contrast to intramitochondrial heteroplasmy where the two forms of mtDNAs co-exist in every organelle. Intramitochondrial heteroplasmy is believed to explain the stable heteroplasmy of a *D. subobscura* mutant (Petit *et al.*, 1998). Similarly, the heteroplasmy in our *uaDf5* strain may be intramitochondrial, which could explain the stability of the heteroplasmy and the

inheritance patterns we observe. The *uaDf5* mutation has been maintained for over 100 generations in the absence of selection. Over this time, we have not detected homoplasmic wild-type animals suggesting that the 2 forms of mtDNA do not segregate from each other. This is also consistent with the faithful nucleoid model, which proposes that mtDNA molecules are grouped into polyploid nucleoids whose compositions do not change through replication and the nucleoid is the segregating unit (Jacobs *et al.*, 2000). The variation in *uaDf5* levels seen within the brood of a hermaphrodite with intermediate proportions of mutated mtDNA would presumably arise because of the random inheritance of nucleoids with variable mtDNA compositions.

When the heteroplasmy of the parent hermaphrodite is shifted to more extreme levels, the *uaDf5* contents of the offspring form skewed distributions. When the proportion of *uaDf5* in the hermaphrodite is high, the average and the median of the *uaDf5* contents of the progeny are significantly lower. Conversely, when the proportion of *uaDf5* in the hermaphrodite is low, the average and the median of the *uaDf5* contents among the progeny increase significantly. Our data strongly suggest the existence of at least two opposing forces in mtDNA inheritance and in the maintenance of a stable heteroplasmy. One force leads to the increased proportion of *uaDf5* molecules when its levels are low, while the second force leads to decreased proportions of *uaDf5* mtDNA when its levels are high. We speculate that the random inheritance of *uaDf5* is achieved when the two opposing forces are balanced. A biased inheritance of *uaDf5* occurs when one of the two forces predominates; this effectively prevents homoplasmic wild type or mutant animals from arising.

What are the possible forces involved in maintaining a stable heteroplasmy? A replicative advantage for shorter mtDNA molecules has been postulated, but there is little experimental evidence to support this idea (Schon *et al.*, 1997). Alternatively, a selection pressure may lead to the preferential replication of *uaDf5* over wild type mtDNA molecules. The mechanism of this selection pressure remains undetermined but may involve nuclear DNA-encoded factors involved in the maintenance or homeostatic regulation of the mitochondrial genome (Battersby and Shoubridge, 2001; Le Goff *et al.*, 2002). The selection pressure may promote the replication of mtDNA molecules in energetically compromised organelles (Yoneda *et al.*, 1992) such as those containing higher proportions of *uaDf5* mtDNA. However, the *uaDf5* mutation does not appear to be under the influence of this selection pressure during larval development and maturation (Figure 4.6). During times of active mtDNA amplification, such as during development from L3 to adult, which involves an ~30-fold increase in mtDNA copy number (Tsang and Lemire, 2002), the proportion of *uaDf5* species does not increase. We thus hypothesize that the selection pressure is exerted at an earlier stage such as germ line or oocyte development.

In animals containing high proportions of *uaDf5* mtDNA, a second force preventing the further accumulation of *uaDf5* molecules is evident. This force may operate through the elimination of germ cells or oocytes with pathologically high (>~80%) *uaDf5* contents, and must operate prior to embryogenesis, since we have not observed embryonic or larval lethality due to the *uaDf5* mutation. Apoptosis, or programmed cell death, is a common fate in the *C. elegans* hermaphrodite germ line (Gumienny *et al.*, 1999), although its potential role in eliminating unfit or unhealthy germ

nuclei remains to be determined. The loss of germ nuclei through apoptosis would not necessarily be manifested in a decreased brood size, since a hermaphrodite is capable of generating ~2,000 germ cells over its lifetime, but only produces 200-300 progeny (Schedl, 1997).

The selection pressure that underlies the increase in *uaDf5* mtDNA contents in broods of hermaphrodites with low *uaDf5* proportions likely prevents the segregation of homoplasmic wild type animals. This pressure may be present because the wild type DNA is not truly wild type but rather has acquired a mutation that would be lethal if homoplasmic. In this scenario, the *uaDf5* mtDNA must be maintained to complement this otherwise lethal mutation. Thus, the heteroplasmy may be stable because both wild type and *uaDf5* mtDNA genomes are needed to produce a fully functional MRC. Alternatively, the selection pressure may be the result of a nuclear mutation that affects replication or segregation. The elimination of oocytes or oocyte precursor cells with high levels of *uaDf5* would also likely be under nuclear control. Thus, it should be possible to isolate nuclear mutations that interfere with either force; these mutations would allow heteroplasmic lines to segregate homoplasmic wild type progeny or to produce phenotypically compromised offspring. Such mutations may lead to a better understanding of the maintenance, expression, and transmission of mtDNA.

Hermaphrodite *% uaDf5*	Progeny Sample Size	Progeny Mean *% uaDf5*	Progeny Median *% uaDf5*	*z* test p value[a]	Kolmogorov-Smirnov p value[b]
50	162	47	48	NS[c]	NS
65	29	64	66	NS	NS
81	28	69	71	<0.01	<0.01
75	36	66	67	<0.01	<0.01
29	25	38	38	<0.01	<0.01
24	34	37	38	<0.01	<0.01

Table 4.1: Heteroplasmy levels of six different hermaphrodites and their progeny

[a] The mean *% uaDf5* of the progeny is significantly different from the hermaphrodite *% uaDf5* based on a two-tailed *z* test using the Student's *t*-distribution

[b] The distribution of the heteroplasmy levels of the progeny is significantly different (skewed) from a normal distribution based on a Kolmogorov-Smirnov goodness-of-fit test

[c] Not significant

Strain	CB1489 *him-8(e1489)*[a]	LB138 *him-8(e1489); uaDf5*[a]
Swimming (cycles / min)	106 ± 6 (n=20)	107 ± 6 (n=20)
Pharyngeal Pumping (contractions / min)	274 ± 10 (n=20)	275 ± 8 (n=20)
Defecation (sec / cycle)	55 ± 6 (n=20)	53 ± 7 (n=20)
Brood Size (progeny / adult)	246 ± 34 (n=11)	231 ± 38 (n=11)
Lifespan (days)	12.7 ± 2.8 (n=37)	12.3 ± 2.1 (n=27)

Table 4.2: Heteroplasmy for *uaDf5* does not impair fitness
[a] Values are means ± SD (number of animals scored)

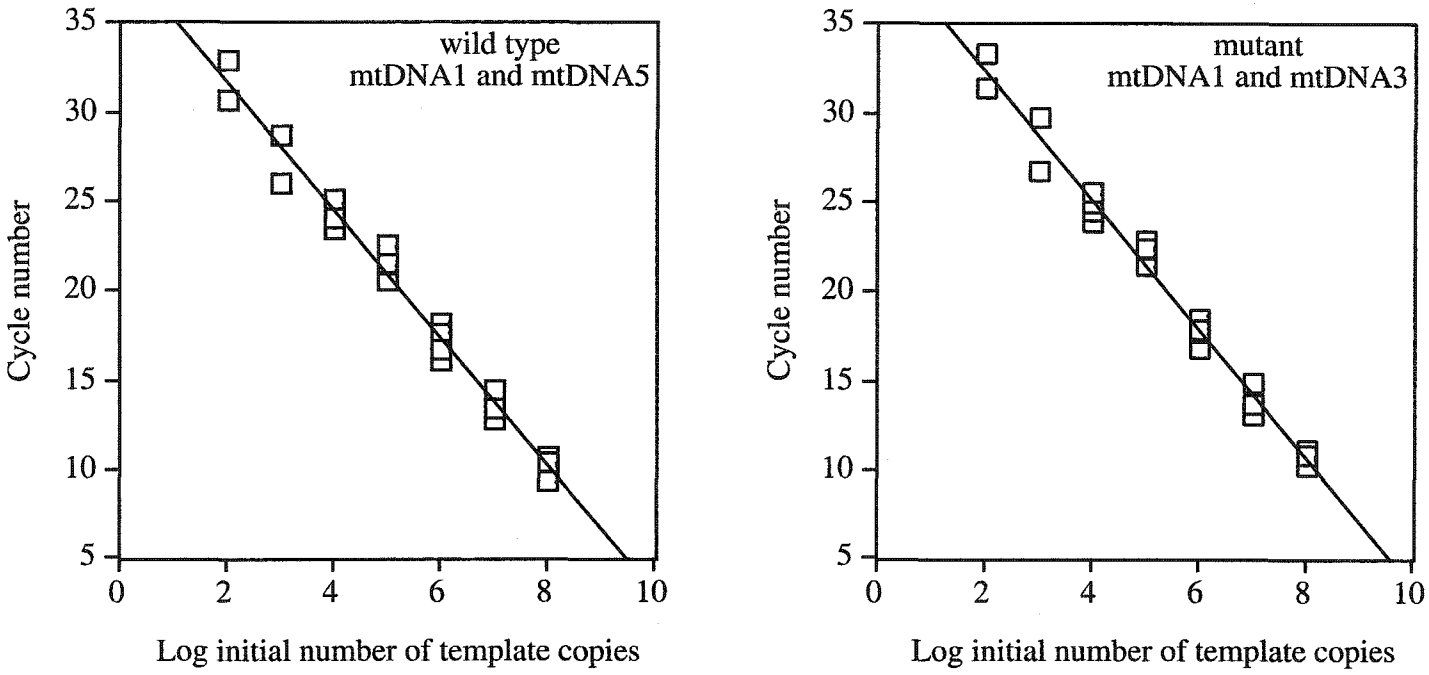

Figure 4.1: Construction of standard curves for the determination of wild type and mutant mtDNA levels Quantitative PCR was performed as described in 'Materials and Methods'. The inital number of template copies refers to the initial number of copies of the input template used. The pair of primers used and the amplified product is indicated. The correlation coefficients (r^2) are 0.982 (left graph) and 0.984 (right graph).

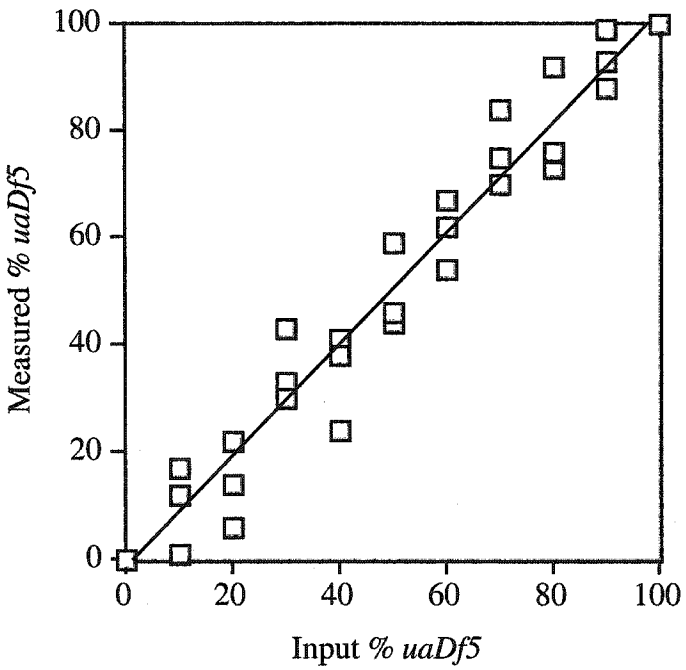

Figure 4.2: Reliability and reproducibility of the quantitative PCR method
Quantitative PCR was performed as described in 'Materials and Methods'. The input and measured proportions of *uaDf5* agree with a correlation coefficient (r^2) of 0.959.

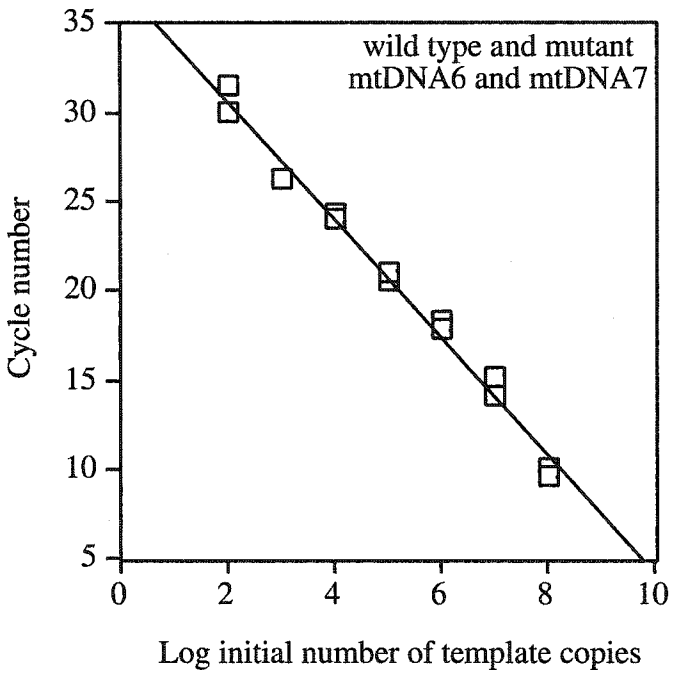

Figure 4.3: Construction of a standard curve for the determination of total (wild type and mutant) mtDNA copy number

Quantitative PCR was performed as described in 'Materials and Methods'. The inital number of template copies refers to the initial number of copies of the input template used. The pair of primers used and the amplified product is indicated. The correlation coefficient (r^2) is 0.987.

