

# Phenotypic and Suppressor Analysis of Defecation in *clk-1* Mutants Reveals That Reaction to Changes in Temperature Is an Active Process in *Caenorhabditis elegans*

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## ABSTRACT

Mutations in the *Caenorhabditis elegans* maternal-effect gene *clk-1* affect cellular, developmental, and behavioral timing. They result in a slowing of the cell cycle, embryonic and postembryonic development, reproduction, and aging, as well as of the defecation, swimming, and pharyngeal pumping cycles. Here, we analyze the defecation behavior in *clk-1* mutants, phenotypically and genetically. When wild-type worms are grown at 20° and shifted to a new temperature, the defecation cycle length is significantly affected by that new temperature. In contrast, we find that when *clk-1* mutants are shifted, the defecation cycle length is unaffected by that new temperature. We carried out a screen for mutations that suppress the slow defecation phenotype at 20° and identified two distinct classes of genes, which we call *dsc* for defecation suppressor of *clk-1*. Mutations in one class also restore the ability to react normally to changes in temperature, while mutations in the other class do not. Together, these results suggest that *clk-1* is necessary for readjusting the defecation cycle length in response to changes in temperature. On the other hand, in the absence of *clk-1* activity, we observe temperature compensation, a mechanism that maintains a constant defecation period in the face of changes in temperature.

MUTATIONS in the *Caenorhabditis elegans* gene *clk-1* are highly pleiotropic, affecting the rates of physiological traits that occur over a wide range of timescales (WONG *et al.* 1995). They result in a mean lengthening of the cell cycle of early embryos, embryonic and postembryonic development, as well as the defecation, swimming, and pharyngeal pumping cycles of adults. *clk-1* mutations also affect reproductive features, like the egg-production rate and self-brood size, which are both reduced, and lead to an increased life span.

A number of observations suggest that the phenotypes of *clk-1* mutants are the result of an inability to appropriately set the rate of physiological processes (WONG *et al.* 1995; BRANICKY *et al.* 2000). One example is that many of the features affected by *clk-1* mutations are more variable, in addition to being slower on average. For instance, although the average length of embryogenesis of *clk-1* mutants is slower than that of the wild type, some *clk-1* embryos can develop faster than wild-type embryos, while others take more than two times longer, which suggests that timing is deregulated in the mutants (WONG *et al.* 1995). Also, we observed that *clk-1* mutant embryos are unable to properly adjust their rate of development in response to changes in temperature. Briefly, when wild-type embryos are cultured to the two-cell

stage at a particular temperature and are then transferred to a new temperature, they immediately develop at a rate corresponding to that new temperature. In contrast, when *clk-1* mutant embryos are transferred to a new temperature, the rate of development at the new temperature is strongly influenced by the temperature experienced before the shift (WONG *et al.* 1995). This suggests that *clk-1* might also be needed to reset physiological rates in response to changes in temperature. Finally, all of the phenotypes affected in *clk-1* mutants can be maternally rescued; that is, homozygous mutant progeny issued from a heterozygous hermaphrodite are phenotypically wild type. This maternal rescue extends to adulthood, such that all adult behaviors and even the long life of *clk-1* mutants are rescued (HEKIMI *et al.* 1995; WONG *et al.* 1995). We interpreted this to mean that *clk-1* affects a regulatory process that is somehow involved in setting physiological rates in the worm (WONG *et al.* 1995; FELKAI *et al.* 1999; BRANICKY *et al.* 2000). In the presence of maternally supplied *clk-1* product, timing can be set appropriately early in development so that homozygous *clk-1* mutants can subsequently develop and behave like the wild type.

*clk-1* encodes a mitochondrial protein that is highly conserved, structurally and functionally, among eukaryotes (PROFT *et al.* 1995; JONASSEN *et al.* 1996; EWBANK *et al.* 1997; VAJO *et al.* 1999) and encodes a putative hydroxylase (STENMARK *et al.* 2001) that is required for the biosynthesis of ubiquinone [UQ; also called coenzyme Q (CoQ)], a prenylated benzoquinone lipid that

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functions as a transporter of electrons in complexes II and III of the respiratory chain. Mutants of the yeast homolog of *clk-1*, *coq7*, do not produce UQ and therefore cannot grow on nonfermentable carbon sources (MARBOIS and CLARKE 1996). Mitochondria isolated from *clk-1* mutants also do not contain detectable levels of UQ but instead accumulate the UQ biosynthetic intermediate, demethoxyubiquinone (DMQ; MIYADERA *et al.* 2001). In *clk-1* mutants this compound functions as an electron carrier, such that the mitochondria can maintain respiration despite the complete absence of UQ (FELKAI *et al.* 1999; MIYADERA *et al.* 2001). However, DMQ cannot substitute entirely for UQ as *clk-1* mutants cannot complete development when they are fed *Escherichia coli* strains that do not produce UQ (JONASSEN *et al.* 2001).

It is, however, not clear how the absence of UQ relates to the other mutant phenotypes as there is no correlation between this biochemical phenotype and the severity of the overall phenotype. Indeed, the quinone phenotype is identical for all three known *clk-1* alleles (*e2519*, *qm30*, and *qm51*): UQ is totally absent from mitochondria in all three cases, and all three accumulate the same amount of DMQ. Yet, most of the features affected in *clk-1* mutants are slowed down much more severely in the putative null alleles *qm30* and *qm51* than they are in the partial loss-of-function allele *e2519* (WONG *et al.* 1995; FELKAI *et al.* 1999). Also, by various measures of energy metabolism in intact worms, *clk-1* mutants have been shown to have metabolic capacities and ATP levels comparable to the wild type (BRAECKMAN *et al.* 1999). Together, these observations suggest that much of the phenotype of *clk-1* mutants may not be the direct consequence of an absence of UQ in mitochondria or a decreased level of energy production.

One of the features affected in *clk-1* mutants is the defecation cycle. In *C. elegans*, defecation is effected by a stereotyped defecation motor program (DMP). The DMP consists of three distinct steps: the posterior body muscle contraction (pBoc), the anterior body muscle contraction (aBoc), and the expulsion (Exp), which consists of the enteric muscle contractions (EMC; THOMAS 1990). In the presence of adequate food, the defecation cycle period of 56 sec is regular in single animals over time and among animals, with a standard deviation of only a few seconds. In addition to its tight periodicity, the defecation cycle has other properties that suggest that it might be controlled by an endogenous "clock." For example, the phase of the cycle can be reset by lightly touching the animal, and the rhythm is maintained even in the absence of expression of the DMP (LIU and THOMAS 1994).

