CLK-1 controls respiration, behavior and aging in the nematode *Caenorhabditis elegans*

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Mutations in the *clk-1* gene of the nematode *Caenorhabditis elegans* result in an average slowing of a variety of developmental and physiological processes, including the cell cycle, embryogenesis, post-embryonic growth, rhythmic behaviors and aging. In yeast, a CLK-1 homologue is absolutely required for ubiquinone biosynthesis and thus respiration. Here we show that CLK-1 is fully active when fused to green fluorescent protein and is found in the mitochondria of all somatic cells. The activity of mutant mitochondria, however, is only very slightly impaired, as measured *in vivo* by a dye-uptake assay, and *in vitro* by the activity of succinate cytochrome *c* reductase. Overexpression of CLK-1 activity in wild-type worms can increase mitochondrial activity, accelerate behavioral rates during aging and shorten life span, indicating that *clk-1* regulates and controls these processes. These observations also provide strong genetic evidence that mitochondria are causally involved in aging. Furthermore, the reduced respiration of the long-lived *clk-1* mutants suggests that longevity is promoted by the age-dependent decrease in mitochondrial function that is observed in most species.
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Introduction

In *clk-1* mutants, the timing of a wide range of physiological processes is deregulated (Wong *et al.*, 1995). This leads to an average lengthening of such diverse processes as the cell cycle, embryogenesis, post-embryonic development, life span and the period of rhythmic adult behaviors of *Caenorhabditis elegans*, such as swimming, pharyngeal pumping and defecation. A number of observations suggest that *clk-1* plays a regulatory role in these processes: (i) in spite of their dramatic phenotype, mutant animals do not appear sick in any way by visual inspection; (ii) the deregulation of the rate of embryogenesis leads occasionally to embryonic developmental times that are shorter than those of wild-type worms; and (iii) all phenotypes, including those measured in large adults such as behavior and life span, are fully rescued when homozygous mutants originate from a mother which carries a wild-type copy

of the gene. These observations and other evidence (Wong *et al.*, 1995) suggest that *clk-1* is involved in a pervasive mechanism (a physiological clock) that times many aspects of the worm's life.

clk-1 encodes a protein of 187 residues with high similarity to yeast Coq7p, as well as to predicted mammalian proteins (Jonassen *et al.*, 1996; Ewbank *et al.*, 1997). The protein sequences can each be split and aligned to reveal a tandemly repeated core domain of 82 residues, the TRC (tandemly repeated in CLK-1) domain. The CLK-1 protein must be phylogenetically very ancient because much of the divergence between the two domains has also been conserved in yeast, worms and rat, so that most of the similarity between the proteins in the different organisms is not due to the residues that define the TRC domain. This implies that in a common ancestor of worms, yeast and rats, the two halves of a new gene, each coding for a TRC domain, had time to diverge significantly in their function. The only prokaryotic genome in which a *clk-1* homologue has been identified so far is that of the intracellular parasite *Rickettsia prowazekii* (Andersson *et al.*, 1998), whose genome is closely related to that of mitochondria. It is likely, therefore, that the *clk-1* gene has been acquired by eukaryotes via the mitochondrial genome.

The yeast Coq7p has recently been shown to be a mitochondrial protein (Jonassen *et al.*, 1998) and is required for respiration in yeast, where it is necessary for the biosynthesis of the lipid co-factor coenzyme Q (CoQ) (also called ubiquinone). Indeed, no or very little CoQ is found in these mutants (Marbois and Clarke, 1996). However, Coq7p does not resemble any of the enzymes known to be involved in the biosynthesis of CoQ in *Escherichia coli*. The biochemical function of CLK-1 or Coq7p is therefore unknown but appears to be conserved, as both the worm and the rat genes are capable of complementing a $\Delta coq7$ yeast mutant (Jonassen *et al.*, 1996; Ewbank *et al.*, 1997). However, the phenotype of *clk-1* mutants, which are slow-living but healthy, cannot be reconciled with the notion of a total absence of respiration in these worms.

One aspect of the phenotype of *clk-1* mutants which has attracted particular attention is their increased life span (Wong *et al.*, 1995), in particular in combination with mutations in other longevity increasing pathways (Lakowski and Hekimi, 1996; Hekimi *et al.*, 1998). Indeed, although the physiological and molecular consequences of aging have been studied in great detail, it has been difficult to establish a causal relation to life span for any specific function or molecule. Very few genes, in any organism, have been rigorously shown to affect the normal process of aging. One difficulty is that aging is a phenotype of the organism as a whole, and thus the involvement of a gene in the aging process can only be rigorously established in intact organisms whose entire life span can

Table I. A CLK-1::GFP fusion protein containing the entire CLK-1 amino acid sequence and expressed from an extrachromosomal array rescues the mutant phenotype

Strain	Chromosomal genotype	Extrachromosomal array	Post-embryonic development (h) ^a	Defecation cycle length (s) ^a	Brood size (eggs) ^a	Life span (days) ^a
N2	<i>clk-1(+)</i>		53.8 ± 0.1 (99)	53.9 ± 0.6 (20)	268 ± 10 (11)	22.0 ± 0.3 (358)
MQ130	<i>clk-1(qm30)</i>		71.0 ± 0.5 (228)	87.5 ± 3.8 (20)	87 ± 12 (10)	25.1 ± 0.5 (399)
MQ563	<i>clk-1(qm30)</i>	<i>qmEx130[clk-1::gfp]</i>	54.2 ± 0.4 (114)	59.8 ± 0.8 (20)	267 ± 16 (10)	21.2 ± 0.4 (200)

^aPhenotypes were measured as described previously (Wong *et al.*, 1995; Lakowski and Hekimi, 1996, for life span) at 20°C. The mean ± SEM is shown and the sample sizes are in parentheses.

be scored. The other major difficulty is that alteration of the function of many genes leads to a shortening of life span. When life span is shortened, however, it is very difficult to establish that the altered gene function has indeed accelerated the normal aging process, rather than having induced a specific life-shortening pathology. *clk-1* is one of the very few genes that can lengthen animal life span when mutant (Hekimi *et al.*, 1998), and thus is likely to be involved in a process that determines the normal rate of aging.