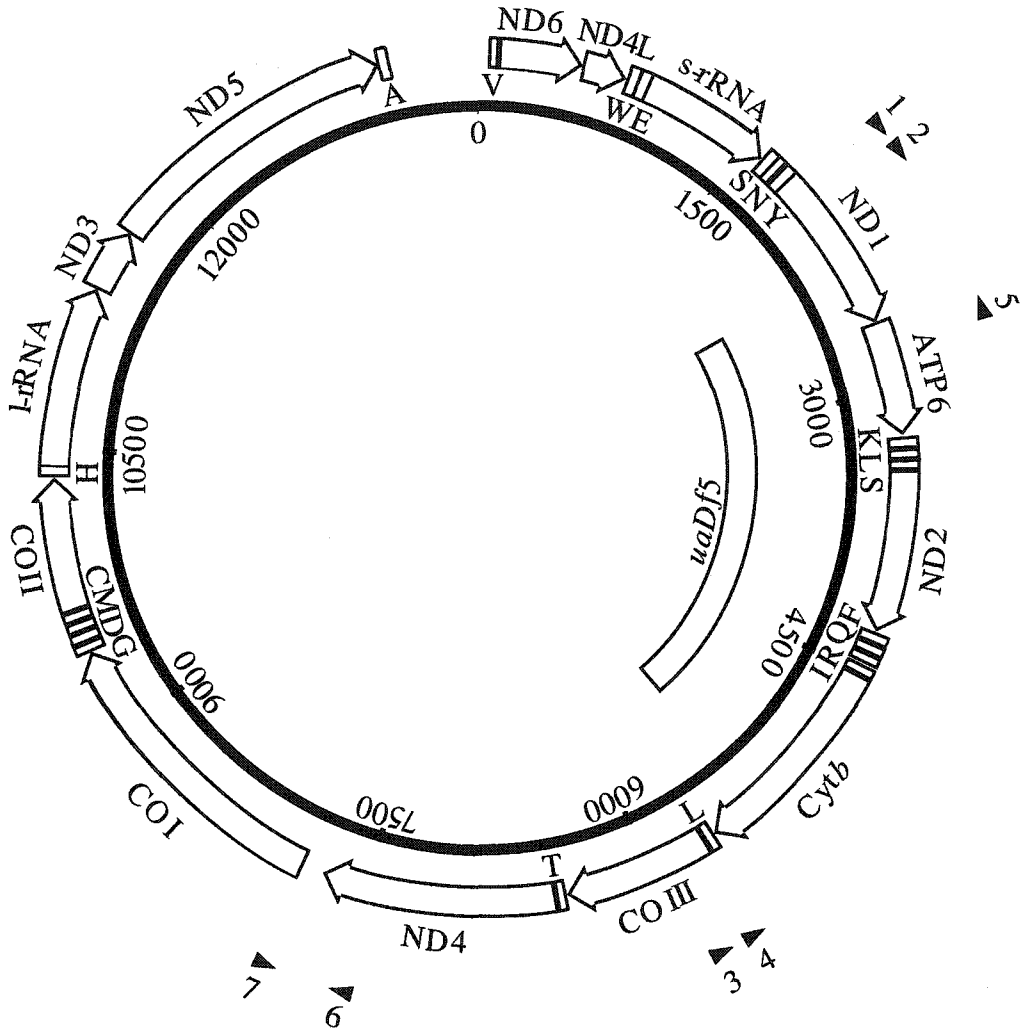

Figure 4.4: A map of the *C. elegans* mtDNA
The nematode mtDNA is a 13.8-kbp molecule encoding 12 MRC subunits (subunits of the NADH-ubiquinone oxidoreductase: ND1, ND2, ND3, ND4, ND4L, ND5, ND6; cytochrome *b* of the ubiquinol-cytochrome *c* oxidoreductase: Cyt b; subunits of the cytochrome *c* oxidase: COI, COII, COIII; and a subunit of the ATP synthase: ATP6), 2 ribosomal RNAs (l-rRNA and s-rRNA), and 22 tRNAs (Okimoto *et al.* 1992). Each tRNA gene is identified by the one letter amino acid code. The position of the *uaDf5* deletion is shown within the circle. The positions and directions of oligonucleotide primers (mtDNA1 to mtDNA7) are indicated as numbers beside the arrowheads.

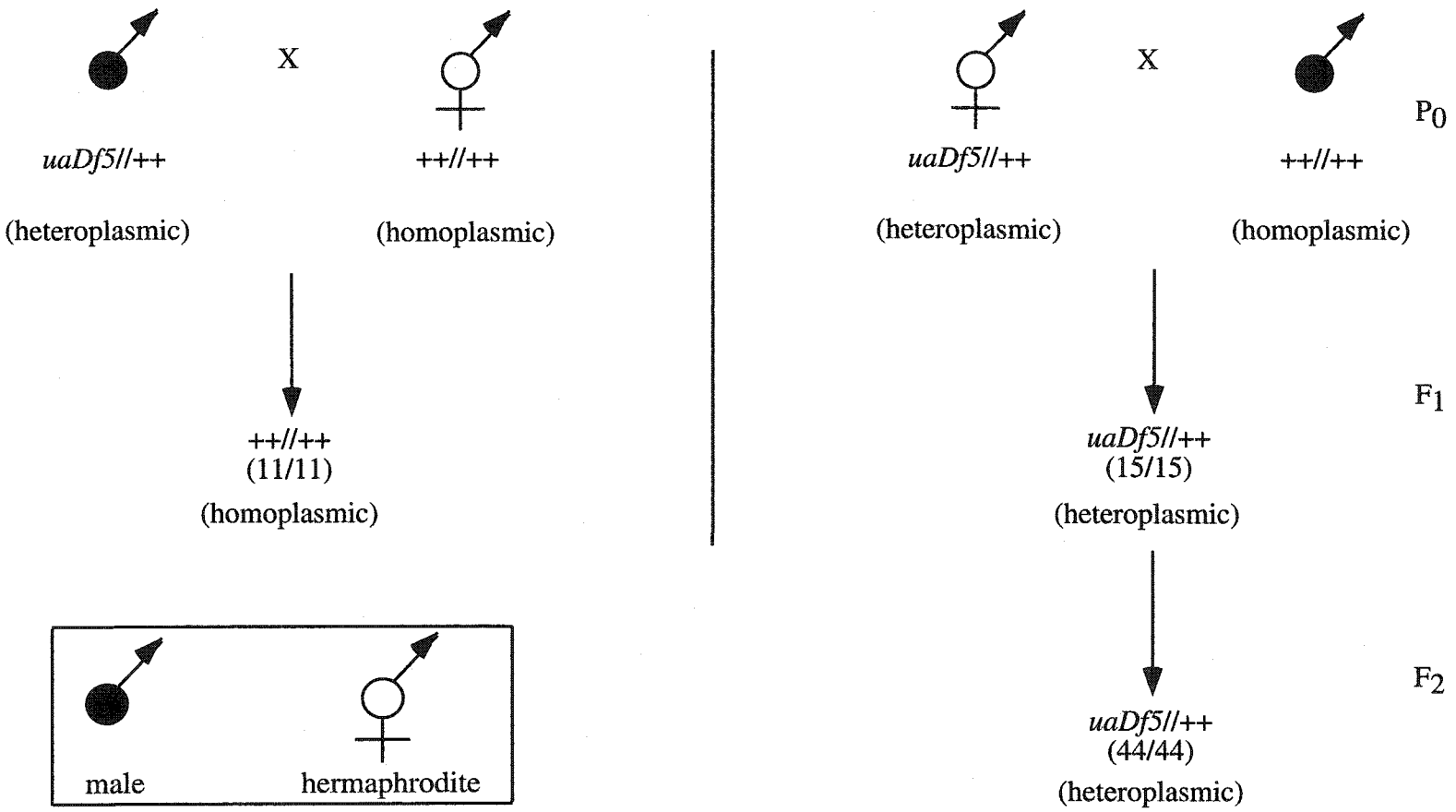

Figure 4.5: Maternal inheritance of *uaDf5* mtDNA
Crosses were performed as described in 'Materials and Methods'. The number of animals analyzed of a particular genotype is indicated in parentheses.

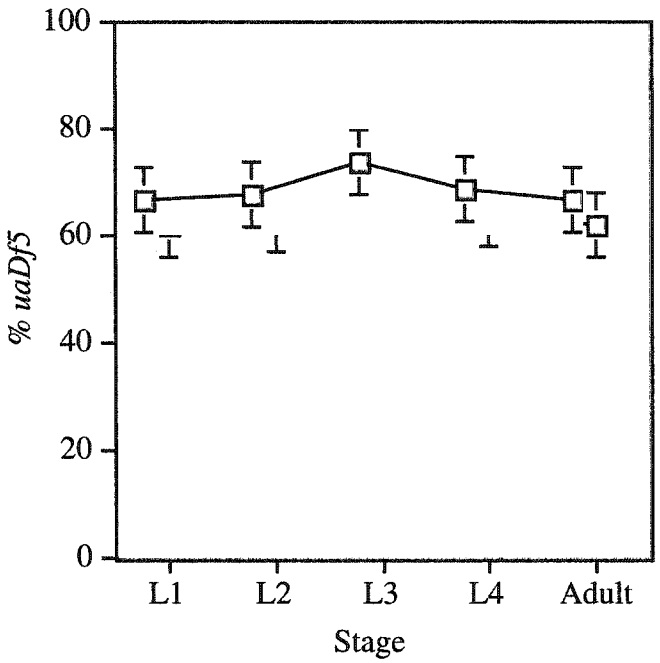

Figure 4.6: The level of heteroplasmy remains unchanged throughout development

Portions of a synchronized population of LB138 animals were analyzed for *uaDf5* content at each of the four larval stages (L1-L4) and as adults. At least four replicates were performed on each population.

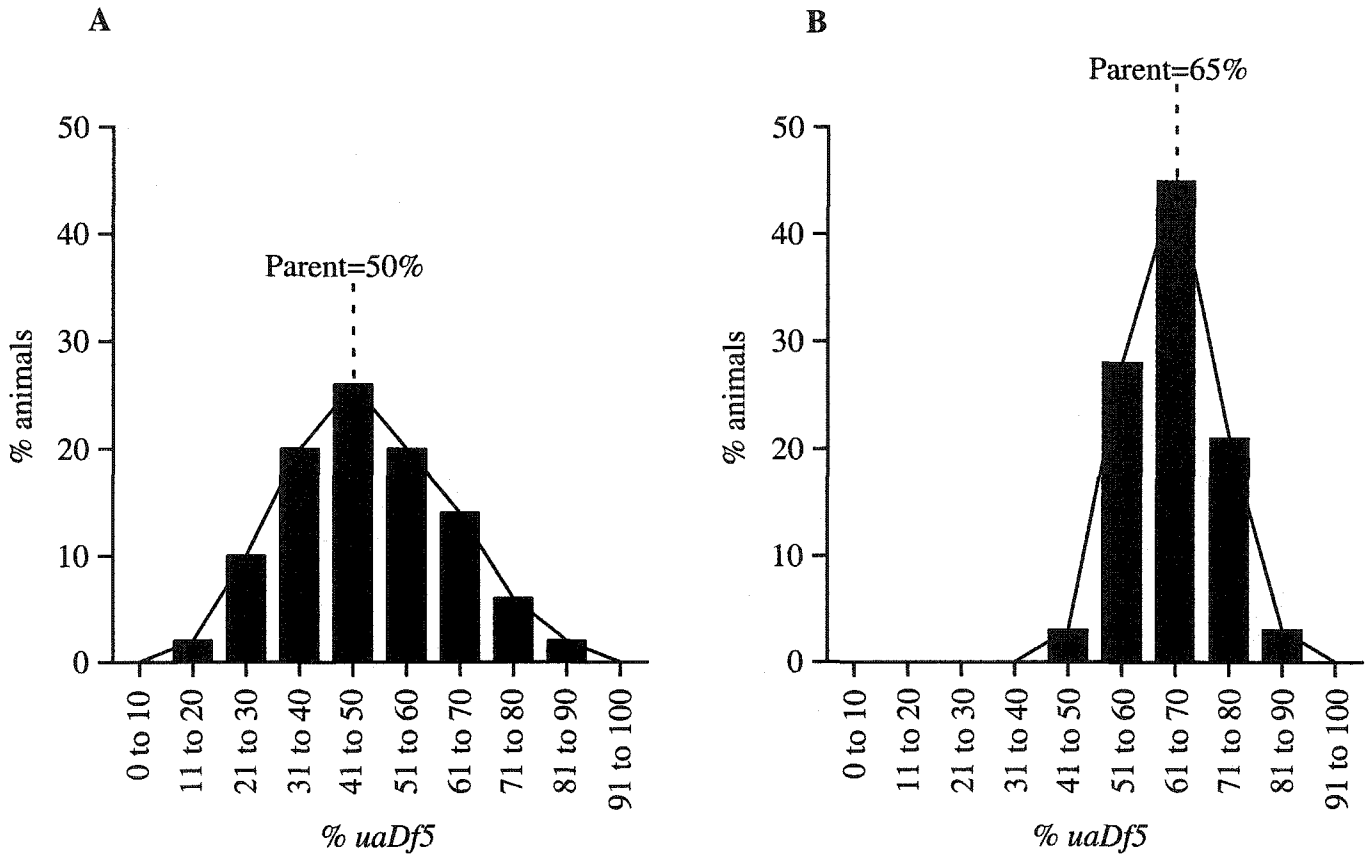

Figure 4.7: Random inheritance of *uaDf5* mtDNA

A) A single adult LB138 hermaphrodite containing 50% *uaDf5* mtDNA was allowed to self-fertilize. 162 progeny of a total brood size of 195 were analyzed for their mtDNA content as described in 'Materials and Methods'. (B) A single adult LB138 hermaphrodite containing 65% *uaDf5* mtDNA was allowed to self-fertilize for a short period of time. All the hermaphrodite progeny were analyzed for mtDNA content. The percentage of animals having *uaDf5* contents within a 10% range is plotted against the percentage ranges used.

