The Caenorhabditis elegans clk-1 mutants lack coenzyme Q₈ and instead accumulate the biosynthetic intermediate demethoxy-Q₉ (DMQ₉). clk-1 animals grow to reproductive adults, albeit slowly, if supplied with Q₈-containing Escherichia coli. However, if Q is withdrawn from the diet, clk-1 animals either arrest development as young larvae or become sterile adults depending upon the stage at the time of the withdrawal. To understand this stage-dependent response to a Q-less diet, the quinone content was determined during development of wild-type animals. The quinone content varies in the different developmental stages in wild-type fed Q₈-replete E. coli. The amounts peak at the second larval stage, which coincides with the stage of arrest of clk-1 larvae fed a Q-less diet from hatching. Levels of the endogenously synthesized DMQ₉ are high in the clk-1(qm30)-arrested larvae and sterile adults fed Q-less food. Comparison of quinones from animals fed a Q-replete or a Q-less diet establishes that the Q₈ present is assimilated from the E. coli. Furthermore, this E. coli-specific Q₈ is present in mitochondria isolated from fertile clk-1(qm30) adults fed a Q-replete diet. These results suggest that the uptake and transport of dietary Q₈ to mitochondria prevent the arrest and sterility phenotypes of clk-1 mutants and that DMQ₉ is not functionally equivalent to Q.

Ubiquinone, or coenzyme Q (Q),¹ is a polyprenylated benzoquinone lipid that is predominantly associated with the inner mitochondrial membranes of eukaryotes. The number of prenyl groups in the tail is species-specific. Escherichia coli contains Q₈ and Caenorhabditis elegans contains Q₉, where the subscript designates the number of isoprene units in the tail. Q has an essential role in mitochondrial electron transport from complex I and II to III (1). Q also acts as the following: a lipid-soluble antioxidant (2); a component of plasma membrane electron transport (3); a component of uridine synthesis (4); and as a component of the proton-pumping function of the uncoupling proteins UCP1, UCP2, and UCP3 (5, 6). Q is synthesized within mitochondria in a series of steps that is initiated by linking the polyisoprenyl tail to 4-hydroxybenzoic acid. The head group is then modified in a series of hydroxylation, methylation, and decarboxylation steps to form the final product (7). A defect in hydroxylation, late in the biosynthetic pathway, causes accumulation of demethoxy-Q₈ (DMQ₈) in E. coli (8), and a similar function was attributed to the COQ7/CAT5 locus in Saccharomyces cerevisiae (9–11). Studies on prokaryotic homologues of COQ7 suggest that the encoded polypeptide is a membrane-bound di-iron carboxylate protein that catalyzes the hydroxylation of DMQ to demethyl-Q (12).

Mutation of the clk-1 gene in C. elegans, a homologue of COQ7, leads to slow embryonic and post-embryonic development, slow adult behaviors, and reduced brood sizes under standard culture conditions (13, 14). clk-1 mutants have a defect in Q biosynthesis, and they accumulate the biosynthetic intermediate demethoxy-Q₉ (DMQ₉) instead of producing Q₉ (15). However, mitochondrial function in clk-1 mutants fed a standard E. coli diet is only slightly diminished compared with wild-type when assayed by Rhodamine-6G uptake, NADH-cytochrome c reductase, and succinate-cytochrome c reductase activities (15, 16). These results have been interpreted as indicating that DMQ₉ is a functional substitute for Q in the respiratory chain and that the CLK-1 phenotype results from some non-mitochondrial function of Q. These conclusions contrast with previous analyses of DMQ₉ function in E. coli in which partial function of complex I is observed, but DMQ₉ is inactive in succinate dehydrogenase (complex II) activity (17). Significantly, the C. elegans studies employed nematode cultures that were grown on media containing OP50 E. coli, which provides a dietary source of Q₈. The clk-1 mutant animals fed a Q-less diet as hatchlings arrest development as L2 larvae (18). If the Q-less diet is fed at a later stage, the clk-1 mutant dauer larvae develop into sterile adults.

