Molecular Mechanism of Maternal Rescue in the clk-1 Mutants of Caenorhabditis elegans*

Received for publication, August 4, 2003, and in revised form, September 24, 2003 Published, JBC Papers in Press, September 29, 2003, DOI 10.1074/jbc.M308507200

Jason Burgess, Abdelmadjid K. Hibi‡, Claire Y. Bénard‡, Robyn Branicky¶, and Siegfried Hekimi

From the Department of Biology, McGill University, Montréal, Quebec H3A 1B1, Canada

The clk-1 mutants of Caenorhabditis elegans display an average slowing down of physiological rates, including those of development, various behaviors, and aging. clk-1 encodes a hydroxylase involved in the biosynthesis of the redox-active lipid ubiquinone (co-enzyme Q), and in clk-1 mutants, ubiquinone is replaced by its biosynthetic precursor demethoxyubiquinone. Surprisingly, homozygous clk-1 mutants display a wild-type phenotype when issued from a heterozygous mother. Here, we show that this maternal effect is the result of the persistence of small amounts of maternally derived CLK-1 protein and that maternal CLK-1 is sufficient for the synthesis of considerable amounts of ubiquinone during development. However, gradual depletion of CLK-1 and ubiquinone, and expression of the mutant phenotype, can be produced experimentally by developmental arrest. We also show that the very long lifespan observed in daf-2 clk-1 double mutants is not abolished by the mitochondrial HPLC. This suggests that, like developmental arrest, the increased lifespan conferred by daf-2 allows for depletion of maternal CLK-1, resulting in the expression of the synergism between clk-1 and daf-2. Thus, increased adult longevity can be uncoupled from the early mutant phenotypes, indicating that it is possible to obtain an increased adult lifespan from the late inactivation of processes required for normal development and reproduction.

The clk-1 gene of Caenorhabditis elegans encodes a 187-amino acid protein that is orthologous to yeast Coq7p as well as to rodent and human sequences (1). CLK-1 is necessary for ubiquinone (coenzyme Q, or Q) biosynthesis. Q, is a prenylated benzoquinone (the numerical subscript denotes the number of isoprene repeats, which is a species-specific trait) that is primarily involved in numerous redox reactions in the cell, including those in the mitochondrial electron transport. In its reduced form, Q is an antioxidant, preventing lipid peroxidation in biological membranes (2, 3). In the absence of CLK-1, worms and mice are defective in Q biosynthesis (4–6) and accumulate the Q precursor, demethoxyubiquinone (DMQ9) (5, 6). Yet, mitochondrial respiration is not strongly affected in clk-1 mutants, indicating that DMQ9 can substitute for some, but not all, of the Q functions in vivo (5–9).

Despite the presence of DMQ9, clk-1 mutants require Q from their bacterial food source to proceed through development and become fertile adults (4, 10). Indeed, when clk-1 mutants are transferred onto a Q9-deficient E. coli strain before the early larval stages, they arrest development at the second larval stage, and when the animals are transferred onto Q9-deficient bacteria later in development, they become sterile adults. This fact strongly suggests that DMQ9 cannot functionally replace Q9 for all of its cellular roles, and that at least some Q is required, which can be provided as Q8 in the diet. Thus, the viability and overall good health of the clk-1 mutants seems to rely on a combination of endogenously produced DMQ9 and dietary Q8.