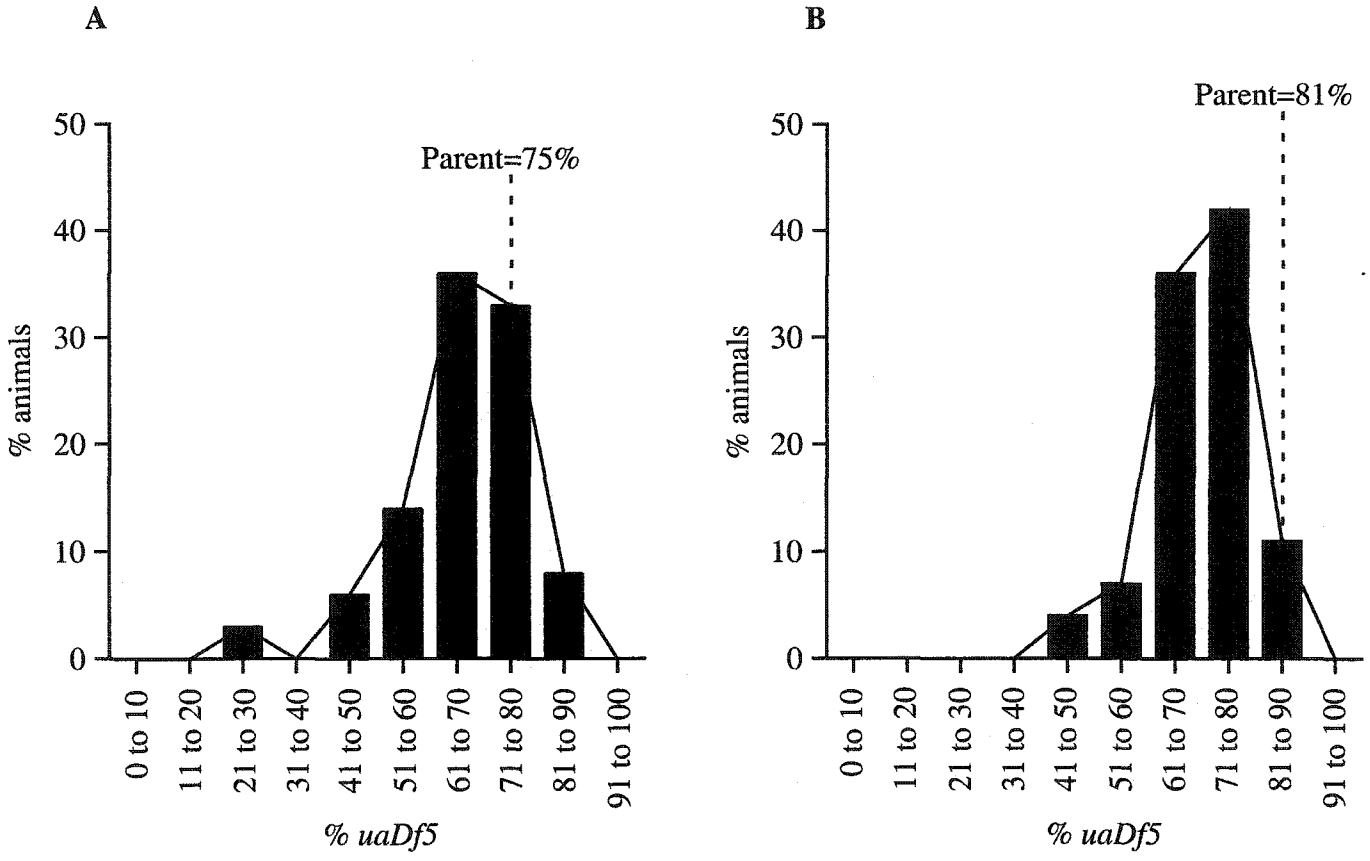

Figure 4.8: Biased inheritance of *uaDf5* mtDNA at high levels of heteroplasmy
Single adult LB138 hermaphrodites containing 75% (A) or 81% (B) *uaDf5* mtDNA were allowed to self-fertilize for a short period of time. All the hermaphrodite progeny were analyzed for mtDNA content. The percentage of animals having *uaDf5* contents within a 10% range is plotted against the percentage ranges used.

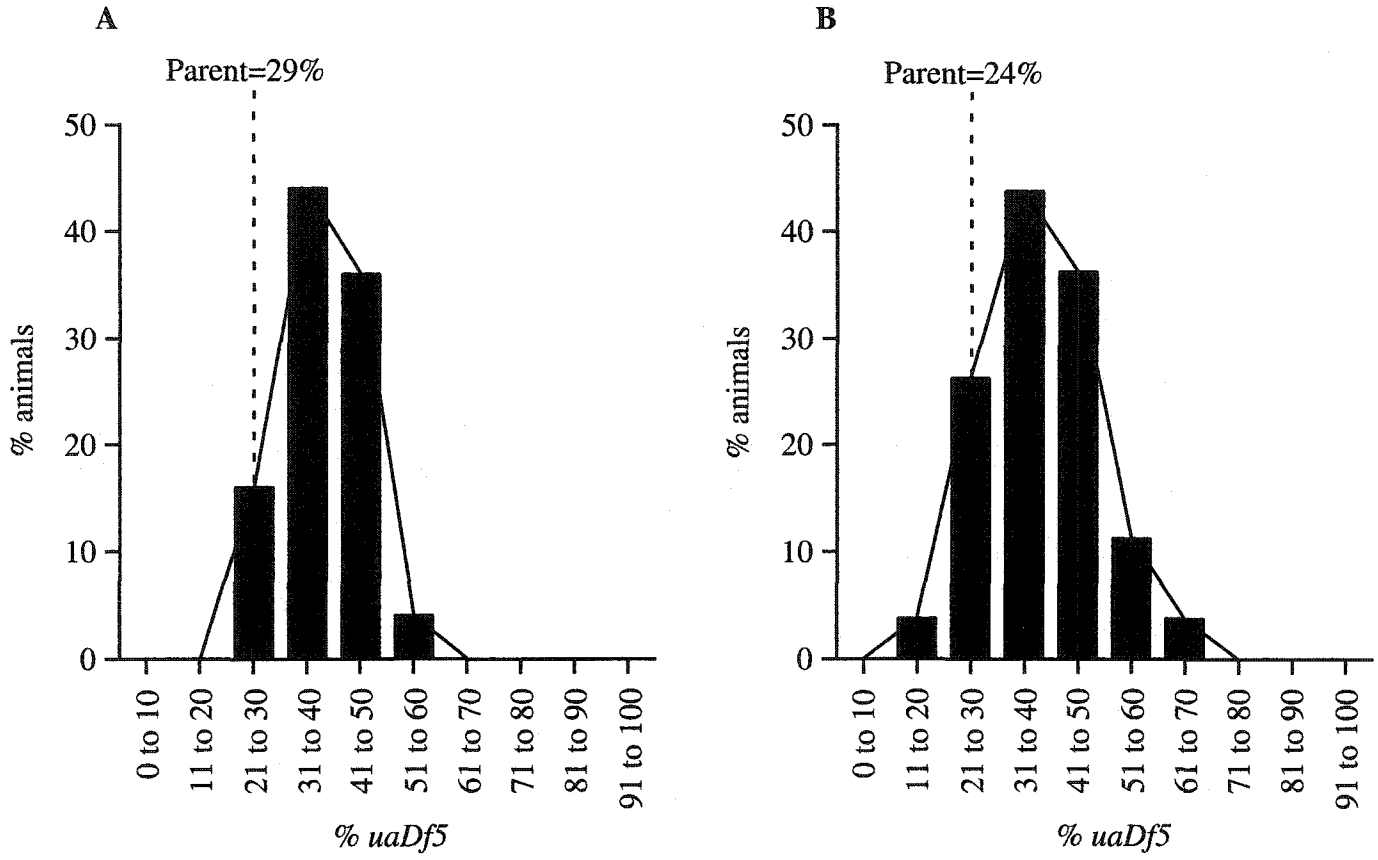

Figure 4.9: Biased inheritance of *uaDf5* mtDNA at low levels of heteroplasmy
Single adult LB138 hermaphrodites containing 29% (A) or 24% (B) *uaDf5* mtDNA were allowed to self-fertilize for a short period of time. All the hermaphrodite progeny were analyzed for mtDNA content. The percentage of animals having *uaDf5* contents within a 10% range is plotted against the percentage ranges used.

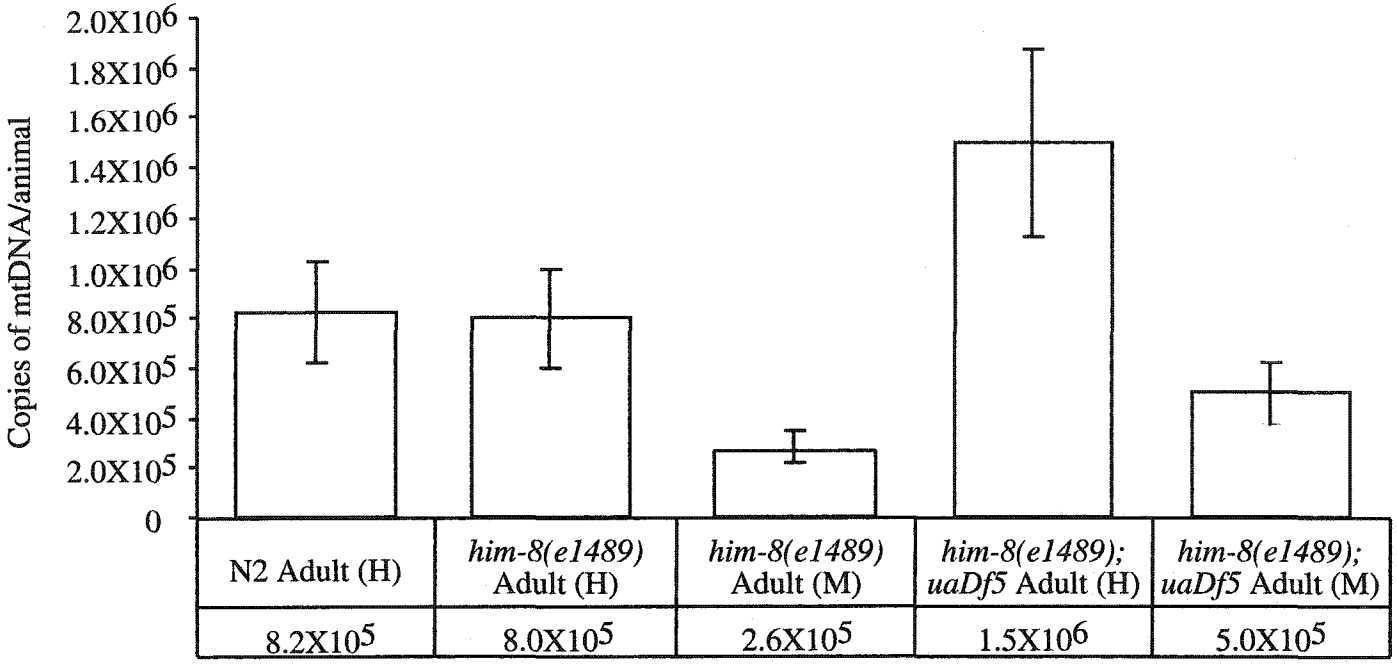

Figure 4.10: The total mtDNA copy numbers of wild type (N2), *him-8*, and *uaDf5* animals
The numbers below the bars are the mtDNA copy numbers per animal averaged over 20 synchronized animals. The letters 'H', and 'M' indicate hermaphrodite and male, respectively. The value for *uaDf5* adult hermaphrodites is significantly higher that of *him-8* adult hermaphrodites ($p < 0.05$) based on a two-tailed Student's *t* test. The mtDNA content of *uaDf5* adult males is significantly higher than *him-8* adult males ($p < 0.05$) based on a two-tailed Student's *t* test. At least three replicates were performed for each condition.

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Chapter 5

Mitochondrial Genome Content is Regulated During Nematode Development

A version of this chapter has been published. Tsang, W. Y. and Lemire, B. D. (2002) *Biochem. Biophys. Res. Commun.* **291**, 8-16.

5.1 Introduction

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 (Clayton, 1991) and is maternally inherited, being transmitted through the egg cytoplasm (Wallace, 1993). The *Caenorhabditis elegans* mtDNA, which encodes 12 MRC subunits, is similar in size and gene content to its human counterpart (Okimoto *et al.*, 1992).

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 (Attardi and Schatz, 1988). 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 (Consortium, 1998).

The role of the mitochondrial genome in cellular energy metabolism and in organismal development is under active investigation (Jenuth *et al.*, 1996; Larsson *et al.*, 1998; Wallace, 1999). 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 (Zeviani and Antozzi, 1997). Ethidium bromide (EtBr), a DNA-intercalating dye, is a

potent inhibitor of mtDNA transcription and replication in mammalian, avian, and yeast cells (Hayakawa *et al.*, 1998; King and Attardi, 1996; Morais, 1996). The depletion (Piko and Chase, 1973) or elimination (King and Attardi, 1996) 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 five-fold copy number increase from the L3 to the L4 stage, and a further six-fold 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 contains the majority of organellar genomes and only a small component is linked to somatic development. With sperm- (*fem-3(gf)*) 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 (Austin and Kimble, 1987; Beanan and Strome, 1992) 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 lifespans 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.

5.2 Materials and Methods

Strains

Worms were cultured as described (Lewis and Fleming, 1995). 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)(gf)*; 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 (Figure 5.5). SS104 (*glp-4(bn2)*), JK1107 (*glp-1(q224)*) and JK509 (*glp-1(q231)*) are temperature-sensitive germline deficient strains. JK816 (*fem-3(q20)(gf)*) 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 (Koekemoer *et al.*, 1998). The standard curve was obtained as follows. N2 worm lysates were prepared by incubating worms in lysis buffer (50 mM KCl, 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 *u*g/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 1,064-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) 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, California), 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 sec annealing at 61 °C, and 30 sec 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 1,064-bp product was quantified with a Gel Doc 1000 system (BioRad, Hercules, California). Non-specific 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 (Figure 5.1). 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) before lysis. To confirm the reliability and reproducibility of the PCR, we also established a standard curve for nuclear DNA copy number (Figure 5.2A), using primers that recognize wild type sequences present on the nuclear *atp-2* gene (Tsang *et al.*, 2001); F1B2, 5'-ACGTTCAGCCTGAACTGAGAC-3' and F1B9, 5'-CTCCGATGACAGCGACAATG-3' amplify a 978-bp product. Quantitative PCR was performed for each initial template copy number at least twice, and the results were reproducible. For sample analysis, quantitative PCR was performed on lysates containing known numbers of N2 worms from which the input nuclear DNA copy numbers can be calculated (Figure 5.2B). In all cases, the input nuclear DNA copies and the measured copies agree closely (Figure 5.2B), suggesting that nuclear DNA is quantitatively released during lysis.