The relationship between the withdrawal of dietary Q₈ and the severe clk-1 mutant phenotypes of sterility and larval arrest is not understood. To investigate this question, we quantified the amounts of accumulating quinones at different developmental stages of C. elegans. These experiments establish the normal developmental pattern of Q accumulation in wild-type nematodes and provide a basis for the analysis of defects present in the clk-1 mutants. The quinone content of N2 and clk-1(qm30) animals at two different stages of development fed either Q-replete (OP50) or Q-less E. coli was determined. We found that Q₈ is present in the lipid extracts of OP50-fed nematodes and the levels diminish greatly when they are moved to a Q-less diet. This decline in Q₈ levels is observed when either hatchlings or dauer larvae are fed Q-less E. coli. In the first case, Q₈ is only supplied maternally in the egg, whereas in the second the Q₈ derives from a Q-replete diet fed prior to dauer formation. In both situations, the clk-1(qm30)
mutants fed the Q-less diet contain large amounts of DMQ9 yet are developmentally arrested in the first case or are sterile adults in the second. This suggests that DMQ9 is inadequate to sustain larval growth or germ line development. The clk-1(qm30) mutants complete development to fertile adults only when adequate levels of Q8 are attained. Finally, we found that mitochondria isolated from both N2 and clk-1(qm30) animals fed OP50 contain diet-derived Q8. Therefore, uptake of dietary Q8 and its delivery to mitochondria appear to be essential for both development and fertility of clk-1 mutants.

EXPERIMENTAL PROCEDURES

Culture Conditions—C. elegans were cultured in S medium (liquid) (0.1 M sodium chloride, 0.05 M potassium phosphate, pH 6, 5 mg/L cholesterol, 0.01 M potassium citrate, pH 6, 3 mM calcium chloride, 3 mM magnesium sulfate, 50 mM EDTA, 25 mM ferrous sulfate, 10 mM manganese chloride, 10 mM zinc sulfate, 1 mM copper sulfate) and fed E. coli (19). Handling methods and solutions were standard (19). C. elegans strains used were N2 (Bristol strain) (20) and MQ130 clk-1(qm30) (13). The E. coli strains used as food sources are listed in Table I.

Isolation of Nematode Developmental Stages— Cultures of gravid adults were treated with alkaline hypochlorite solution to obtain eggs (19). The collected eggs were allowed to hatch without food to obtain syncytial larvae. For the N2 developmental stages, L1 larvae were fed OP50 and grown to the desired stage. To confirm the developmental stages, cells in the gonadal or vulval lineage were scored using differential interference optics (21, 22). The N2 strain used in the study of the above developmental stages was obtained from the Caenorhabditis Genetics Center. All subsequent analyses were done with N2 animals obtained from Dr. Hekimi (McGill University, Canada).

For the timed larval feeding experiments, starved N2 and clk-1(qm30) L1 larvae were fed either Q-replete or Q-less E. coli for 8 h at 20 °C and then harvested. In addition, a sample of arrested clk-1(qm30) larvae was harvested after 30 h of feeding Q-less E. coli. For samples of post-dauer 2 (PD2) and young adult animals, dauer larvae were isolated with 1% SDS, and young adults were isolated from the bacterial pellet as in Ref. 18 and separated by HPLC, grown in LB with high aeration overnight. Total lipid extracts were collected from the bacterial pellet as in Ref. 18 and separated by HPLC, and the peak fraction corresponding to DMQ9 was collected and dried down. The sample was differentially extracted by MeOH/petroleum ether, and the petroleum ether layer was removed, dried, and resuspended in 100 μl of 9:1 MeOH/EtOH. The concentration of DMQ9 was calculated by recording the absorbance of an ethyl acetate solution at 271 nm and by using the extinction coefficient ε271 = 14,500 (15). The chromatographic areas recorded for DMQ9 were used to extrapolate the amount of DMQ9 in clk-1(qm30) animals, because the mole/area ratios are identical. The relative ratios comparing milligrams of wet weight to milligrams of dry weight and milligrams of wet weight to milligrams of protein were determined. Wet weights were measured for L2, L4, and young adult stages, and then these samples were dried overnight in a Speedvac. Dry weights were determined. Finally, the pellets were resuspended in 1 mM NaOH and assayed for protein concentration by the bicinchoninic acid assay. The ratios of milligrams of wet weight to milligrams of dry weight were 6.6 ± 0.6, and the milligrams of wet weight to milligrams of protein ratios were 11.8 ± 0.5. These suggest that for these various stages, the animals have similar water content.

