

Mutations in the *clk-1* Gene of *Caenorhabditis elegans* Affect Developmental and Behavioral Timing

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ABSTRACT

We have identified three allelic, maternal-effect mutations that affect developmental and behavioral timing in *Caenorhabditis elegans*. They result in a mean lengthening of embryonic and postembryonic development, the cell cycle period and life span, as well as the periods of the defecation, swimming and pumping cycles. These mutants also display a number of additional phenotypes related to timing. For example, the variability in the length of embryonic development is several times larger in the mutants than in the wild type, resulting in the occasional production of mutant embryos developing more rapidly than the most rapidly developing wild-type embryos. In addition, the duration of embryonic development of the mutants, but not of the wild type, depends on the temperature at which their parents were raised. Finally, individual variations in the severity of distinct mutant phenotypes are correlated in a counterintuitive way. For example, the animals with the shortest embryonic development have the longest defecation cycle and those with the longest embryonic development have the shortest defecation cycle. Most of the features affected by these mutations are believed to be controlled by biological clocks, and we therefore call the gene defined by these mutations *clk-1*, for "abnormal function of biological clocks."

A number of features in living organisms appear to be precisely timed. Frequently, the processes that regulate timing are called biological clocks. In the mechanical world, clocks are machines that measure time. Do organisms possess devices that fulfill the same function? Most organisms appear indeed to have a circadian clock, an internal mechanism that allows them to anticipate, and thus to keep in register with, the phenomena of the external world, such as day and night or the seasons. Numerous observations strongly support the existence of internal circadian clocks. Among the most important is the existence of free-running rhythms, for example, in behavioral activity patterns. Vertebrates and invertebrates show peaks of locomotory activity at particular times of the day or night, that still are present in the absence of external stimuli, for example when animals are kept in constant darkness or constant light.

Another insight into the nature of the circadian clock mechanism has come from genetics (DUNLAP 1993). In a number of organisms including *Drosophila* (KONOPKA and BENZER 1971), *Neurospora* (FELDMAN and HOYLE 1973), the hamster (RALPH and MENAKER 1988) and the mouse (VITATERNA *et al.* 1994), mutations have been isolated that alter the period of the free-running rhythms. Furthermore, distinct mutations can either shorten or lengthen that period. Molecular cloning of the *per* locus of *Drosophila* and the *frq* locus of *Neuro-*

spora subsequently demonstrated that these changes could be brought about by single amino acid changes in the protein products of these loci.

It has been suggested that the action of *per* is involved very directly in the circadian clock mechanism. The abundance of the *per* mRNA and PER protein product cycle with a circadian periodicity (HARDIN *et al.* 1990; ZERR *et al.* 1990) and there is negative feedback of the PER protein on the abundance of the *per* mRNA (HARDIN *et al.* 1990). In addition, PER possesses dimerization sequence motifs shared with a number of basic-helix-loop-helix transcription factors (HUANG *et al.* 1993). This suggests that the activity of the PER protein and the regulatory properties of the *per* mRNA and gene might, together, constitute the clock mechanism. Essentially similar findings and conclusions have been reached for the *Neurospora* clock gene *frq* (ARONSON *et al.* 1994).

Circadian clocks are responsible for the activation of behaviors at particular times during the day-night cycle. Many behaviors are also periodic in a completely different sense: as reiterated activation of a particular motor program. Walking, breathing, gut peristalsis, heart rate and sleep patterns are only a few familiar examples among the large number of such ultradian rhythms. Some of these rhythms are very regular, and the mechanisms underlying these stable oscillations are being investigated at numerous levels (GLASS and MACKEY 1988; FRIESEN *et al.* 1993). For the most complex motor patterns, it generally is believed that neuronal clocks, also called central pattern generators, drive these rhythms

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(COHEN *et al.* 1988). A central pattern generator can be visualized as a neuronal network with an oscillatory output. The neuronal bases of pattern generators generally have been investigated at the physiological level. Insights at the genetic level have come from studies on the effects of *per* mutations on the *Drosophila* courtship song (KYRIACOU and HALL 1980) and more recently from studies on the defecation cycle in the nematode *Caenorhabditis elegans* (THOMAS 1990; LIU and THOMAS 1994). This defecation cycle consists of the successive contraction of three distinct sets of muscles, repeated at regular intervals and resulting in the expulsion of gut contents. A number of mutations that can alter the period of this cycle now have been identified (D. LIU and J. THOMAS, personal communication). As for mutations in circadian clock genes, they include mutations that lengthen, as well as shorten, the cycle period.

The ontogeny of multicellular organisms also requires mechanisms that ensure that this extraordinarily complex sequence of events is coordinated correctly. Indeed, much effort in the field of developmental biology is directed at determining the spatial and temporal patterns of activation of genes defined by mutations affecting development. In *C. elegans*, this has included the isolation of heterochronic mutations that alter the succession of larval stage-specific developmental events (AMBROS and HORVITZ 1984; AMBROS 1989). However, in spite of the striking regularity in the timing of developmental events, including the total length of development, no evidence for a general mechanism that would measure developmental time has been presented yet (GILBERT 1991). In the absence of such a developmental clock, developmental timing would have to be seen as resulting entirely from the sheer precision of cascades of inductive interactions.

In multicellular organisms, development is accompanied by, and largely based on, an increase in the number of cells composing the organism. The length of the cell cycle therefore could play a central role in determining the outcome of many developmental events. Indeed, in some instances regulation of the cell cycle period appears to be used as an effector of developmental timing. For example, in many organisms a sudden lengthening of the early embryonic cell cycles is concomitant with a number of developmental events at the so-called midblastula transition (NEWPORT and KIRSCHNER 1982a,b). There appears to be a threshold in the steadily increasing nuclear/cytoplasmic ratio during embryonic cleavage that triggers this transition (EDGAR *et al.* 1986; KIMELMAN *et al.* 1987). Here, regulation of the duration of the cell cycle clearly is used as a type of developmental clock.