Here we have taken several approaches to investigate how *clk-1* affects cellular processes and to address the question of whether *clk-1* can be said to control the rate of worm physiology, including the rate of aging.

Results

A CLK-1::GFP fusion protein is fully active

A reporter gene expressing a CLK-1::GFP fusion protein containing the entire CLK-1 amino acid sequence was constructed (see Materials and methods), and tested for rescue of the phenotype of *clk-1(qm30)* mutant worms by germline microinjection (Mello and Fire, 1995). The construct fully rescues all tested mutant phenotypes, including the duration of post-embryonic development, the length of the defecation cycle, the brood size and life span, indicating that the fusion protein conserves most or all of the properties of CLK-1 (Table I).

clk-1 is expressed in the mitochondria of all somatic cells

Examination by fluorescence microscopy of live animals expressing CLK-1::GFP reveals an accumulation of the fusion protein in mitochondria (Figure 1A and B). This was confirmed by co-localization of the fluorescence originating from CLK-1::GFP with that of 6G rhodamine, a mitochondria-specific vital dye (Figure 2). The reporter gene was expressed in all tissues except the germline, where no fluorescence could be detected. Fluorescence could easily be scored in all gut, epidermal and muscle cells, including the vulval muscles and the pharynx, as well as in the cells of the excretory system, in the somatic gonad and in many neurons. Given the large number of anatomically similar neurons we cannot be certain that all neurons express the gene, but it seems likely (see Materials and methods). We also found that the gene was expressed in a very similar manner at all developmental stages, including embryos and dauer larvae. Note that although no transgene expression could be seen in the germline, the very low brood size of the mutants is rescued to the wild-type level by the transgene (Table I). At this stage,

however, we cannot tell whether this means that very low levels of expression, below the limit of detection of GFP, are sufficient for full function in the germline, or whether the brood size phenotype of the mutant is due to a defect in the somatic gonad.

clk-1 mutations affect mitochondrial function *in vivo*

The fact that the CLK-1::GFP fusion protein is capable of fully rescuing the mutant phenotype and is localized to mitochondria suggests that its primary action is in mitochondria. Using 6G rhodamine, which requires active mitochondria for uptake (Johnson *et al.*, 1981), we examined the morphology of mitochondria in the wild type and in *clk-1(qm30)* mutants at different ages. No difference of morphology between the genotypes was observed in young animals. Over time, however, the mutant, but not the wild-type mitochondria lost the ability to accumulate the dye, resulting in its diffuse distribution throughout the cytoplasm (Figure 1C and D). By day 7 of adulthood, this process was almost complete with 39/42 of the mutants, but only 1/41 of the wild-type animals, appearing like the animal in Figure 1D. These observations indicate that mitochondria from *clk-1* worms are active, but that their activity gradually falls below a threshold sufficient for dye accumulation. Although these observations represent a somewhat indirect way of determining mitochondrial activity, they are fully *in vivo* observations, and show the state of intact mitochondria in live, active animals of known chronological age. The use of activity-dependent dyes to assess the condition of mitochondria during aging is also being used in other systems (Hagen *et al.*, 1997).

clk-1(qm51) and *clk-1(qm30)* are null mutations

The findings that *clk-1* mutants are alive and have active mitochondria during most of their lives are difficult to reconcile with the known fully respiration-deficient phenotype of *coq7* mutants. One possibility is that all the available *clk-1* mutants are only partial loss-of-function mutations. The two strongest mutations are *qm30* which is a 590 base pair deletion encompassing the entire last exon of CLK-1, and *qm51*, which alters the obligate terminal G in the splice acceptor of intron 2, while the third mutation, *e2519*, is a missense mutation with a weaker phenotype (Ewbank *et al.*, 1997). Several lines of investigation suggest that the stronger mutations are null or close to null. For example, *qm51* mutants appear to produce no *clk-1* mRNA (Figure 3B). To confirm this, we also performed reverse transcription PCR (RT-PCR) on RNA extracted from *qm51* (see Materials and methods). Sets of primers were used to yield amplicons correspond-

ing to the two first exons, the three first exons and the full-length product (5 exons), respectively. All three products were readily amplified from wild-type RNA, but only a small amount of the smallest product corresponding to the first two exons could be detected in *qm51* RNA (data not shown). Therefore, *qm51* must be very close to being a null mutation, as only a very small amount of a highly truncated *clk-1* message is produced in this mutant.

***clk-1* mutations affect mitochondrial function in vitro**

To investigate further how the activity of *clk-1* differs in worms from that of *COQ7* in yeast, we tested the activity of succinate cytochrome *c* reductase, i.e. electron transfer from succinate to CoQ and from CoQ to cytochrome *c*.