Mitochondrial Isolation—New cultures were inoculated at a concentration of 2000 dauer larvae/ml S medium, fed OP50, and incubated at 20 °C until the animals reached adulthood. The animals were cleaned by sucrose flotation and washed thoroughly. The nematodes were resuspended in isolation buffer (IB: 210 mM mannitol, 70 mM sucrose, 0.1 mM EDTA, 5 mM Tris, pH 7.4) with 1 mM PMSF at 10 ml per 5 g of nematodes. The samples were kept cold on ice throughout the fractionation procedure. The animals were homogenized with a Kontes ground glass tissue grinder (Fisher catalogue number KSS6450-0025) using 15 strokes. The volume was increased to 25 ml with IB + 1 mM PMSF and centrifuged at 750 × g for 10 min. The supernatants were saved, and another 10 ml of IB + 1 mM PMSF was added to the pellets. The pellets were resuspended and homogenized again with another 15 strokes, and the volumes were increased to 25 ml and centrifuged at 750 × g for 10 min. The homogenates were microscopically examined to verify disruption. An aliquot of the combined supernatants was saved as total lysate. The supernatants were centrifuged at 12,000 × g for 10 min. An aliquot of the resulting supernatant was saved as post-mitochondrial supernatant. The mitochondrial pellets were gently resuspended in 12 ml of IB. The mixture was centrifuged at 750 × g × 10 min. The supernatants were collected, avoiding the pellets, and were centrifuged at 12,000 × g for 10 min. The final mitochondrial pellets were resuspended in IB. All subcellular fractions were stored at −20 °C until use.

Western Analysis—Fractions were assayed for protein concentration by the bicinchoninic acid assay. Western analysis of 40 μg of protein from the total lysates, the post-mitochondrial supernatants, and the mitochondrial fractions from N2 and clk-1(qm30) and 2.5 μg and 1 μg samples of protein from an OP50 lysate were performed by electrophoresis on 12% Tris-glycine gels, followed by transfer to Hybond ECL nitrocellulose. The primary antibody to yeast Fb2-α-ATPase was used at a 1:5000 dilution, and the primary antibody to E. coli cytochrome c oxidase was used at a 1:200 dilution. Although generated against the holocytochrome c oxidase, this antibody only reacted well with subunit II, at 35 kDa. Horseradish peroxidase-linked secondary antibodies to rabbit IgG were used in a 1:2000 dilution.

Identification of Quinones by Atmospheric Pressure Chemical Ionization Mass Spectrometry—A PerkinElmer Life Sciences Sciex (Thornhill, Canada) API III triple quadrupole mass spectrometer was tuned and calibrated as previously described with an Ion Spray™ source using a solution of polyethylene glycol (29). The manufacturer-supplied atmospheric pressure chemical ionization (APCI) source consisting of a heated nebulizer and a corona discharge needle was then installed and operated at 450 °C in the flow injection mode using methanol as the flowing solvent (0.2 ml/min). Dried HPLC fractions were redissolved in MeOH/water/petroleum ether (1/0.5/1, all v/v), vigorously mixed, and then centrifuged, and the petroleum ether layer was removed to a clean