The periodicity of the defecation cycle can be altered by mutations in at least 13 genes (Dec phenotype). These mutations fall into two major classes: short Dec (Dec-s), for mutations that decrease the cycle length, and long Dec (Dec-L), for mutations that increase the cycle length (IWASAKI *et al.* 1995). The molecular identi-

fication of the Dec-L gene, *dec-4* (*lef-1/itr-1*), as the inositol triphosphate (IP<sub>3</sub>) receptor, a protein involved in regulating intracellular calcium levels, suggests that calcium oscillations contribute to the regulation of the rhythm. Indeed, DAL SANTO *et al.* (1999) showed that calcium levels peak in the intestine just prior to the first muscle contraction of the DMP and that expression of the IP<sub>3</sub> receptor in the intestine was sufficient for normal rhythm generation. Although it is not yet clear how the different Dec genes might be interacting to regulate the defecation cycle, the molecular characterization of two other genes, *flr-1* and *unc-43/dec-8*, also supports roles for calcium and the intestine in rhythm regulation. *flr-1* mutants, originally identified on the basis of their resistance to fluoride (KATSURA *et al.* 1994), have, among other defecation phenotypes, a very short defecation cycle length (IWASAKI *et al.* 1995). *flr-1* encodes an ion channel of the degenerin/epithelial sodium channel superfamily, which is expressed only in the intestine from embryos to adults (TAKE-UCHI *et al.* 1998). Mutations in *unc-43* result in multiple behavioral defects, including defecation phenotypes (LIU and THOMAS 1994; REINER *et al.* 1999). Loss-of-function mutations result in an increased frequency of defecation, usually the result of a repetition of the DMP ~13 sec after the initiation of the primary motor program, whereas the gain-of-function mutation results in a decreased frequency of defecation. *unc-43* encodes the *C. elegans* CaM Kinase II, which is widely expressed in neurons, muscles, and the intestine (REINER *et al.* 1999).

Here, we analyzed the defecation behavior of *clk-1* mutants, with particular emphasis on the response of the mutants to changes in temperature. We find that when wild-type worms are grown at 20° and then shifted to a new temperature, the defecation cycle length is significantly affected by that new temperature. In contrast, when *clk-1* mutants are shifted, the defecation cycle length is the same at the new temperature as it is at 20°. We reasoned that if *clk-1* is actively involved in regulating the timing of the defecation cycle it might work through other gene products. We carried out a screen for suppressor mutations at 20° and identified 10 mutations, which correspond to seven different complementation groups. We find that the suppressor mutations fall into two distinct classes. In addition to suppressing the slow defecation at 20°, one class also suppresses the inability of *clk-1* mutants to adjust their defecation cycle length after a temperature shift, while the other class does not. One general conclusion that can be drawn from this study is that adjusting the rate of defecation in response to changes in temperature is an active process in *C. elegans*, which requires *clk-1*.

## MATERIALS AND METHODS

**General methods and strains:** Most strains were derived from the wild-type *C. elegans* N2 Bristol strain and were cul-

tured as described (BRENNER 1974). The wild-type RW7000 strain was used for some of the linkage analyses, using sequence-tagged sites (STS). All animals were grown at 20° unless otherwise indicated. The genes, alleles, and STSs used in this work are as follows:

LGI: *bli-4(e937)*, *stP124*;

LGII: *rol-6(e187)*, *rol-1(e91)*, *unc-52(su250ts)*, *maP1*;

LGIII: *daf-2(e1368)*, *dpy-17(e164)*, *clk-1(qm30)*, *unc-32(e189)*, *dec-7(sa296)*, *vab-7(e1562)*;

LGIIV: *flr-3(ut9)*, *dpy-9(e12)*, *unc-33(e204)*, *unc-5(e53)*, *unc-31(e928)*, *dpy-4(e1166sd)*, *sp4*;

LGV: *unc-34(e315)*, *dpy-11(e224)*, *stP192*; and

LGX: *lin-15(n765)*, *flr-4(ut7)*, *unc-3(e151)*, *lon-2(e678)*, *unc-2(e55)*, *stP103*.

**Isolation of suppressor mutations:** *clk-1(qm30)* animals were mutagenized with 25 mM ethyl methanesulfonate (EMS), as described (SULSTON and HODGKIN 1988). Groups of five mutagenized hermaphrodites (P0) were plated on 60-mm petri dishes and left to self-fertilize. Groups of 25 F<sub>1</sub> animals were transferred to 90-mm plates as young adults and were left to lay eggs for ~24 hr. F<sub>2</sub> animals were scored for one defecation cycle each at 20°. On the basis of the assumption that specific suppressor mutations would not cause morphological or other behavioral defects, only wild-type-looking animals were scored. A maximum of 50 F<sub>2</sub> animals were scored from each plate to minimize the probability of scoring multiple worms carrying the same mutation. Animals that had a defecation cycle length of <65 sec were picked to 60-mm plates, singled, and left to self-fertilize. The progeny (F<sub>3</sub>) of the singled candidate worms were scored for defecation; only those strains that had a significant proportion of fast defecating worms in the F<sub>3</sub> generation were kept for further genetic and phenotypic analysis. A total of 5421 F<sub>2</sub> animals were screened, a number equivalent to ~2134 haploid genomes (ELLIS and HORVITZ 1991).

**Mapping of suppressor mutations:** Mutations were linked to chromosomes in one of three ways. For mutations that could be mapped on wild-type [*clk-1(+)*] backgrounds, linkage analysis was performed either by using the DA438 strain (for *qm133*, *qm141*; AVERY 1993) or by using the wild-type RW7000 strain and testing linkage to STSs by PCR analysis (for *qm142*, *qm183*) as described (WILLIAMS 1995). For mutations that had less obvious phenotypes on wild-type backgrounds or that could not be linked to an STS, linkage to each chromosome was tested separately, using strains that contained visible marker mutations in the *clk-1(qm30)* background (for *qm178*, *qm179*, *qm180*, *qm182*, and *qm184*). Formal linkage analysis was not needed for *qm166*, as it was found to be tightly linked to *clk-1(qm30)*. Once linkage to a chromosome was established, mutations were mapped more precisely using two- and three-point mapping strategies.

To avoid marker effects, for most three-point mapping experiments, homozygous recombinant progeny were isolated from F<sub>2</sub> recombinant animals and were crossed with homozygous mutant males. Defecation was scored in the F<sub>1</sub> cross-progeny. Because *qm133* has other phenotypes that could be scored in addition to defecation cycle length, including a thin, starved appearance, all mapping of *qm133* was done using conventional methods (by singling recombinant F<sub>2</sub> animals and scoring for the presence of ¼ *qm133* animals in the F<sub>3</sub> generation). All mapping of *qm133*, *qm141*, and *qm183* was done with strains in a *clk-1(+)* background and was scored at 20°; all mapping of *qm179*, *qm180*, *qm182*, and *qm184* was done with strains in a *clk-1(qm30)* background and was scored at 25°. Mapping of *qm142* was also done in a *clk-1(qm30)* background but was scored at 20°. As *qm142* has a dominant effect, it was mapped by crossing homozygous recombinant progeny with *clk-1(qm30)* males and scoring the F<sub>1</sub> animals after 48 hr

after they had reached adulthood, when the dominant effect of *qm142* is strongest. The mapping data have been submitted to WormBase (<http://www.wormbase.org/>), and the critical data are summarized in Table 2.