Mitochondria from *clk-1(qm30)* and *clk-1(qm51)* mutants display an altered rate of respiration versus the wild type by this assay (Figure 3A). However, in contrast to the situation in *coq7* mutants, respiration is only slightly reduced, consistent with the observation that young mutant mitochondria can accumulate 6G rhodamine normally. To test whether a relative depletion of CoQ is the primary cause of the reduction in respiration, we tested the activity of succinate cytochrome *c* reductase with an added excess of CoQ₁ (25 µg/ml). Adding exogenous CoQ₁ is effective at stimulating the enzymatic activity of both wild-type and mutant mitochondria. However, the effect on the mutant mitochondria is not greater than on the wild-type mitochondria (Figure 3A). These findings suggest that a relatively mild reduction in respiration is the null phenotype for *clk-1*. It also makes it unlikely that *clk-1* encodes an enzyme of CoQ biosynthesis or that it is required for the function of such enzymes (Jonassen *et al.*, 1998), and is consistent with the viable phenotype of the worms. The difference between yeast and worms, therefore, is probably explained by differences in other regulatory mechanisms also impinging on respiration and the production of CoQ. Indeed, wild-type yeast cells grow efficiently in the absence of oxygen and strongly repress respiration in the presence of glucose. A complete absence of respiration is

Fig. 2. Co-localization of CLK-1::GFP with 6G rhodamine, a mitochondria-specific dye (see also Materials and methods). The structure shown is the terminal bulb of the pharynx. The terminal bulb is mainly composed of six large myo-epithelial cells rich in mitochondria. (A) The pattern of fluorescence produced by 6G rhodamine. (B) The pattern of fluorescence produced by CLK-1::GFP. The two patterns can be virtually superimposed indicating the CLK-1 is localized mostly to mitochondria.

Fig. 1. Mitochondrial expression of CLK-1 and decrease of mitochondrial function during aging in *clk-1* mutants. (A) Expression of a CLK-1::GFP fusion protein in a neuron. The arrow points to the cell body where fluorescent mitochondria can be seen throughout the cytoplasm, and the arrowheads point to individual mitochondria along the axonal process. (B) Mitochondrial expression of CLK-1::GFP in a single muscle cell (arrow). Long, thin mitochondria can be seen to follow the known direction of the contractile elements. In both (A) and (B), the cells are visualized in animals that are mosaic for an extrachromosomal array which is expressing the transgene. Single cells expressing the transgene can therefore be seen clearly on a non-expressing background. The diffuse green color is non-specific autofluorescence from underlying tissue. (C) Mitochondrial structure in the hypodermis of a 7-day-old wild-type animal. The mitochondria are labeled with G6 rhodamine, a dye that requires active mitochondria for uptake into mitochondria (Johnson *et al.*, 1981). The arrowhead points to a hypodermal nucleus. (D) Absence of mitochondrial uptake of G6 rhodamine in the hypodermis a 7-day-old *clk-1(qm30)* mutant. The dye is filling the cytoplasm rather than being taken up by the mitochondria. The dye appears excluded from the nuclei (arrowhead) as well as from a number of spherical cytoplasmic structures, presumably storage granules.

Fig. 3. The level of CLK-1 activity controls the activity of the respiratory chain over a narrow range. **(A)** The activity of the mitochondrial succinate cytochrome *c* reductase was measured in several genotypes (see Materials and methods). This activity catalyzes the flow of electrons from succinate to CoQ and from CoQ to cytochrome *c*, and CoQ is thus an obligatory co-factor for this activity. Purified mitochondria were incubated with an excess of succinate and of cytochrome *c*. Oxidation of cytochrome *c* was blocked by sodium azide and the rate of reduction of cytochrome *c* was monitored colorimetrically. The blue bars represent the activity measured without added CoQ₁; the red bars represent the activity measured with an excess added CoQ₁ (25 µg/ml). The error bars represent the standard errors of the mean (*N2*, *n* = 14; *clk-1(qm51)*, *n* = 4; *clk-1(qm30)*, *n* = 4; MQ678, *n* = 6). The genotype of the strain MQ678 is *clk-1(+); qmIs10 [clk-1::gfp]*, and this strain thus overexpresses CLK-1 activity (see main text). *clk-1(qm51)* and *clk-1(qm30)* are the most severe alleles of *clk-1* and both lead to an average 2-fold slowing of development and behavior. **(B)** Northern blot analysis of the expression of *clk-1* from wild-type and mutant loci (the genetic lesions are described in Ewbank *et al.*, 1997). *clk-1(e2519)* is a missense mutation resulting in a partial loss-of-function. Normal levels of mRNA are produced in the *e2519* mutant. *clk-1(qm51)* is a point mutation in a splice acceptor site and virtually no message appears to be produced in the mutant. *clk-1(qm30)* is a 590 bp deletion which removes the last exon of the gene and a truncated mRNA is produced. The virtual absence of mRNA in *qm51* suggests that both *qm51* and *qm30* mutations are null or close to null mutations.

therefore a normal alternative metabolic state for these cells. For worms, however, *clk-1* appears to regulate respiration over a much narrower range.