Table I

| Strain   | Genotype | Reference |
|----------|----------|-----------|
| OP50     | ura      | (20)      |
| GD1      | H2W722, ubiG | KanR | (49) |
| JC7623A-1| JC7623, ubiE | KanR | (50) |
| JC831    | ubiF     | (49) |
| MU1227   | MM386, ubiA | CamR | (51) |
| AN78     | Hfr, metB, ubiF | (8) |
container and dried in a stream of nitrogen. The dried sample was redissolved in an appropriate volume of methanol/ethanol (9/1, v/v), typically 100 µl for the putative Q₈ fraction and 400 µl for the putative DMQ₈ fraction) and injected into the methanol stream that was flowing into the heated nebulizer (20 µl/injection). Positive ion spectra were collected (profile mode) by scanning quadrupole 3 from m/z 150–850 (0.3-Da step size, 2.46 s/scan, orifice 70 V). Fragment ion spectra under MS/MS conditions were obtained for quadrupole 1 pre-selected parent ions by scanning quadrupole 3 (m/z 50–850, 0.3-Da step size, 2.81 s/scan, orifice voltage 70 V) while the collision chamber (quadrupole 2) was flooded with argon (CGT instrumental setting of 200). Representative spectra were computed as the average of all the spectra acquired from each sample injection using instrument-supplied software (MacSpec, version 3.3, PE Sciex, Ontario, Canada).

RESULTS

Quinone Levels Vary with Development and Temperature—To establish the normal pattern of quinone accumulation throughout development, N2 populations were fed standard Q-replete OP50 and harvested at various life stages, lipids were extracted, and quinone content was quantified. C. elegans larvae proceed through four molts, designated as the four larval stages L1, L2, L3, and L4, before reaching young adulthood. Fig. 1A shows the quinone content of N2 harvested at the stages of L1, L2, L3, and L4, before reaching young adulthood. When expressed as picomoles of quinone per mg wet weight, there was an overall increase in the levels of all the quinones at the L2 larval stage, followed by a decrease at L4. Quinone content is higher in young adults than in L4. Analyzed on a separate day, Fig. 1B shows that the quinone content in L1 larvae feeding for 4 h is higher than that in eggs. The absolute response from the HPLC/ECD system for the same standard samples varied from day to day, as is typical (24). However, within one working day the response variation was smaller, as indicated by the error bars. Consequently, samples were analyzed in batches that could be accommodated within one working day and are presented together in the graphs.

Quinone amounts were found to be affected by temperature. N2 animals were reared at either 16.5 °C or 25 °C for multiple generations to avoid a maternal effect (Fig. 1C). Q₈ levels in young adults were higher at 25 °C as compared with 16.5 °C (6.94 ± 0.08 pmol/mg wet weight at 25 °C versus 4.33 ± 0.12 pmol/mg wet weight at 16.5 °C). Rhodoquinone-9 (RQ₉) levels at 25 °C were 8.90 ± 0.28 pmol/mg wet weight, whereas the level was lower, 5.47 ± 0.14 pmol/mg wet weight, at 16.5 °C. Q₉ amounts were approximately two times higher at 25 °C at 49.45 ± 1.90 pmol/mg wet weight as compared with 22.80 ± 0.39 pmol/mg wet weight at 16.5 °C.

Long-term Feeding of Q-less Food to wild-type populations—To investigate the extent to which the diet contributes to Q₈ content, wild-type animals were fed Q-less E. coli for several generations. N2 were divided into cultures containing either Q-replete OP50, or the Q-less foods GD1 or JCGΔ-1. Small populations were expanded two times sequentially in Q-less food to avoid lingering effects from the Q-replete food. The lipids were extracted from dauer larvae harvested from these cultures, and the quinones were quantified by HPLC/ECD. Representative data from animals fed either OP50, GD1, or JCGΔ-1 are displayed in Fig. 2A. In the presence of Q-less food, Q₈ levels dropped to 0.24 ± 0.02 pmol/mg wet weight for the GD1 or JCGΔ-1-fed strains, respectively. In contrast, RQ₈ and Q₉ amounts increased dramatically as compared with those fed OP50. For instance, the OP50-fed animals contained 23.56 ± 0.65 pmol Q₈/mg wet weight, whereas N2 fed GD1 or JCGΔ-1 accumulated 32.25 ± 0.70 pmol Q₈/mg wet weight and 32.77 ± 1.64 pmol Q₉/mg wet weight, respectively. Interestingly, if one compares the combined pmol Q₈ + Q₉ totals, there is no difference in total amount of Q in these animals (Fig. 2B). There may be a compensatory response to the lack of dietary Q₈ by which wild-type animals increase endogenous Q₉ production.