Exposure to and removal from EtBr

EtBr was added at a final concentration of 125 *u*g/ml of agar to seeded NGM plates (Lewis and Fleming, 1995) and synchronized animals were placed directly onto the plates. All experiments were performed at room temperature (23 °C) unless otherwise stated. For recovery, EtBr-induced L3 arrested worms were washed off at the indicated times and transferred onto seeded NGM plates without EtBr. Animals were scored as recovered when they developed into gravid adults within five days.

Measurement of lifespan

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.

5.3 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 hours after fertilization, an egg hatches as a first stage larva or L1 (Lewis and Fleming, 1995). 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 (Figure 5.3). A 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

(Wood, 1988); 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 non-disjunction 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 (Figure 5.4). 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) as compared to adult hermaphrodites (800,000) (Figure 5.4).

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 (Schedl, 1997). 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 (Austin and Kimble, 1987). Wild type L4 larvae have 130,000 copies of mtDNA (Figure 5.3) compared to the 67,000 copies in the

glp-1(q339) mutant (Figure 5.5). That number remains relatively unchanged as the *glp-1* animals mature (Figure 5.5). 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 (Figure 5.5). 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 (Figure 5.5). At the restrictive temperature of 25 °C, the mutants have only 60,000-70,000 copies as L4 larvae (Figure 5.5) 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 (Beanan and Strome, 1992). The *glp-4(bn2)* mutant mtDNA copy number profile is similar to the *glp-1(q224)* and *glp-1(q231)* profiles (Figure 5.5). 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 three-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 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(gf)*) and sperm-deficient (*fem-1*) animals. The FEM proteins are required for spermatogenesis in both males and hermaphrodites (Schedl, 1997). Loss of function *fem-1* mutations transform hermaphrodites into females with no sperm production (Nelson *et al.*, 1978). In contrast, gain of function *fem-3* mutations masculinize the hermaphrodite germ line, resulting in a vast excess of sperm produced and no oocytes (Barton *et al.*, 1987). At the restrictive temperature of 25 °C, *fem-3(gf)* 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(gf)* and of *fem-1* hermaphrodites at 15 °C are similar to those of wild type (Figure 5.6). At 25 °C, *fem-3(gf)* and *fem-1* L4 larvae have copy numbers comparable to the wild type L4 (Figure 5.6). However, we observed a large difference in mtDNA copy numbers between adult *fem-3(gf)* and *fem-1* hermaphrodites at 25 °C. *fem-3(gf)* mtDNA copy numbers do not increase much between the L4 and adult stages (from 170,000 to 190,000; Figure 5.6). With *fem-1* animals, the copy number increases approximately six-fold as it does in the wild type (from 140,000 to 800,000; Figure 5.6). 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 laid by hermaphrodites raised at 25 °C and found it to be roughly 18,000 copies per oocyte (Figure 5.6).

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 (Figure 5.7). Above 83 μg/ml EtBr, all embryos hatched, developed through 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 (Figure 5.7). 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 (Table 5.1). 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 5.2). 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 hours after egg laying, EtBr-treated animals are not readily distinguishable from control animals; both have typical L2-sized gonads (Figure 5.8A, B). 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 (Figure 5.8D). In contrast, EtBr-treated animals are slightly smaller in size than control animals and their gonads remain unchanged from the 24-hour point (Figure 5.8C). 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 (Figure 5.3). 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 (Figure 5.9). 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 (Figure 5.9). 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 (Figure 5.10). Their progeny arrest as embryos or as L3 larvae (Figure 5.9). 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 (Figure 5.11). 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 (Figure 5.11) and does not undergo the five-fold increase observed in untreated animals (Figure 5.3). 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 (Figure 5.11). The progeny have significantly reduced mtDNA complements and develop to and arrest as L3 larvae (Figure 5.11). In untreated controls, the copy number exhibits a six-fold increase from L4 to adult and remains high in older adults after they have laid their broods (Figure 5.3).

5.4 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, 2002), 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* (Chase and Dawid, 1972; Piko and Chase, 1973; Rubenstein *et al.*, 1977). 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 (Figure 5.11).

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 six-fold 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 three-fold somatic copy number 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(gf)*, 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* female, which only makes oocytes at 25 °C, has 800,000 copies of mtDNA. In contrast, an adult *fem-3(gf)* animal, 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(gf)* 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 (Hecht *et al.*, 1984; Piko and Taylor, 1987). 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 (Beanan and Strome, 1992). The difference of ~60,000 germline associated copies of mtDNA seen between a wild type L4 (130,000 copies; Figure 5.3) and a *glp-1* or *glp-4* L4 at 25 °C (~70,000 copies of mtDNA; Figure 5.5) 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 eucaryotic cells (Shadel and Clayton, 1997).

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 (Figure 5.11). 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 lifespan extension may arise because of caloric restriction due to improper feeding behavior or impaired pharyngeal pumping (Lakowski and Hekimi, 1998). Mutations in two nuclear encoded MRC subunits significantly impair pharyngeal pumping rates and similarly extend lifespan (Tsang *et al.*, 2001).

We have proposed that an energy-related developmental checkpoint may control the L3 to L4 transition (Tsang *et al.*, 2001). 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 (Figure 5.11). 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 (Tsang *et al.*, 2001). Fourth, mutations affecting nuclear encoded MRC subunits of complexes I or V also quantitatively result in L3 arrested larvae (Tsang *et al.*, 2001). 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 (Fraser *et al.*, 2000).

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.

L3 (days arrested)	% Recovery[a]	Number of animals scored
0	94	268
1	91	219
3	70	177
6	22	277
9	13	151
12	0	248

Table 5.1: Recovery of L3 arrested animals after removal from EtBr at 25°C
[a] Defined as the percentage of animals developing into gravid adults within five days

Strain	Lifespan (days)	SD (days)[a]	Number of animals
N2	11.9	1.6	30
EtBr-N2	13.8	2.0	26

Table 5.2: Lifespan is extended by ethidium bromide

[a] $p < 0.01$ based on a two-tailed Student's *t* test to + / +

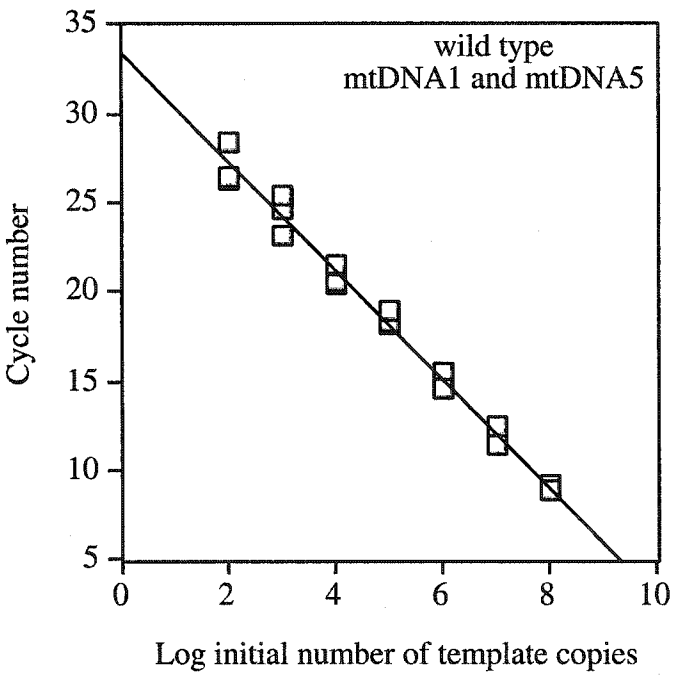
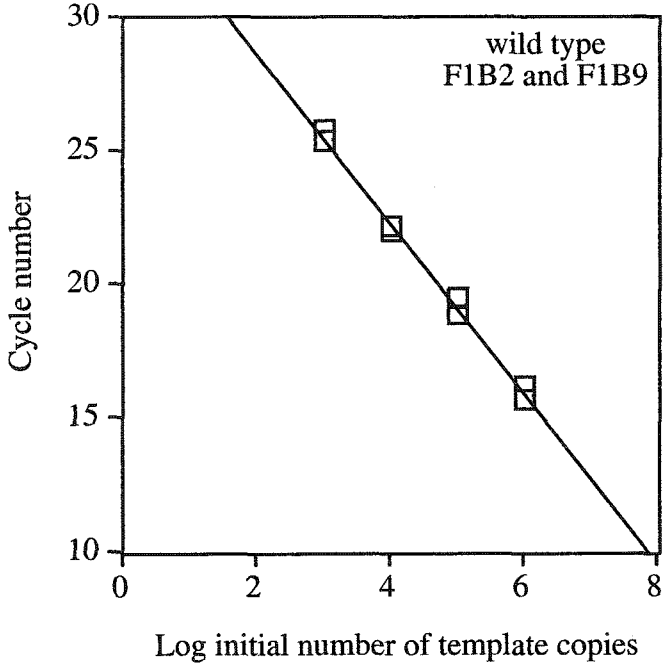


Figure 5.1: Construction of a standard curve for the determination of mtDNA copy number

Quantitative PCR was performed as described in 'Materials and Methods'. The initial number of template copies refers to the initial number of copies of the input template used. The pair of primers used and the amplified product is indicated. The correlation coefficient (r^2) is 0.989.

A



B

Input number of worms	Input nuclear DNA copy number	Measured nuclear DNA copy number
295 L1	$3.3x10^5$	$2.8X10^5$
46 L1	$5.2X10^4$	$4.7X10^4$
95 L4	$1.9x10^5$	$2.4X10^5$
51 L4	$1.0x10^5$	$1.5X10^5$

Figure 5.2: Construction of a standard curve for the determination of nuclear DNA copy number
(**A**) Quantitative PCR was performed as described in 'Materials and Methods'. The inital number of template copies refers to the initial number of copies of the input template used. The pair of primers used and the amplified product is indicated. The correlation coefficient (r^2) is 0.995. (**B**) The input nuclear DNA copy number is calculated by assuming that a L1 and a L4 animal have 560 and 1000 cells, respectively.

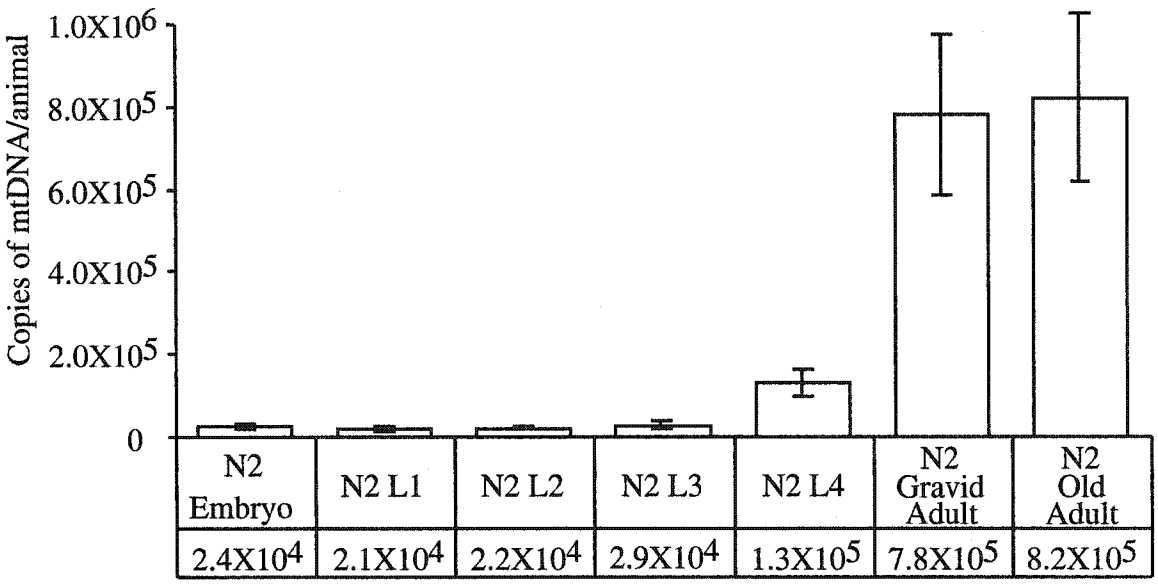


Figure 5.3: The mtDNA copy numbers of the *C. elegans* developmental stages Values represent the mtDNA copy numbers per animal averaged over 20 synchronized animals. At least three replicates were performed for each developmental stage.