**Complementation tests:** Complementation tests were performed when different mutations appeared to map to the same genetic region or when a mutation mapped to a region where a Dec gene had previously been mapped. Generally, males homozygous for one mutation were mated to hermaphrodites homozygous for the other mutation, and defecation was scored in the *trans*-heterozygous F<sub>1</sub> animals. In this manner it was found that *qm166* and *qm178* fail to complement each other and the previously identified mutant *dec-7(sa296)*, which are therefore all likely to be allelic. Similarly, *qm179*, *qm184*, and *qm180* all fail to complement each other and are therefore all likely to be allelic. *qm133* maps in the region of *flr-4* but complements *flr-4(ut7)*, suggesting that they define distinct genes. Similarly, *qm182* maps in the region of *flr-3* but complements *flr-3(ut9)*, suggesting that they define distinct genes.

**Behavioral analyses:** Defecation was scored in hermaphrodites on their first day of adulthood at 20°, unless otherwise indicated. The defecation cycle length was defined as the duration between the pBoc steps of two consecutive defecations. Each animal was scored for five consecutive cycles (six consecutive pBocs), and the mean and standard deviation were calculated. To prevent the animals from being heated by the microscope lamp during the scoring session, the plates were placed on "heat sinks" (petri dishes filled with water) and animals were scored from them for a maximum of only 15 min.

**Temperature shift experiments:** Animals were grown at 20° and transferred to either 15° or 25° as young adults. Animals were then scored at 15° or 25° 2–6 hr after being transferred to that temperature. As it was difficult to maintain the temperature of the plates at 15°, animals were scored for only three consecutive cycles at this temperature, and plates were kept on the microscope for only as long as was required to score one animal.

***qm142* time course studies:** To generate heterozygous *dsc-2(qm142)/+* animals, *dsc-2(qm142) dpy-11(e224)* animals were mated with N2 males. To generate *qm30; qm142/+* animals, *clk-1(qm30); unc-5(e53)* hermaphrodites were mated with *clk-1(qm30); dsc-2(qm142)* males. Late L4 stage F<sub>1</sub> generation animals were picked to plates and examined 3 hr later. Animals that had molted to adults during this period were used for the experiment and were considered to be 1.5-hr-old adults at the end of the interval. The sample size of each genotype for each time point is ~10. The same sets of animals were scored at the different time points.

**Statistical analyses:** We performed two-sample Student's *t*-tests, taking into account the unequal variances of the samples. We tested whether N2 and *clk-1(qm30)* mutants were different when grown and scored at 15° and 25° from when they were grown and scored at 20°. Highly significant differences ( $P < 0.05$ ) were found for both N2 and *clk-1(qm30)* at 20° vs. at 15°. Significant differences were not detected for N2 at 20° vs. at 25° ( $P = 0.40$ ) or for *clk-1(qm30)* at 20° vs. at 25° ( $P = 0.27$ ). We also tested whether N2 and *clk-1(qm30)* mutants were different when grown at 20° and scored at 15° and 25° from when they were grown and scored at 20°. Highly significant differences ( $P < 0.001$ ) were found in all comparisons except for *clk-1(qm30)* at 20° vs. at 15° ( $P = 0.29$ ) and at 20° vs. at 25° ( $P = 0.99$ ). We also tested whether each suppressor mutation had a significant effect on the defecation cycle by comparing, at every temperature, each *clk-1(qm30)* double mutant strain with *clk-1(qm30)* and every *clk-1(+)* mutant strain with *clk-1(+)*. Highly significant differences (generally  $P < 0.001$ ) were found for all comparisons except the following:

*clk-1(qm30) dec-7(qm166)* vs. *clk-1(qm30)* at 15° ( $P = 0.34$ ) and at 25° ( $P = 0.97$ ); *clk-1(qm30) dec-7(qm178)* vs. *clk-1(qm30)* at 15° ( $P = 0.83$ ) and at 25° ( $P = 0.15$ ); *clk-1(qm30); dsc-3(qm184)* vs. *clk-1(qm30)* at 15° ( $P = 0.80$ ); and *clk-1(qm30); dec(qm183)* vs. *clk-1(qm30)* at 20° ( $P = 0.12$ ) and at 25° ( $P = 0.31$ ).

## RESULTS

***clk-1* mutants have a lengthened defecation cycle that is not affected by changes in temperature:** Defecation in *C. elegans* is achieved by the periodic activation of a stereotyped motor program. In wild-type animals, the defecation cycle length is 56 sec, with a standard deviation of only 3.4 sec (at 20°). As previously described (WONG *et al.* 1995; FELKAI *et al.* 1999), in *clk-1* mutants the defecation cycle is both increased in length and more irregular: In *clk-1(qm30)* animals, the cycle length is 88 sec, with a standard deviation of 14 sec, and in the weaker allele *clk-1(e2519)*, it is 77 sec, with a standard deviation of 7 sec (at 20°; Figure 1, Table 1).

To examine the effect of temperature on the defecation cycle length of wild-type and *clk-1* mutant worms, we raised the worms for two generations at 15° or 25° and scored them at the temperature at which they had been raised (Figure 1A). We found that *clk-1* mutants are slower than the wild type at all temperatures and that both genotypes have significantly longer cycles when grown and scored at 15° than when grown and scored at 20°. In contrast, when wild-type or mutant worms are grown and scored at 25° the defecation rates are not significantly different from those at 20°. These results agree with, and extend, those of IWASAKI *et al.* (1995).

To examine the effect of temperature shifts on the defecation cycle length of wild-type and *clk-1* mutant worms, we raised the worms at 20°, shifted them to 15° and to 25°, and scored them at that new temperature (Figure 1B). We found that when wild-type animals are shifted to either 15° or 25°, the defecation cycle length is significantly affected by the change in temperature. When the worms are transferred from 20° to 25°, the mean cycle length is decreased by 13 sec; when the worms are transferred from 20° to 15°, the mean cycle length is increased by 26 sec. This adjustment happens very rapidly. For example, we found that when wild-type worms are scored 5 min after they are transferred to 25°, they are already as fast as they are when scored 2–6 hr later (data not shown). Note that the defecation cycle of wild-type worms raised and scored at 25° (Figure 1A) is in fact slower than when the worms are raised at 20° and then shifted to 25° for scoring (Figure 1B). This suggests that the worms have become adapted or acclimated to the higher temperature during development and are thus less affected when scored at that temperature. However, whether the worms are raised at 15° or 20° does not appear to affect the length of the defecation cycle when it is scored at 15°.