The clk-1 Developmental Arrest Results from Insufficient Q—All of the clk-1 mutant alleles fail to grow beyond the L2 stage when fed Q-less E. coli from hatching (18). To determine the types and levels of Q isomers present at the point of growth arrest, synchronized starved L1 larvae were transferred to either Q-replete or Q-less food and analyzed as a function of...
time. Concurrent cultures of clk-1(qm30) and control N2 eggs were collected and allowed to hatch overnight in the absence of food. In N2 animals, the level of Q₈ dropped to 0.09 ± 0.02 pmol/mg wet weight and the amount of RQ₉ and Q₉ increased in these starved L1 larvae relative to the amounts in the eggs (Fig. 3A). The RQ₉ levels increased from 0.28 ± 0.02 to 6.82 ± 0.15 pmol/mg wet weight while the Q₉ amounts increased from 7.12 ± 0.17 to 27.33 ± 0.72 pmol/mg wet weight. Upon feeding with OP50 for 8 h, Q₈ levels increased to 1.38 ± 0.18 pmol/mg wet weight while Q₉ levels decreased to 11.87 ± 1.41 pmol/mg wet weight. N2 fed either of two Q-less E. coli strains, GD1 (ubiG⁻) or MU1227 (ubiA⁻), showed slightly greater amounts of Q₈ than those fed OP50 (20.25 ± 0.15 and 14.53 ± 0.85 pmol/mg wet weight, respectively), while the Q₉ levels remained below the limit of detection. clk-1(qm30) mutants displayed a similar pattern, but produced DMQ₉ rather than Q₉ (Fig. 3B). When the clk-1(qm30) larvae remained in the Q-less food beyond 8 h, they arrested at the L2 stage, as previously described (18), which allowed a later time point to be collected. Quinone measurements of the arrested larvae that had been fed Q-less E. coli for 30 h showed a distinct increase in RQ₉ and DMQ₉ levels, from 4.25 ± 0.39 pmol RQ₉/mg wet weight and 9.98 ± 0.03 pmol DMQ₉/mg wet weight at the 8-h time point to 9.05 ± 0.19 pmol RQ₉/mg wet weight and 15.85 ± 0.06 pmol DMQ₉/mg wet weight after 30 h. The Q₈ levels on the ubiG⁻ Q-less food were 0.15 ± 0.05 and 0.12 ± 0.04 pmol/mg wet weight for the 8- and 30-h time points, respectively. Q-less E. coli strains with defects in other genes necessary for E. coli biosynthesis were fed to the clk-1(qm30) larvae, because these E. coli accumulated different biosynthetic intermediates. The clk-1(qm30) larvae fed MU1227 (ubiA⁻) and JC7623-1 (ubiE⁻) showed similar RQ₉ and DMQ₉ increases at the 30-h time point (data not shown). In contrast, clk-1(qm30) mutants fed Q₉-replete E. coli contained 0.58 ± 0.02 pmol Q₉/mg wet weight and less RQ₉ (2.82 ± 0.26 pmol/mg wet weight) and DMQ₉ (7.02 ± 0.11 pmol/mg wet weight) at the 8-h time point and were able to progress through development. Therefore, the critical difference that enables growth of clk-1 mutants appears to be the uptake of sufficient Q₉ from the E. coli diet.

Sterility of clk-1 Adults Results from Inadequate Q—clk-1 mutants will form dauer larvae under crowded culture conditions if fed OP50. If these dauer larvae are fed Q-less food, they will recover and grow to sterile adults (18). The quinone levels of these sterile adults were compared with quinone levels present in N2 dauer larvae fed either Q-replete or Q-less E. coli during recovery. Fig. 4 shows data from animals harvested at PD2 and young adulthood. PD2 morphologically resemble L4 (25). The quinone contents of N2 animals fed either Q-less E. coli (GD1 (ubiG⁻), JC7623-1 (ubiE⁻), or JCG1 (ubiG⁻)) or OP50 were similar, except for the 10-fold decline in Q₈ accumulation in nematodes cultured on the Q-less foods (Fig. 4, A}

**Fig. 3. Larval feeding of Q-replete and Q-less food.** N2 wild-type (A)- and clk-1(qm30) (B)-starved larvae were isolated and fed either Q-replete (OP50) or the Q-less GD1 (ubiG⁻) or MU1227 (ubiA⁻) food for either 8 h or 30 h, as noted. The picomoles of quinones/mg wet weight was quantified by HPLC/ECD. Error bars represent standard deviation from three separate injections of each sample, and data shown are representative of six experiments for N2 and five experiments for clk-1(qm30) experiments. The clk-1(qm30) larvae remained arrested at the L2 stage through the 30-h time point.

