Mitochondrial complex I mutations in *Caenorhabditis elegans* produce cytochrome c oxidase deficiency, oxidative stress and vitamin-responsive lactic acidosis

Leslie I. Grad and Bernard D. Lemire*

Canadian Institutes of Health Research Group in Membrane Protein Research, Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

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Mitochondrial dysfunction, with an estimated incidence of 1 in 10 000 live births, is among the most common genetically determined conditions. Missense mutations in the human *NDUFV1* gene, which encodes the 51 kDa active site subunit of the NADH–ubiquinone oxidoreductase or complex I, can lead to severe neurological disorders. Owing to the rare and often sporadic nature of mitochondrial disorders, the mechanisms of pathogenesis of most mutations remain poorly understood. We have generated transgenic strains of *Caenorhabditis elegans* that express disease-causing mutations in the *nuo-1* gene, the *C. elegans* homolog of the *NDUFV1* gene. The transgenic strains demonstrate hallmark features of complex I dysfunction such as lactic acidosis and decreased NADH-dependent mitochondrial respiration. They are also hypersensitive to exogenous oxidative stress, suggesting that cellular defense mechanisms against reactive oxygen species are already taxed by an endogenous stress. The lactic acidosis induced by the *NDUFV1* mutations could be partially corrected with the vitamins riboflavin and thiamine or with sodium dichloroacetate, an activator of the pyruvate dehydrogenase complex, resulting in significant increases in animal fitness. Surprisingly, cytochrome c oxidase activity and protein levels were reduced, establishing a connection between complexes I and IV. Our results indicate that complex I mutations exert their pathogenic effects in multiple ways: by impeding the metabolism of NADH, by increasing the production of reactive oxygen species, and by interfering with the function or assembly of other mitochondrial respiratory chain components.

INTRODUCTION

The primary function of mitochondria, mediated by the mitochondrial respiratory chain (MRC), is to provide energy via oxidative phosphorylation. This energy is essential to most eucaryotic cells as it drives the majority of cellular processes. The MRC is composed of four membrane-bound electron-transporting protein complexes (I–IV) that generate a proton gradient across the mitochondrial inner membrane and the ATP synthase (complex V) that uses the proton gradient for ATP synthesis. Defects in one or more of these complexes can result in a large variety of diseases often with multisystemic presentations. Mitochondrial dysfunction occurs with an estimated incidence of 1 in 10 000 live births and is often caused by a deficiency in complex I, the NADH–ubiquinone oxidoreductase (1,2). Mammalian complex I is the largest MRC complex, containing at least 46 nuclear- and mitochondrial-DNA encoded subunits, a flavin mononucleotide (FMN) cofactor, and up to eight iron–sulfur clusters (3–5). The enzyme couples electron transfer from NADH to ubiquinone to proton pumping across the mitochondrial inner membrane. Complex I dysfunction is linked to myopathies, encephalomyopathies, and neurodegenerative disorders such as Parkinson’s disease and Leigh syndrome (1,2,6,7).

*To whom correspondence should be addressed. Tel: +1 7804924853; Fax: +1 7804820886; Email: bernard.lemire@ualberta.ca

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presented with muscular hypotonia, myoclonic epilepsy, brain atrophy and elevated lactate and pyruvate concentrations in the blood and cerebrospinal fluid. Both died before the age of 18 months (9). A patient homozygous for the A341V mutation presented with similar symptoms, as well as macrocystic leukodystrophy (9). At the time of the report, this patient, age 10, was severely spastic and blind (9). A more recent investigation identified six additional mutations including a deletion, an inversion, and the four single amino-acid substitutions Y204C, C206G, E214K and A432P (10). Patients presented with hypotonia, ataxia, psychomotor retardation or Leigh syndrome, and most died before the age of 3 years as a result of acute metabolic acidosis (10). Each of the six missense mutations in NDUFV1 affect highly conserved amino acids, suggesting these may be functionally important. It seems unlikely that low cellular energy can be entirely responsible for the diversity and severity of the NDUFV1-related disease and of mitochondrial disorders in general, implying other molecular mechanisms are involved in pathogenesis. Unfortunately, due to the rarity of most mitochondrial disorders, these mechanisms remain poorly understood.

In this study, we have used the free-living soil nematode, Caenorhabditis elegans, to investigate the pathogenesis of complex I disorders. C. elegans offers numerous advantages for studying mitochondrial dysfunction (11,12). The organism has a life cycle of 3–4 days at 20°C and features a simple anatomy with distinct tissue systems. Mitochondrial dysfunction can often present in a tissue-specific fashion, affecting those tissues with the greatest demand for MRC-generated ATP. C. elegans is transparent; living animals can be readily examined by light microscopy for morphological or physiological abnormalities. The sequences of the nuclear and mitochondrial genomes have been determined (13,14). Finally, the structure and bioenergetics of the nematode MRC are very similar to that of the mammalian MRC (15). The C. elegans model system has garnered increasing popularity for understanding the mechanisms of human disease such as muscular dystrophy and Alzheimer disease (16,17), as well as other types of mitochondrial dysfunction (18–21).

Here, we investigate the mechanisms of pathogenesis of three NDUFV1 missense mutations. We have previously isolated and characterized a mutation in the C. elegans nuo-1 gene, the homolog of the NDUFV1 gene (22). The nuo-1(ua1) allele is a large deletion that is homozygous lethal, resulting in developmental arrest at the third larval stage (22). We generated transgenic strains lacking endogenously expressed nuo-1, but expressing nuo-1 carrying A352V, T434M and A443F mutations, which correspond to the residues A341, T423 and A432 of the NDUFV1 gene, for which disease-causing mutations have been reported (9,10). All three transgenes rescue the lethality of the nuo-1 knockout background. The transgenic strains exhibit a number of morphological defects, including premature tissue degeneration and abnormal development of the reproductive system. The complex I mutations result in reduced brood sizes, decreased respiration, lactic acidosis and hypersensitivity to oxidative stress. The lactic acidosis was responsive to the vitamins riboflavin and thiamine and to sodium dichlorooracetate, an activator of the pyruvate dehydrogenase complex. Surprisingly, mutants also displayed significant decreases in complex IV activity and in steady-state levels of COX I, a mitochondrial DNA-encoded subunit. Taken together, our data suggest that complex I mutations are pathogenic not only because they impair the oxidation of NADH, but also because they increase the production of reactive oxygen species and interfere with the function or assembly of other MRC complexes.