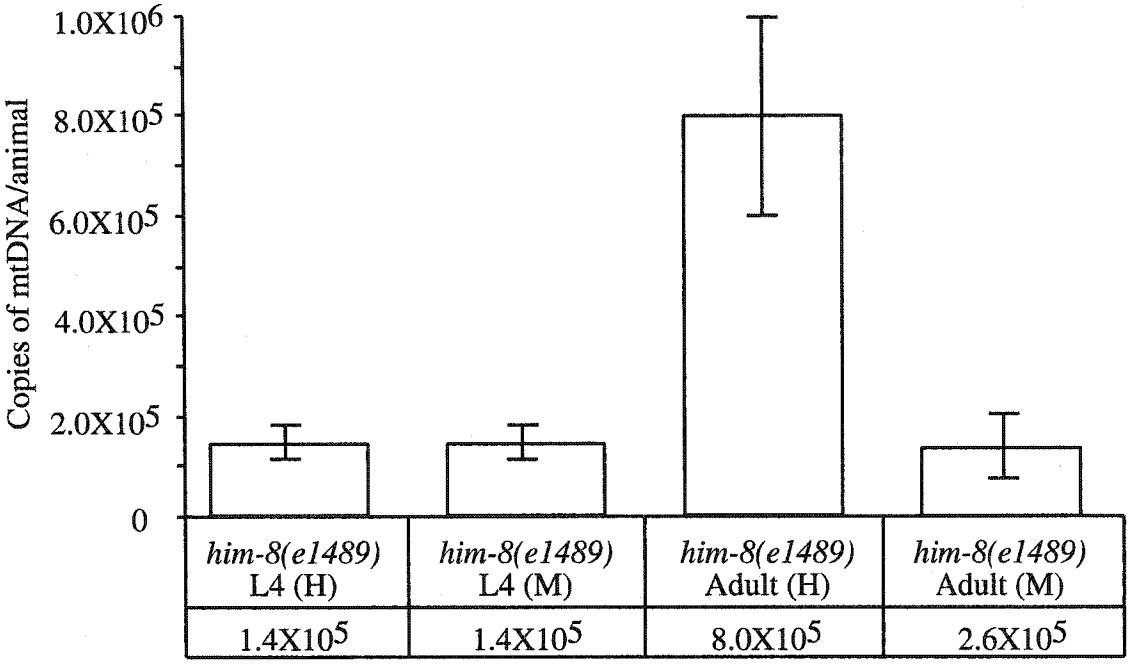


Figure 5.4: The mtDNA copy numbers of a *him-8* mutant
Values represent the mtDNA copy numbers per animal averaged over 20 synchronized animals. At least three replicates were performed for each developmental stage. (H) and (M) indicate hermaphrodite and male, respectively.

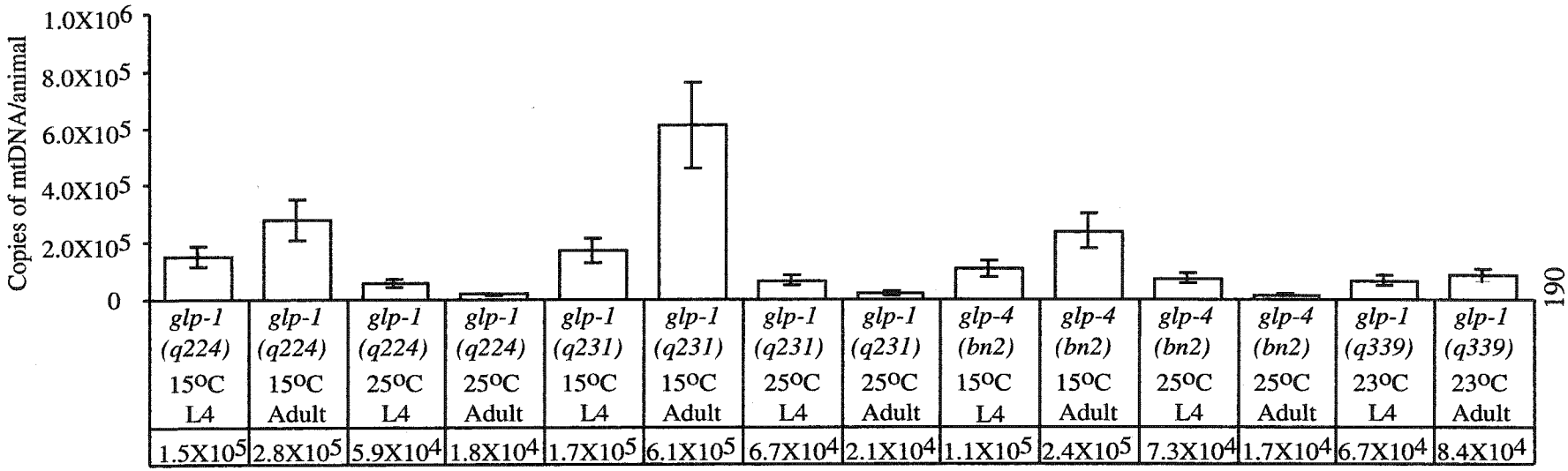


Figure 5.5: The mtDNA copy numbers of *glp-1* and *glp-4* mutants
Values represent the mtDNA copy numbers per animal averaged over 20 synchronized animals. At least three replicates were performed for each developmental stage. The *glp-1(q339)* mutation is not temperature-sensitive.

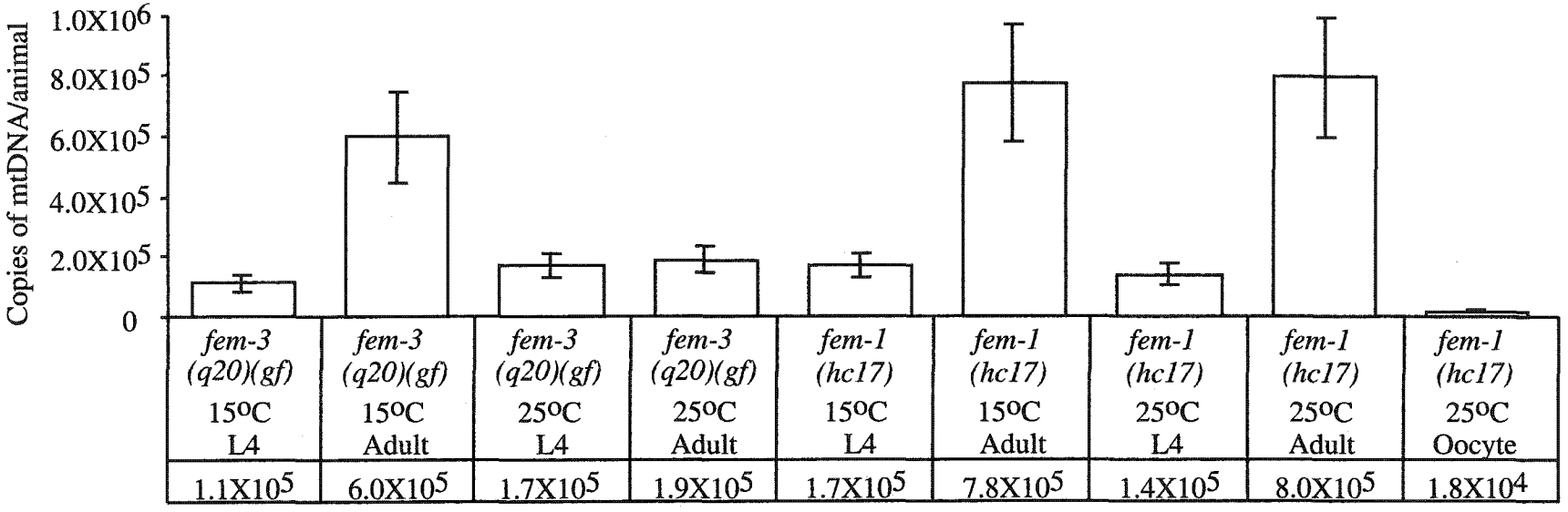


Figure 5.6: The mtDNA copy numbers of *fem-3(gf)* and *fem-1* mutants
Values represent the mtDNA copy numbers per animal averaged over 20 synchronized animals. At least three replicates were performed for each developmental stage.

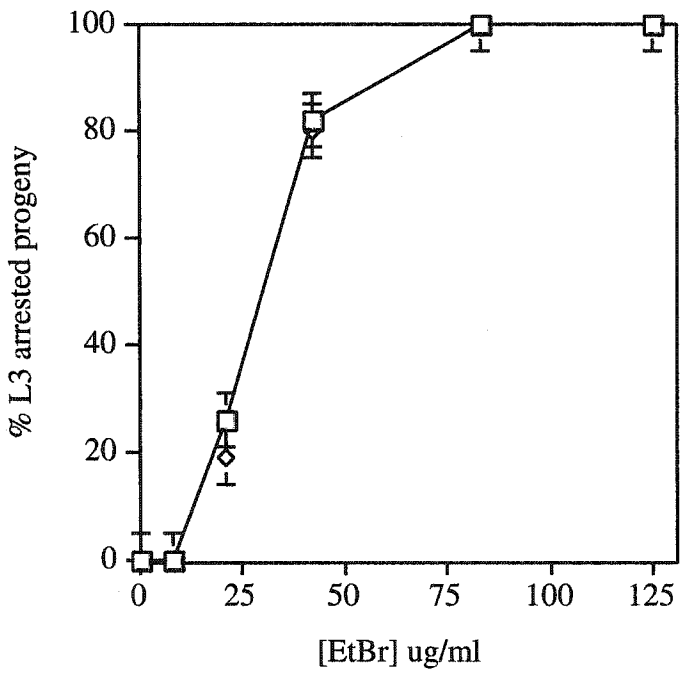


Figure 5.7: Exposure of wild type and CB1489 hermaphrodites to EtBr Gravid hermaphrodites were transferred to plates with increasing concentrations of EtBr. The ratio of L3 arrested progeny over the total number of progeny scored (>80) is expressed as a percentage. The symbols used are: squares, N2; diamonds, CB1489.

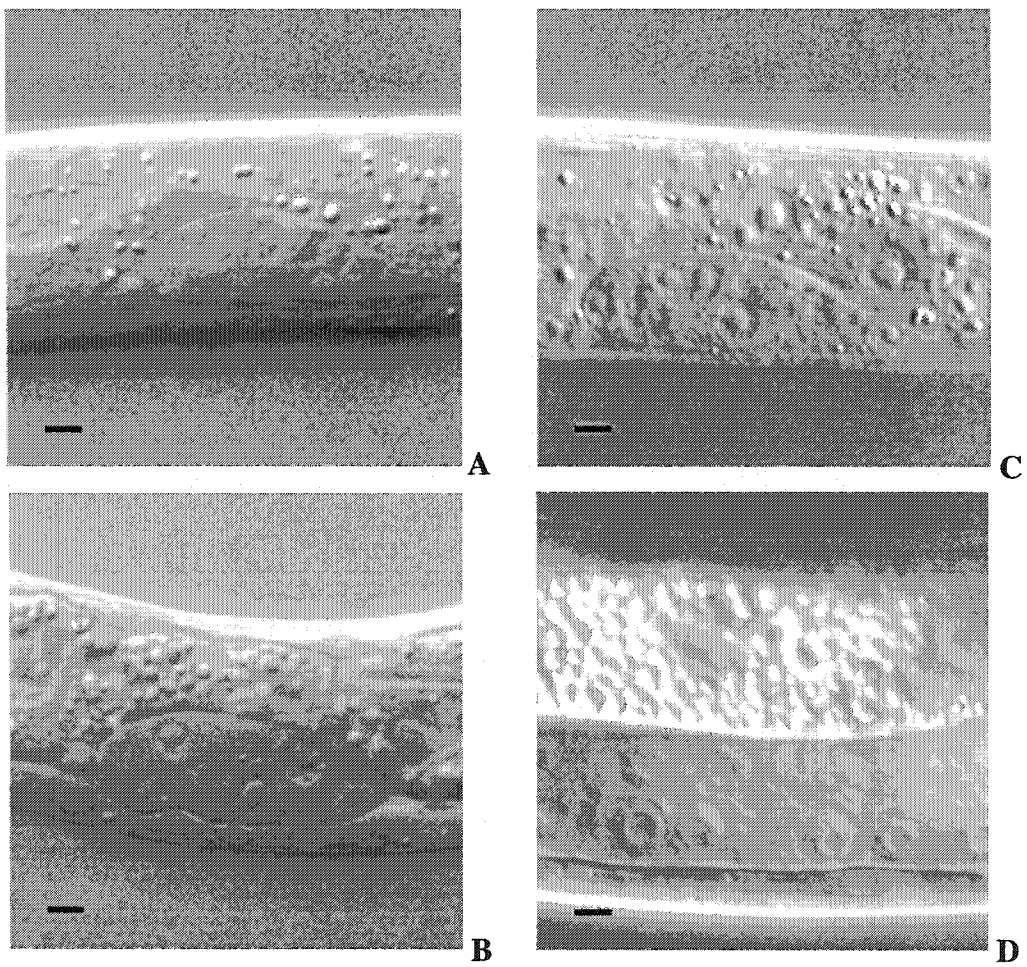


Figure 5.8: Nomarski photographs of control and EtBr-treated N2 animals (**A**) EtBr, at 27 h; (**B**) control, at 27 h; (**C**) EtBr, at 33 h; (**D**) control, at 33 h; Bar, 3-μm. Worms were cultured at 25 ^{o}C.

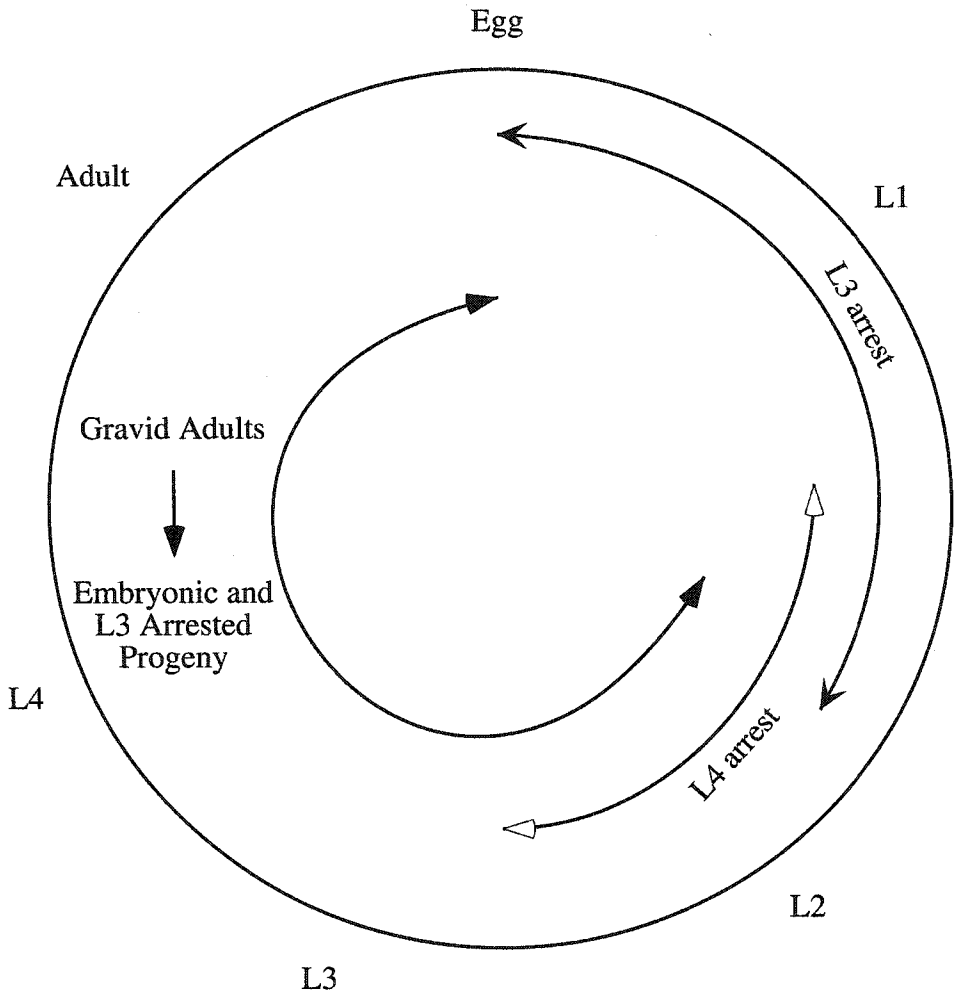


Figure 5.9: Exposure of N2 animals to EtBr at different stages of development at 25°C

Each curved line with double arrows indicates the period of time during with EtBr exposure leads to the indicated phenotype.