It is currently unclear how the substitution of Q9 for DMQ9 relates to the Clk phenotype, especially given the relatively high mitochondrial respiration observed in these mutants. However, the increased lifespan of clk-1 mutants could be directly caused by the absence of Q9. Indeed, the altered redox properties of DMQ9 might reduce the production of reactive oxygen species (11), whose most abundant source is the Q reaction intermediate semiquinone (12). Moreover, the level of ROS production has previously been implicated in lifespan determination in the worm (13, 14). Alternatively, the effect on lifespan could be secondary to the severe developmental and reproductive phenotypes caused by the lack of endogenous Q production. Indeed, clk-1 mutant worms develop slowly and have a markedly reduced brood size (15), whereas disruption of mouse clk-1 results in embryonic lethality, suggesting that Q is essential for mammalian development as well (6, 16). Thus, one of the difficulties in interpreting the consequences of manipulating endogenous Q production to obtain effects on lifespan is that the effect on lifespan could be only secondary. Here, we propose that, in maternally rescued animals, the effect of clk-1 mutations on adult lifespan is uncoupled from their effects on development and reproduction. We show that this uncoupling is produced by the presence of maternally contributed CLK-1 protein and endogenously produced Q9 during larval development and early adult life, followed by a depletion of ubiquinone later in life, which leads to an increased adult lifespan. Given that almost all developmental and reproductive phenotypes are fully wild type in maternally rescued animals and that a long lifespan is still observed, this excludes the early phenotypes as being necessary for longevity.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† Supported by the Swiss National Foundation.
‡ Supported by scholarships from the Natural Sciences and Engineering Research Council of Canada and the Faculty of Graduate Studies of McGill University.
¶ Supported by a McGill Major Fellowship.
* A Canadian Institutes of Health Research Investigator. To whom correspondence should be addressed; Dept. of Biology, McGill Univ., 1205 Dr Penfield Ave., Montréal, QC H3A 1B1, Canada. Tel.: 514-398-6440; Fax: 514-398-1874; E-mail: Siegfried.Hekimi@McGill.ca.
1 The abbreviations used are: Q, ubiquinone; Q, prenylated benzoquinone with n = the number of isoprene repeats; DMQ9, demethoxyubiquinone; HPLC, high pressure liquid chromatography; DMQ92, Q9-deficient ubiB knockout bacterial strain; GD1, Q-deficient ubiG knockout bacterial strain; OP50, standard E. coli strain.
The brood sizes of all other genotypes on all bacterial strains, except for the clk-1 m+ brood sizes on OP50, which are not significantly different (p = 0.78).

The durations of development of clk-1 m+ animals on OP50, which are considered to be the wild-type durations, are not significantly different (p < 0.05) from those of ubiG (both clk-1 m+ and ubiG), which have a wild-type phenotype, were singled and allowed to lay eggs for 10 h on an unseeded plate supplemented with ampicillin (50 μg/ml) to prevent bacterial growth. The parents were washed off the plate using sterile M9 medium supplemented with 0.5% glucose and 50 μg/ml kanamycin. When working with DM123 and GD1, nematode growth media plates were supplemented with 50 μg/ml kanamycin to prevent contamination from other bacterial strains. The following strains and mutations were used: N2 (Bristol strain), clk-1(qm30) III, dpy-17(e164) III, unc-32(e189) III, and daf-2(e1370) III.

Phenotypic Characterization—clk-1 m+ animals were identified as those animals that produced slow growing progeny. All animals were raised at 20 °C and switched to 25 °C just after the final molt into adulthood. Aging was performed as described (21).

Western Blot Analysis—Approximately 2000 clk-1 m+ animals were used to separate the samples for Western blot analysis. Approximately 2000 clk-1 qm30 mutant animals were also collected as negative controls.

Animals were denatured in SDS sample buffer and 100 μl dithiothreitol by boiling at 85 °C for 5 min. Protein samples were run on an acrylamide gel and transferred onto a nitrocellulose membrane (Bio-Rad). The membrane was incubated with the primary anti-CLK-1 antibody 2774 (22) (1:1000), and subsequently with donkey anti-rabbit antibody (The Jackson Laboratory, 1:2500). The secondary antibody was detected by using the ECL kit (Amersham Biosciences).