Cell division plays a central role in all life cycles, and the mechanisms driving the cell cycle, (sometimes called the cell-cycle clock) are being studied in great detail (NORBURY and NURSE 1992). Much attention has focused on the interactions between cellular kinases

and phosphatases and the cyclins. At least some cyclins are clearly timing devices; they are unstable molecules that slowly accumulate during various phases of the cell cycle, and their sudden destruction corresponds to transitions from one phase to the next. Most work with cyclins and associated molecules has focused on their involvement in the mechanisms that ensure the proper succession of events of the cell cycle rather than the length of the cycle as a whole (HARTWELL and WEINERT 1989). However, recent work with new types of cyclins, such as the D cyclins, is addressing the role of cyclins as transducers of signals that set the length of the cell cycle period (SHERR 1993).

The final developmental events for most organisms are senescence and death. Research into the determinants of life span is uncovering environmental effects as well as genetic components contributing to the duration of life. In *C. elegans* both avenues are being actively pursued (DECUYPER and VANFLETEREN 1982; FRIEDMAN and JOHNSON 1987; JOHNSON 1987; HONDA *et al.* 1993; VANFLETEREN 1993). In particular, the possibility of an internal mechanism determining life span is being investigated at the genetic level (FRIEDMAN and JOHNSON 1987; JOHNSON 1987; KENYON *et al.* 1993).

Above, a number of processes that appear to be, or could be, using timing devices have been outlined. These regulatory mechanisms appear to possess very different characteristics and no evidence points to a common mechanism. Here, however, we show that mutations in the gene *clk-1* of *C. elegans* affect the timing of most of these features. We discuss how this pattern of phenotypes could be produced by mutations in a single gene, and speculate that *clk-1* might be involved in the function of a central biological clock that would ensure the temporal coordination of all features of the organism.

MATERIALS AND METHODS

General methods and strains: *C. elegans* was cultured as described by BRENNER (1974). Standard genetic methods for *C. elegans* were used (SULSTON and HODGKIN 1988). Animals were cultured at 20° unless otherwise stated. Wild-type animals were N2 Bristol strain. Mutant animals used were CB4876 *clk-1(e2519)*, MQ28 *clk-1(qm11)*, MQ50 *clk-1(qm30)*, CB1978 *dpy-17(e164) unc-32(e189)*, MQ27 *dpy-17(e164) clk-1(e2519)* and CB4512 *gro-1(e2400)*.

Isolation of *clk-1* mutants: Wild-type (N2) animals were mutagenized with ethyl methane sulfonate (EMS) following the standard protocol (SULSTON and HODGKIN 1988). Groups of five mutagenized hermaphrodites (P_0) were plated on 9-cm petri dishes and allowed to self-fertilize. They were removed after having laid ~200 eggs (40 eggs each). The resulting F1 animals were left on the plate until they had laid a total of 6000–10,000 F2 eggs and then were removed. When most of the resulting F2 animals had grown to adulthood, 50 adults with no detectable morphological or behavioral mutant phenotype were picked and transferred onto individual small plates. Those animals that produced an entire brood of morphologically, behaviorally or developmentally (slow growth) mutant worms were analyzed further. Removing P_0 and F1

animals from the plates after they had laid a limited number of eggs had several advantages. It ensured that the F2 worms were sufficiently staged to make it unlikely that a slowly developing F2 animal that had been laid early would be mistaken for an animal developing at the normal rate. It also allowed us to start the procedure with several mutagenized animals without having to deal with overcrowded plates at the screening stage. The full results of this screen will be presented elsewhere (P. BOUTIS, B. LAKOWSKI and S. HEKIMI, unpublished data). Five mutations, out of 15,000 F2 animals screened in the way described above, were isolated, tested for complementation and mapped.

Complementation tests: After isolation, all mutants were crossed to wild-type (N2) males and the progeny of these animals examined. For each mutant, all male progeny as well as an approximately similar number of hermaphrodites were found to develop and defecate at wild-type rate, indicating that the presence of a wild-type copy of the gene in the zygote is sufficient to obtain a wild-type phenotype. The progeny (F2) of these rapidly growing (F1) hermaphrodites also was found to grow at wild-type rate. A number of such rapidly growing F2 animals were picked singly onto fresh plates and approximately a quarter of these animals produced only slow-growing progeny. These experiments indicate that all our mutations are fully recessive maternal effect mutations for the postembryonic growth rate and defecation-cycle length phenotypes. The possibility of a paternal effect was tested for *clk-1(e2519)*: when *e2519/+* males were crossed to *e2519/e2519* hermaphrodites, one half of the male progeny was slow growing and showed a slow defecation cycle. Notice, however, that for *clk-1(e2519)/+* derived from *e2519/e2519* mothers a slight maternal effect lengthening of the duration of embryogenesis might have been detected (see RESULTS). Similarly, slow-growing males sired by *e2519/+* fathers might have a slightly shorter embryogenesis than expected (see RESULTS).

Two types of complementation tests were performed. First, *e2519* homozygous males were mated to homozygous mutant hermaphrodites of the other slow-growing strains, and the male cross-progeny were tested for slow postembryonic growth and slow defecation cycle. Two mutations, *qm11* and *qm30* failed to complement *e2519* by this test. Second, self-progeny of *trans*-heterozygotes for mutations that failed to complement by the first test were examined. It was found that all self-progeny were slow growing. This indicates that these mutations failed to complement for the maternal effect as well; that is, *trans*-heterozygous mothers failed to produce maternal rescue. The first time these tests were performed, non-complementing double heterozygous hermaphrodites could not be identified formally because no other genetic markers were used. Instead, the approximate number of hermaphrodite cross-progeny on a particular plate was inferred from the number of males, and a sufficiently large number of single hermaphrodites were picked to ensure that a majority should be cross-progeny. Later, these tests were repeated and confirmed by crossing *qm11* or *qm30* males into a *dpy-17(e164) clk-1(e2519)* double mutant.