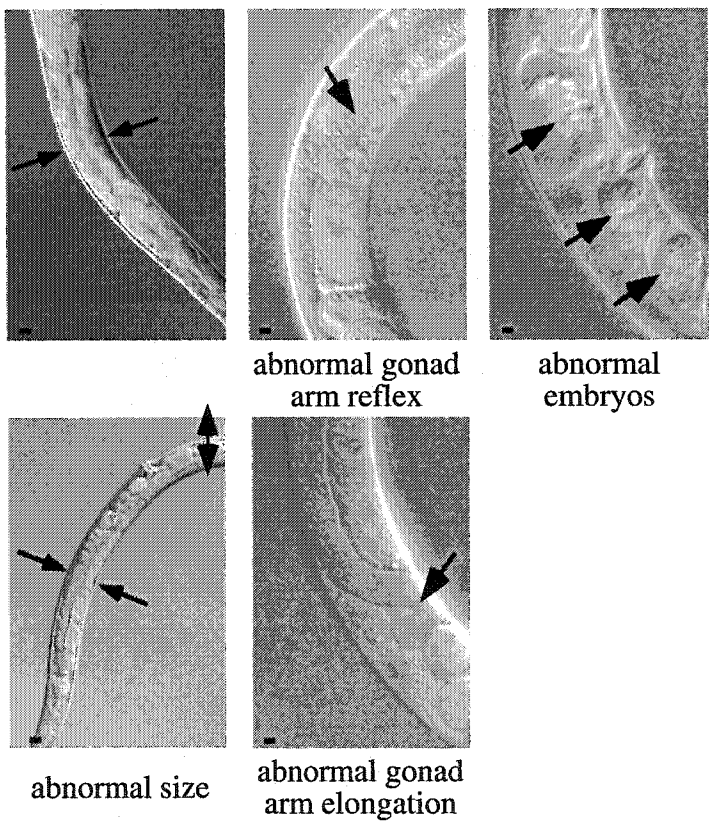


Figure 5.10: Nomarski photographs showing the various defects associated with N2 animals five days after the EtBr exposure at the L2 (26-28h) stage
Bar, 15-μm (for two pictures on left); and 5-μm (other pictures).

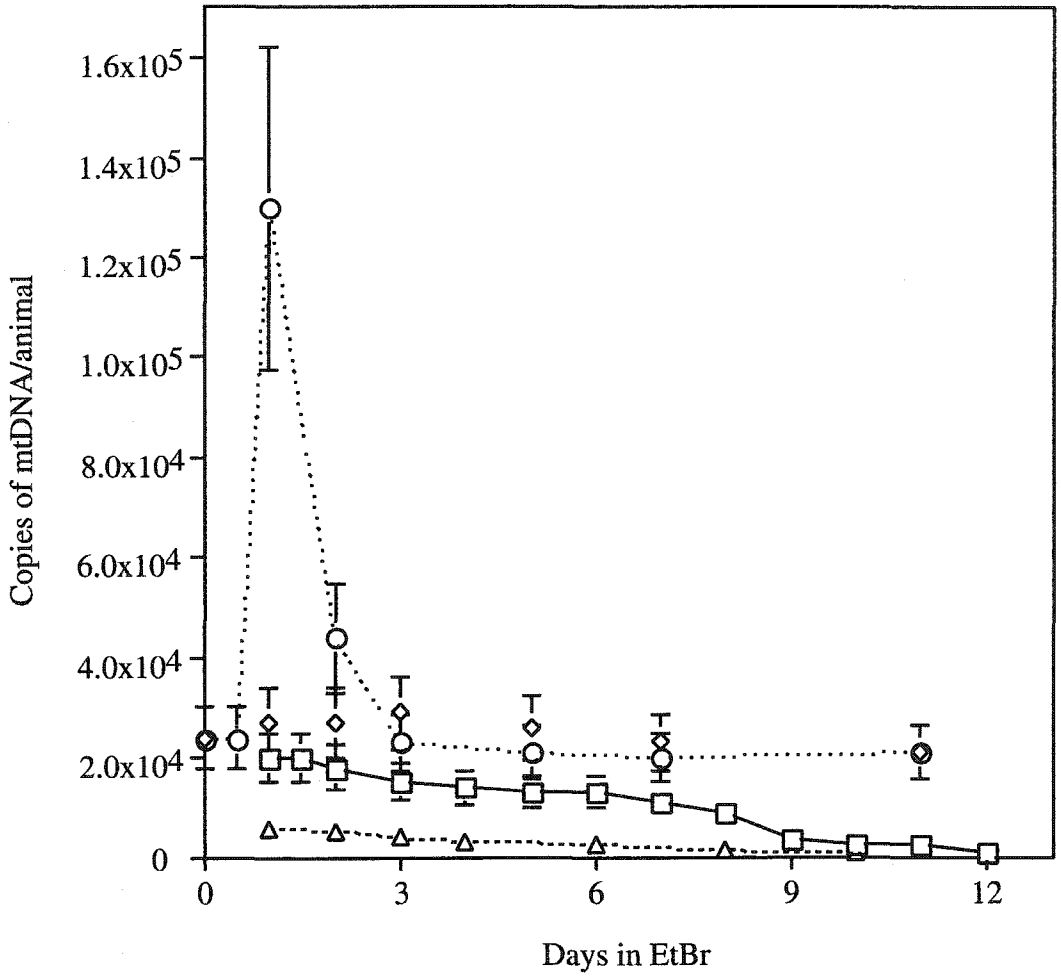


Figure 5.11: The mtDNA copy numbers of EtBr-treated wild type animals

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.

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Chapter 6

General Discussion and Conclusions

6.1 Conclusion

The aim of my thesis work was to gain a better understanding of mitochondrial biogenesis in the nematode, *Caenorhabditis elegans,* by exploring how mutations in the MRC lead to mitochondrial dysfunction and disease. The nematode model system has numerous advantages over the other model systems, including the yeast, *Saccharomyces cerevisiae* and the fungus, *Neurospora crassa*, two systems traditionally used for studying mitochondrial function and biogenesis. The advantages include a sophisticated genetic and developmental system, a mammalian-like MRC, and multiple tissue types. The *C. elegans* model is amenable to the investigation of a number of fundamentally intriguing problems in mitochondrial research and medicine. In the following sections, I will discuss a few of these problems, summarize and discuss relevant results from my thesis work, and offer some directions for future studies.

6.2 Relationship between Genotype to Phenotype

In humans, defective mitochondrial energy production is characterized by a bewildering set of disease phenotypes (Wallace, 1999). The symptoms are often multi-systemic with a predominant involvement of the heart, skeletal muscle, and the nervous system (Beal, 1995; Luft and Landau, 1995). Mitochondrial disorders can be induced by defects in subunits of the MRC complexes, or in factors controlling their biogenesis and assembly (DiMauro and Andreu, 2000; Sue and Schon, 2000). These diseases can be inherited in a Mendelian fashion if the nuclear genome is involved or maternally if the mtDNA is affected. The molecular natures of these defects are quite diverse and can include point mutations, inversions, deletions, and duplications. A major challenge in the

field of mitochondrial medicine is to understand the correlation between genotype and phenotype. How does a MRC mutation lead to a diverse set of human mitochondrial diseases? How can a particular disease phenotype be caused by different MRC mutations? This problem can be difficult to address in some model organisms such as yeast or *Neurospora* since a loss of MRC function can often be tolerated. A more sophisticated developmental system like *C. elegans* is more suitable because it has a more complex life cycle, multiple tissue types, and a highly conserved MRC. All severe losses of MRC function in the nematode are likely to be lethal as in humans, provided that the mutation is expressed in every tissue.

We have examined the correlation between genotype and phenotype in *C. elegans* by isolating and characterizing three MRC mutations as described in chapters 2 and 4. The first two are deletions in the nuclear *nuo-1* and *atp-2* genes encoding the active site subunits of complexes I and V, respectively (Tsang *et al.*, 2001). Both mutations are maintained in balanced heterozygotes that give rise to 1/4 homozygous mutant progeny. These hatch from eggs, develop past the L1 and the L2 stages, but arrest at the L3 stage without further development prior to sexual maturation. Thus, both mutations are lethal. The L3 arrested animals exhibit a multi-systemic disorder, with defects including an L2-stage gonad arrest, impaired mobility, slower pharyngeal pumping, and an extended defecation cycle. Thus, we have demonstrated that two different null MRC mutations can lead to the same phenotype. It would be interesting to generate less severe *nuo-1* or *atp-2* mutations and introduce them into the respective deletion backgrounds. In this way, we could begin to understand how specific mutations contribute to the overall phenotype. For example, it is possible that mutations within the NUO-1 NADH-binding site are more

severe than mutations affecting other domains. In humans, mutations in the *nuo-1* homolog, *NDUFV1*, can result in two distinct phenotypes, myoclonic epilepsy and leukodystrophy, or Leigh syndrome (Benit *et al.*, 2001; Schuelke *et al.*, 1999). The *NDUFV1* mutations, when introduced into the *nuo-1* gene provide an excellent opportunity to examine the effects of hypomorphic MRC mutations on worms.

The third mutation isolated is the *uaDf5* deletion in the mtDNA, which affects multiple genes, including genes for four MRC subunits and seven tRNAs (Tsang and Lemire, 2002b). In contrast to the nuclear mutations, animals bearing the *uaDf5* deletion are healthy and are not compromised. Interestingly, all of the nematode mitochondrial genome mutations reported in the literature so far have had no or only subtle phenotypes (Denver *et al.*, 2000; Feng *et al.*, 2001). The *uaDf5* mtDNA is maintained in a stable heteroplasmic state with the wild type mtDNA with the proportion of *uaDf5* mtDNA never exceeding ~85%. This observation suggests that the presence of a small fraction of wild type mtDNA copies (~15%) is sufficient to support normal development and that the threshold level of pathogenesis for the *uaDf5* genome exceeds 85%. It would to desirable to manipulate and increase the proportion of *uaDf5* genomes and determine at what level and what types of pathogenic conditions can be produced. The heteroplasmic state can be altered with mtDNA inhibitors such as doxycycline (Spelbrink *et al.*, 1997) and ethidium bromide (King, 1996), by the introduction of a restriction endonuclease that specifically recognizes and cleaves one form of the mtDNA (Srivastava and Moraes, 2001; Tanaka *et al.*, 2002), or by the introduction of potential anti-sense agents such as peptide nucleic acids that are specifically designed to recognize and interact with only one form of the mtDNA (Chinnery *et al.*, 1999). At present, we do not know what the phenotype of

worms carrying pathogenic levels of *uaDf5* would be. However, the results we generated from the *nuo-1* and *atp-2* studies suggest that these *uaDf5* worms might arrest at the L3 stage of development. Alternatively, these animals may arrest as embryos, and there are two lines of evidence to support this notion. First, progeny with reduced mtDNA complements generated by exposure to ethidium bromide demonstrate embryonic lethality (Tsang and Lemire, 2002a). Second, maternal mRNA and/or protein contribution is required for development of *nuo-1* and *atp-2* mutant worms to the L3 stage (Tsang *et al.*, 2001). In its absence, as seen with RNAi treatments, an embryonic arrest phenotype results.

Due to the lack of phenotype in *uaDf5* animals, I explored the consequences of pharmacologically inhibiting mtDNA expression. In chapters 2 and 5, results using three drugs, chloramphenicol (Piko and Chase, 1973), doxycycline (Nijtmans *et al.*, 1995), and ethidium bromide (King and Attardi, 1996; Morais, 1996), which have effects on mtDNA expression in mammalian cell lines, are presented. The first two are translation inhibitors, while the third is an inhibitor of replication/transcription. Curiously, exposure of wild type embryos to each of these three drugs results in a developmental arrest at the L3 stage characterized by a gonad arrest at the L2 stage (Tsang *et al.*, 2001; Tsang and Lemire, 2002a). Thus, the drug-treated animals mimic the nuclear MRC mutants.