HPLC Assay for Ubiquinone Quantification—Collecting Worms—To obtain a large number of clk-1 m+ animals for HPLC analysis, the strain clk-1(qm30) unc-32(e189) was used to separate maternally rescued animals from their heterozygous and wild-type sib worms by selecting for the severe Unc phenotype. Approximately 1000 heterozygous hermaphrodites of the genotype clk-1(qm30) unc-32(e189) were picked and allowed to perform a 12-h limited egg laying (100 animals/large plate). These parents were then washed off the plate leaving the eggs behind. The synchronized progeny were raised to the adult stage and were then collected by rinsing plates with M9 buffer. To separate the maternally rescued animals from their heterozygous and wild-type sibs, a total of 150 μl of worms suspended in M9 buffer was applied to the center of a large plate (15 cm) that was seeded with food around the edges. Wild-type and heterozygous animals were able to move toward the food, while homozygous clk-1(qm30) unc-32(e189) m- animals were left in the center of the plate because of their severe Unc phenotype. These worms were collected by cutting out the center of the plate and washing the worms off the agar on to a clean plate.

The selection strategy was then repeated a second time, resulting in a worm prep that contained over 95% clk-1 m+ maternally rescued animals. Worm pellets were then frozen at −80 °C until ubiquinone extraction was performed.

The worm strain unc-32(e189) was used as a Qa biosynthesis control and the strain clk-1(qm30) unc-32(e189) was used as a DMQ biosynthesis control. Control strains used were synchronized by allowing 100 gravid hermaphrodites to lay eggs for 10 h on a single large plate, with a total of 40 large plates used for each genotype. At the end of the 10-h limited laying period, parents were washed off the plate with M9 buffer, leaving the eggs behind on the plate. Progeny were then allowed to grow to adulthood and were collected by washing the plates with M9.

<table>
<thead>
<tr>
<th>Bacterial food source</th>
<th>clk-1+/+</th>
<th>clk-1 m+</th>
<th>Developmentb</th>
<th>Developmentb</th>
</tr>
</thead>
<tbody>
<tr>
<td>OP50</td>
<td>310 ± 36</td>
<td>114 ± 23</td>
<td>60.2 ± 7.0e</td>
<td>80.7 ± 3.5e</td>
</tr>
<tr>
<td>ubiB</td>
<td>253 ± 30</td>
<td>(12')</td>
<td>58.9 ± 7.0</td>
<td>n.a.</td>
</tr>
<tr>
<td>(DM123)</td>
<td>(12)</td>
<td>(&gt;100)</td>
<td>(57)</td>
<td>(33)</td>
</tr>
<tr>
<td>ubiG</td>
<td>278 ± 40</td>
<td>0</td>
<td>57.7 ± 4.43</td>
<td>n.a.</td>
</tr>
<tr>
<td>(GD1)</td>
<td>(12)</td>
<td>(100)</td>
<td>(56)</td>
<td>(23)</td>
</tr>
</tbody>
</table>

* The brood sizes of animals whose parent was transferred on the named food source and who have thus undergone their entire development on this food source.

* The length of development from egg to the adult molt was measured.

* The brood sizes of clk-1 m+ on OP50 bacteria, which are considered to be the wild-type brood sizes, are significantly different at p < 0.05 from those of all other genotypes on all bacterial strains, except for the clk-1 m+ brood sizes on OP50, which are not significantly different (p = 0.78).

* The sample size is large because no animal was observed to produce progeny.
The process of determining the elution profile for this compound was described (33). Worm extracts were completed to 1 ml with water and raised on either food source, even after prolonged periods of L1 arrest. As described previously, clk-1 m− mutants showed a reduced brood size when raised on a Q-replete food source, independently of the length of arrest, and were fully sterile on a Q-deficient food source. clk-1 m− maternally rescued animals were fully rescued for brood size when raised on a Q-replete food source, and prolonged arrest did not affect brood size. clk-1 m− animals raised on a Q-deficient food source were able to produce a wild-type brood size but exhibited a progressive decline in brood size after increasing length of L1 arrest.

Worms were then washed as outlined above to remove contaminating E. coli. Worm pellets were then frozen at −80 °C until ubiquinone extraction was performed.