Transgenic expression of CLK-1 activity produces a gain-of-function phenotype

By analyzing the mitochondria from worms expressing the *clk-1::gfp* transgene in a *clk-1(+)* background, we found that respiration was increased in a small but signi-

ficant way over that of wild-type mitochondria (Figure 3A). This observation suggested that the expression of the CLK-1::GFP fusion protein might also induce a gain-of-function phenotype at the level of the whole organism. We scored all life history and behavioral parameters of young transgenic animals to test for gain-of-function features. We found that development as well as the behavior and reproductive functions of young adult transgenic animals are not measurably different from those of the wild type (data not shown). We reasoned that other mechanisms than those affected by *clk-1* function probably also limit the maximal rates of these processes. However, it had been noted previously by others that the defecation cycle period, one of the physiological rates that is altered by *clk-1* mutations, lengthens when worms age (Croll *et al.*, 1977; Bolanowski *et al.*, 1981). We tested the possibility that increased *clk-1* activity could prevent this slowdown by comparing the defecation cycle length of wild-type animals, *clk-1* mutants and animals overexpressing *clk-1* activity at different times during adult life (Figure 4; Table II). We found that between day 1 and day 4 of adult life there is a dramatic slowing of the defecation cycle in the wild type, but a much lesser slowing in *clk-1* mutants. In fact, on day 4, the average cycle length of wild-type and mutant animals is indistinguishable. This suggested that a mechanism involving *clk-1* is responsible for the slowdown in the wild type on day 4. Furthermore, expression of CLK-1::GFP fully prevents the slowdown in a large subset of the transgenic animals (Figure 4C; Table II). Indeed, 24% of the 4-day-old animals expressing the transgene are as fast on day 4 as they were on day 1, while <3% of the wild-type animals remain fast (Figure 4C; Table II). The fact that the defecation of only a subset of the transgenic animals is as efficient on day 1 as on day 4 might be due to the existence of an additional, *clk-1*-independent mechanism of slowdown; possibly a non-specific degradation resulting from premature aging (see below).

To control for non-specific effects of the transgene or the fusion protein, we tested animals carrying a reporter construct identical to that producing the gain-of-function except for a E→K mutation at residue 148 of the protein (see Materials and methods). This mutation, *clk-1(e2519)*, is known to produce a partial loss-of-function of the CLK-1 protein (Wong *et al.*, 1995; Ewbank *et al.*, 1997). Expression of the mutant E2519::GFP fusion protein was visible in mitochondria but, as expected, was not capable of rescuing the phenotype on day 1, nor had any detectable effect on day 4 (Table II). This indicates that the CLK-1 part of the fusion protein and no other element of the reporter construct or of the expressed protein was responsible for the observed effects.

Transgenic expression of CLK-1 activity increases the rate of aging

The life span of mutant and *clk-1(+)* animals expressing transgenic CLK-1 activity was scored (Figure 5; Table III). Animals expressing the CLK-1::GFP protein from a free extrachromosomal array in a wild-type background have a significantly reduced life span (compare strain MQ599 with the wild type) (Figure 5; Table III). The short-lived transgenic animals do not appear to be sick or otherwise different from the wild type except for the absence of the

Fig. 4. The age-dependent slowing of the defecation cycle can be prevented by overexpression of CLK-1 activity. The defecation cycle was measured at 20°C as described previously (Wong *et al.*, 1995). Five inter-defecation periods were measured for each worm and the average of five is shown. The blue bars represent animals on the first day of adulthood and the red bars animals on the fourth day of adulthood. (A) Defecation cycle in wild-type worms. (B) Defecation cycle in *clk-1(qm30)* mutants. (C) Defecation cycle in animals from strain MQ599. The genotype of this strain is *clk-1(+); qmEx133 [clk-1::gfp]* and overexpresses CLK-1 activity (see main text).

behavioral decline after day 4 (see above), which suggests that their shorter life span is the result of an increase in their rate of normal aging and not the result of a novel pathology. In contrast, expression of the mutant E2519::GFP protein has no effect on life span (compare strain MQ597 to MQ130) (Figure 5; Table III), indicating that it is essential for the transgene to express a functional CLK-1 moiety and that the expression of the GFP moiety per se is not deleterious. To ensure that the shortened life

span of MQ599 was indeed the result of the presence of the array in the strain, we scored the life span of a strain derived from MQ599 but from which the mitotically unstable free extrachromosomal array had been lost. The life span of these animals was found to be indistinguishable from the wild type (see Table III).

Transgenic animals expressing *clk-1* reporter genes are obtained by co-injection with a dominant marker that confers an easily scored 'roller' phenotype. Therefore, all

Table II. Defecation cycle length on day 1 and day 4 of adulthood for different genotypes

Strain	Genotype	Average defecation cycle length (s) ^a		Worms with fast cycles on day 4 ^b (% total)
		day 1	day 4	
N2 ^c	<i>clk-1(+)</i>	54.9 ± 0.6 (70)	106.8 ± 3.5 (68)	2.9
MQ130 ^c	<i>clk-1(qm30)</i>	81.5 ± 1.6 (80)	103.0 ± 1.7 (80)	0
MQ599 ^c	<i>clk-1(+); qmEx133</i> <i>[clk-1::gfp]</i>	57.1 ± 0.6 (68)	90.3 ± 3.6 (67)	24.0
MQ597 ^d	<i>clk-1(qm30); qmEx132</i> <i>[clk-1(e2519)::gfp]</i>	73.7 ± 1.3 (49)	107.9 ± 4.1 (49)	0

^aThe defecation cycle was measured at 20°C as described previously (Wong *et al.*, 1995). The mean ± SEM is shown and the sample size is in parentheses.

^bWorms were considered to have fast defecation on day 4 when their defecation cycle length fell within the range of values found for the wild type on day 1.