RESULTS

Generation of transgenic C. elegans strains expressing nuo-1 mutations

The C. elegans NUO-1 protein shares 75% amino acid sequence identity with the NDUFV1 protein. Mutated versions of the nuo-1 gene carrying the A352V, T434M or A443F mutations were generated. These three amino acids are conserved in the NDUFV1 protein and correspond to A341, T423 and A432, respectively. The A352V and T434M mutations exactly mimic the disease-causing NDUFV1 A341V and T423M mutations. The nuo-1 A443F mutation, previously created in our laboratory, is similar to the NDUFV1 A432P mutation. Each mutated nuo-1 gene was transformed with an unc-119(+) co-transformation marker into LB77 [nuo-1(ua1)]/+ via microparticle bombardment (23). LB77 is also homozygous for the unc-119(ed3) mutation, which renders the animals partially paralyzed and unable to form dauer larvae, an alternate life stage that allows C. elegans to survive for months without feeding. Successful transformants regain mobility and the ability to enter the dauer state. Transformation by microparticle bombardment produces extrachromosomal arrays of variable mitotic and meiotic stability (24) or, at a lower frequency, the DNA can be integrated into the genome (23). Three independent transformants were selected by complementation of the unc-119 defects for each mutation. Homozygous nuo-1(ua1) progeny carrying the nuo-1 transgenes were subsequently isolated. The genotypes of all transgenic lines were confirmed by PCR analysis. For all three mutations, each independent line gave similar results and one was chosen for more detailed examination. The nuo-1 A443F and A352V transgenes were present as extrachromosomal arrays with transmission rates of 67±27% and 60±19%, respectively. The T434M transgene was integrated and maintained in the homozygous state.

Characterization of transgenic strains

Each of the nuo-1 transgenes complemented the lethality of the nuo-1(ua1) background and supported full development to reproductive adults. We conclude that the mutant NUO-1 proteins are expressed and functionally assembled into complex I, which was sufficiently active to support the energy generation needed for development. Progeny of the A443F and A352V transgenic lines that had lost the extrachromosomal arrays arrested as embryos, a phenotype similar to that seen with nuo-1 RNA interference (22).

Growth, reproduction and aging are biological processes that depend on the integrity and function of the MRC (18,22,25,26). We observed that the three transgenic mutants had variably decreased reproductive capabilities as measured by their brood sizes; the A443F mutation has the most severe effect (Fig. 1A).
and early adult stages. Normally, by the late L4 stage, the arms of the gonad are fully extended anteriorly and posteriorly along the ventral surface and reflexed along the dorsal surface, forming a U-shaped structure (27). The distal portion of each gonad arm contains syncytial germline nuclei incompletely surrounded by membranes (Fig. 2A). As *C. elegans* matures into a reproductive adult, membranes surround individual nuclei as they migrate towards the proximal ends of the gonad, forming oocytes that are fertilized while passing through the spermatheca. Fertilized oocytes are then expelled from the uterus through the vulva. Somatic gonad development was severely affected in transgenic animals expressing the A443F and A352V mutations and moderately affected in the T434M mutant. In early adult animals, gonad arms were usually shorter than normal, suggesting difficulties with distal tip cell migration, and appeared shriveled, leaving space between the gonad and the body wall (Fig. 2B–D). Furthermore, the syncytial germline nuclei were difficult to identify and more disorganized than in wild-type gonads. As the transgenic animals matured into gravid adults, oocyte formation proceeded but more slowly than normal, suggesting that meiotic germline division or oogenesis is compromised by mitochondrial dysfunction (Fig. 2F–H). Vulval development was slower in more severely affected mutant animals but otherwise appeared complete. The decreases in brood sizes are not due to an egg-laying defect but rather are the result of impaired germline development.

MRC function is closely connected to lifespan determination and aging (28). Both germline and somatic tissue deterioration increase significantly with age (29). The overall health of *nuo-1* transgenic animals declined rapidly once they had reached reproductive maturity. The transgenic mutants accumulated a dark pigment, a biomarker of aging, within 2 days of becoming gravid adults; pigment accumulation occurs in wild-type *C. elegans* towards the end of their reproductive period, which is approximately 4 days into adulthood (29). Microscopic analysis revealed prominent vacuolar structures that appeared throughout the body, usually beneath the cuticle, suggesting the degeneration of body wall muscle tissue (29,30). The vacuolar structures in the transgenic mutants became apparent on the first day of adulthood (Fig. 3B–D) about 4 days earlier than in wild-type animals (Fig. 3A and E) and accumulated as the transgenic animals declined rapidly once they had reached reproductive maturity. The transgenic mutants accumulated a dark pigment, a biomarker of aging, within 2 days of becoming gravid adults; pigment accumulation occurs in wild-type *C. elegans* towards the end of their reproductive period, which is approximately 4 days into adulthood (29). Microscopic analysis revealed prominent vacuolar structures that appeared throughout the body, usually beneath the cuticle, suggesting the degeneration of body wall muscle tissue (29,30). The vacuolar structures in the transgenic mutants became apparent on the first day of adulthood (Fig. 3B–D) about 4 days earlier than in wild-type animals (Fig. 3A and E) and accumulated as the animals aged (Fig. 3F–H). The A352V and A443F mutants were the most severely affected, while the T434M mutant displayed a milder phenotype.