**Quinone Extraction**—A worm sample of around 500 μl was sufficient to obtain enough Q for HPLC analysis. Q extraction was performed as described (33). Worm extracts were completed to 1 ml with water and then transferred to a 50-ml plastic tube. 50 μl of 2,6-di-tert-butyl-4-methyl-phenol (Aldrich) suspended in ethanol (10 mg/ml) was added to the worm sample to prevent auto-oxidation of Q. This sample was homogenized for several minutes at maximum speed by using a handheld homogenizer. 1 ml of 0.1% SDS was then added to the mixture to denature the sample. A second round of homogenization was then performed. After this, 2 ml of ethanol were added to the tube to collect the homogenized, denatured worm mix by vortexing for 30 s. This mixture was then transferred to a corex tube. 2 ml of hexane was added, and the tightly capped test tube was vigorously vortexed for 2 min. The sample was then centrifuged for 5 min at 2200 rpm, and 1.5 ml of the hexane organic supernatant layer was transferred to a 1.5-ml Eppendorf tube. The hexane extraction was then repeated a second time. The combined hexane extracts were then evaporated using a heated speed vacuum. Samples were then re-suspended in a total of 70 μl of the methanol/ethanol (70:30) mobile phase for HPLC analysis. Samples were kept on ice and run within 2 h of extraction to keep ubiquinone degradation to a minimum.

**HPLC Analysis**—HPLC analysis was performed by using a methanol/ethanol (70:30) mobile phase solvent. This mixture was boiled for 30 min to eliminate air bubbles and then allowed to cool to 40 °C, which was then maintained throughout the HPLC experiment using a heat plate. Samples were eluted at a rate of 2 ml/min and were analyzed at a wavelength of 275 nm. The ubiquinone standard Q9 (Sigma) was used to determine the elution profile for this compound.

**RESULTS**

**Maternally Rescued clk-1 Mutants Can Complete Larval and Reproductive Development When Raised on Q-deficient Bacteria**—clk-1 mutants raised on E. coli strains defective in Q8 biosynthesis arrest development or become sterile, depending on the time of transfer onto the Q-deficient food source (4, 10). This phenotype is likely the most direct consequence of the Q9 biosynthesis defect of clk-1 mutants, as the wild-type and other long-lived mutant strains have normal growth and reproduction on a Q-deficient food source (10). Thus, the ability to complete growth and produce progeny on Q-deficient E. coli reflects the endogenous Q status of the animal (10, 23). Consequently, we assayed the ability of clk-1 maternally rescued animals to complete growth and produce a full brood on a Q8-deficient food source.

As reported previously, we found that clk-1(qm30) mutants raised from embryogenesis on the Q8-deficient E. coli strains DM123 (a ubiB knockout strain) or GD1 (a ubiG knockout strain) arrest at the L2 stage (4, 10). Although this arrest is fully penetrant, it is not absolute, as the clk-1 mutants start to exit the L2 arrest after ~1 week, and almost all animals subsequently become sterile adults. The late recovery from arrest has not been reported previously. Yet, we observed this phenomenon consistently for all conditions under which the mutants arrest. Presumably, this was previously missed because, as the animals do not proceed through the developmental stages slowly but are fully arrested for at least a week, there was no reason to suspect that they would resume development and eventually all reach adulthood. On the other hand, clk-1(qm30)/+ heterozygotes and maternally rescued clk-1(qm30) mutants raised on DM123 or GD1 are able to complete larval development at a normal rate under these conditions (Table I). Moreover, there is no obvious developmental lag for the maternally rescued mutants when raised on DM123 or GD1. This strongly suggests that maternally rescued clk-1 mutants possess sufficient amounts of endogenous Q9 to complete larval development.