In contrast to the observations with the wild type, we found that *clk-1* mutants are unable to readjust the

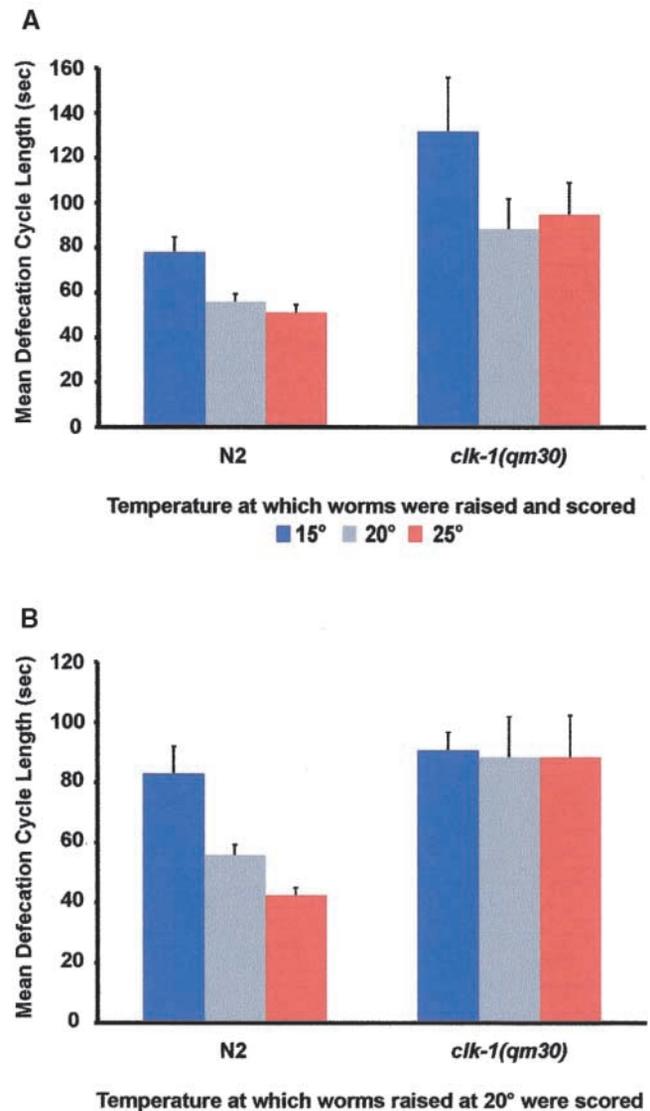


FIGURE 1.—The effects of temperature on the defecation cycle length of the wild-type (N2) and *clk-1* mutants. The bars represent the means of animals that had each been scored for five consecutive defecation cycles at 20° and 25° and for three consecutive cycles at 15°. The error bars represent the standard deviations of the means. The sample sizes are as follows: for 20°,  $n \geq 50$ ; for 15° and 25°,  $n \geq 25$ . (A) Animals were raised at 15°, 20°, and 25° and were scored at the temperature at which they had been raised. The numerical values are as follows: for N2 at 15°, 20°, and 25°, respectively,  $78.0 \pm 6.7$ ,  $55.9 \pm 3.4$ , and  $51.2 \pm 3.4$ ; for *clk-1(qm30)* at 15°, 20°, and 25°, respectively,  $131.9 \pm 24.0$ ,  $88.4 \pm 13.5$ , and  $94.8 \pm 14.3$ . (B) Animals were raised at 20° and scored at 15°, 20°, and 25°. The numerical values can be found in Table 1.

length of their defecation cycle after they are shifted to a new temperature. When *clk-1* mutants are transferred either from 20° to 25° or from 20° to 15° there is no change in the mean cycle length (Figure 1B, Table 1). This suggests that after the mutants have become adapted to 20°, they cannot readjust the defecation cycle length upon a temperature shift, indicating that *clk-1(+)* activity is required for this adjustment to occur.

**A screen for suppressors of *clk-1*:** To identify genes

TABLE 1  
Quantitative phenotypic analysis of suppressor mutations on wild-type and *clk-1* backgrounds

Mutation	Length of defecation cycle (sec)						
	Wild type			<i>clk-1(qm30)</i>			<i>clk-1(e2519):</i> 20°
	15°	20°	25°	15°	20°	25°	
+	82.8 ± 9.2	55.9 ± 3.4	42.5 ± 2.5	90.7 ± 6.0	88.3 ± 13.5	88.4 ± 13.8	77.1 ± 6.7
<i>dsc-1(qm133)</i>	62.3 ± 9.6	46.9 ± 4.5	33.4 ± 4.0	76.7 ± 10.6	64.7 ± 12.2	72.9 ± 18.4	62.2 ± 12.9
<i>dsc-2(qm142sd)</i>	61.1 ± 5.0	45.1 ± 15.5	31.2 ± 3.7	76.7 ± 21.1	60.0 ± 14.0	74.6 ± 10.0	58.8 ± 6.7
<i>dec-7(qm166)</i>	55.1 ± 3.2	35.6 ± 3.2	34.1 ± 4.7	86.2 ± 22.2	58.9 ± 27.8	88.6 ± 13.2	ND
<i>dec-7(qm178)</i>	ND	ND	ND	91.8 ± 23.9	63.2 ± 24.0	92.3 ± 11.4	ND
<i>dsc-3(qm179)</i>	75.6 ± 4.4	51.9 ± 2.8	35.3 ± 3.5	76.8 ± 9.1	63.0 ± 6.2	41.6 ± 4.3	56.4 ± 3.0
<i>dsc-3(qm180)</i>	74.4 ± 5.8	52.7 ± 2.9	33.7 ± 2.9	85.7 ± 9.2	68.5 ± 6.9	60.0 ± 20.7	66.7 ± 5.1
<i>dsc-3(qm184)</i>	73.9 ± 4.7	53.0 ± 3.2	37.0 ± 2.2	90.3 ± 5.3	66.0 ± 10.3	53.3 ± 7.6	61.4 ± 5.7
<i>dsc-4(qm182)</i>	68.1 ± 5.6	47.8 ± 2.5	33.9 ± 2.0	75.8 ± 7.5	60.7 ± 6.3	48.0 ± 7.7	60.2 ± 3.4
<i>dsc-5(qm141)</i>	64.7 ± 8.1	46.3 ± 7.0	36.5 ± 1.8	79.5 ± 6.9	75.5 ± 9.6	74.8 ± 7.4	56.7 ± 5.1
<i>dec(qm183)</i>	69.7 ± 5.2	49.9 ± 3.0	33.3 ± 1.2	102.2 ± 11.6	84.8 ± 12.2	84.4 ± 13.9	83.1 ± 15.8

Animals were raised at 20° and scored at 15°, 20°, or 25°. The numbers given are the means ± the standard deviations of animals that had each been scored for five consecutive defecation cycles at 20° and 25° and for three consecutive cycles at 15°. The sample sizes are as follows: for N2 and *clk-1(qm30)* strains at 20°,  $n \geq 50$ ; for N2 and *clk-1(qm30)* strains at 15° and 25°,  $n \geq 25$ ; for *clk-1(e2519)* strains,  $n \geq 25$ . As *dec-7* is tightly linked to *clk-1*, the defecation cycle lengths of some *dec-7* strains are not determined (ND). Phenotypic analyses of the *clk-1(+)* *dec-7(qm166)* strain were carried out in the background of the *dpy-17(e164)* mutation, which does not affect defecation.

that interact with *clk-1* to regulate the defecation cycle length, we carried out a screen to isolate mutations that could suppress *clk-1*, that is, mutations that could restore the length of the defecation cycle of *clk-1* mutants to that of the wild type. *clk-1(qm30)* worms were mutagenized with EMS and second-generation ( $F_2$ ) animals were directly scored for one defecation cycle each at 20°. Animals that had a cycle length of <65 sec were kept for further analysis. In this manner, we screened 5421  $F_2$  animals (an equivalent of ~2134 haploid genomes) and identified eight suppressor mutations, which correspond to five different complementation groups. Seven of these mutations are recessive and one mutation, *qm142*, has dominant effects (described in detail below). On the basis of the mapping and complementation tests we performed (see MATERIALS AND METHODS and Table 2), it is likely that these mutations define four new complementation groups, which we called *dsc* for defecation suppressor of *clk-1*. Two mutations, *qm166* and *qm178*, are alleles of the previously identified Dec-s gene *dec-7*. From our screen, we also isolated two other recessive mutations, *qm141* and *qm183*, which affect defecation, but which do not suppress *clk-1(qm30)* (discussed below). None of the mutations can suppress other *clk-1* phenotypes, such as slow growth or pharyngeal pumping (data not shown).