The second type of complementation test described above was also a test for second-site noncomplementation: *trans*-heterozygotes between two unlinked noncomplementing recessive mutations should produce 5/16 phenotypically wild-type progeny. In contrast, there should be no wild-type progeny if the two mutations are closely linked. By this test, *e2519*, *qm11* and *qm30* were found to be closely linked.

Mutation frequency of *clk-1*: The conditions of our screen are such that the frequency at which *clk-1* alleles were recovered represent a slight underestimate of the mutation frequency. We picked and examined 50 single animals out of

each plate of 10,000 F2s issued from 200 F1s. Only 1/4 of the 50 F2s issued from each F1 will be homozygous and identical by descent at a given locus. Therefore, on average, only 12.5 out of the 10,000 animals, from which 50 are picked, are identical by descent at a given locus. Under these conditions there is only a small probability that animals identical by descent at a particular locus will be chosen twice (or more).

Genetic mapping of *clk-1*: Three-point mapping against *dpy-17* and *unc-32* was performed for *clk-1(e2519)* with the following results: *dpy-17* (3/63) *clk-1* (60/63) *unc-32*. Two-point mapping was performed using MQ27 *dpy-17(e164) clk-1(e2519)* and four recombinant chromosomes were found out of 1450 chromosomes scored. Together these results indicate that *clk-1* is ~0.2 map units to the right of *dpy-17*.

Assays for developmental phenotypes: *Embryonic development:* Adult hermaphrodites were placed in a drop of M9 buffer and cut open with a razor blade. Two-celled eggs were chosen and placed singly onto fresh plates. The embryos were monitored every 30 min until hatching.

Post-embryonic development: Unstaged eggs were placed at 20° and left for 1 hr to hatch. Larvae that had hatched during that period were placed singly on fresh plates and monitored every 3 hr until maturity. Animals were scored as mature adults after they had undergone the final molt and a vulva could be observed.

Egg-production rate: Ten hermaphrodites that had reached adulthood 24 hr before were picked and placed on fresh plates for 4 hr at 20°. The 10 hermaphrodites then were removed and the number of eggs laid was counted.

Self-brood size: Larvae were placed singly onto fresh plates and incubated at 20° until they had matured and laid the first few eggs. The hermaphrodites then were transferred onto fresh plates daily to prevent overcrowding until egg-laying ceased. The progeny were counted 3 days after removal of the parents.

Life span: Unstaged eggs were placed at 20° and left for 1 hr to hatch. Larvae that had hatched during that period were placed singly onto fresh plates, and monitored once daily until death. The animals were transferred once daily while producing eggs to keep them separate from their progeny. Animals were scored as dead when they no longer responded with movement to light prodding on the head.

Time-lapse video microscopy: Embryos at the pronuclear migration phase were mounted on a 2% agar pad in M9 buffer. They were visualized with a Zeiss Axiovert Microscope with a ×100 oil immersion objective. Recordings were made with a Hamamatsu CCD camera model C2400, and a Panasonic time-lapse video cassette recorder model G720A. The temperature on the surface of the microscope slide was monitored and kept at 21°.

Assays for behavioral phenotypes: Behavioral phenotypes were scored at 20° in hermaphrodites that had reached adulthood ~24 hr before.

Defecation: Hermaphrodites were placed singly onto fresh plates. They were maintained at 20° and the defecation cycle length of the animals was measured. Defecation cycle length was defined as the duration between the first muscular contraction of one defecation and the first muscular contraction of the next defecation. For each animal, five defecation cycles were measured with a stopwatch and the mean and standard deviation of the five values were calculated. The duration of the three muscular contractions which constitute the defecation behavior was timed separately. The stopwatch was started at the first sign of the anterior muscular contraction and stopped after expulsion. Three defecations were measured for each animal.

Pumping: Pharyngeal pumping was visualized through the

dissecting microscope. The animals were scored for 1 min and each animal was scored twice.

Swimming: Animals were placed in a drop of M9 buffer and the simple rhythmic thrashing of the animals was counted. The animals were scored for 1 min and each animal was scored twice.

Effects of temperature on embryonic development: Animals were cultured at three different temperatures, 15, 20 and 25°. Gravid hermaphrodites from each temperature were cut open with a razor blade and two-celled embryos were chosen and returned to plates stored either at the same temperature at which the parents had been cultured or at 20° for the remainder of embryogenesis. These embryos were monitored every 30 min until they hatched.

Correlation assays: Embryonic development of two-celled embryos at 20° was monitored as above. "Quickly developing" and "slowly developing" embryos were kept for further post-embryonic development and defecation cycle period analyses. Quickly developing embryos were defined as those that hatched before 13 hr for N2 and those that hatched before 12.5 hr for *clk-1* (*e2519*). Slowly developing embryos were defined as those that hatched after 13.5 hr for N2 and those that hatched after 21.75 hr for *clk-1* (*e2519*). Postembryonic development and the adult defecation cycle period were scored for each of these embryos.

RESULTS

A genetic screen for maternal-effect mutations affecting development and behavior: To identify new genes affecting development, we performed a genetic screen to isolate mutants that present a morphological or behavioral phenotype when derived from a homozygous mutant parent, but that are phenotypically rescued when derived from a parent carrying a wild-type allele of the affected gene. Our rationale for this screen is that only gene products required early in development or in very small amounts could be deposited in the egg in sufficiently large amounts to be able to rescue the phenotype of adults, which are ~500 times more voluminous than the egg.