We next asked if maternally rescued clk-1 mutants raised from embryogenesis onward on a Q8-deficient diet would be able to produce progeny. In agreement with previous results, we found that clk-1(qm30) mutants raised on the standard OP50 bacterial strain have a reduced mean brood size compared with the wild type (15), but that clk-1(qm30) mutants raised on the Q8-deficient strains DM123 or GD1 are fully sterile (Table I, Fig. 1, Refs. 4, 10). Moreover, we found that clk-1 maternally rescued animals are able to produce a large brood (>200) when raised on either OP50, DM123, or GD1 (Table I). Thus, maternally rescued clk-1 mutants can complete reproductive development when raised on Q-deficient bacteria.
clk-1 Maternally Rescued Animals Have Measurable Levels of Ubiquinone—The observation that clk-1 maternally rescued animals are fully rescued for both growth and fertility on a Q-deficient food source strongly suggests that clk-1 maternally rescued animals have amounts of endogenous Q₉ that are sufficient for normal function. To investigate this further, we performed HPLC analysis to determine the Q₉ and DMQ₉ content of maternally rescued clk-1 mutants as well as of the wild-type and clk-1 mutant controls at the young adult stage (Fig. 2). Wild-type control animals show a peak eluting at ~690 s, which corresponds to the elution time of the Q₉ standard (682 s). The clk-1 mutants exhibit a peak eluting earlier at ~651 s, which corresponds to DMQ₉. clk-1 maternally rescued animals exhibit two peaks at 650 and 686 s, corresponding to DMQ₉ and Q₉, respectively.

In summary, it seems that maternally rescued clk-1 mutants have Q₉ and are likely able to produce it. The presence of DMQ₉ could suggest that there may not be sufficient CLK-1 activity at the adult stage to fully catalyze the conversion of DMQ₉ to Q₉. Consistent with the latter view, clk-1 maternally rescued animals are fully rescued for all adult behaviors with the exception of the egg-laying rate, which is similar to that of clk-1 mutants (15).

**Fig. 2.** Q₉ is detected in clk-1 maternally rescued animals. HPLC chromatograms of quinones eluted at a rate of 2 ml/min in a mobile phase of methanol/ethanol (70:30) and measured at λ₂₇₅ nm and expressed in mV are shown. A total of 500 μl of unc-32(e189) (wild-type controls), 400 μl of clk-1(qm30) unc-32(e189) m⁻ (clk-1 mutants), and 400 μl clk-1(qm30) unc-32(e189) m⁻ (clk-1 maternally rescued animals) were used. unc-32 animals were used as a Q₉ control. The vertical line indicates the elution time of the Q₉ standard. unc-32 controls exhibit a peak eluting at ~690 s, which corresponds to the elution time of the Q₉ standard (682 s). The clk-1 mutants exhibit a peak eluting earlier at ~651 s, which corresponds to DMQ₉. clk-1 maternally rescued animals exhibit two peaks at 650 and 686 s, corresponding to DMQ₉ and Q₉, respectively.
Maternal Rescue in clk-1 Mutants

CLK-1 Protein Is Present throughout Larval Development in Maternally Rescued clk-1 Mutants—The level of Q9 present in maternally rescued mutants is similar to that of the wild type, suggesting that this Q9 cannot, in its majority, correspond to the Q9 found in the oocyte that gave rise to the animal. Therefore, either CLK-1 protein or some alternative enzyme must be present in maternally rescued mutants. To explore this, we used an anti-CLK-1 antiserum and carried out immunoblot analysis to detect CLK-1 in developmentally staged animals. We collected the same number of worms at each developmental stage and for each genotype to compare the absolute amounts of CLK-1 protein per animal, rather than the amounts relative to total protein content (Fig. 3). We found that CLK-1 protein was clearly detectable in maternally rescued animals at all four larval stages. The amount of CLK-1, however, was considerably lower than in wild-type animals. Moreover, in the wild type, the amount of CLK-1 per animal increased during development, indicating that CLK-1 is actively synthesized. In contrast, the total amount of CLK-1 in maternally rescued animals appeared constant, indicating that there is little degradation of CLK-1 during larval development, despite the very active larval growth. This suggests that the detected protein is contributed maternally. Although no clk-1 mRNA can be detected in maternally rescued mutants (data not shown), it remains possible that CLK-1 was produced in maternally rescued animals because of the presence of undetectable levels of a maternally derived transcript. However, none of the CLK-1 protein detected in maternally rescued clk-1 mutants could be produced from the zygotic genome, as the qm30 mutation is a partial deletion of the gene that would result in a truncated protein, and no CLK-1 protein at all is detected in clk-1(qm30) mutants (Fig. 3), as previously described (22). In conclusion, the presence of maternally derived wild-type CLK-1 protein in clk-1 maternally rescued animals likely accounts for the maternal rescue.