**Analysis of suppressor mutants:** We analyzed the phenotype of these suppressor mutants in a number of different ways (Table 1). We reisolated all of the mutations on a wild-type [*clk-1(+)*] background to determine how the mutations affect defecation in the presence of *clk-1(+)* activity. To see if the suppressors could also

suppress the inability of *clk-1* mutants to react to changes in temperature, we scored every *clk-1(+)* and *clk-1(qm30)* strain at 20° as well as after shifts to 15° and 25°. We also reisolated the mutations on the background of *clk-1(e2519)*, which has partial *clk-1* activity.

**There are two classes of suppressors:** By analyzing the effect of the mutations on the *clk-1(qm30)* background, we find that the suppressors fall into two distinct classes on the basis of their differential abilities to suppress *clk-1(qm30)* after temperature shifts, particularly to 25° (Figure 2). The class I mutants *dsc-3(qm179)*, *qm180*, *qm184* and *dsc-4(qm182)* strongly suppress *clk-1(qm30)* at 20° as well as after shifts to 25° (Figures 2A and 3A). At 25°, *dsc-3(qm179)*, the strongest mutant in this respect, can even shorten the defecation cycle length of *clk-1(qm30)* mutants to less than the wild-type length. Thus, the profile of defecation in the class I *dsc; clk-1(qm30)* double mutants at the different temperatures is just like that of the wild type: slowest at 15°, fastest at 25°. Therefore, these mutants can suppress the long defecation cycle of *clk-1(qm30)* mutants when grown and scored at 20° as well as the temperature insensitivity when grown at 20° and then shifted to another temperature.

In contrast to class I mutants, the class II mutants suppress only weakly [*dsc-1(qm133)* and *dsc-2(qm142)*] or not at all [*dec-7(qm166)*, *qm178*] after a temperature shift. This suggests that although these mutants can suppress the long defecation cycle of *clk-1(qm30)* mutants at 20°, they cannot suppress the inability of *clk-1(qm30)* mutants to readjust their defecation cycle length after a temperature shift. *dec-7*, however, might be a special case, as the mutants' slow mean defecation cycle after shifts to 15°

**TABLE 2**  
Summary of genetic mapping of mutants isolated in the suppressor screen

Mutation	Genetic mapping data <sup>a</sup>
<i>dsc-1(qm133)</i> X	[ <i>unc-3 lin15/dsc-1</i> ] <i>unc-3</i> (15/51) <i>dsc-1</i> (36/51) <i>lin-15</i> Complements <i>flr-4(ut7)</i>
<i>dsc-2(qm142d)</i> V	[ <i>clk-1; unc-34 dpy-11/dsc-2</i> ] <i>unc-34</i> (6/41) <i>dsc-2</i> (35/41) <i>dpy-11</i>
<i>dsc-3(qm179)</i> IV	[ <i>clk-1; unc-33 dpy-4/dsc-3</i> ] <i>unc-33</i> (17/61) <i>dsc-3</i> (44/61) <i>dpy-4</i>
<i>dsc-4(qm182)</i> IV	Fails to complement <i>qm180</i> and <i>qm184</i> [ <i>clk-1; dpy-9/dsc-4</i> ] 0/40 <sup>b</sup>
<i>dsc-5(qm141)</i> II	Complements <i>flr-3(ut9)</i> [ <i>rol-1unc-52/dsc-5</i> ] <i>rol-1</i> (7/37) <i>dsc-5</i> (30/37) <i>unc-52</i>
<i>dec(qm183)</i> X	[ <i>lon-2 unc-2/dec</i> ] <i>lon-2</i> (3/45) <i>dec</i> (42/45) <i>unc-2</i>
<i>dec-7(qm166)</i> III	Fails to complement <i>dec-7(sa296)</i> and <i>qm178</i>

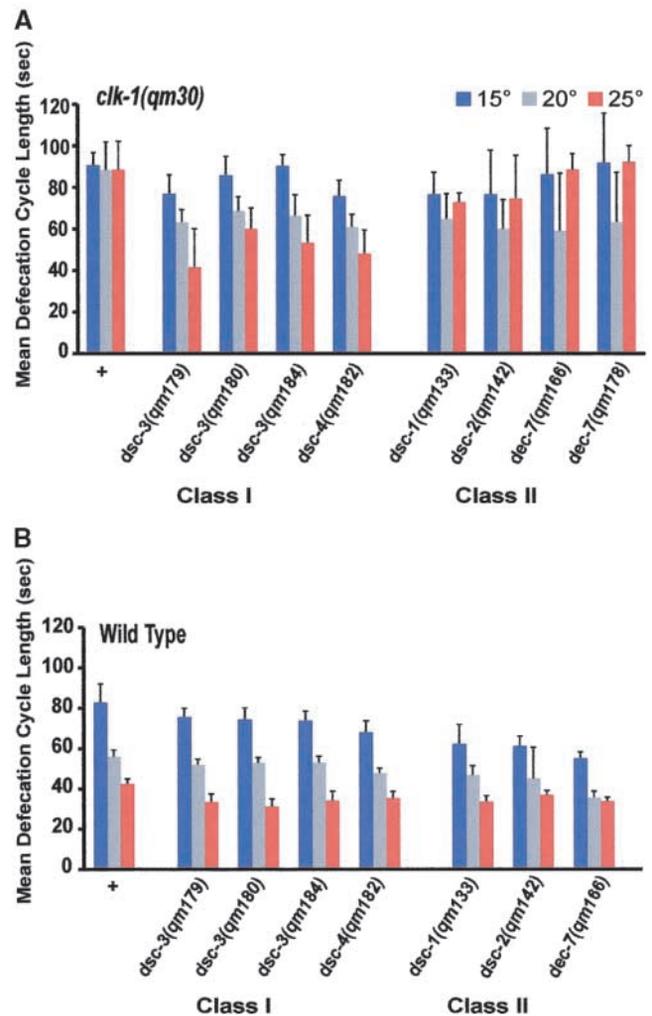
<sup>a</sup> The genotypes given in brackets are those of the F<sub>1</sub> animals whose descendants were scored to obtain two- and three-factor mapping data.

<sup>b</sup> Non-Dpy F<sub>2</sub> progeny were scored for the presence of the *qm182* mutation; the denominator represents the number of *qm182* animals that were isolated and the numerator represents the number of *qm182* animals that were also heterozygous for the *dpy-9* mutation.

or 25° could be due to an inability to carry out the DMP rather than to intrinsically slow cycling (see below).