Wild-type (N2) worms were mutagenized with ethyl methane sulfonate (EMS) and allowed to self-fertilize for two generations. Animals resulting from the second round of self-fertilization (F2) are likely to be homozygous carriers of a number of new mutations with or without visible phenotypic effects. Such animals were examined and those which displayed wild-type growth rate as well as overall wild-type morphology and behavior were selected, placed singly onto fresh plates and allowed to self-fertilize. Those strains that then produced an entirely mutant brood (F3) were analyzed further (see MATERIALS AND METHODS for details of the screening procedure).

Out of 15,000 F2 animals and their progeny analyzed in this way, three allelic mutations (*e2519*, *qm11* and *qm30*), which confer slow developmental rates and display a slow defecation cycle, were identified. They define the *clk-1* gene. Very slowly growing strains have generally a very sick appearance, the animals being thinner and more transparent than the wild type. *clk-1* mu-

tants, however, have an essentially wild-type appearance. The first *clk-1* mutation to be identified (*e2519*) was originally kept and studied because the mutants displayed this surprising association of apparent health and slow growth. It was later found that these mutants also displayed a slow defecation cycle. Only two other mutants (*qm37* and *qm38*) were found that are not allelic to *clk-1* or to each other but have features in common with the *clk-1* mutants, including slow growth, a wild-type general appearance, a slow defecation cycle and maternal rescue of the defects. These findings suggest that only a small number of loci can mutate toward a phenotype resembling that of *clk-1* mutants. *clk-1* maps to chromosome III, ~0.2 map units to the right of *dpy-17* (see MATERIALS AND METHODS). This position suggested possible allelism to *gro-1* (*e2400*), which similarly confers overall slow growth and had been isolated from a wild strain by JONATHAN HODGKIN (personal communication). Our examination of the phenotype of *gro-1* (*e2400*), revealed a number of additional similarities to *clk-1* mutants, including maternal rescue and a slow defecation cycle. However, *gro-1* (*e2400*) complements all three *clk-1* alleles.

The three *clk-1* mutations are probably loss-of-function alleles, as they arose at a frequency (1/5000) expected for loss-of-function mutations when using the standard EMS mutagenesis protocol (BRENNER 1974; GREENWALD and HORVITZ 1980; AVERY 1993). Moreover, as shown below, the same set of features is affected to various degrees, but in a qualitatively similar way in all three mutants. In addition, the postembryonic development of the *trans*-heterozygote *e2519/qm30* appears intermediate in severity between the two.

***clk-1* mutations are pleiotropic:** We examined the development and behavior of *clk-1* mutant and wild-type worms and found that *clk-1* mutations have broadly pleiotropic effects (Table 1). All observed phenotypic effects are related to timing and result in a mean lengthening of the duration of developmental stages and of the period of cyclic behaviors. This mean lengthening is sometimes, but not always, associated with an increase in the variability of the measured value, as indicated by an increase in the standard deviation of the sample. A detailed examination of the animals by differential interference contrast microscopy did not reveal any morphological abnormalities. The same set of timing features is affected in all three alleles of *clk-1*.

The duration of embryonic development was scored from the two-cell stage to hatching. Eggs were dissected from hermaphrodites and observed continuously (every 30 min) until hatching. The two-cell stage was chosen as the start point for the ease with which it can be identified under the dissecting microscope. The mean duration of embryonic development is affected in all three alleles up to a near twofold mean lengthening in the strongest allele (*qm30*). However, even the slowest embryos of any allele (>30 hr at 20°) proceed to fur-

TABLE 1
Quantitative phenotypic analysis

Phenotypes	Genotypes				
	N2	<i>e2519 m⁺z⁻</i> (maternal rescue)	<i>e2519 m⁻z⁻</i>	<i>qm11</i>	<i>qm30</i>
Embryonic development (hours)	13.3 ± 1.0 (n = 500)	13.6 ± 0.8 (n = 50)	17.1 ± 3.9 (n = 500)	16.1 ± 1.0 (n = 100)	22.8 ± 5.0 (n = 100)
Postembryonic development (hours)	46.6 ± 3.0 (n = 500)	50.6 ± 3.1 (n = 100)	70.6 ± 4.8 (n = 500)	81.0 ± 5.6 (n = 100)	99.2 ± 6.2 (n = 100)
Egg production rate (eggs/hour)	6.0 ± 1.2 (n = 200)	3.8 ± 0.9 (n = 25)	2.7 ± 1.0 (n = 200)	2.1 ± 0.5 (n = 200)	0.9 ± 0.1 (n = 200)
Self-brood size (no. of progeny)	302.4 ± 30.5 (n = 20)	340.9 ± 77.2 (n = 22)	191.1 ± 33.0 (n = 20)	191.1 ± 17.6 (n = 10)	87.2 ± 37.2 (n = 10)
Life span (days)	18.6 ± 5.2 (n = 50) (max: 27)	19.9 ± 5.3 (n = 50) (max: 33)	26.0 ± 9.0 (n = 50) (max: 45)	28.5 ± 10.3 (n = 50) (max: 46)	22.8 ± 9.8 (n = 50) (max: 46)
Defecation (mean of five cycle periods, in sec)	50.8 ± 5.6 (n = 100)	54.7 ± 8.4 (n = 11)	69.4 ± 9.9 (n = 100)	77.9 ± 6.9 (n = 25)	92.4 ± 15.0 (n = 25)
Pumping (cycles/min)	259.0 ± 23.7 (n = 25)	232.3 ± 26.7 (n = 11)	156.0 ± 29.0 (n = 25)	169.2 ± 25.0 (n = 25)	170.3 ± 26.9 (n = 25)
Swimming (cycles/min)	120.7 ± 6.5 (n = 25)	120.3 ± 13.2 (n = 11)	91.7 ± 5.7 (n = 25)	87.5 ± 9.8 (n = 25)	75.6 ± 4.9 (n = 25)

Phenotypic comparison of wild-type (N2) animals, *clk-1* mutants and maternally rescued *e2519* mutants. Developmental and behavioral phenotypes were compared among wild type (N2) and mutants carrying either of three *clk-1* alleles: *e2519*, *qm11* and *qm30*. The numbers are means ± SD and the sample size is given in parentheses. In the header, *m* represents the maternal and *z* the zygotic contribution of *clk-1* activity, respectively; + and -, wild-type and mutant form of the gene, respectively. All phenotypes were scored at 20°C. The maximum life span observed is also given in the life span row. Data used to obtain the values for defecation and embryonic development are shown in full form as Figures 1 and 2, respectively.

ther development after hatching. In all three alleles, this lengthening of embryonic development is also reflected in a slowing down of the cell cycle (analyzed in more detail in a later section). The high variability of the developmental times of the mutants and the effects of temperature are described in more detail in later sections.