^cThe data for N2, MQ130 and MQ599 is also shown in graphic form in Figure 3.

^dSee Materials and methods for some additional controls.

Fig. 5. CLK-1 activity controls the rate of aging in *C.elegans*. The graph shows the percentage of worms alive on a given day after having been laid during a 4 h period on day 0. The wild type ($n = 158$) is shown as blue squares, *clk-1(qm30)* ($n = 199$) in green diamonds, animals from strain MQ599 ($n = 168$) in red triangles, and animals from strain MQ597 ($n = 87$) with purple circles. The genotype of MQ599 is *clk-1(+); qmEx133 [clk-1::gfp]* and expresses additional CLK-1 activity in a wild-type background which results in a shorter life span (the difference in life span between MQ599 and wild type is highly significant, at $p < 0.005$ by a Student's t -test). The genotype of MQ597 is *clk-1(qm30); qmEx132[clk-1(e2519)::gfp]*, and thus expresses a CLK-1::GFP fusion protein carrying the *e2519* mutation. The difference between the fusion proteins expressed in MQ599 and MQ597 consists of only a single amino acid (Ewbank *et al.*, 1997), yet the E2519::GFP protein shows no life-span shortening activity. This indicates that it is the activity of the CLK-1 part of the fusion protein which is responsible for the life span shortening in MQ599, and no other property of the transgene.

the arrays described in this work also contain DNA from the plasmid pRF4 that expresses a mutant form of the ROL-6 protein (Mello and Fire, 1995). For clarity, this is omitted in the description of the genotypes in the text and the tables. However, several observations show that the effects we observe are not due to the presence of pRF4 in the array or to the roller phenotype. Animals from strain MQ597 have a Clk-1 and a Rol-6 phenotype, yet their life span is statistically indistinguishable from that of the non-rolling *clk-1* mutants of MQ130 (see Table III; Materials and methods). Similarly, animals from strain MQ680 have a Rol-6 but otherwise wild-type phenotype

and their life span is not different from that of non-rolling wild-type animals (see Table III; Materials and methods). In addition, in a separate experiment we have tested *clk-1(+)* animals with arrays containing only pRF4 and detected no effects on life span or defecation (see Materials and methods).

We have argued previously that the long life of *clk-1* mutants is due to their slow life (Lakowski and Hekimi, 1996; Hekimi *et al.*, 1998). Indeed, if life span is set by the relation between the rate of sustaining molecular or other injuries and their repair, then slowing down the rate of living could favor repair and thus lead to a slower

Table III. Mean life span of strains expressing *clk-1* transgenes

Strain	Genotype	Array ^a	Life span (days) ^b
N2	<i>clk-1</i> (+)		22.0 ± 0.3 (358)
MQ130	<i>clk-1(qm30)</i>		25.1 ± 0.5 (399)
MQ599	<i>clk-1</i> (+)	<i>qmEx133</i>	20.5 ± 0.3 (368)
MQ678	<i>clk-1</i> (+)	<i>[clk-1::gfp]</i>	
		<i>qmIs10</i>	19.7 ± 0.3 (200)
MQ680	<i>clk-1(qm30)</i>	<i>[clk-1::gfp]</i>	
		<i>qmEx133</i>	21.6 ± 0.4 (201)
		<i>[clk-1::gfp]</i>	
Derived from MQ599 ^c	<i>clk-1</i> (+)	<i>none</i>	22.3 ± 0.2 (150)
Derived from MQ678 ^c	<i>clk-1</i> (+)	<i>qmIs10/+</i>	19.3 ± 0.4 (150)
		<i>[clk-1::gfp]</i>	
MQ597 ^c	<i>clk-1(qm30)</i>	<i>qmEx132</i>	25.7 ± 1.0 (87)
		<i>[clk-1(e2519)::gfp]</i>	

^aArray numbers with a *qmEx* prefix designate free extrachromosomal arrays; array numbers with a *qmIs* prefix designate arrays which have been integrated into the chromosomal genome by γ -ray treatment. The expression in square parentheses describes the type of fusion protein that is expressed by the array.

^bLife span was measured as described previously (Lakowski and Hekimi, 1996). The mean $\pm$ SEM is shown and the sample size is in parentheses.

^cSee Materials and methods for additional controls.

accumulation of unrepaired damage. Consistent with this model, the transgenic animals overexpressing CLK-1 activity live faster by at least one measure (behavior), age more quickly and have a shorter life span.

The increase in the rate of aging is due to overexpression of CLK-1 activity

A number of observations suggest that it is overexpression of CLK-1 activity that accelerates aging in these strains, and not some other aspect of abnormal expression from a transgene. We transferred, by simple genetic methods, the array (*qmEx133*) initially generated in a wild-type background [*clk-1*(+)] into a mutant [*clk-1(qm30)*] background and found that although the overall phenotype was fully rescued, the life span of the mutant was not different from wild type (compare MQ680 with the wild type in Table III). This suggests that it is the increase over wild-type levels in the total amount of CLK-1 activity (from either the chromosomal locus or the transgene) that is crucial to life span shortening in MQ599.