**Impaired respiration and lactic acidosis in transgenic mutants**

We investigated the effects of the three complex I mutations on respiration by monitoring oxygen consumption of live animals. Respiration values were normalized to rates per 1000 animals or to protein content. The percentages of respiratory activity in the transgenic lines as compared with the wild-type are very similar for both methods of calculation because the sizes of transgenic and wild-type animals are not significantly different (data not shown). The A352V and A443F mutants consumed oxygen at rates approximately half the wild-type rate, while the T434M mutant had a rate ~65% of the wild-type rate (Table 1). These rates correlate well with the severity

**Gonadal abnormalities and premature aging in transgenic mutants**

We observed the first signs of impaired gonadal development when the reproductive system is rapidly maturing during the L4
of the mutations as measured by brood sizes or lifespan. We also determined the rotenone-sensitive NADH-oxidase activities of isolated mitochondria. The mitochondria were disrupted and a membrane fraction isolated to allow access of NADH to the matrix-facing NADH-binding site of complex I. Rotenone is a specific inhibitor for complex I and rotenone-sensitive respiration is attributable to complex I activity. In agreement with the live animal respiration rates, the A443F and A352V mutations had the most severely affected rotenone-sensitive respiration rates (23 and 37% of wild-type, respectively; Table 2). Complex I-mediated respiration in the T434M transgenic strain was reduced to 52% of wild-type. The relative oxygen consumption rates of live animals (Table 1) are higher than the complex I-specific rates (Table 2), possibly indicating the preferential use of other respiratory substrates over NADH in live animals.

Impaired complex I-dependent respiration will lead to excess cellular NADH, favoring the reduction of pyruvate to lactate. We found that pyruvate concentrations were decreased and lactate concentrations were increased in the mutants compared with the wild-type, resulting in greatly elevated lactate/pyruvate ratios (Table 3). Again, the A443F mutant demonstrated the largest change with a ratio 6.3-fold higher than the wild-type. These data suggest that the *nuo-1* mutations may indirectly impede the citric acid cycle and oxidative phosphorylation by depleting the cellular pools of pyruvate. In addition, the pyruvate dehydrogenase complex is inhibited by phosphorylation as a result of the activation of the pyruvate dehydrogenase kinase by NADH (31). Thus, the increased levels of NADH stimulate the reduction of pyruvate to lactate and result in lactic acidosis.

**Pharmacological treatment of transgenic mutants**

A variety of pharmacological agents, including vitamins, have been used to treat mitochondrial diseases (32–34). We picked
L4-staged transgenic animals to individual plates supplemented with sodium dichloroacetate, thiamine or riboflavin, activators of the pyruvate dehydrogenase complex that are used clinically to treat lactic acidosis (32–34) and allowed them to lay their broods. Riboflavin, as a precursor of FMN, is also essential for complex I function. We also investigated the effects of ascorbate, a free-radical scavenger, and oxaloacetate, an intermediate of the citric acid cycle. All three mutant strains, but not the wild-type, showed significant increases in brood size when supplemented with 1 \( \mu g/ml \) riboflavin (Fig. 4). Significant increases in brood size were also observed for the A352V and A443F mutants when supplemented with 5 \( \mu g/ml \) sodium dichloroacetate or 0.1 mM ascorbate. Thiamine supplementation (1 \( \mu g/ml \)) significantly increased the brood size of the A352V mutant. None of the mutants benefited significantly from 1 \( \mu g/ml \) oxaloacetate. The reproductive fitness of all three nuo-1 mutant strains can be improved by pharmacological intervention. Each mutant strain benefits from a specific set of supplements, suggesting that the metabolic consequences of each mutation may not be identical in each of the three transgenic strains.

To investigate further the mechanisms by which the supplements exert their effects, we examined the lactate/pyruvate ratios of sodium dichloroacetate or riboflavin-treated A352V and A443F mutants. These two supplements were chosen because they consistently showed the greatest benefit. Lactate concentrations were significantly decreased and pyruvate concentrations significantly increased by sodium dichloroacetate, resulting in markedly lower lactate/pyruvate ratios for both mutants (Table 4). Supplementation with riboflavin showed similar results for the A443F mutant, but had no significant effect on the A352V mutant strain (Table 4). These results suggest that lactic acidosis is responsible for at least some of the pathogenic consequences of all three nuo-1 mutations. Treatments that ameliorate the acidosis can markedly decrease the severity of the phenotype. Interestingly, the beneficial effects of riboflavin on the brood sizes of the A352V mutant (Fig. 4) do not appear to be associated with a reduction in lactate/pyruvate ratios (Table 4). We suggest that riboflavin improves A352V mutant metabolism in a manner that is mechanistically different than sodium dichloroacetate.

**Table 1.** Transgenic mutants have decreased rates of oxygen consumption

<table>
<thead>
<tr>
<th>Strain</th>
<th>Oxygen consumption rate nmol O(_2)/min/1000 animals(^a)</th>
<th>Oxygen consumption rate nmol O(_2)/min/mg protein(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2</td>
<td>1.3 ± 0.12 (100%)</td>
<td>8.6 ± 1.1 (100%)</td>
</tr>
<tr>
<td>A352V</td>
<td>0.66 ± 0.11 (51%)*</td>
<td>4.6 ± 0.9 (54%)*</td>
</tr>
<tr>
<td>T434M</td>
<td>0.86 ± 0.01 (66%)*</td>
<td>5.4 ± 0.4 (64%)*</td>
</tr>
<tr>
<td>A443F</td>
<td>0.62 ± 0.02 (48%)*</td>
<td>4.3 ± 0.4 (51%)*</td>
</tr>
</tbody>
</table>

\(^a\)Values are the means ± SD of a minimum of five trials.