Postembryonic development was scored by monitoring the developmental stage of the worms every 3 hr, and we observed a lengthening of the duration of all four larval stages. The mean developmental time is altered in all three alleles up to a more than twofold lengthening in *qm30* (Table 1). However, even the slowest animals (>100 hr) became fertile adults and had a generally wild-type appearance. The lengthening of postembryonic development is the phenotype by which the mutations were isolated originally and which is followed in genetic crosses. For all mutations, the distribution of developmental times does not overlap that of wild type and is much broader than that of the wild type (Table 1). However, the percentage increase in standard deviation in the mutants is not significantly greater than the percentage increase in mean developmental times. This suggests that whatever is responsible for the variability in postembryonic development is the same in the mutants as in the wild type.

The egg production rate is one of the most strongly

affected features in *clk-1* mutants, with a more than sixfold decrease in *qm30*. Self-brood size is also strongly affected, with a more than threefold decrease in *qm30* (Table 1). The self-brood size is a measure of the number of sperm produced by the hermaphrodite (WARD and CARREL 1979). All three mutants can produce a much larger number of progeny when mated by males. However, self-brood size is only an indirect measure of sperm number, and we cannot exclude the possibility that the mutants make some nonfunctional sperm. In spite of the slowdown of gametogenesis, the adult gonad appears wild type in the distribution of mitotic germ cells *vs.* maturing oocytes. This suggests that mitotic germ line proliferation and gametogenesis are slowed down in a concerted manner. However, the reduction in usable sperm suggests that the slowing down of sperm production is not compensated by a corresponding lengthening of the duration of spermatogenesis.

The mean and maximum life span were found to be lengthened in all three mutants (Table 1). This is not due to a delayed onset of mortality. Rather, the rate of dying of the population of mutants is slower than the wild-type rate at all chronological ages (data not shown).

Defecation is effected by a series of three muscular contractions involving three distinct groups of muscles.

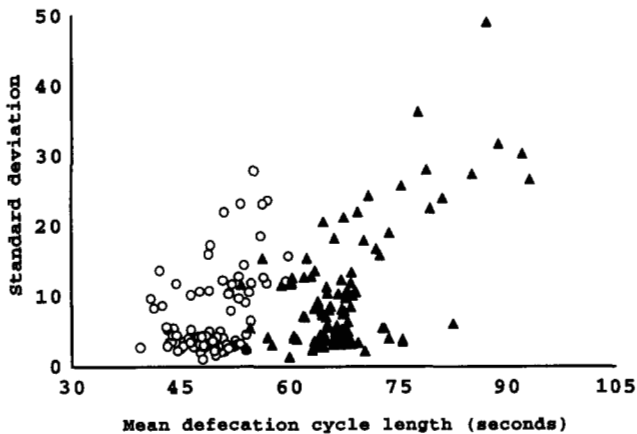


FIGURE 1.—Defecation cycle length of wild-type (N2) and *clk-1* (*e2519*) animals. Defecation cycle length of individual animals was scored at 20° for five consecutive defecations. Defecation is effected by a series of three muscular contractions. A defecation cycle was defined as the time between the first muscular contraction of one defecation and the first muscular contraction of the next defecation. Each point represents one animal. Sample size is 100 for each genotype. Wild-type animals (○) and *e2519* animals (▲) are represented. The ordinate corresponds to the mean length of five defecation cycles and the abscissa represents the standard deviation for that mean cycle length.

The series of three contractions is repeated at regular intervals (THOMAS 1990). Animals were scored for five interdefecation periods and the mean and standard deviation of the five periods were calculated for each animal. Figure 1 shows the relationship between the mean and the corresponding standard deviation for each of the 100 N2 and *e2519* animals. The values shown in Table 1 for N2 and *clk-1* alleles are the mean and standard deviation of the means of all individual animals scored. The cycle period is strongly affected in all alleles, with a 1.8-fold lengthening in *qm30*, the strongest allele. However, this lengthening does not result in constipation as in other defecation-cycle mutants (THOMAS 1990). In addition, the *clk-1* mutants appear to be not only slower than the wild type but also more variable in two distinct ways. First, as can be seen in Figure 1 and as indicated by the larger standard deviation given in Table 1, the distribution of the means is broader in the mutant: the mutant animals are more different from each other than are the wild-type animals. Second, individual mutant animals have, on average, a more irregular cycle period (the abscissa of Figure 1). The average standard deviation for individual animals is 6.4 seconds for N2, while it is 10.4 sec for *e2519*.

In *clk-1* mutants, pharyngeal pumping is slowed down 1.4-fold. However, these mutants do not appear starved, in contrast to other mutants known to affect pumping frequency (AVERY 1993). For this phenotype, as for life span, all three alleles are of similar severity. This is true both for the mean rate and the standard deviation of the sample. Pumping rate is the cyclic feature with the highest frequency that we have examined.