The phenotypes of class I and class II mutants are much more similar on the wild-type background than on the *clk-1(qm30)* background, although the class I mutants generally have weaker effects on the wild type than the class II mutants, particularly at 15° and 20° (Figure 2B). All the mutations significantly decrease the length of the defecation cycle at all temperatures and both the class I and class II mutants react to changes in temperature like the wild type (*i.e.*, defecation is fastest at 25°, slowest at 15°). This indicates that the lack of effect of the class II mutants at 25° on the *clk-1(qm30)* background cannot be due to any of the *dsc* mutations being intrinsically temperature sensitive. Overall, the observation that on the *clk-1(qm30)* background there are two distinct classes of interactions suggests that the genes of the different classes interact with *clk-1* in different ways.

**The effect of the *dsc* mutations on the *clk-1(e2519)* background:** We reisolated the *dsc* mutations on the background of *clk-1(e2519)*, which has a weaker phenotype than *clk-1(qm30)*, to see if they could speed up *clk-1(e2519)* more than *clk-1(qm30)*. Although we did consistently find that the defecation cycle lengths of the *clk-1(e2519); dsc* double mutants were shorter than those of the *clk-1(qm30); dsc* double mutants, the difference was generally very small [Table 1; see below for a description



**FIGURE 2.**—There are two classes of *clk-1* suppressor mutants. Animals were raised at 20° and scored at 15°, 20°, and 25°. The bars represent the means of animals that had each been scored for five consecutive defecation cycles at 20° and 25° and for three consecutive cycles at 15°. The error bars represent the standard deviations of the means. The sample sizes are as follows: at 20°,  $n \geq 50$ ; at 15° and 25°,  $n \geq 25$ . (A) Suppressor mutations on the *clk-1(qm30)* background. The class I mutations suppress the increased cycle length of *clk-1(qm30)* mutants and restore the ability to react to changes in temperature. The class II mutations suppress the increased cycle length at 20° but do not restore the ability to react to changes in temperature. (B) Suppressor mutations on the wild-type background. The class I mutants generally have weaker effects than the class II mutants, particularly at 15° and 20°, but both classes of mutants react to changes in temperature like the wild type. The numerical values can be found in Table 1.

of the special case of *dsc-5(qm141)*]. The exact significance of this is unclear at present; however, it could suggest that *clk-1* also has effects on the defecation cycle that do not depend on the *dsc* genes. Possibly, part of the residual activity of *e2519*, which allows the mutants to have a faster defecation cycle than *qm30* mutants, acts in such a *dsc* gene-independent fashion.

**Mutations that cannot suppress *clk-1(qm30)*:** While

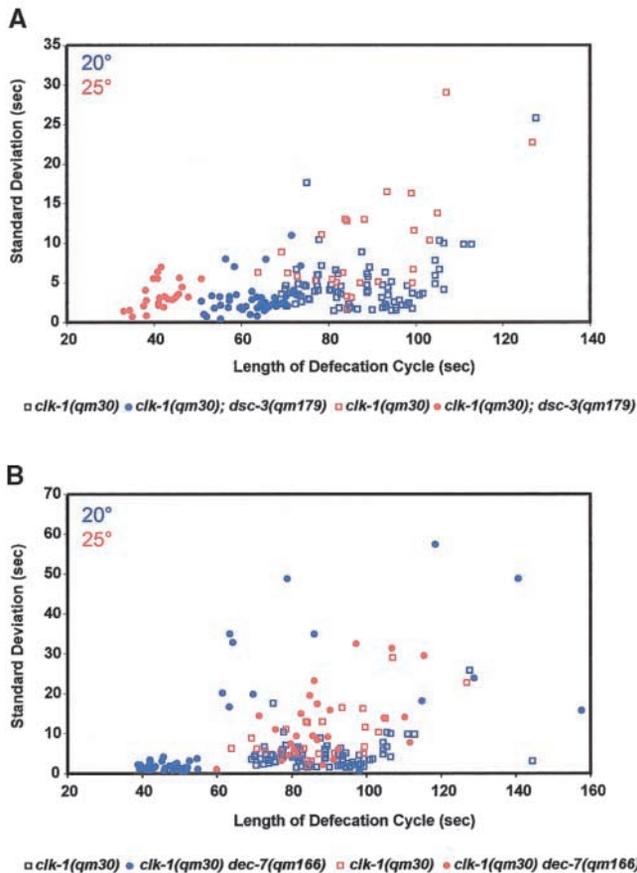


FIGURE 3.—Examples of class I and class II suppressors. Each point represents the mean (on the ordinate) and standard deviation (on the abscissa) of one animal, scored for five consecutive defecation cycles. (A) *dsc-3(qm179)* is an example of a class I mutant: It suppresses *clk-1(qm30)* at 20° and after a shift to 25°, such that in the double mutant, defecation is faster at 25° than at 20°. (B) *dec-7(qm166)* is an example of a class II mutant: It suppresses *clk-1(qm30)* at 20° but cannot suppress after a shift to 25°, such that the defecation rate of the double mutant at 25° is not faster than the defecation rate of *clk-1(qm30)*.

screening for suppressors, we isolated a number of candidate strains from which we could not subsequently isolate mutations that strongly suppress *clk-1(qm30)*. In two cases, however, the candidate strains did segregate mutations that significantly affect the rate of defecation. One of these mutations, *dsc-5(qm141)*, has a strong effect on the defecation cycle of wild-type worms at all temperatures, as well as on *clk-1(e2519)* mutants, but only a very weak, but significant, effect on *clk-1(qm30)* mutants at all temperatures. This suggests that *dsc-5(qm141)* might be acting largely through *clk-1*, as it affects the null mutant (*qm30*) only slightly, whereas it fully suppresses the partial loss-of-function mutation (*e2519*). The other mutation we isolated, *qm183*, also has significant effects on the defecation cycle of wild-type worms at all temperatures, but it cannot suppress either *clk-1(e2519)* or *clk-1(qm30)* mutants. It is clear, therefore, that the slow defecation of *clk-1(qm30)* mutants cannot simply be sup-

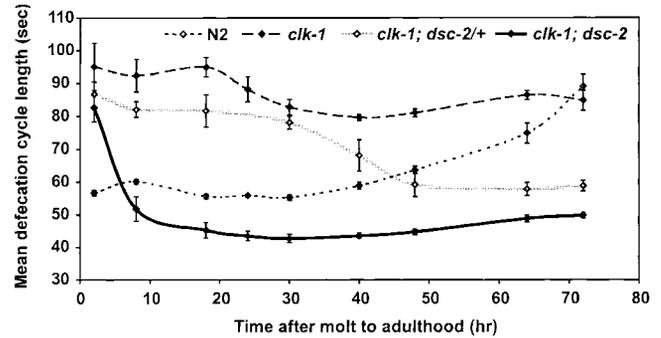


FIGURE 4.—The severity of phenotype of *dsc-2(qm142)* mutants is semidominant and time-dependent. A time-course study of mutants heterozygous and homozygous for the *dsc-2(qm142)* mutation in the *clk-1(qm30)* background is shown. Animals were scored at different time points after having molted to adults (at time 0 hr). Each point represents the mean of ~10 animals, scored for three consecutive defecation cycles at 20°; the error bars represent the standard errors of the means. *clk-1* designates the *clk-1(qm30)* allele; *dsc-2*, the *dsc-2(qm142)* allele; and +, the *dsc-2(+)* allele.

pressed by every mutation that decreases the defecation cycle length of the wild type. In fact, some mutations may require full (*i.e.*, *qm183*) or at least partial (*i.e.*, *qm141*) *clk-1(+)* activity to affect the defecation cycle.