**Fig. 4. Recovery and growth from the dauer stage in Q-less versus Q-replete food.** N2 (A and B) and clk-1(qm30) (C and D) dauer larvae were allowed to recover in either Q-replete (OP50) or Q-less (GD1 (ubiG⁻), JCG1-1 (ubiG⁻), JC7623-1 (ubiE⁻)) food and were harvested at the PD2 and young adult stages. Their lipids were extracted, and the quinones were quantified by HPLC/ECD as in Fig. 1.
and B). OP50-fed N2 young adults contained 2.31 ± 0.02 pmol of Q9/mg wet weight. GD1-fed young adults contained 0.33 pmol ± 0.01 of Q9/mg wet weight, and the JC7623Δ-1 (ubiE') and JCGA1 (ubiG')-fed strains contained only 0.09 ± 0.01 and 0.03 ± 0.01 pmol of Q9/mg wet weight, respectively. Q9 levels were slightly growing from PD2 to young adulthood. This same trend was seen when the animals were fed Q-less JC7623Δ-1 (ubiE') or JCGA1 (ubiG') E. coli (Fig. 4B). Interestingly, uptake and/or retention of compounds from the diet may be fairly specific. Q9 was detected in animals fed Q-replete E. coli, but the quinone intermediate (2-methoxy-6-octaperyl-1,4-benzoquinone) that accumulated in the bacterial strain JC7623Δ-1 (ubiE') was not detected in either N2 or clk-1 nematodes (data not shown). The clk-1(qm30) animals produced profiles similar to N2, but DMQ9 was synthesized rather than Q9 (Fig. 4, C and D). The amount of DMQ9 ranged from 25–32 pmol/mg wet weight when the clk-1(qm30) animals were fed Q-less food. The fertile clk-1(qm30) animals contained 4.61 ± 0.48 pmol of Q9/mg wet weight, whereas the sterile animals contained 0.24 ± 0.03, 0.10 ± 0.01, or 0.34 ± 0.02 pmol of Q9/mg wet weight when fed the Q-less foods GD1 (ubiG'), JC7623Δ-1 (ubiE'), or JCGA1 (ubiG'), respectively. The data indicate that fertility of the clk-1 mutants depends upon uptake of dietary Q9.

clk-1 Mitochondria Contain Q9—N2 and clk-1(qm30) adults fed OP50 were isolated, homogenized, and fractionated to separate a mitochondria-enriched fraction from the total nematode lysate. Antibodies generated to the yeast Atp2 polypeptide (the β subunit of F1-ATPase) were previously found to recognize the homologous ATP2 polypeptide of C. elegans (26). Western analysis showed that the mitochondrial protein F1-ATPase present in the total lysate was absent from the post-mitochondrial supernatant and was enriched in the mitochondrial fraction (Fig. 5A). In addition, the nematode subcellular fractions did not show detectable levels of the E. coli marker cytochrome o oxidase, indicating that the samples are substantially free of E. coli contamination. Hence, the quinones present in these samples do not derive from E. coli cells either present in the gut or adhering to the surface of the nematodes. Lipids from each fraction were then separated by HPLC and analyzed by ECD for its quinone content per mg protein (Fig. 5, B and C). The mitochondrial fraction contained the majority of the quinones, with Q9 levels in N2 reaching 3.2 nmol/mg of protein and DMQ9 amounts in clk-1(qm30) at 2.9 nmol/mg. The Q9 levels in the mitochondria are substantial, at 0.65 nmol/mg of protein in N2 and 0.45 nmol/mg of protein in clk-1(qm30). Therefore, not only is dietary Q9 present in total lipid extracts of whole animals, but Q9 is also present in isolated mitochondria.