On an agar surface *C. elegans* moves forward by propagating rearward sinusoidal waves. For technical reasons, however, we measured locomotory performance by scoring swimming, a simple rhythmic thrashing, in a drop of M9 buffer. All three alleles show a significant slowing of swimming frequency, with *qm30* being the most severe mutation (Table 1).

***clk-1* mutations can be maternally rescued:** *clk-1* homozygous mutants (*clk-1/clk-1*) descending from self-fertilizing *clk-1/+* parents display a profound maternal rescue of their development and behavior (Table 1), which was the basis for selection in the mutant screen. To obtain the scores shown in Table 1, whole broods of self-fertilizing *e2519/+* mutants were scored for all phenotypes and homozygous mutant animals identified later by their entirely mutant brood. Most phenotypes are totally or partially rescued. The egg-production rate is the least well rescued phenotype, with a value intermediate between that of mutant and wild type. In spite of the fact that adults are ≥ 500 times larger than the egg, an almost complete phenotypic rescue extends to postembryonic development and life span (shortened in rescued animals) as well as to behaviors such as defecation, which are scored in adults. Furthermore, in maternally rescued animals the self-brood size is excessive relative to the wild type (Table 1). The increased mean brood size also is associated with a major increase in variability, so that the maximal score recorded (580) is far greater than ever seen in the wild type (357).

In contrast to the strong maternal effect, we observed only a very minor paternal effect, which we investigated using *e2519.clk-1/clk-1* animals derived from *clk-1/clk-1* mothers and *clk-1/+* fathers appear entirely mutant except that embryonic development appears to be slightly shortened relative to *clk-1/clk-1* self-progeny (<10%). Although it is very strong, the maternal effect is not strict: one zygotic copy of *clk-1(+)* is also sufficient for a wild-type phenotype: *clk-1/+* animals derived from *clk-1/clk-1* mothers appear almost entirely wild type, except for a small (<10%) delay in the embryonic development time compared to animals with a fully wild-type pedigree (data not shown).

The profound maternal rescue suggests that either *clk-1* is required early in development or that the *clk-1* product is required in vanishingly small amounts and that the perdurance throughout development of maternal product is sufficient for phenotypic rescue in adults. In later sections, we describe experiments in which we use temperature shifts to manipulate the rate of development and which support the former hypothesis.

The length of embryonic development is highly variable in *clk-1* mutants, resulting in animals which develop more rapidly than the wild type: We scored the embryonic development of all three *clk-1* mutants and N2 animals; they are shown as histograms in Figure 2. The distribution of N2 animals centers around a single modal value. The distribution of *qm11*, the *clk-1* allele

with the shortest mean embryonic development (Table 1), similarly centers around a single value: the embryonic development of these animals is slow but as regular as the wild type. In contrast, the distribution for *e2519* and *qm30* are extremely broad and do not seem to follow a single defined distribution. For *e2519*, a proportion of the values cluster around the same value as *qm11* but the remainder are spread on either side. The phenotype of *qm30* is even more severe in the sense that no clear clustering of values can be identified.

Figure 2 also shows that a number of *e2519* embryos developed as rapidly as the most rapidly developing wild-type animals, and indeed, a small number appeared to develop faster than the fastest wild type. To confirm this observation, we followed the development of an additional 500 embryos of *e2519* and N2 up to 12.5 hr after the two-cell stage. Figure 3 shows hatching times for the fastest of the total of 1000 embryos for each genotype. Three percent of *clk-1* embryos hatch earlier than 10 hr, whereas only 0.3% of wild-type embryos do so.

A number of observations suggest that the variability in the embryonic developmental rates of *e2519* is not due to genetic inhomogeneity. First, *e2519* has been back-crossed >20 times to the wild type; this suggests that hypothetical modifier mutations would have to be linked relatively tightly to *clk-1*. Second, particularly slow or rapid development of *clk-1* mutant embryos is not heritable as indicated by the following experiment. We scored the embryonic developmental times of 179 *e2519* animals, which were then left to grow to adulthood. We then dissected one embryo from each adult animal and scored its developmental time. We found no correlation between the two sets of times (correlation coefficient $r = 0.026$). Together these observations suggest that *clk-1* mutations result in a deregulation of the duration of embryonic development and that the degree of deregulation is an intrinsic aspect of the severity of each allele. *qm11* is the allele with the fastest and the most regular development and therefore can be considered to be the allele with the weakest effect, at least on embryonic development.

The cell cycle period is affected in *clk-1* mutants: We used microscopy to observe embryos from fertilization to hatching and found that the cell cycles in all lineages are lengthened in mutant embryos compared with the wild type. We analyzed nine early N2 and *e2519* embryos by time-lapse video microscopy to determine if a slowing down of a particular phase of the cell cycle is responsible for the delays observed. Table 2 lists the observed durations of three distinct phases of the three earliest divisions. We call "mitosis" the time from nuclear breakdown to the start of cytokinesis. We found the duration of cytokinesis itself in *e2519* mutants to be indistinguishable from the wild type. We found mitosis to be slightly lengthened on average in the mutant. However, the average lengthening (of the mitoses in

P0, AB and P1) is only 1.1-fold, and most of the difference we observed was because of an apparent lengthening of the AB mitosis only. By far the most significant delay was observed for the interphases of AB and P1, which were lengthened 1.6-fold compared with the wild type. This lengthening is of similar magnitude to the effect of the mutation on the other features we have scored (Table 1). In the wild type, the interphases of the early cell cycles are occupied entirely by DNA synthesis with neither G1 nor G2 observable (EDGAR and MCGHEE 1988). In the future, it will be informative to determine if the lengthening we observe is because of a slowdown of DNA replication or to the appearance of a G1 and/or G2 phase.

In addition, we observed that not only the cell cycle but all phases of embryonic development appear lengthened, as monitored by observation of the stage-specific changes in the shape of the embryo. These delays have, however, not been precisely quantified.