\(^*\)P < 0.05 compared with the N2 wild-type strain using two-sample \( t \)-test.

**Table 2.** Transgenic mutants have decreased rotenone-sensitive NADH-dependent oxygen consumption rates

<table>
<thead>
<tr>
<th>Strain</th>
<th>Oxygen consumption rate (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2</td>
<td>187 ± 45 (100%)</td>
</tr>
<tr>
<td>A352V</td>
<td>70 ± 20 (37%)*</td>
</tr>
<tr>
<td>T434M</td>
<td>97 ± 21 (52%)*</td>
</tr>
<tr>
<td>A443F</td>
<td>43 ± 24 (23%)*</td>
</tr>
</tbody>
</table>

\(^b\)Values are expressed as nmol O\(_2\) min \(^{-1}\) mg protein \(^{-1}\) and represent the means ± SD of a minimum of five trials.

\(^*\)P < 0.02 compared with the N2 wild-type strain using a two-sample \( t \)-test.
Complex IV deficiency in mutant nuo-1 transgenic strains

Impaired metabolism caused by a defect in one MRC complex is often associated with altered levels of the other MRC complexes (26,35–37). We investigated the levels of cytochrome c oxidase activity in mutant and wild-type animals by histochemical staining; the complex IV-dependent oxidation of diaminobenzidine produces a brown pigment. Cytochrome c oxidase activity was diminished in the body wall and pharyngeal muscles of L3 larvae from all three transgenic mutants (Fig. 5A). The A352V and A443F mutants were more severely affected than the T434M mutant. As the mutants matured, cytochrome c activity increased, but failed to reach wild-type levels (Fig. 5B).

To determine whether the observed decreases in cytochrome c oxidase activity are due to diminished levels of assembled complex IV, we performed western blot analyses with an antiserum directed against human COXI, a mitochondrial DNA-encoded subunit of the complex IV catalytic core. COXI levels were consistently lower in the nuo-1 mutant strains than in the wild-type (Fig. 5C). When normalized to ATP-2 levels, a subunit of the ATP synthase, the A443F, A352V and T434M mutants had COXI levels of 43±12, 51±17 and 90±3% of the wild-type, respectively.

To further assess the effects of the complex I mutations on the MRC, we measured the enzymatic activities of the individual or pairs of MRC complexes in wild-type and transgenic mitochondrial membranes (Table 5). As expected, the rotenone-sensitive NADH-decylubiquinone reductase activities in all three mutants were significantly reduced. The cyanide-sensitive cytochrome c oxidase activities were also severely affected. The complex II and complex III activities were either modestly affected or unchanged with respect to the wild-type. Several lines of evidence [histochemical staining (Fig. 5A and B), respiration measurements (Table 2), western blots (Fig. 5C), and activity measurements (Table 5)] indicate that the complex I mutations impair the assembly of functional complex IV.

**nuo-1 mutants are hypersensitive to oxidative stress**

Reactive oxygen species are generated as a normal by-product of mitochondrial respiration and can damage cellular proteins, lipids and DNA (38). Mitochondrial dysfunction can result in accelerated rates of reactive oxygen species generation (39).

We subjected the nuo-1 mutants to two forms of oxidative stress, hyperoxia and the herbicide, paraquat. Paraquat is believed to increase the production of superoxide, although its site of action is not known. All three mutants are hypersensitive to hyperoxia, with the A352V mutant being particularly so (Fig. 6A). Similarly, mutant animals were significantly more sensitive to 0.2 M paraquat than the wild-type (Fig. 6B). The sensitivity of the A352V mutant but not the T434M or the A443F mutants to hyperoxia could be significantly reduced by supplementation with either sodium dichloroacetate or riboflavin (Fig. 6C). Sodium dichloroacetate increased the survival of all three mutants challenged with paraquat (Fig. 6D), while riboflavin improved survival of the A352V and the A443F mutants.

### DISCUSSION

For most mitochondrial diseases, the molecular mechanisms of pathogenesis are only poorly understood, in part because of the rarity of these diseases, which limits the availability of materials for detailed investigations. In this study, we mimicked pathogenic human mutations of the NDUFV1 gene by using the *C. elegans* homolog, the *nuo-1* gene. The *nuo-1* A352V,
T434M and A443F mutations complement the lethality of the *nuo-1* deletion background, resulting in viable strains that could be analyzed in detail. The ability to tolerate mutations that would otherwise be lethal in more complex organisms may be an additional advantage of the nematode model system.

Reproduction and development in *C. elegans* are energy-intensive and rely heavily on MRC function (22,25,28,40). All three mutants suffered decreased brood sizes, with the A443F mutation having the most severe effects and the T434M mutation having the mildest. The A352V mutant exhibited a significant developmental lag during larval development. Morphological examination of the mutants revealed somatic gonad arms that had failed to fully extend and disorganization of the germline nuclei. Oogenesis requires the synthesis of large numbers of copies of mitochondrial DNA as well as the storage of materially-derived products needed to support embryonic and early larval development (22,40,41). It is not surprising that a normally functioning MRC is needed for reproductive fitness.

A defect in complex I may create three problems: (1) the MRC will be unable to oxidize NADH and the redox balance of the cell will be perturbed; (2) complex I-mediated proton pumping may be impaired leading to a decreased ability to synthesize ATP; (3) oxygen free radical production may be accelerated (42).