As discussed in the chapter 1, the formation of the MRC is a complex process. The MRC contains more than 80 subunits and requires more than 100 additional factors for its assembly and biogenesis. These factors include chaperones, assembly factors, the mitochondrial protein import machinery, factors for mtDNA replication, transcription and translation, and ions and coenzymes. We have hypothesized that inhibition of any one of

these assembly or biogenesis pathways will lead to common developmental arrest at the L3 stage (Figure 6.1). This hypothesis is supported by results presented in chapters 2 and 5: the L3 arrest phenotype is produced either by mutations in the nuclear-encoded MRC subunits (*nuo-1* and *atp-2*), or by interfering with the mtDNA replication, transcription, and translation machineries with drugs. Recently, Jonassen *et al.* reported that mutations in the *clk-1* gene, which disrupt the biosynthesis of ubiquinone, have a conditional lethal phenotype (Jonassen *et al.*, 2001). When grown on a ubiquinone-deficient food source, *clk-1* mutants suffer an L2 stage developmental arrest. The L2 arrest, which was judged by gonad development, is remarkably similar to the *nuo-1* and *atp-2* induced arrests. Thus, the *clk-1* data suggest that a larval arrest can be induced when coenzyme biosynthesis and intake are impaired. In addition, RNAi knockdown of MRC genes frequently results in larval or embryonic arrest (Fraser *et al.*, 2000; Gonczy *et al.*, 2000). Embryonic arrest is not a phenotype unique to MRC dysfunction. A forward genetic screen using the L3-stage arrest as an endpoint might be a fruitful way of isolating additional MRC gene mutations. Alternatively, additional MRC gene mutations could be isolated through reverse genetic screens.

Why do animals with MRC dysfunction arrest at the L3 stage of development? We know that aerobic metabolism (Vanfleteren and De Vreese, 1996), mtDNA copy number (Tsang and Lemire, 2002a), and ATP-2 protein levels (Tsang *et al.*, 2001) increase substantially from L3 to L4. The L3 to L4 transition may be particularly sensitive to MRC defects because it is the first developmental point associated with substantial mitochondrial biogenesis in the new larva. In theory, worms with an impaired MRC could arrest at earlier stages of development when their energy status fails to meet

a certain minimum threshold level. For example, animals with reduced mtDNA complements (Tsang and Lemire, 2002a) or maternal mRNA/protein contributions (Tsang *et al.*, 2001) demonstrate an embryonic arrest phenotype. Likewise, the nematode *coq-3* mutation, which encodes an enzyme that catalyzes an early step of ubiquinone biosynthesis, can lead to sterility and a maternally-rescued lethality at the L1 stage (Hihi *et al.*, 2002). Thus, the minimum energy threshold level required for development may be quite high for the L3-to-L4 transition, surpassing the capacity for energy production provided by maternally contributed mitochondrial components.

It is reasonable to speculate that the expression or function of certain heterochronic genes involved in development may be affected by MRC dysfunction. The *nuo-1* and the *atp-2* mutants, and the drug-treated animals arrest at the L3 stage of development as judged by their overall body size. The somatic gonads of these animals arrest at an earlier L2 stage. Do these animals possess a mixture of L2 and L3-staged cells? Are they really arrested at an L3 stage of development, or are they L2 larvae? A number of heterochronic genes, which control the relative timing of stage-specific events such as cell division patterns, cell cycle progress, developmental arrest, etc., have been identified and characterized in worms (Ambros, 1997; Ambros, 2000). Heterochronic gene mutations lead to precocious, retarded, or reiterated development of certain cell lineages, resulting in temporal mosaics with cells or tissues at different stages of development. For example, the heterochronic gene *lin-14* is involved in the generation of the postdeirid neuroblast, PDNB, which is the anterior granddaughter of the hypodermal V5 cell (Ambros and Horvitz, 1984). LIN-14 levels are high in wild type L1 larvae, and the generation of the PDNB neuroblast is prevented. The PDNB neuroblast is born in the

L2 stage when the level of LIN-14 is downregulated. However, in *lin-14* mutants, the PDNB neuroblast is produced precociously at the L1 stage (Ambros and Horvitz, 1984). If heterochronic genes are involved in determining the MRC mutant phenotype, we might expect to see reiteration, retardation, or advanced stage-specific developmental programs in certain tissues. We have ruled out a delay of stage-specific programs because our L3 arrested animals do not show signs of further development after they have arrested. Animals that have arrested for one day are morphologically similar to animals that have arrested for many days. We have not observed any precocious L4-specific or adult-specific development. For example, we have not observed L4-sized or adult-sized vulva or gonads in the nuclear mutants or the drug-treated animals. We also do not believe that stage-specific programs are reiterated in any tissues for two reasons. First, normal somatic gonad development at the L2 stage is characterized by the formation of two gonad arms and the generation of two distal tip cells at the distal ends of each arm. The gonads in the L3 arrested animals do not show any abnormal features; they simply fail to develop further, suggesting that the gonads are arrested on the normal developmental pathway. Second, preliminary observations have indicated that vulval development, another commonly used stage-specific marker, appears to be arrested at an early L3 stage in the *nuo-1* and *atp-2* animals as judged by the presence of two daughter cells associated with each of the vulval precursor cells, P3.p to P8.p. The total number of vulval precursor cells remains unchanged even after several days of arrest. Thus, stage-specific cell divisions are not being reiterated.

We believe that the arrested animals are indeed developmentally at an L3 stage. In addition to the expected number of vulval precursor cells, two further lines of evidence

support this notion. First, *nuo-1* homozygotes are capable of entering the dauer state, which is an alternative L3 developmental stage, suggesting that they are physically and developmentally L3 animals. This also makes the possibility that the arrested animals are developmentally L2 larvae unlikely. Second, there is precedence for an L3-staged animal to possess an L2-sized gonad. For example, dauer larvae, considered to be in an alternate L3 stage developmentally, have gonads with an L2-staged appearance (Wood, 1988). Interestingly, the *C. elegans daf-12* gene, which encodes a member of the nuclear hormone receptor gene family, plays a major role in the control and advancement of the L2- to the L3-specific developmental programs and in dauer formation (Antebi *et al.*, 1998; Antebi *et al.*, 2000). Mutations in *daf-12* frequently result in animals having a mixture of L2, L3, and dauer-like features in different tissue types (Antebi *et al.*, 1998; Antebi *et al.*, 2000). Our MRC mutants are phenotypically different from *daf-12* mutants. We have not observed any dauer-like characteristics, such as alae formation, a long and thin body size, or a darkened intestine in the MRC mutants. That we have observed both L3 and dauer stages in *nuo-1* animals, suggests that the commitment or the decision to enter either the dauer or the normal developmental pathways is not impaired by MRC dysfunction. We postulate that development beyond the L3 stage ceases because the minimum energy threshold level required exceeds the capacity for energy production provided by maternally contributed mitochondrial components. New mitochondrial components must be synthesized in order to meet the energy requirements for development to proceed.

The common L3 arrest phenotype we have observed suggests that the transition from the L3 to the L4 stage may involve an energy-sensing mechanism that invokes a

developmental checkpoint when an energy deficit arises (Figure 6.1). An energy sensor could respond to one or more metabolites, whose concentrations communicate information about the status of mitochondrial energy production. Recently, a number of candidate proteins for the role of energy sensor have been described. Each of these proteins responds to a certain metabolite that is linked to growth and development. For example, the corepressor CtBP or carboxyl-terminal binding protein is a transcriptional regulator that may act as a redox sensor by virtue of its NAD^+/NADH binding motif (Zhang *et al.*, 2002). A high NADH/NAD^+ ratio, which is often associated with mitochondrial dysfunction (King and Attardi, 1996), may lead to the transcriptional repression of a set of genes involved in the L3 to L4 transition. Since genomic silencing is associated with histone deacetylation via the NAD-dependent histone deacetylase, Sir2p (Imai *et al.*, 2000; Lin *et al.*, 2000), measuring and comparing the state of acetylation of histones and the concentrations of NADH and NAD^+ in wild type and mutant animals may be informative. If a redox sensor participates in the developmental checkpoint, then a wild type animal would be expected to have a low NADH/NAD^+ ratio and increased histone acetylation. A mutant animal, on the other hand, would have a high NADH/NAD^+ ratio and decreased histone acetylation. Modulating the organism's redox state may prove to be an informative way for investigating this hypothesis. For example, the yeast single subunit NADH dehydrogenase encoded by the *NDI1* gene can be introduced into *nuo-1* or *atp-2* mutant animals (Seo *et al.*, 1998; Seo *et al.*, 2000). Ndi1p differs from a mammalian complex I in that it does not translocate protons across the inner mitochondrial membrane, but is capable of oxidizing NADH to NAD^+, which is needed for both glycolysis and the tricarboxylic acid cycle. If excess NADH contributes

to the MRC mutant phenotype, then *NDI1* expression should ameliorate the severity of the larval arrest phenotype. The mammalian Target of Rapamycin (mTOR), which functions to regulate ribosome biosynthesis and cell growth, is a protein kinase that directly senses the intracellular concentration of ATP (Dennis *et al.*, 2001). Interestingly, mutations in the *C. elegans* mTOR homolog are lethal and result in an L3 developmental arrest (Long *et al.*, 2002). Thus, the nematode mTOR may be the developmental checkpoint or energy sensor that controls the transition from the L3 stage to the L4 stage. Experimentally measuring decreases in the levels of ATP or ribosome number in our mutant worms could be a first step in establishing mTOR as the energy sensor for the L3-to-L4 transition. Finally, a suppressor screen that identifies animals that no longer arrest at the L3 stage could be performed to identify energy-sensing and developmental checkpoint components.

If CtBP or mTOR is indeed the energy sensor for the L3-to-L4 transition checkpoint, what developmental or genetic process does it control? Developmentally, the L3-to-L4 transition is characterized by a substantial increase in body size, the maturation of the somatic gonad and vulva, and a proliferation of the germ cells. The energy sensor may modulate the transcription or translation of a number of genes involved in these processes. We speculate that CtBP or mTOR normally functions to positively regulate the production and/or secretion of a signaling molecule (see next section). The concentration of this signaling molecule has to reach a certain level in order for development to proceed to the L4 stage. Developmental transition from the L3 stage to the L4 stage may ultimately require the expression of certain genes, such as heterochronic genes, leading to the termination of the L3-specific developmental program and the initiation of the L4-

specific programs. When the MRC is impaired, production of the signaling molecule drops below a certain defined threshold level, leading to an arrest at the L3 stage without further development. The L3 arrest phenotype is reversible: when EtBr was removed from the EtBr-induced L3 arrested worms, these animals were capable of developing beyond the L3 stage (Tsang and Lemire, 2002a). This situation is analogous to diapause caused by nutrient starvation where the starvation-induced arrest is reversible (Arasu *et al.*, 1991). The molecular identification of the targets of CtBP and mTOR involved in the L3-to-L4 transition should greatly contribute to our understanding of the relationship between mitochondrial function and development.

6.3 Tissue Specificity

In addition to the diversity of phenotypes they produce, mitochondrial diseases are often tissue-specific in their effects. Tissue-specificity has been ascribed to the differential energy requirements of each tissue, to fluctuations in mtDNA heteroplasmy, and to the variable expression patterns of MRC subunit isoforms in each tissue type (Chinnery and Turnbull, 1997; Grossman and Shoubridge, 1996; Poyton and McEwen, 1996). Investigation of tissue-specificity in mitochondrial dysfunction requires a model that possesses distinct and identifiable tissue types; such studies are impossible to execute in the yeast or *Neurospora* systems.

The results in chapter 3 describe the use of a technique called mosaic analysis to study the tissue-specificity of the *atp-2(ua2)* mutation in *C. elegans*. In genetic mosaics, some cells or tissues are *atp-2(+)* and others are *atp-2(ua2)*. The *atp-2* gene is nuclear-encoded and unique; no other *atp-2* genes are known to be in the genome. The tissue-

specific effects observed in mosaics are likely related to that tissue's reliance on the ATP synthase for its energy requirements. My data suggest that the *atp-2* gene is essential in all tissues for optimal development (Tsang and Lemire, 2003). The loss of *atp-2(+)* in most tissues or lineages is lethal and results in L3 arrested larvae. Rare mosaics with losses of the *atp-2(+)* gene in some neuronal, hypodermal, and pharyngeal cells, and/or in all intestinal cells can develop past the L3 stage to the L4 or the adult stages. One interpretation of these results is that these cells or tissues rely less heavily on the MRC for energy production and are thus less sensitive to MRC defects. A testable corollary is that these tissues have fewer mitochondria, which could be estimated from serial electron micrographs, or from measuring the fluorescence intensity of different tissue-specific, mitochondrially-targeted GFP constructs.

An alternative explanation is that some tissues have less important roles in controlling animal development. Except for the gonad, development appears to be an all-or-none phenomenon. In any single *atp-2* mosaic, either all or none of the cells or tissues develop past the L3 stage, despite the presence of a mixture of genotypically wild type and mutant cells (Tsang and Lemire, 2003). Thus, the *atp-2* gene is functioning in a cell non-autonomous or tissue non-autonomous manner; the genotype of a cell does not necessarily determine its phenotype or developmental history. Cell non-autonomy suggests that development beyond the L3 stage is controlled by a global mechanism in which all cells contribute to reach a consensus decision to continue development or not. It seems unlikely that this consensus mechanism relies directly upon *atp-2* function but rather upon the production of a signal that reflects the signaling cell's energy status. Candidate signaling molecules include hormones (Chitwood, 1999), neuropeptides (Li *et*

al., 1999), or other metabolites whose concentrations determine whether the checkpoint is invoked or not. Thus, the candidate energy sensors, CtBP and mTOR, may act to positively regulate the production of a signaling molecule that controls the transcription of a set of genes required for the L3-to-L4 transition. The molecular identification of suppressors of the L3 arrest phenotype may shed some light onto the precise nature of the signaling molecule. Alternatively, a decrease in the cellular ATP levels may impair the secretion of a signaling molecule, as occurs in diabetic patients whose pancreatic ß-cells carry a mtDNA mutation (Suzuki *et al.*, 1997).

The mosaic analysis data suggest that the presence of *atp-2(+)* in multiple tissues is required for the L3-to-L4 transition. To further determine which tissue or combination of tissues is critical for development, the phenotypes of transgenic animals carrying the wild type *atp-2* transgene driven under the control of tissue-specific promoters could be examined.