Prolonged Arrest of Maternally Rescued clk-1 Mutants at the L1 Larval Stage Results in a Partial Clk Phenotype—Given the above, the maternal effect observed with clk-1 mutants is likely based on wild-type clk-1 product being contributed to the embryo by the mother. To test this hypothesis further, we devised a scheme to deplete maternally derived clk-1 activity. When worms hatch from the egg, they are developmentally arrested until they succeed in feeding. In the absence of food, they remain arrested and can survive for relatively long periods of time in this state without damage. We arrested clk-1 maternally rescued worms and controls by withholding food for 3, 6, and 9 days (see “Experimental Procedures”). Beyond 9 days of arrest, the health of even wild-type animals started to deteriorate markedly. Surprisingly, even after 9 days of arrest, maternally rescued clk-1 mutants were still able to complete post-embryonic development at a rate comparable with that of wild-type animals, although the overall rate of development was markedly slower for all genotypes. In contrast, we could observe a gradual shortening of the defecation cycle period of maternally rescued mutants as a function of the numbers of days of arrest, although arrest did not affect the length of the defecation period of either wild-type animals or clk-1 mutants (Fig. 4). These observations indicate that, when given sufficient time, gradual loss of clk-1 activity occurs and produces a gradual phenotypic effect, suggesting that maternally rescued aging animals gradually develop the CLK-1 phenotype.

It is unclear why even 9 days of arrest does not affect the maternal rescue of the rate of post-embryonic development. One possibility is that post-embryonic development is intrinsically less sensitive to the level of clk-1 activity than the defecation cycle. Additionally, the effects of L1 arrest (longer periods of arrest result in longer developmental rates for all genotypes) might mask subtle effects on the rate of development. Another possibility is that the process of post-embryonic growth itself is required to dilute clk-1 activity sufficiently (there is a >500-fold increase in volume from the L1 stage to the adult) to produce mutant phenotypes in the fully grown animal.

Prolonged Arrest of Maternally Rescued clk-1 Mutants at the L1 Stage Results in a Gradual Depletion of Q9.—The technique of depleting maternally derived product by arresting at the L1 stage would not allow for the collection of a sufficient number of maternally rescued animals to measure directly the level of CLK-1 or Q9. However, it is reasonable to assume that the inability of clk-1 mutants to grow on a Q-deficient food source is a direct reflection of their content of endogenous quinones. Therefore, we tested the ability of maternally rescued mutants and controls to produce progeny when fed only on Q-deficient bacteria since the beginning of post-embryonic development and having previously undergone L1 arrest for various periods of time (Fig. 1). Arrest at the L1 stage produced a small decrease in brood size for all genotypes after 3 days (p < 0.01), even when grown on Q-replete bacteria, but the duration of the arrest had only a negligible effect. As described above, when raised on OP50, clk-1 mutants have a much reduced brood size, whereas clk-1/+ animals and maternally rescued mutants have a wild-type brood size. Furthermore, as expected, when grown on Q-deficient bacteria, clk-1/+ have a wild-type brood size that is insensitive to the duration of L1 arrest, and clk-1 homozygotes produce no progeny at all. The brood size of maternally rescued mutants, however, gradually decreases with the length of L1 arrest, such that the brood size significantly decreases with each 3 days of arrest (p < 0.05). This indicates that endogenous ubiquinone, and the ability to synthesize ubiquinone, is exhausted over time in these animals.