Our data strongly support a role for oxidative damage in the pathogenesis of the *nuo-1* mutations. Complex I is a major site of oxygen radical production in the MRC (38,43). The oxidation of reduced flavin by molecular oxygen can produce superoxide or peroxide products (44). The NUO-1 subunit harbors an FMN cofactor and the mutations we introduced may increase access of oxygen to the reduced flavin. All three mutants are hypersensitive to the oxidative stresses, hyperoxia and paraquat, with the A352V mutant being the most affected. We believe the mutations increase the endogenous production of oxygen free radicals making the cell’s defense mechanisms less able to deal with the additional stresses. The beneficial effects of supplementation with ascorbate, an antioxidant, on the reproductive capabilities of the A352V and A443F mutants also suggest that oxidative stress contributes to the pathogenesis of these mutations. All three transgenic mutants suffered shortened lifespans and exhibited signs of premature aging; vacuolar structures that appear to be associated with the degeneration of the body wall muscle became apparent in 1-day-old mutant adults. These structures are only found in wild-type adults near the end of their egg laying period, roughly 4 days into adulthood. Along with the early accumulation of dark pigment in the mutants, the vacuolar structures serve as biomarkers of premature senescence and are particularly prominent in the A352V and A443F mutants. Premature aging and oxidative damage are closely linked in *C. elegans* (18,39,45,46) and other organisms (47). Determining the exact site of oxygen radical production in the MRCs of the mutants will further increase our understanding of the molecular mechanisms of pathogenesis.

Inefficient oxidation of cellular NADH also contributes to pathogenesis. Lactic acidosis resulting from impaired utilization of pyruvate by the citric acid cycle, increased glycolytic utilization, and increased glycolytic activity, is a hallmark of complex I dysfunction (48) and of mitochondrial disease in general (49). The three-dimensional structure of the NUO-1 protein is not known, but the probable locations of the NADH, FMN, and iron–sulfur cluster binding sites in this protein have been suggested (3). None of the three mutations we studied are located within these sites, making it difficult to assign them a probable mechanism of action. The mutations may destabilize the 51 kDa subunit, may impede its ability to assemble into the complex, or they may affect catalysis by impairing substrate binding or electron flow. The lack of an antibody against a subunit of complex I has hampered our investigation of complex I assembly. Whatever the mechanism of action of the mutations, the oxygen consumption data indicate that the complex I mutations significantly reduce the rates of NADH-dependent respiration via the MRC. Consequently, the *nuo-1* mutants have significantly higher lactate concentrations and lactate/pyruvate ratios (3- to 6-fold; Table 3). Significant improvements in reproduction of all three transgenic mutants are seen in the presence of sodium dichloroacetate, riboflavin or thiamine. These three supplements are activators of the pyruvate dehydrogenase complex, which converts pyruvate to acetyl-coenzyme A for further metabolism in the citric acid cycle. In the presence of high levels of NADH, the pyruvate dehydrogenase complex is inactivated by the NADH-stimulated phosphorylation of the pyruvate dehydrogenase complex by the pyruvate dehydrogenase kinase (31,34). Sodium dichloroacetate inhibits the kinase. Riboflavin and thiamine are vitamin precursors for pyruvate dehydrogenase complex cofactors. Each of the supplements may stimulate the complex and lead to an increase in substrate for the citric acid cycle and a decrease in pyruvate available for reduction to lactate. Riboflavin, as a precursor to FMN, may also benefit the three mutants by directly improving complex I function. That riboflavin supplementation benefits the A352V mutant (Fig. 4) without significantly reducing its lactate/pyruvate ratio (Table 4) may be indicative of such a mechanism, but a more direct investigation of complex I catalytic properties will be needed to confirm this.

The inefficient oxidation of cellular NADH may also contribute to the hypersensitivity of the mutants to hyperoxia and paraquat. The more reducing cellular environment of the mutants is likely to facilitate the inappropriate reduction of oxygen by reduced electron carriers. The beneficial effects of sodium dichloroacetate and riboflavin on mutant survival under oxidative stress may result from the ability of these

### Table 4. Supplementation can decrease the lactate/pyruvate ratios

<table>
<thead>
<tr>
<th>Strain</th>
<th>Supplement</th>
<th>[Lactate]$^{\text{a}}$</th>
<th>[Pyruvate]$^{\text{a}}$</th>
<th>Lactate/pyruvate</th>
</tr>
</thead>
<tbody>
<tr>
<td>A352V</td>
<td>None</td>
<td>3.6±0.77</td>
<td>0.090±0.008</td>
<td>40±9.3</td>
</tr>
<tr>
<td>A352V</td>
<td>NaDCA$^{\text{b}}$</td>
<td>3.01±0.05</td>
<td>0.138±0.004</td>
<td>21.8±0.7**</td>
</tr>
<tr>
<td>A352V</td>
<td>Riboflavin</td>
<td>3.16±0.03</td>
<td>0.089±0.009</td>
<td>36±3.6</td>
</tr>
<tr>
<td>A443F</td>
<td>None</td>
<td>4.4±1.7</td>
<td>0.057±0.011</td>
<td>77±34</td>
</tr>
<tr>
<td>A443F</td>
<td>NaDCA</td>
<td>1.92±0.04</td>
<td>0.091±0.005</td>
<td>21±1.9**</td>
</tr>
<tr>
<td>A443F</td>
<td>Riboflavin</td>
<td>2.86±0.08</td>
<td>0.123±0.018</td>
<td>23±3.5**</td>
</tr>
</tbody>
</table>

$^{\text{a}}$Values are expressed in mM and represent the means±SD of a minimum of four trials.

$^{\text{b}}$NaDCA, sodium dichloroacetate.

**P < 0.01 compared with the unsupplemented sample using a two-sample t-test.
Figure 5. Analysis of cytochrome c oxidase activity and assembly in mutants and wild-type. Cytochrome c oxidase activity appears as a dark stain and is seen predominantly in the pharyngeal muscles and intestine, with some staining in the body wall muscles. Anterior and central regions of nematodes are shown. As a control, N2 animals were also treated with 10 mM KCN (N2 + inh) to inhibit COX activity. (A) Staining of L3-staged animals. Bars = 12 μm. (B) Staining of L4-staged animals. Bars = 15 μm. (C) Western blot analysis of whole-animal lysates showing relative amounts of COX I protein. Each lane represents the proteins from approximately 5000 animals. The blot was stripped and re-probed with antiserum against ATP-2. COX I levels were quantified by densitometry and normalized to ATP-2 levels. The relative abundances of COX I are indicated.
IId 132
IVh 58
minimum of five trials were performed for each assay and strain.