**The *dsc-2(qm142)* mutation has a semidominant time-dependent effect:** We found that the strength of the *qm142* mutant phenotype changes with the age of the animal, in both heterozygotes and homozygotes, albeit at very different rates (Figure 4). We performed a time course study in which we scored defecation in the same animals at different time points after they had molted to adults. In a *clk-1(qm30)* background, the homozygous *dsc-2(qm142)* animals are almost as slow as the *clk-1(qm30)* animals 2 hr after molting to adults, but by 8 hr, the defecation cycle length is restored to the wild-type length, and by 18 hr, the defecation cycle length is significantly shorter than that of the wild type. The defecation cycle length of *clk-1(qm30); dsc-2(qm142)/+* animals is very similar to that of *clk-1(qm30)* animals until about 40 hr. In fact, it takes ~48 hr after molting to adults for the defecation cycle length of the *dsc-2(qm142)/+* heterozygous animals to become as fast as the age-matched wild-type animals. As the *dsc-2(qm142)/+* heterozygotes never become as fast as the homozygotes at any time point and take longer than the homozygotes to speed up significantly, the effect of the *qm142* mutation is incompletely dominant over the wild-type allele. This is also confirmed by observations of *qm142* heterozygotes and homozygotes on the wild-type background (data not shown), although the effects are much less dramatic. One way in which the *dsc-2(qm142)* allele could have this semidominant time-dependent effect is that the mutation results in a protein that can interfere with the function of the wild-type DSC-2 protein. An accumulation of the mutant product with time could increase the severity of the mutant phenotype.

**Mutations in *dec-7* result in multiple discrete defecation cycle lengths:** We characterized all the suppressor mutants by analyzing the mean defecation cycle length of a number of animals that had each been scored for five defecation cycles (Table 1). We also calculated the standard deviations of individual animals. We noted that animals carrying *dec-7* mutations in a *clk-1(qm30)* back-

ground have very high standard deviations at 15° and 20° but not at 25° [see Figure 3B for the example of *dec-7(qm166)* at 20° and 25°]. To analyze this variability further we plotted the frequency of single defecation cycle lengths of *clk-1 dec-7* animals at four different temperatures (Figure 5). At all temperatures, there is only one frequency peak for *clk-1(qm30)* mutants, but there are two peaks for both *clk-1 dec-7* double mutant strains at the three temperatures <25° (Figure 5, A–C). One of the peaks occurs at a cycle length that is two times that of where the other peak occurs. At 25°, however, there is only one peak, which coincides with the *clk-1(qm30)* peak (Figure 5D).

One interpretation of this pattern is that *clk-1* and/or *dec-7* have a role in coupling the activation of the DMP to the cycle, such that the coupling increasingly fails in *clk-1 dec-7* double mutants with increasing temperature. This would result in double cycle lengths and could mean that at 25° every cycle observed is actually a double cycle. Multiple discrete cycle lengths are not observed in *dec-7* mutants on a *clk-1(+)* background or in other class II mutants, and thus this phenomenon appears to be specific to *clk-1 dec-7* mutants.

Another interpretation is that, with increasing temperature, there is a decrease in the penetrance of the suppression of *clk-1* by *dec-7*. This is suggested by the observation that at 20°, 22.5°, and 25° the main peak coincides with the unsuppressed *clk-1* peak (Figure 5). However, this does not happen at 15°, which is difficult to explain with this interpretation.

## DISCUSSION

Mutations in *clk-1* affect numerous features of the worm including the rates of rhythmic behaviors, as well as growth and reproductive features (WONG *et al.* 1995; BRANICKY *et al.* 2000). Results from earlier experiments, which looked at the effect of temperature shifts on the growth rate of embryos, suggested that *clk-1* mutants are impaired in their ability to sense or react to changes in temperature (WONG *et al.* 1995). Here we have focused on the defecation cycle to further explore this phenomenon.