Maternally Rescued daf-2 clk-1 Double Mutants Have Increased Adult Longevity Despite Fast Physiological Rates during Development—Most phenotypes of clk-1 mutants are almost completely rescued in homozygous mutant animals issued from heterozygous mothers (15). However, the increase in adult lifespan of clk-1 mutants is not dramatic (~30%), and previous experiments that examined the consequences of the maternal effect on adult lifespan were inconclusive (15). To better investigate this question, we studied clk-1 daf-2 double mutants (21). The effect of clk-1 mutations on lifespan is greatest in combination with mutations in daf-2, as mutations in clk-1 can approximately double the lifespan increase of daf-2 mutants (14, 21, 24).

We first tested whether the clk-1 phenotype could be maternally rescued in daf-2 clk-1 double mutants. We found that both the duration of post-embryonic development and the pe-
riod of the defecation cycle of homozygous daf-2 clk-1 double mutants issuing from mothers homozygous for daf-2 but heterozygous for clk-1 was indistinguishable from those of daf-2 (Fig. 5, A and B). This finding is consistent with the otherwise wild-type phenotype of clk-1(qm30)/clk-1+) heterozygote animals. However, for lifespan, the synergism between clk-1 and daf-2 was still observed when the clk-1 mutant phenotypes were maternally rescued (Fig. 5C). clk-1(qm30) still increases the lifespan of daf-2 mutants by a considerable amount (>9 days). However, the lifespan increase is not as large as in the fully mutant situation (>16 days).

These findings suggest that the increased lifespan conferred by daf-2 allows for the depletion of maternal CLK-1 in adults, which results in the expression of at least part of the synergism between clk-1 and daf-2. We found that although there was Q in maternally rescued adults, only a few days of developmental arrest were sufficient to deplete it completely (Figs. 1 and 4). We can therefore speculate that after a few days of adult life, DMQ has replaced Q. Because daf-2 mutants live for a long time by themselves, the double mutants can benefit from the presence of DMQ for a large fraction of their lives and thus live even longer.

The difference of lifespan between double mutants and maternally rescued double mutants may also be due to the considerably greater fertility of maternally rescued daf-2 clk-1 animals in comparison to the full mutants. Indeed, we observed that the rescued double mutants showed high fertility, whereas the non-rescued mutants are frequently almost sterile (after being shifted to 25 °C). Fertility per se is not believed to have much effect on C. elegans lifespan. However, egg laying is likely somewhat traumatic and could have a greater effect on the lifespan of very long-lived mutants that endure damage from the environment for a longer period of time.

**DISCUSSION**

The nematode C. elegans has been widely employed as a model system to investigate the molecular mechanisms of aging, particularly because mutations that increase lifespan can be readily identified in this organism (24–26). One class of such long-lived mutants is that of the maternal-effect clk genes (27), which produce a highly pleiotropic phenotype that can be described as an average slowing down of a number of physiological processes (15). The affected processes include embryonic and larval development, rhythmic behaviors such as defecation and pharyngeal pumping, as well as reproduction. Strikingly, all of these phenotypes can be fully rescued maternally, that is, the first generation of homozygous clk mutants descended from a heterozygous mother appear phenotypically wild-type (27).

At this time, 10 genes have been isolated that can mutate to give a Clk phenotype (Ref. 27 and Y. Meng and S. H., unpublished observations). It is interesting to note that although the presence of a long lifespan was not included in the screening criteria for the isolation of these mutants, all strains are long-lived. Moreover, there is a strong positive correlation between
the magnitude of the effect of clk mutations on slowing physiological rates (as determined by the rate of larval development) and on adult lifespan extension (21). For instance, double mutant combinations of clk genes result in a further lengthening of development as well as of adult lifespan. Thus, it seems that a slow life is sufficient for an increased lifespan. This finding is consistent with the rate of living theory of aging (28, 29), which predicts that aging is the consequence of an imperfect balance between molecular damage production and its repair. This suggests that when physiological rates are slowed down, as in the case of clk mutants, molecular damage accumulation will occur at a slower rate, resulting in an increased lifespan (24). However, given that a number of critical life history parameters are affected in clk mutants, including the rate of development and brood size, it is not clear which (if any) of these alterations are necessary for the increase in adult lifespan of clk mutants, or whether these traits can be uncoupled from adult lifespan altogether. We have investigated this question by studying the effect of the maternal rescue on the lifespan of clk-1 mutants.