Individual variations in the severity of *clk-1* mutant phenotypes are correlated in a nonintuitive way: As described in previous sections, we found a high variability among individual mutant animals in a variety of features. For example, the standard deviation of the sample of embryonic developmental times of *e2519* mutants is almost four times larger than that of the wild type (N2) (Table 1 and Fig. 2). Similarly, for the defecation cycle period, the variability among mutant animals is approximately two times larger than that of the wild type (Table 1 and Figure 1). The distribution of hatching times of the mutants appears not to follow a normal distribution, suggesting that the rapidly and slowly developing animals are actually different from each other in some unknown, nonheritable way. Do the animals with widely different hatching times remain recognizably different during later development and adulthood? To investigate this, we selected wild-type and mutant animals with very fast or very slow embryonic development and scored the duration of their postembryonic development and defecation cycle (Table 3). For both these features, we observed a striking inverse correlation with embryonic development time. Animals with fast embryonic development develop slowly during postembryonic development and have a slow defecation cycle, and animals with slow embryonic development develop rapidly during postembryonic development and have a fast defecation cycle. Both slow and fast phenotypes fall within the normal *clk-1* mutant range. None of the other phenotypes that we have shown to be affected in *clk-1* mutants appears to correlate in severity with the speed of embryonic development (data not shown). An additional observation is that the total time taken to develop (the sum of embryonic and postembryonic development) is constant, regardless of the cohort. That is, fast embryogenesis is offset by slow larval growth and *vice versa*. These phenomena are apparent in the wild type as well as in the mutant, but the effects

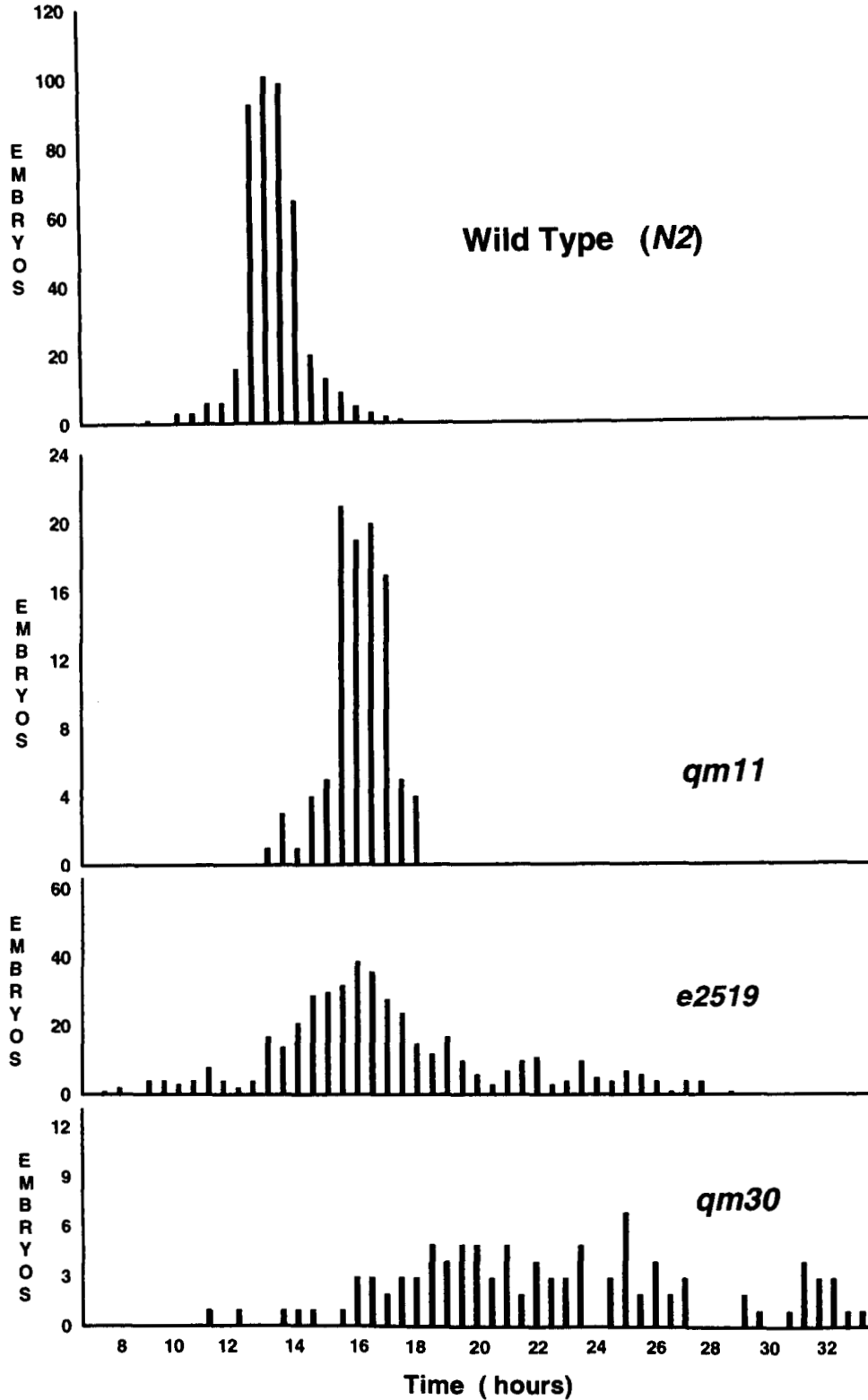


FIGURE 2.—Duration of embryonic development of wild-type (N2) and *e2519*, *qm11*, *qm30* animals. Two-celled embryos were dissected from gravid hermaphrodites, placed singly onto fresh plates and monitored every 30 min until they hatched. Each bar represents the number of animals that hatched within a 30-min period. The embryos were maintained at 20° throughout development. The sample size is 500 for N2 and *e2519* and 100 for *qm11* and *qm30*.