Another observation also pointed to the importance of the level of expression of CLK-1 activity for life span. Extrachromosomal arrays form spontaneously upon DNA injection in *C.elegans* and are unstable elements which can be lost at each cell division. Consequently, animals carrying such arrays are mosaics, containing the array in only a subset of their cells (Mello *et al.*, 1991). One way to increase the overall level of expression from such arrays is to stabilize them by integration into the chromosomal genome by γ -ray treatment (Mello and Fire, 1995). We found that the integrated form of *qmEx133* (now called *qmIs10* in strain MQ678; Table III) shortens life span significantly more than the free form (compare MQ599 with MQ678), presumably because CLK-1 activity is expressed in all the cells of the worm. Although *qmIs10* has been backcrossed to the wild type, it is possible that the γ -ray treatment has generated mutations linked to *qmIs10*. We found, however, that the life span of animals heterozygous for the *qmIs10* insertion (obtained by mating wild-type males into MQ678; Table III) had a life span as short as that of homozygotes, indicating that no fortuitous recessive mutation could account for the accelerated aging.

Discussion

Mitochondrial function and aging

Many avenues of research have implicated mitochondrial function in the aging process (Harman, 1972; Linnane *et al.*, 1989; Shigenaga *et al.*, 1994; Miquel, 1998). Mitochondria are the major source of damaging oxygen radicals and are also the first compartment in the cell to be damaged by these radicals. Indeed, aging is accompanied by a decrease in mitochondrial function, which has led to the hypothesis that the resulting deficit in energy production could be a cause of aging. *clk-1* is one of the very few genes for which we know that it can lengthen animal life span when mutant, and is thus likely to be involved in a process that determines the normal rate of aging. We have shown that CLK-1 is localized to mitochondria where its function affects the level of respiration. We believe this finding to be one of the strongest demonstrations to date that mitochondrial function is causally related to aging. The respiratory capacity of long-lived *clk-1* mutant mitochondria is slightly depressed in young animals (as observed with purified mitochondria) and decreases more rapidly during aging than that of wild-type mitochondria (as observed by dye uptake in intact animals). This suggests that *clk-1* mutants may live longer than the wild type as the result of a decreased production of oxygen radicals, and that the age-dependent decrease in mitochondrial function observed in many organisms, rather than being a cause of aging, is a process promoting long life. This conclusion is strengthened by our findings that overexpression of CLK-1 activity has the reverse effect: it increases respiration, prevents the physiological slowing down during aging, and shortens life span.

Our results can be compared with recent findings with mutants in the *C.elegans* gene *mev-1*, which encodes a subunit of the enzyme succinate dehydrogenase cytochrome *b* (Ishii *et al.*, 1998). *mev-1* mutants have severely decreased activity of succinate cytochrome *c* reductase activity and a shortened life span. *mev-1* mutants are also hypersensitive to oxygen, which suggests that the shortened life span of the mutants is the result of an increased production of reactive oxygen radicals (Ishii

et al., 1998). The same step of the respiratory chain is slightly affected in *clk-1* mutants but results in a lengthened life span. How can we reconcile such different observations? The MEV-1 protein is an integral part of the enzyme reducing CoQ and its absence or alteration is likely to result in abnormal as well as reduced activity. In fact, Ishii *et al.* (1998) hypothesize that MEV-1 participates directly in transporting electrons from succinate dehydrogenase to CoQ and that the *mev-1(kn1)* mutation could affect the ability of a heme iron in the enzyme to accept and relinquish electrons, and thus promote the formation of free radicals. On the other hand, the biochemical function of CLK-1 is unknown and CLK-1 is not a known subunit of any respiratory enzyme. CLK-1 might therefore affect respiration only indirectly, in a regulatory manner that does not enhance the production of oxygen radicals. Furthermore, the effect on respiration might not be the only or the principal action of *clk-1* (see below).

What is the function of CLK-1 in mitochondria?

Saccharomyces cerevisiae cells that lack Coq7p cannot manufacture the essential respiratory lipid co-factor CoQ, and consequently cannot grow on non-fermentable carbon sources (Marbois and Clark, 1996). Supplementation of the growth medium with CoQ partially restores growth (Jonassen *et al.*, 1998), indicating that the mutant's absolute substrate requirement is a consequence of its deficit in CoQ synthesis. It remains unclear, however, what the exact biochemical activity of CLK-1 might be. Although it is possible that CLK-1 is an enzyme in the biosynthesis of CoQ, it does not resemble any biosynthetic enzyme identified in *E.coli* or yeast, or any other gene product of known function (Marbois and Clarke, 1996; Ewbank *et al.*, 1997). The pathway of CoQ biosynthesis in yeast has not yet been fully characterized. Notably, the functional homologue of the *E.coli* gene product encoded by *ubiF* has not been identified and could correspond to Coq7p. However, inspection of the *E.coli* genomic sequence in the region identified by genetic studies (Felton *et al.*, 1980) reveals the the predicted protein o391 (described in DDBJ/EMBL/GenBank accession No. ECAE000170) almost certainly corresponds to *ubiF* (J.J.Ewbank, unpublished observation). The yeast protein it most closely resembles, the ubiquinone biosynthesis monooxygenase Coq6p, bears no resemblance to either Coq7p or CLK-1 (unpublished observation). Furthermore, our findings show that CLK-1 is not strongly required for one of the main steps in mitochondrial function for which CoQ is necessary (Figure 3). In addition, *clk-1* mutants show maternal rescue: homozygous mutants which are derived from a heterozygous mother display a wild-type phenotype throughout their lives, despite the fact that their body size increases by a factor of ~1000 over their lifetime (Wong *et al.*, 1995). Such a phenotype is difficult to associate with null mutations in a structural enzyme, and suggests that *clk-1* has a regulatory function that, in yeast, but not strongly in worms, also affects CoQ biosynthesis.