The structural assignments of DMQ9 and Q9 isolated from clk-1(qm30) mitochondrial lipid extracts were verified by APCI mass spectrometry and tandem mass spectrometry. The APCI mass spectra of authentic Q9 (Sigma) and DMQ9 (purified from AN78) yielded intense signals at m/z 795.6 and 697.5, respectively, corresponding to their protonated molecular ions (calculated 795.6291 and 697.5560 Da, respectively, Fig. 6, A and B). MS/MS on these parent ions (Fig. 6, A and B, insets) yielded intense diagnostic fragment ions at m/z 197.0 and 167.0, respectively, corresponding to tropolone ions of the polar head groups (calculated 197.0814 and 167.0708, respectively). The APCI mass spectra of the putative Q9 and DMQ9 samples (Fig. 6, C and D) isolated from clk-1(qm30) mitochondrial lipid extracts showed the analogous ions at m/z 727.5 and 765.6, respectively, corresponding to the predicted protonated molecules (calculated 727.5665 and 765.6186 Da, respectively). Furthermore, MS/MS on these parent ions (Fig. 6, C and D, insets) yielded the expected fragment ions at m/z 197.3 and 167.0, respectively, in agreement with the predicted corresponding head groups.

DISCUSSION

Knowledge of quinone production and uptake throughout C. elegans development is necessary for understanding the phenotypic differences of clk-1 mutants fed a diet lacking or containing a source of Q. Here we present the temporal analysis of the quinone types and amounts in wild-type animals from
transition after the L1 stage (29). There is high activity of the glyoxylate pathway during embryogenesis, but this decreases during the L1 stage (30). Upon maturation to L2 larvae, there is an increased reliance on trichloroacetic acid cycle metabolism, which continues through the L3 and L4 stages (29). The arrest at the L2 larval stage of the clk-1 mutant fed Q-less food from hatching coincides temporally with this metabolic shift. The L2 developmental arrest, with L2-like gonads, is not a unique phenotype for the Q-deficient clk-1 mutants, because similar arrests are seen in animals harboring null mutations in genes encoding a subunit of complex I, *nuo-1(ua1)*, or of complex V, *atp-2(ua2)*) (26). Other phenotypic similarities for *clk-1, *nuo-1, and *atp-2* mutant animals include slowed movements, slowed pharyngeal pumping, and slowed defecation. In addition, a maternal effect has been described for each of these mutants. Maternal contributions allow survival through embryogenesis for the *nuo-1* and *atp-2* mutants, because the phenotype produced by RNA-mediated interference of these genes is embryonic lethality (26). Thus, *clk-1* mutant animals reared on Q-less food contain DMQ9 and RQ9 and behave as if they are respiratory-deficient.

However, other investigators have offered a different interpretation. The assertion has been made that DMQ9 supports mitochondrial respiration in *clk-1* mutant mitochondria (15, 31). These studies demonstrated the function (or partial function) of DMQ9 in complexes I, II, and III and emphasized that oxygen consumption rates are nearly normal in mitochondria isolated from *clk-1* mutant nematodes. The rescue of growth arrest of *clk-1* mutant nematodes by a Q-replete diet is attributed to the inability of DMQ to replace Q at a non-mitochondrial site, because Q derived from the diet was considered incapable of being transported into mitochondria (31, 32). Although activity of respiratory complexes in mitochondria isolated from *clk-1* mutant nematodes was found to be nearly normal (15, 16), these animals had been fed a standard diet containing Q8, and neither the possible contribution of dietary Q8 nor endogenously produced RQ9 to assays of respiration was considered. Assays performed with *melk-1* mutant ES mouse cell extracts (which contain DMQ9 but no RQ9) showed profound defects in complex II + III activity (32). Homozygous

The L2 developmental arrest of *clk-1* mutants fed Q-less food from hatching also coincides with increased Q production in wild-type, but there is no corresponding increase in mitochondria reported for this stage. Energy metabolism in wild-type *C. elegans* is regulated during larval development, with a key

**Fig. 6. Identification of quinones isolated from clk-1 (qm30) mitochondria.** APCI mass spectrometry and MS/MS of (A) authentic Q8 purchased from Sigma, and (B) DMQ9 isolated from AN78 used as a standard. Fractions collected from HPLC separation of lipids isolated from *clk-1(qm30)* mitochondria were identified as (C) Q8 and (D) DMQ9. Displayed here are the molecular an regions of each spectra, and in the insets are the regions of the MS/MS spectra showing the corresponding diagnostic headgroup fragment ions.
clk-1 mutant animals display an embryonic lethal phenotype (32, 33).