...complex I mutations result in a complex IV assembly defect in a model organism. We do not understand how, we believe that this is the first observation. The nematode mutants displayed features that parallel those elicited by NDUFV1 mutations, such as increased lactate/pyruvate ratios and decreased NADH-dependent mitochondrial respiration. In addition, the C. elegans mutants revealed aberrant tissue degeneration and allowed us to explore the molecular mechanisms of pathogenesis of these complex I mutations through supplementation experiments. Finally, we report a previously unrecognized connection between complex I integrity and complex IV assembly.

Table 5. Electron transport chain assays

<table>
<thead>
<tr>
<th>Assay</th>
<th>N2</th>
<th>A352V</th>
<th>T434M</th>
<th>A443F</th>
</tr>
</thead>
<tbody>
<tr>
<td>I&lt;sup&gt;b&lt;/sup&gt;</td>
<td>81 ± 5</td>
<td>24 ± 4** (30%)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>36 ± 3** (44%)</td>
<td>26 ± 4** (32%)</td>
</tr>
<tr>
<td>II&lt;sup&gt;b&lt;/sup&gt;</td>
<td>132 ± 9</td>
<td>97 ± 7* (73%)</td>
<td>119 ± 5 (90%)</td>
<td>101 ± 9* (77%)</td>
</tr>
<tr>
<td>III&lt;sup&gt;c&lt;/sup&gt;</td>
<td>58 ± 4</td>
<td>46 ± 2 (79%)</td>
<td>71 ± 5* (122%)</td>
<td>44 ± 4* (76%)</td>
</tr>
<tr>
<td>II + III&lt;sup&gt;d&lt;/sup&gt;</td>
<td>54 ± 4</td>
<td>43 ± 3 (80%)</td>
<td>76 ± 3* (141%)</td>
<td>35 ± 4* (65%)</td>
</tr>
<tr>
<td>G1P + III&lt;sup&gt;e&lt;/sup&gt;</td>
<td>24 ± 1</td>
<td>19 ± 1 (79%)</td>
<td>25 ± 2 (104%)</td>
<td>11 ± 2** (46%)</td>
</tr>
<tr>
<td>IV&lt;sup&gt;f&lt;/sup&gt;</td>
<td>58 ± 4</td>
<td>27 ± 3** (47%)</td>
<td>44 ± 3* (76%)</td>
<td>33 ± 3* (57%)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Enzyme activities (nmol substrate min<sup>-1</sup> mg<sup>-1</sup> protein) are means ± SD. A minimum of five trials were performed for each assay and strain.
<sup>b</sup> Complex I function was measured as the rotenone-sensitive NADH-decylubiquinone reductase activity.
<sup>c</sup> Complex II function was measured as the malonate-sensitive succinate-decylubiquinone reductase activity.
<sup>d</sup> Complex III function was measured as the antimycin A-sensitive succinate-cytochrome c oxidase activity.
<sup>e</sup> Complex IV function was measured as the antimycin A-sensitive glycerol-1-phosphate-dichlorophenolindophenol reductase activity.
<sup>f</sup> The combined function of the glycerol–phosphate dehydrogenase and complex I were measured as the antimycin A-sensitive decylubiquinone reductase activity.
<sup>g</sup> The combined function of complexes II and III were measured as the antimycin A-sensitive succinate–cytochrome c reductase activity.
<sup>h</sup> The combined function of the glycerol–phosphate dehydrogenase and complex III were measured as the antimycin A-sensitive glycerol-1-phosphate-cytochrome c reductase activity.
<sup>i</sup> Complex IV function was measured as the cyanide-sensitive cytochrome c oxidase activity. *P < 0.001; **P < 0.0001 compared with N2 wild-type activity using two-sample t-test.

Supplements to stimulate NADH oxidation and produce a less reducing cellular environment (Fig. 6C and D).

Surprisingly, the nuo-1 mutations result in a complex IV deficiency as judged by decreased cytochrome c oxidase activities (Table 5) and by lower steady-state levels of COXII subunit (Fig. 5). How can missense mutations in complex I affect complex IV activity? In yeast mutants that affect the assembly of complex IV, lower levels of the mitochondrial DNA-encoded COXI, II and III are often observed (50,51). In mammalian mitochondria, the majority of complex I is physically associated with complex III and a minority is present in a supercomplex of complexes I, III and IV (52). Supercomplexes have been postulated to enhance substrate channeling, catalysis and the sequestration of reactive intermediates that may lead to the production of oxygen free radicals (52). Complex III levels in the mutants are not severely affected. This argues against a role for supercomplex formation in the complex IV decreases we observed. Although we do not understand how, we believe that this is the first demonstration of defined complex I mutations causing a complex IV assembly defect in a model organism.

In conclusion, we have shown that human disease-causing mutations in the 51 kDa subunit of complex I can be successfully modeled in C. elegans. The nematode mutants displayed features that parallel those elicited by NDUFV1 mutations such as increased lactate/pyruvate ratios and decreased NADH-dependent mitochondrial respiration. In addition, the C. elegans mutants revealed aberrant tissue degeneration and allowed us to explore the molecular mechanisms of pathogenesis of these complex I mutations through supplementation experiments. Finally, we report a previously unrecognized connection between complex I integrity and complex IV assembly.

**MATERIALS AND METHODS**

### Strains

Worms were cultured as described (12). We used the following C. elegans strains: wild-type N2 Bristol; LB77, nuo-1(ua1)/mhl1 [dpv-10(e128) mls14]/Il, unc-119 (ed3)/III.