6.4 Mitochondrial Genetics

The genetics of mtDNA mutations differ from nuclear mutations because of their mode of inheritance, the number of copies per cell, and the presence and variability of a heteroplasmic state. Heteroplasmy can vary, increasing or decreasing as cells divide (Grossman and Shoubridge, 1996). Pathogenic conditions arise when the proportion of the mutant mtDNA reaches a certain threshold level that is usually around 60 to 90%. Due to the importance of heteroplasmy in human disease, it is vital to understand its fluctuation and the segregation and transmission of mtDNA.

In humans, the segregation of mtDNA sequence variants can be extremely rapid, sometimes within a single generation (Degoul *et al.*, 1997; Howell *et al.*, 1991; Howell *et al.*, 1994), or it can be unexpectedly slow, with the maintenance of a stable and persistent intergenerational heteroplasmy (Howell *et al.*, 1992). In contrast, heteroplasmy in yeast or *Neurospora* is generally unstable and shifts rapidly (Rickwood *et al.*, 1988). Since these organisms can tolerate the loss of mtDNA under certain growth conditions, the transmission and maintenance of their mtDNAs are likely to be regulated in mechanistically different manners than in humans (Lecrenier and Foury, 2000).

In Chapter 4, I described the isolation and characterization of a large-scale mtDNA deletion called *uaDf5* (Tsang and Lemire, 2002b). This mutation is maternally inherited and exists in a stable heteroplasmic state with the wild type mtDNA. The proportion of *uaDf5* is maintained at around 60% throughout development. The heteroplasmic levels of individuals bearing the *uaDf5* deletion can vary from ~20% to ~80%. Homoplasmic wild type or *uaDf5* animals have not spontaneously segregated from the heteroplasmic strain. This situation is consistent with the heteroplasmy being intra-mitochondrial, with each mitochondrion containing both wild type and mutant mtDNA molecules. Intra-mitochondrial heteroplasmy could be examined by using fluorescent oligonucleotides that specifically recognize each of the two forms of mtDNA. The wild type mtDNA could be detected with a probe complementary to the region deleted by the *uaDf5* mutation, while the *uaDf5* mtDNA could be detected with a probe that spans the deletion junction. With this approach, it may also be possible to determine the proportions of *uaDf5* mtDNA in different tissues. In a stable heteroplasmic strain of *Drosophila*, the levels of heteroplasmy vary between different tissues (Béziat *et al.*,

1997). Alternatively, stable heteroplasmy may arise because mtDNAs do not exist as independent entities but are rather packaged with other mitochondrial proteins into nucleoids (Jacobs *et al.*, 2000); the composition of these nucleoids may be maintained during cell division. In yeast cells, nucleoids can be stained and visualized with DAPI (4'-6-diamidino-2-phenylindole) as punctate spots (Hobbs *et al.*, 2001). Similar experiments can be performed in *C. elegans* to address if worm mtDNAs are assembled into nucleoids.

I have shown that the *uaDf5* genome can be inherited randomly or non-randomly. Single, self-fertilized hermaphrodites with intermediate levels of *uaDf5* mtDNA (50% or 65%) have broods that form random, non-skewed distributions centered on the parental mean. However, hermaphrodites with more extreme proportions of *uaDf5* mtDNA (~20% or ~80%) have broods with skewed distributions. The proportions of *uaDf5* mtDNA in the offspring have a tendency to shift down when the heteroplasmy of the parent is high. Conversely, when the heteroplasmy of the parent is low, the proportions of *uaDf5* mtDNA in the progeny tend to go up. Thus, the intergenerational stability of the *uaDf5* heteroplasmy appears to be controlled by two opposing forces, one that increases the proportion of *uaDf5* mtDNA when it is low and a second decreasing it when proportions are high. At intermediate heteroplasmic levels, the two forces are in equilibrium with each other, and *uaDf5* levels in the offspring reflect the maternal state.

What are the forces involved in maintaining a stable heteroplasmy and when do they act? It has been suggested deletion-containing mtDNA molecules may have a replicative advantage over the wild type mtDNA molecules because they are shorter. In the case of the *uaDf5* deletion, there does not appear to be a replicative advantage, since

the proportion of *uaDf5* is constant at ~60% through development from the L1 to the adult. This includes times of intense mtDNA replication from L3 to adult. Alternatively, nuclear DNA-encoded factors may confer a replicative advantage by exerting a selection pressure at the level of replication or maintenance of the mitochondrial genome prior to hatching. The molecular nature of these nuclear-encoded factors are currently under active investigation (Battersby and Shoubridge, 2001; Battersby *et al.*, 2003; Le Goff *et al.*, 2002), but may include mtDNA transcription and translation factors, mtDNA topoisomerases, mtDNA repair proteins, or any factors that bind and interact with the mtDNA. Recently, it was demonstrated that the CLK-1 protein has the ability to bind and interact with one of the replication origins of the mtDNA (Gorbunova and Seluanov, 2002). A *clk-1* mutation may differentially affect the replication rates of wild type and *uaDf5* mtDNA and thus may alter the heteroplasmic levels of *uaDf5* animals. Alternatively, a respiratory advantage may account for the maintenance of a stable heteroplasmic state. The wild type DNA may have acquired an undetected mutation that would severely affect respiratory function. If this were the case, the *uaDf5* mtDNA must be maintained to complement the undetected lethal mutation in the 'wild type' genome. Sequencing the 'wild type' mitochondrial genome should address this possibility. Respiratory selection must operate prior to embryogenesis, possibly through the elimination of germ nuclei or oocytes with very high or low *uaDf5* contents, since there is no observed phenotype, including inviable embryos, associated with the *uaDf5* mutation. Elimination of germ nuclei via apoptosis has been observed in the *C. elegans* hermaphrodite germline and is also be under the control of the nuclear genome (Gumienny *et al.*, 1999). Mutations in genes that prevent the elimination of germ nuclei

in the germline, such as *ced*-3 and *ced-4*, can be crossed into a *uaDf5* background. If apoptosis is involved in the elimination of germ nuclei with extreme *uaDf5* contents, then such a construct would lead to the production of unhealthy progeny which may be phenotypic. The molecular nature of these nuclear-encoded factors could also be established via a genetic screen for homoplasmic wild type or mutant animals.

6.5 Mitochondrial Biogenesis

The number of mitochondria and mtDNA copies are precisely maintained within a cell, vary between tissues (Bogenhagen and Clayton, 1974; Robin and Wong, 1988), and can respond to a wide variety of stimuli such as temperature or stress. In normal conditions, the number of mitochondria and the mtDNA copy number correlate loosely with the energy requirements or demands of a cell (Moraes, 2001; Williams, 1986). An increase in energy demand can be due to somatic requirements or germline requirements associated with sexual maturation. However, a complete description of the changes in the number of mitochondria or in the number of mtDNA copies has not been reported in any single eucaryotic species.

In chapter 5, I have measured the mtDNA copy numbers of the various developmental stages of *C. elegans* as an indirect indicator of energy demands (Tsang and Lemire, 2002a). Roughly 25,000 copies of mtDNA are inherited maternally from an oocyte and this complement is sufficient to support early development from the embryonic stage to the L3 stage. A five-fold increase in copy number is associated with the L3-to-L4 transition, while a six-fold increase is observed from the L4 stage to the adult. The first increase in mtDNA copy number correlates with the L3 arrest phenotype

of the *nuo-1* and *atp-2* mutants and with the drug-treated animals, thus supporting the hypothesis for an energy sensor or checkpoint between the L3 and L4 stages. Developmentally, the L3-to-L4 transition is characterized by a substantial increase in body size, the maturation of the gonad and the vulva, a rapid proliferation of the germline, and the initiation of spermatogenesis. Thus, the mtDNA copy number increase may be associated with somatic and/or germline maturation processes.

By measuring the mtDNA copy numbers of several mutant strains defective in germ line, sperm, or oocyte production, I estimated the mitochondrial genome contents of different cells (Tsang and Lemire, 2002a). With germline deficient mutants, which show markedly reduced mtDNA contents, I demonstrate that the majority of mtDNA in adult worms is germline associated. With sperm- or oocyte-deficient mutants, I confirmed that mtDNA amplification is primarily associated with oocyte production. I have estimated that a somatic cell has ~45 to ~70 mtDNA copies per cell. It remains to be determined whether different somatic cells or tissues have distinct mtDNA copy numbers. For instance, cells that are highly oxidative, such as myocytes and neurons, may contain large numbers of mitochondria and mtDNA. Undifferentiated germline cells and sperm have ~250 and 30-40 copies of mtDNA per cell, respectively. 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 eucaryotic cells (Shadel and Clayton, 1997). It is possible that *C. elegans* may possess fewer mitochondria than higher eucaryotes. The number of mitochondria in worms should be determined.

The number of copies of mtDNA is tightly regulated not only in different tissue types, but also in different stages of development. What are the mechanisms involved in

the regulation of mtDNA copy number? In mammals, it is known that the mtDNA copy number is controlled in part by the mitochondrial transcription factor A (Larsson *et al.*, 1998), which in turn is regulated by the nuclear respiratory factor 1, or NRF1 (Wu *et al.*, 1999). NRF1 can be induced by PGC-1, a master transcriptional co-activator for the regulation of mitochondrial biogenesis in brown adipose tissue and skeletal muscle (Wu *et al.*, 1999). Exposure to cold or exercise triggers the expression of PGC-1. We believe that such a mechanism for mitochondrial biogenesis, and the players involved in this pathway may also exist in worms. A genetic screen for mutants with a decrease or an increase in copy number would be a desirable goal.

6.6 Concluding Remarks

The work presented in this thesis represents a starting point for further investigation of the principles governing mitochondrial biogenesis and dysfunction. While much of the data is genetic, it provides an important conceptual framework for further investigations into the molecular and biochemical mechanisms of mitochondrial function. In particular, what are the factors involved in the communication between mitochondria and the nucleus? What is the molecular identity of the energy sensor? What are the set of genes that respond to mitochondrial function, and how do they affect development? What regulates the maintenance, segregation, and transmission of mtDNA? My work has clearly demonstrated the utility of the nematode model and has opened up new avenues for addressing the above questions. We believe that *C. elegans* will continue to be a powerful experimental system with which to study the genetics,

physiology, and developmental biology of mitochondrial biogenesis, dysfunction, and inheritance.

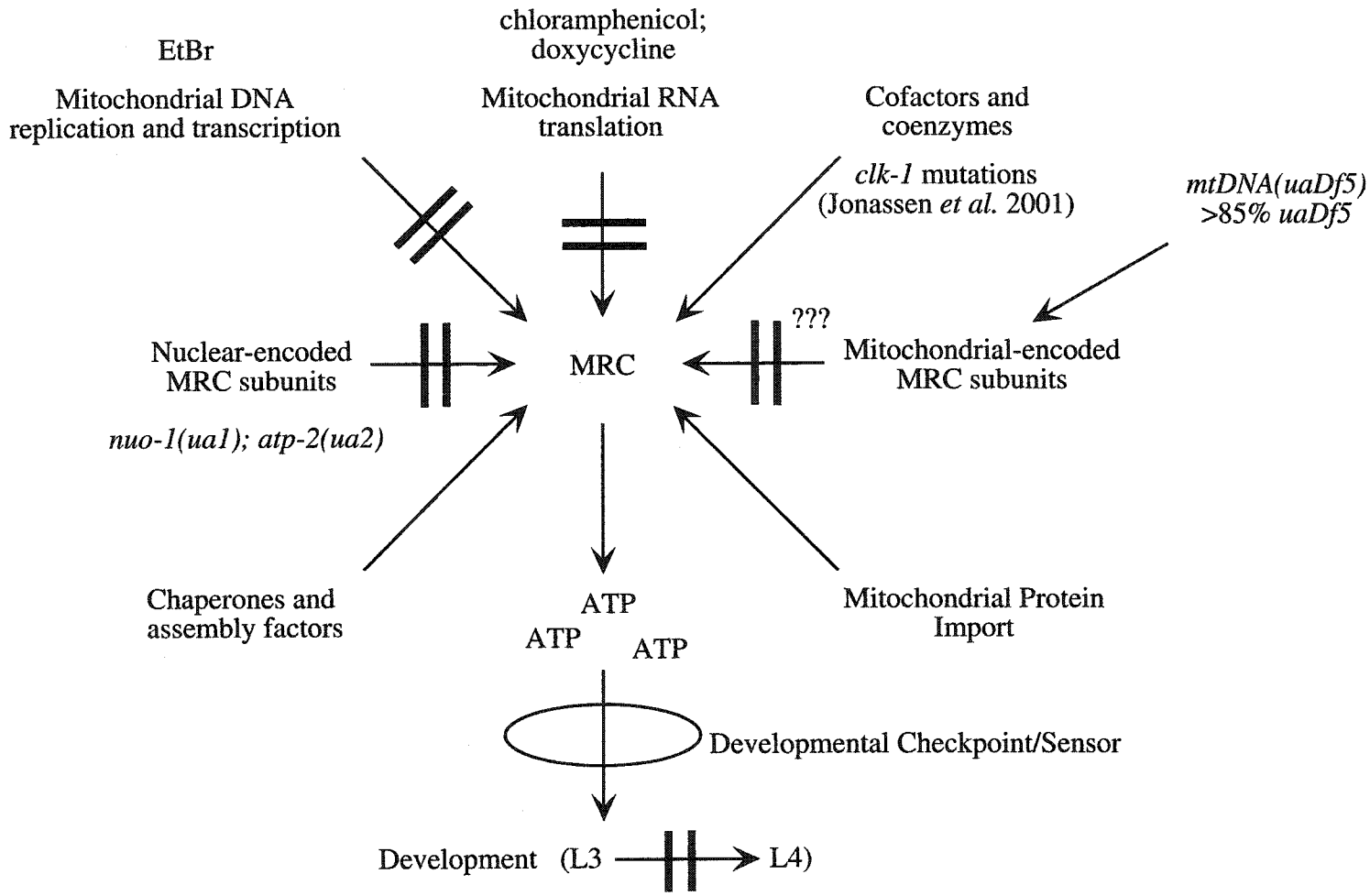

Figure 6.1: The role of the MRC in *C. elegans* development

6.7 Bibliography

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