### Reaction of defecation to changes in temperature:

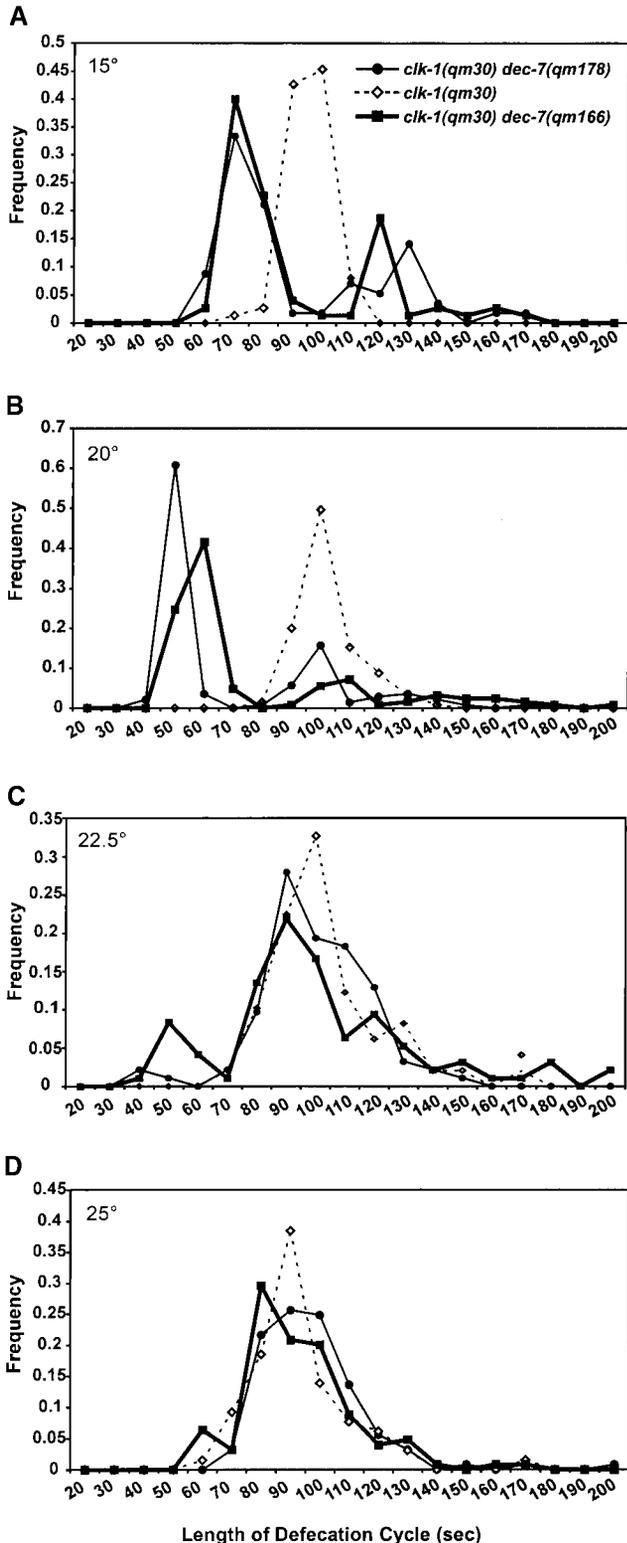


FIGURE 5.—Mutations in *dec-7* result in multiple discrete defecation cycle lengths. An analysis of the frequency of single defecation cycle lengths of *dec-7* mutants in the *clk-1(qm30)* background is shown. The sample sizes for all genotypes at 15°, 20°, and 25° are  $n \geq 25$ ; at 22.5°,  $n = 10-25$ . At all temperatures, there is only one frequency peak for *clk-1(qm30)* mutants. At 15° (A), 20° (B), and 22.5° (C), there are two frequency peaks for both *clk-1(qm30) dec-7(qm166)* and *clk-1(qm30) dec-7(qm178)* double mutants, the second peak occurring at a cycle length double that of the first peak. At 25° (D), there is only one frequency peak for the *clk-1(qm30) dec-7* double mutants, which coincides with the *clk-1(qm30)* peak.

When we examined the reaction of wild-type animals to changes in temperature we found that when they are raised at 20° and are shifted to either 15° or 25° the defecation cycle length is profoundly altered by the shift. When shifted to 15°, the defecation cycle is significantly lengthened, and when shifted to 25°, the defecation cycle is significantly shortened. The specifics of assay conditions or temperature-shift protocol may affect these results: One previous report found little change in cycle length with temperature (LIU and THOMAS 1994) but results similar to those reported here have also been obtained recently by K. IWASAKI and J. THOMAS (personal communication). Our findings do, however, indicate that there is a temperature compensation mechanism. Indeed, we have found that, in contrast to the wild type, *clk-1* mutants do not adjust the length of their defecation cycle when they are shifted to a new temperature. Because worms are poikilothermic animals and the rate of the biochemical reactions that underlie physiological processes must be temperature dependent, this implies that there is a temperature compensation mechanism that can maintain the cycle length even at different temperatures. In summary, our findings suggest that the reaction to temperature we observe in the wild type is an active process that requires *clk-1* activity. In the absence of *clk-1* activity, we can observe a temperature compensation mechanism, which presumably also acts in the wild type, such that the wild-type condition appears to be a combination of temperature compensation and active adjustment.

**Suppressors of the defecation phenotypes of *clk-1* mutants:** We reasoned that if *clk-1* is involved in regulating the timing of the defecation cycle it might work through other gene products. To identify such genes we carried out a screen for mutations that could suppress *clk-1(qm30)* at 20°. We identified eight suppressor mutations, which fall into five different complementation groups. We found that these mutations also significantly decrease the defecation cycle length of the wild-type and *clk-1(e2519)* mutants at 20°. If we consider the degree to which the mutations decrease the cycle length in the different backgrounds, (*i.e.*, the percentage by which the mutation decreases the cycle length in each background), all of the *dsc* mutations [except *dsc-5(qm141)* and *dec(qm183)*] have the greatest effect on the *clk-1(qm30)* background and the weakest effect on the wild-type background (Table 1). In some cases, this is very striking. For example, the *dsc-3* mutants decrease the rate of defecation ~25% on the *clk-1(qm30)* background but only ~5% on the wild-type background. This effect is so small that these mutations could probably not have been found in a wild-type background. On the other hand, only the *dec-7(qm166)* mutation affects the wild type to the same degree as it affects *clk-1*. It is therefore not surprising that *dec-7* was the only gene we identified that was also found in a previous screen aimed at finding

mutations that affect the defecation cycle length in a wild-type background (IWASAKI *et al.* 1995).

When we examined how defecation in the *dsc* or *dec*; *clk-1(qm30)* double mutants is affected by changes in temperature, we found that the suppressors fall into two distinct classes. Class I mutants completely suppress the defecation phenotypes of *clk-1* mutants, such that they suppress *clk-1* at 20° and after a switch to a new temperature. Double mutants carrying class I mutations have a profile of defecation very much like that of the wild type with respect to temperature: shorter after a shift to 25° and longer after a shift to 15°. In contrast, although the class II mutants suppress as well as the class I mutants when grown and scored at 20°, they cannot overcome the inability of *clk-1* mutants to react to changes in temperature. After a switch to 15° or 25° these mutations cannot, or only very poorly, suppress the slow defecation cycle. The existence of the class II mutants therefore indicates that the slow defecation and the inability to adjust the cycle length in response to changes in temperature are separable phenotypes of *clk-1* mutants.

Although it is not yet clear how the class I mutants restore the reaction to temperature, one interesting possibility is that these genes are actually components of the temperature compensation mechanism. These mutations might therefore be acting by abolishing the mechanism of temperature compensation observed in the absence of *clk-1*. In this model, the reaction to temperature observed in the *clk-1*; *dsc* double mutants might be a more passive (thermodynamic) reaction than the combination of compensation and active adjustment we believe to be the wild-type condition. This would imply that the combined mechanisms of temperature compensation and active adjustment increase the defecation cycle length from what it would be if it was determined passively by temperature. A mechanism that prevents the organism from functioning permanently at the highest possible rate at a given temperature might be adaptive.

**Toward understanding the pleiotropic *clk-1* phenotype:** Although *clk-1* affects ubiquinone biosynthesis (JONASSEN *et al.* 2001; MIYADERA *et al.* 2001; STENMARK *et al.* 2001), it is not clear how this is linked to the other phenotypes of *clk-1* mutants. Indeed, *clk-1* mutant mitochondria can function properly using DMQ, the biosynthetic intermediate they accumulate, and the severity of the ubiquinone phenotype is not correlated with the severity of the organismal phenotypes. The results presented here also support the notion that the phenotype of *clk-1* mutants, specifically the slow defecation phenotype, cannot simply be explained by a lack of ubiquinone. Indeed it is unlikely that the suppressors, which do not affect any other phenotype, act by overcoming the ubiquinone deficiency in only those cells that are responsible for the slow defecation cycle of the *clk-1* mutants. One possibility is that *clk-1* functions in

other processes in addition to the biosynthesis of ubiquinone. Alternatively, it could be that although DMQ can partially replace UQ in the respiratory chain, it is unable to replace UQ for some of its other functions, whose resulting impairment is responsible for some aspects of the *clk-1* phenotype, such as the slow and temperature-invariant defecation cycle we described here. UQ is found in almost all biological membranes (DALLNER and SINDELAR 2000) and is known to be a cofactor of the uncoupling proteins (UCP) in the mitochondria (ECHTAY *et al.* 2000, 2001), to regulate the permeability transition pore (FONTAINE *et al.* 1998), and to function in plasma membrane and lysosomal oxido-reductase systems (SANTOS-OCANA *et al.* 1998; GILLE and NOHL 2000). Identifying the molecular nature of the *dsc* genes and their cellular and intracellular localization should thus help us understand the basis of the *clk-1* phenotypes and the regulation of the defecation cycle in general.

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