### Plasmid constructs

The nuo-1(ua1) mutation has been described previously (22). The A352V, T434M and A443F mutations were constructed by oligonucleotide-directed mutagenesis using a megaprimer mutagenesis method by introducing the following codon conversions: GCC to GTC, ACT to ATG and GCA to TTC, respectively (53). All mutations were confirmed by sequencing. The nuo-1 gene cassette consisted of a 5.4 kb XhoI genomic

**Figure 6.** The effects of oxidative stress on wild-type and transgenic mutant strains. Survival was scored after 5 days and was defined as the percentage of L1 larvae that developed into adults. A minimum of 50 animals were scored. Each value is the mean of five trials. (A) L1 larvae were placed onto seeded NGM plates and incubated at 22°C in either atmospheric (gray bars) or 100% oxygen (black bars). (B) L1 larvae were placed onto seeded NGM plates with no (gray bars) or 0.2 m parquat (black bars) and incubated at 20°C. (C) L1 larvae were placed onto seeded NGM plates and incubated at 22°C in 100% oxygen without added supplement (black bars), with 5 µg/ml sodium dichloroacetate (light gray bars), or with 1 µg/ml riboflavin (dark gray bars). (D) L1 larvae were placed onto seeded NGM plates with 0.2 m parquat without added supplement (black bars), with 5 µg/ml sodium dichloroacetate (light gray bars), or with 1 µg/ml riboflavin (dark gray bars). *P-values were derived using two-sample t-test. **P < 0.001 compared with wild-type in (A) or (B) or to the non-supplemented control in (C) or (D).
fragment from cosmid C09H10. The mutated nuo-1 genes, including the promoter, were cloned at the unique XbaI site into pDP#MM016b, which carries an unc-119(+) gene (23,54).

Generation of transgenic C. elegans

LB77 was transformed by microparticle bombardment using a BioRad Biostic PDS-1000/HE (BioRad Laboratories, Hercules, CA, USA) as described (23). Following bombardments, worms were allowed to recover at 20°C. unc-119 mutants, which are paralyzed, cannot form dauer larvae and die in the absence of food. After 7–14 days, plates were examined for dauer animals with wild-type motility. We confirmed transformation with unc-119(+) and the nuo-1 transgenes by PCR analysis. Three independent transgenic lines were selected for each mutation.

Phenotypic analyses

For lifespan measurements, gravid adults were allowed to lay eggs on unseeded plates for 6 h at 20°C. Once hatched, L1 animals were transferred to separate plates and incubated at 20°C. Animals were monitored daily and scored as dead when they no longer responded to gentle prodding on the head. Animals were transferred daily during egg laying to keep them separate from their progeny.

To determine brood sizes, individual L4 animals were transferred to separate plates, monitored daily during egg laying, and transferred to fresh plates to keep them separate from their progeny. Plates with eggs were incubated an additional 24 h to allow hatching and unhatched eggs and larvae counted. This continued until adult animals no longer laid eggs.

Freshly hatched L1 animals were transferred to separate plates and incubated at 20°C. Animals were monitored every 12 h and scored as L4 larvae when the crescent-shaped immature vulval structure was observed. For morphological analysis, animals were mounted on 2% agarose pads and observed under a Zeiss Axioskop-2 research microscope with a SPOT-2 digital camera (Carl Zeiss Canada Ltd, Calgary, Canada).

Isolation of mitochondria

All procedures were performed at 0–4°C. Worms were harvested from liquid culture and washed repeatedly in S Basal Complete medium to remove bacteria (55). Washed worms were suspended in MSEP buffer (0.2 M mannitol, 70 mM sucrose, 0.1 M EDTA, pH 7.4, 1 mM phenylmethylsulfonyl fluoride) and disrupted with 0.1 mm acid-washed glass beads in a Bead-Beater homogenizer (Biospec Products, Bartlesville, OK, USA) for three 30 s pulses separated by 1 min cooling intervals. Immediately following disruption by the Bead-Beater, the slurry was homogenized by 10 strokes of a glass–glass homogenizer. The homogenate was twice centrifuged (1000g, 10 min) to remove cuticle and large debris. The supernatant was centrifuged (19 200g, 10 min) to pellet mitochondria. The mitochondrial pellet was resuspended in fresh MSE (0.2 M mannitol, 70 mM sucrose, 0.1 M EDTA, pH 7.4) and recentrifuged. The final pellet of mitochondria was resuspended in 0.5 ml MSE. Protein content was determined by the BioRad Protein Assay (BioRad Laboratories, Hercules, CA, USA) using BSA as a standard.

Oxygen consumption rate measurements

Oxygen consumption rates were measured using a Strathkelvin 1302 oxygen electrode with an MT200 Mitocell respiration chamber (Strathkelvin Instruments Ltd, Glasgow, UK). Synchronized worms at the late L4 to early adult stages were washed free of bacteria using M9 buffer (55). Washed worms were resuspended in 1 ml M9 buffer and triplicate aliquots placed on glass coverslips for counting. Worms were diluted to 500 worms per 50 μl volume, five-hundred worms were introduced into the Mitocell chamber, which was maintained at 22°C, and oxygen consumption was measured for a minimum of 10 min. The slopes of the linear portions of the plots were used to calculate oxygen consumption rates. A minimum of three trials were performed for each strain.

Isolated mitochondria were diluted 10-fold in 0.1 M HEPES, pH 7.4 and disrupted by sonication for 2–3 min in a Branson 1200 bath sonicator (Branson Ultrasonics Corp., Danbury, CT, USA) in an ice-water bath. Sonicated mitochondria were centrifuged (18 500g, 30 min, 4°C) and resuspended in fresh 0.1 M HEPES, pH 7.4. Twenty micrograms of mitochondrial protein were diluted to a volume of 60 μl and introduced into the Mitocell chamber. Basal levels of oxygen consumption were recorded for 2–4 min, followed by the addition of NADH to a final concentration of 600 μM. Rotenone was added to a final concentration of 100 nM and oxygen consumption recorded for 5–10 min. Complex I-specific NADH-dependent respiration was calculated as the rate of oxygen consumption in the presence of NADH minus the rate in the presence of NADH and rotenone. A minimum of five experiments was performed for each sample.