Recently, a knockout mutation in the nematode gene coq-3 was observed to be maternal effect larval lethal (31). coq-3 homozygotes, from a heterozygous mother, develop slowly, are small, and most are sterile. The sterility of coq-3 mutants, and of clk-1 mutants fed Q-less E. coli, may be due to a similar mechanism that we believe to be related to biogenesis of germ line mitochondria. The dietary contribution of Q8 does not rescue the lethality of the coq-3 null mutant from homoygous mothers (31). The difference in severity between coq-3 and clk-1 null mutants may derive from the biosynthetic step affected. CoqQp has been shown to be responsible for both O-methylation steps in Q biosynthesis (34) and results in the production of a very early non-redox active intermediate. Additionally, it is likely that these coq-3 mutants lack not only Q9 and DMQ9, but also RQ8, because the biosynthetic pathways of Q9 and RQ8 probably share most early steps (35). Given the potential complete lack of quinones, the coq-3 mutant larvae that do hatch may lack energy to power muscle contraction of the pharynx and thereby fail to take up sufficient Q8 from the diet due to an inability to feed.

As shown here, arrested clk-1 mutants fed a Q-less diet contain DMQ9 and RQ8, and levels of both quinones continue to rise over time (Fig. 4). It is possible that RQ8 could partially compensate for the defect in complex II by operating fumarate reductase in reverse (36). Normally, those eukaryotes that can survive periods of anoxia utilize RQ and fumarate reductase as an essential step of malate dismutation (37). It is presumably the operation of malate dismutation that enables wild-type C. elegans to survive 1–2 days of anaerobic treatment (38). Indeed, it is likely that survival of the clk-1 mutants up to and during the L2 larval arrest depends upon RQ8 and fumarate reductase functioning in both its intended forward direction in malate dismutation and in reverse to produce fumarate from succinate. Importantly, such RQ8 function is still unable to support development of the clk-1 mutant larvae on Q-less diets past the L2 stage or to sustain reproduction. Hence, despite increasing levels of RQ8, the clk-1 mutant larvae remain arrested as L2 larvae until Q8 is supplied. Therefore, although dietary Q8 may indeed serve functions at non-mitochondrial sites, it is reasonable to expect that it also serves crucial functions within the mitochondria.

Q is involved in numerous cellular processes both within and outside of the mitochondria (39), and it is possible that DMQ9 fails to functionally replace Q at multiple sites. In addition to a role in complexes I, II, and III of respiratory electron transport, Q is required for the operation of glycerol-3-phosphate dehydrogenase, fatty acid β-oxidation, pyrimidine synthesis at the dihydroorotate dehydrogenase step (40), and the detoxification of sulfide by sulfide dehydrogenase (40), and it is a substrate of lipoamide dehydrogenase, a component of the Krebs cycle (41) and dihydroorotate dehydrogenase step (4), and the detoxification of sulfide by sulfide dehydrogenase (40), and it is a substrate of lipoamide dehydrogenase, a component of the Krebs cycle (41). The studies presented here indicate that Q content in C. elegans is regulated during development, in response to temperature of cultivation and in response to diet. Dietary Q8 decreased adult lifespan in C. elegans (48), and the model presented relied upon uptake and delivery of Q8 to the mitochondria, which has been substantiated here. The key aspects of metabolism that are altered with the environmental and genetic manipulations that alter Q content remain to be defined in development and aging.

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Development and Fertility in *Caenorhabditis elegans* *clk-1* Mutants Depend upon Transport of Dietary Coenzyme Q to Mitochondria

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