What is the function of CLK-1 in the cell?

clk-1 mutations such as *qm30* and *qm51* which appear to be null, produce a 2-fold average slow down in development and behavioral rates and even stronger effects on reproductive output, which is decreased >6-fold (Wong

et al., 1995). These phenotypes are much stronger than what one would expect from the relatively mild defects we found in the activity of succinate cytochrome *c* reductase. The dramatic slowing down of *clk-1* mutants can also be contrasted with the phenotype of worms whose energy intake is limited. Mutations in *eat* genes perturb the function of the pharynx, the muscular feeding organ of the worm, and result in a lowered food intake (Avery, 1993). *eat* mutants have a typical 'starved' appearance, being more transparent than wild-type worms and often smaller. In spite of this, however, the development of these mutants is only very slightly longer than that of the wild type (Avery, 1993; Lakowski and Hekimi, 1998). This is in strong contrast to *clk-1* mutants, which do not have a starved appearance or reduced size but show delayed development. It suggests that a limitation of energy metabolism is not the primary defect in *clk-1* mutants.

One hypothesis for the function of *clk-1* is that it contributes somehow to the crosstalk between the mitochondria and nuclear gene expression. The nuclear and mitochondrial genomes both contribute gene products to the mitochondrion's energy-producing machinery and these physically separate genomes must, therefore, exchange information to coordinate their contributions (reviewed in Poyton and McEwan, 1996). Furthermore, the energy producing activity of the mitochondria is essential to the rest of the cell, and the needs of a particular cell at a particular time must be conveyed to the organelle to regulate its activity. Similarly, gene expression in the nucleus must be regulated as a function of available energy in the cell. For example, attempts at a high biosynthetic activity could be deleterious in the face of low energy availability. One possible model to explain the severe but healthy phenotype of *clk-1* mutants is that in the absence of *clk-1* function, the nucleus 'believes' that mitochondrial function is depressed and adjusts gene expression accordingly. All the worm's biological parameters are thus depressed in an organized, coordinated and physiologically acceptable way. A similar interpretation is possible for the maternal effect. Early in embryonic life, maternally provided *clk-1* message allows the production of some *clk* gene product. The mitochondria can thus send the message as to their level of activity. Later, when the worm has grown enormously, *clk-1* gene product might be entirely exhausted but the pattern of gene expression corresponding to high mitochondrial activity has been established and does not change in the absence of new information. Given that the mitochondria are in fact fully active, the absence of crosstalk does not result in a depressed phenotype and the animals are fully wild type, at least under stable laboratory conditions. With such a model the small defect in respiration we observe might even be a secondary result of altered nuclear gene expression rather than the primary cause of the overall phenotype.

Materials and methods

Reporter gene construction

The *clk-1* locus was directly amplified from nematode genomic DNA by PCR with *Taq* polymerase and primers SHP121 and SHP122. The PCR product was digested with *Bam*HI and *Sal*I and ligated to pPD95.77 that had also been cut with *Bam*HI and *Sal*I. The vector pPD95.77 was kindly donated by Dr A.Fire; for the structure of pPD95.77 see [ftp://ciw2.ciwemb.edu/pub/FireLabVectors/](http://ciw2.ciwemb.edu/pub/FireLabVectors/). Two plasmids were con-

structed in this manner: pMQ26 was obtained from wild type (N2) genomic DNA, and pMQ27 from *clk-1(e2519)* genomic DNA. The *e2519* mutation results in an E→K change at residue 148 of the protein (Ewbank *et al.*, 1997). These constructs represent an in-frame fusion of the *clk-1* coding sequence with GFP, together with the upstream gene *toc-1* (Ewbank *et al.*, 1997) and its presumptive promoter region (624 bp 5' of the *toc-1* start codon). The sequence of the primers is available upon request. The fusion protein with the *e2519* mutation is also expressed in the mitochondria (data not shown).

Visualization of mitochondria by 6G rhodamine

The dye was obtained from Sigma and dissolved in water and added at a concentration of 10 µg/ml to NGM agar plates, which allows visualization of worm mitochondria (A.S.Badrinath and J.G.White, personal communication). The utility of using dyes that accumulate in mitochondria on the basis of their membrane potential to test mitochondrial function during aging has also been demonstrated by others (Hagen *et al.*, 1997). 6G rhodamine fluoresces in response to green as well as blue excitation light, which can interfere with the visualization of GFP fluorescence. However, the dye bleaches very rapidly when observed in the living animal. We took advantage of this property for the microphotographs shown in Figure 2. The stained cells of the terminal bulb of the pharynx were first observed with green excitation light and a picture taken (Figure 2A). The dye was then bleached by continuous excitation for ~5 min until virtually no fluorescent light was emitted anymore from the structure. The structure was then observed with blue excitation light and the image produced by the GFP-based fluorescence recorded (Figure 2B).

Expression pattern of *clk-1::gfp*

The expression pattern was highly variable from animal to animal. In some animals only a few cells were fluorescent while expression was widespread in others. The expression was also variable in intensity: a given cell could sometimes appear strongly fluorescent in one animal and only very weakly stained in others. Therefore, to assess the expression pattern, we scored positive cells and tissues in a large number of animals (>100). A tissue was considered to be expressing *clk-1::gfp* when cells from that tissue were found to be fluorescent in a significant number of animals (>10). An individual identified cell was considered as positive when it was observed to be strongly expressing in at least two animals. For tissues with many similar cells, such as the nervous system and muscle, not every identifiable cell was individually assessed. However, no preferential staining in certain body regions was observed in these tissues and the staining was often widespread. Therefore, it is possible, but unlikely, that some neurons and muscle cells never express the transgene. Sub-cellular expression pattern appeared mitochondrial by several criteria including (i) similarity to the expression pattern of other mitochondrial reporter genes (e.g. Fire *et al.*, 1998), (ii) similarity to the pattern of fluorescent of 6G rhodamine, and (iii) co-localization with 6G rhodamine (Figure 2).