Electron transport chain assays

Enzymatic activities of individual or pairs of MRC complexes were performed on an Ultraspec 2000 spectrophotometer (Pharmacia Biotech, Cambridge, UK), essentially as described (56) using 25–50 μg of disrupted mitochondrial protein. Rotenone-sensitive NADH-decyIubiquinone oxidoreductase activities were measured at 340 nm using 65 μM 2,3-dimethoxy-5-methyl-6-n-decyl-1,4-benzoquinone as electron acceptor, 2 μg/ml antimycin A, and 0.13 mM NADH. Rotenone was added to 2 μg/ml. Malonate-sensitive succinate– dichlorophenolindophenol reductase activity was measured at 600 nm after preincubating mitochondria in assay medium (2.5 mM KH2PO4, 0.05 mM EDTA, 10 mM succinate, 1.6 mM KCN, 1 mg/ml BSA, pH 7.4) in the presence of 2 μg/ml rotenone and 5 μg/ml antimycin A for 10 min at 30°C. Reactions were started by the addition of 50 μM decylubiquinone and 75 μM dichlorophenolindophenol. Malonate was added to 25 mM. Antimycin A-sensitive decylubiquinol–cytochrome c reductase activity was measured at 550 nm after preincubating mitochondria in assay medium (25 mM KH2PO4, 5 mM MgCl2, 2.5 mg/ml BSA, 2 mM KCN, pH 7.4) in the presence of 50 μM cytochrome c (III), and 2 μg/ml rotenone for 10 min at 30°C. Assays were started by adding 80 μM decylubiquinol. Antimycin A was added to 5 μg/ml. Succinate–cytochrome c reductase activity
was measured at 550 nm after preincubating mitochondria in assay medium (0.1 M Tris–SO₄, pH 7.4, 2 mM KCN, 1 mg/ml BSA) in the presence of 10 mM succinate and 2 µg/ml rotenone for 10 min at 30°C. Assays were started by the addition of 50 µM cytochrome c (III). Antimycin A was added to 5 µg/ml. Glycerol–phosphate–cytochrome c reductase was measured as for succinate–cytochrome c reductase activity, except 20 mM glycerol-1-phosphate was added instead of 10 mM succinate. Cytochrome c oxidase activity was measured at 550 nm using 15 µM cytochrome c (II) as electron donor in the presence of 0.45 mM lauryl maltoside. Potassium cyanide was added to 1 mM.

**Supplementation assays**

Individual L4-staged transgenic animals were transferred to plates supplemented with 5 µg/ml sodium dichloroacetate, 0.1 mM ascorbate, 1 µg/ml thiamine, 1 µg/ml riboflavin, 1 mg/ml oxaloacetate, or without supplement and incubated for 7 days at 20°C in the dark. These concentrations of supplements were chosen as optimal after a series of smaller scale trial experiments. Animals were monitored daily during egg laying and transferred to fresh plates to keep them separate from their progeny. Only hatched progeny were scored. A minimum of 24 broods were counted for each strain and condition.

**Measurements of lactate and pyruvate concentrations**

Worms cultured in liquid medium with or without supplementation were harvested and aliquots counted to determine approximate worm concentrations of cultures. Approximately 1.0 × 10⁷ animals were washed repeatedly to remove bacteria and resuspended in a final volume of 30 ml of 5% trichloroacetic acid. Worms were sonicated on ice with four 30 s pulses interspersed with 30 s cooling periods using a Branson Sonifier 450 (Branson Ultrasonics Corp., Danbury, CT, USA) set to a power level of 450 W. The lysate was centrifuged (10 000 g, 450 (Branson Ultrasonics Corp., Danbury, CT, USA) set to a power level of 450 W. The lysate was centrifuged (10 000 g, 4°C) to pellet precipitate and debris. The supernatant was neutralized with 5 N KOH and clarified with a second centrifugation (10 000 g, 10 min, 4°C) to pellet precipitate and debris. Potassium cyanide was added to 1 mM.

**Cytochrome c oxidase histochemistry**

Cuticles were permeabilized for histochemical staining as described (57). The cytochrome c oxidase staining protocol was adapted for *C. elegans* from Sciaccio and Bonilla (58). Briefly, synchronized animals with permeabilized cuticles were incubated in 1 ml phosphate buffer (5 mM potassium phosphate, pH 7.4) containing 0.1% 3,3’-diaminobenzidine, 0.1% cytochrome c, and 0.02% catalase, for 75 min at 37°C in the dark with constant rotation. Control reactions were incubated in the presence of 10 mM KCN to inhibit complex IV activity. Stained worms were centrifuged for 10 s (360g) and the supernatant was removed. The worm pellet was washed three times for 5 min in phosphate buffer. Stained animals were mounted and photographed.

**Electrophoresis and western blot analyses**

Known numbers of worms were washed, lysed by boiling in loading buffer, and subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (22). Proteins were transferred electrophoretically to nylon membranes. Blots were treated with mouse monoclonal antiserum against the human COXI protein (Molecular Probes, Eugene, OR, USA). Detection was with a peroxidase-labeled goat anti-mouse secondary antibody and the Enhanced Chemiluminescence Western Blotting System (Amersham Biosciences, Bucks, UK). Blots were stripped and re-probed with rabbit polyclonal antiserum raised against *Saccharomyces cerevisiae* ATP2p (22). The relative amounts of protein were quantified from films using the BioRad Gel Doc 1000 image analysis system and Molecular Analyst software (BioRad Laboratories, Hercules, CA, USA).

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