clk-1 mutants fail to synthesize ubiquinone, accumulating the biosynthetic precursor demethoxyubiquinone instead. Almost all phenotypes of clk-1 homozygous mutants, including slow developmental and behavioral rates, and as we have shown now, their dependence on dietary ubiquinone for development and fertility, are rescued by a maternal effect when the homozygous mutant animals originate from a heterozygous mother. We have shown that these animals possess detectable levels of maternally derived CLK-1 protein as well as a substantial amount of ubiquinone. We have also established that the levels of endogenous ubiquinone produced in maternally rescued animals are sufficient for normal growth and fertility, as they can complete larval and reproductive development when raised on Q-deficient bacteria. We have further shown that the capacity of maternally rescued worms to produce functional levels of ubiquinone is exhausted over time, as a period of arrest of maternally rescued clk-1 mutants at the L1 larval stage results in the gradual development of a CLK phenotype, presumably a mirror of an underlying gradual depletion of Q9.

We have shown that maternally rescued clk-1 animals have an increased adult longevity despite fast physiological rates during development. Thus, in these animals, the effect of clk-1 mutations on adult lifespan is likely uncoupled from their effects on development and reproduction. This uncoupling could be produced by the presence of maternally contributed CLK-1 protein and endogenously produced Q9 during larval development and early adult life, followed by a depletion of Q9 later in life, leading to an increased adult lifespan. Thus, our results show that altering the function of clk-1 after reproductive development can result in an extended adult lifespan. Indeed, given that almost all developmental and reproductive phenotypes are fully wild type in maternally rescued animals and that a long lifespan is still observed, the early phenotypes cannot be necessary for longevity. These observations are particularly pertinent to the vertebrate situation. Indeed, mouse embryos are incapable of completing embryogenesis in the absence of nclkl activity (6), presumably because, unlike worms, they cannot subsist without endogenous ubiquinone. Our findings suggest that a late inhibition of nclkl activity that avoids the early deleterious effects, might show lifespan benefits in vertebrates.

Another example of lifespan benefits obtained by the inactivation of a gene function late in development has recently been reported (30). It was shown that the effects of daf-2 on longevity are independent of its effects on reproduction, and that reducing the function of daf-2 in adults throughout the reproductive period is sufficient to increase lifespan. daf-2 encodes an insulin receptor-like tyrosine kinase that controls a signal transduction cascade and is believed to control a regulatory switch that impinges on dauer formation, longevity, and stress resistance (25, 31). Thus, these findings (30) show that even late modulation of a regulatory pathway can have beneficial lifespan effects.

Not all manipulations that have developmental conse-
quences in addition to bringing about lifespan benefits can lead to an extended longevity when the gene function is interfered with only late in development. In fact, it has recently been shown that knocking down the respiratory function of the mitochondria by using RNA interference against nuclearly encoded subunits of the electron transport chain resulted in mostly sterile animals with stunted and slow development and behavior but an increased lifespan (32). In this case, the developmental effects are necessary for the lifespan increase, as the reestablishment of normal expression of the genes of the respiratory chain in adults, after interference early in development (in adults) does not increase lifespan. Also, even the reduction of the function of the respiratory chain late in development does not prevent lifespan increase. These findings suggest that, in this case, interference with metabolic processes increases lifespan only secondarily to a developmental effect. In contrast, the findings we present here suggest that the uncoupling of lifespan extension from undesirable phenotypes can be obtained with a basic metabolic enzyme such as CLK-1 and not only with regulatory pathways such as the daf-2 pathway.

Acknowledgments—We thank Hania Kébir for expert technical assistance and Drs. Phil Rather and Catherine Clarke for bacterial strains. We also acknowledge the Caenorhabditis Genetics Centre, which is funded by the National Institute of Health National Center for Research Resources, for providing some nematode strains.

REFERENCES