PCR

Total RNA was prepared using the TRIzol reagent (BRL) according to the manufacturer's recommendations. Northern blot analysis was then performed as described previously (Labbe *et al.*, 1999). RT-PCR was done as described previously (Ewbank *et al.*, 1997). Briefly, RT was performed on 2.5 µg of total RNA from wild-type and *clk-1(qm51)* strains using AMV reverse transcriptase (Pharmacia), according to the manufacturer's recommendations. Primers SHP10 (exon 2), SHP368 (exon 3) and SHP59 (exon 5) were used in the RT reaction. Subsequently, 30 cycles of PCR were performed on 1 µl of RT product using specific primer pairs for each exon (SL2/SHP10 for exon 2, SL2/SHP368 for exon 3, SL2/SHP59 for exon 5). Thirty cycles of PCR were then performed on 1 µl of the first PCR product using specific nested primer pairs for each exon (SHP168/SHP214 for exon 2, SHP168/SHP369 for exon 3, SHP168/SHP169 for exon 5). The sequence of the primers is available upon request.

Assaying succinate cytochrome *c* reductase

Worms were grown on 9 cm NGM plates with *E.coli* strain OP50 and were harvested before exhaustion of bacteria on the plates to avoid starvation. Mitochondria were isolated by a modification of the method of Murfitt *et al.* (1976), and succinate:cytochrome *c* reductase was assayed according to Brown and Beattie (1977). Approximately 40 plates were necessary per mitochondrial preparation. The soluble ubiquinone analogue CoQ₁ was obtained from Sigma, dissolved in ethanol and added to the reaction mix for a final concentration of 25 µg/ml. CoQ₂

was also used in some experiments with similar results as with CoQ₁ (not shown). The strain [MQ678 *clk-1(+); qmIs10*] used to measure enzymatic activity contains a stably integrated array derived by γ-ray treatment from MQ599 *clk-1(+); qmEx133[clk-1::gfp]*, which was subsequently back-crossed five times. In many other experiments, free arrays are used to avoid confounding effects from possibly deleterious mutations produced by the irradiation. For mitochondrial purification, large number of worms are required and it was therefore necessary to have a homogenous population.

To analyze the significance of the measured difference in enzymatic activity we performed paired *t*-tests calculated using the Microsoft Excel 97 analysis ToolPak™. The addition of CoQ₁ to mitochondria from animals of all genotypes produces increases in activity that are significant at *p* < 0.05. The increase in activity of the overexpressing strain MQ599 was significantly different from wild type at *p* < 0.06 without added CoQ₁, and at *p* < 0.04 with added CoQ₁. The differences in respiration between the wild type and the mutants, with or without added CoQ₁, are not significant. However, given the known respiration deficient phenotype of the equivalent mutants in yeast, and the significant increase observed in overexpressing strains, we believe that the decrease in activity seen in the mutants is likely to be real.

Statistics

We tested the differences in the distributions of defecation times by Mann–Whitney non-parametric tests. Specifically, the distributions of defecation times for *clk-1(qm30)* versus the wild type on day 4 are not different (probability that they are the same, 0.821), while the distributions for MQ599 is significantly different from that of the wild type (probability that they are the same, 0.0004). The comparisons mentioned in the main text between the life spans of different strains were tested by Student's *t*-tests. Specifically, the following pairs of strains have statistically different life spans at *p* ≤ 0.05: MQ599 versus wild type, MQ599 versus MQ678, MQ130 versus the wild type, and MQ597 versus the wild type. The following pairs are not different from each other *p* >> 0.05: MQ597 and MQ130, MQ680 and the wild type, the non-rolling strain derived from MQ599 and the wild type, and the heterozygous strain derived from MQ678 and the wild type.

The Rol-6 phenotype

In a separate series of experiments we tested directly the phenotypic effects of carrying an extrachromosomal array containing only the plasmid pRF4 and thus expressing a mutant form of the ROL-6 protein and showing the mutant phenotype, but not expressing any wild-type or mutant CLK-1 activity. Two rolling lines with free extrachromosomal arrays were established, line #1 and line #2. Line #1 was scored for defecation cycle length. Means ± SEMs were 61.4 ± 0.6 (*n* = 85) on day 1 and 106.8 ± 3.7 (*n* = 80) on day 4. The distribution of defecation times on day 4 was indistinguishable from the wild type (Mann–Whitney probability that they are the same, 0.817), but very different from that of MQ599 (Mann–Whitney probability that they are the same, 0.00053).

Life span was scored simultaneously for animals of both line #1, line #2 and from appropriate controls, that is, non-rolling lines derived from the rolling lines and wild type. The life span of the rolling lines were similar and indistinguishable (Student's *t*-test *p* ≤ 0.56) from that of the cognate non-rolling lines or from that of the wild type. Means ± SEMs were 18.4 ± 0.2 (*n* = 300) for the pooled rollers and 18.6 ± 0.3 (*n* = 250) for the pooled non-rollers.

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