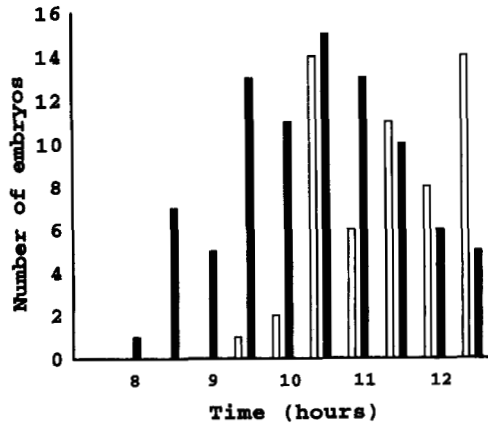


FIGURE 3.—Quickly developing mutant embryos. Some *clk-1* (*e2519*) mutants develop faster during embryogenesis than wild-type (N2) animals. One thousand two-celled embryos of each genotype were dissected from gravid hermaphrodites, placed singly onto fresh plates and monitored every 30 min until 12.5 hr. Each bar represents the number of animals that hatched within a 30-min period. Open bars represent wild-type (N2) embryos and the close bars *e2519* embryos. Three percent of *clk-1* (*e2519*) embryos hatch earlier than 10 hr whereas only 0.3% of wild-type embryos do so.

are much larger in the mutant, which has a 10-hr difference in postembryonic development and a 14-sec difference in the defecation period between the cohorts. Overall, this suggests that animals with different embryonic developmental times remain different from each other throughout later development and as adults.

Effects of temperature on the duration of embryonic development: *C. elegans* is capable of adapting its developmental rate to a relatively broad range of temperatures, from 13 to 25° (HEDGECOCK and RUSSELL 1975). We tested the possibility that this ability was altered in *clk-1* mutants by examining the effect of temperature on embryonic development. N2, all three alleles, and maternally rescued *e2519* were included in the analysis. Two-celled embryos were dissected from animals raised at one of three distinct temperatures (15, 20 or 25°), maintained at that temperature, and the duration of their embryonic development measured (Figure 4 and Table 4). For N2, *qm11*, *e2519* and maternally rescued *e2519* animals, the duration of embryonic development is dependent on temperature, and to a similar degree. In contrast, for *qm30*, the amount of change between any two temperatures is much greater than for N2 or the other alleles. At 25°, however, the three *clk-1* alleles are indistinguishable, producing animals that develop at the same rate, albeit still significantly slower than the wild-type or maternally rescued *e2519*. These observations suggest an altered ability of the *qm30* mutants to respond to various temperatures.

We have shown above that *clk-1* mutants display a profound maternal rescue when derived from mothers carrying a wild-type copy of the gene. One possible interpretation of this phenomenon is that the *clk-1*-sensi-

TABLE 2

Timing of early embryonic cell cycles

Affected cellular events	N2	<i>e2519</i>
P0 (one-cell embryo) mitosis (sec)	171 ± 38	184 ± 31
P0 cytokinesis (sec)	256 ± 53	262 ± 54
AB (ant. daughter of P0) interphase (sec)	596 ± 113	919 ± 232
AB mitosis (sec)	180 ± 33	231 ± 19
AB cytokinesis (sec)	227 ± 57	227 ± 74
P1 (post. daughter of P0) interphase (sec)	729 ± 117	1143 ± 259
P1 mitosis (sec)	207 ± 42	218 ± 53
P1 cytokinesis (sec)	142 ± 56	159 ± 74

Values are means ± SD. Comparison of early cell cycles between wild-type (N2) and *clk-1* (*e2519*) animals. Nine embryos of each genotype were examined. Embryos at the pronuclear migration stage were dissected from gravid hermaphrodites and mounted onto an agar pad on a microscope slide. Their subsequent development was viewed by high-power differential interference contrast (DIC) microscopy and recorded with a time-lapse video system. Mitoses were scored from nuclear breakdown to the start of cytokinesis. Cytokinyses were scored from the earliest sign of the cleavage furrow to complete separation. Interphases were scored from the end of cytokinyses to the start of the next nuclear breakdown.

tive processes are, or can be, set early during development. As embryonic development in the wild type and *clk-1* mutants is sensitive to temperature, we attempted to manipulate the rate of embryonic development with a temperature-shift paradigm. For wild-type and mutant animals, two-celled embryos were dissected from gravid hermaphrodites raised at 15, 20 and 25°, but were then transferred to plates at 20°, and the duration of embryogenesis measured. For N2 and maternally rescued *e2519*, shift to 20° resulted in a length of embryogenesis typical of 20° regardless of the temperature of the zygote up until the two-celled stage (Figure 5 and Table 4). In striking contrast, the rate of embryonic development of *clk-1* mutant zygotes does depend on the temperature experienced up until the two-cell stage. When originating from mothers raised at 15°, the rate of embryonic development at 20° was less than that for embryos derived from mothers raised at 20°. Conversely, when embryos originated from mothers raised at 25°, the rate of embryogenesis at 20° was greater than that of embryos originating from mothers raised at 20°. Notice that, for each allele, the effects produced by early heat (25°) or by early cold (15°) are of very similar magnitude (resulting in straight lines connecting data points in Figure 5).

The results of incubation at constant temperature (Figure 4) might be used to infer that *qm30* shows a graded cold sensitivity. However, two observations suggest a more complex phenomenon. First, early cold (15°) can slow down subsequent development at 20°.

TABLE 3
Correlated variations in the severity of the *clk-1* phenotypes

Phenotype	Quickly developing embryos		Slowly developing embryos	
	N2	<i>clk-1 (e2519)</i>	N2	<i>clk-1 (e2519)</i>
Embryonic development (hr)	12.1 ± 0.7 (29)	11.7 ± 0.7 (29)	14.4 ± 0.5 (29)	22.8 ± 0.7 (29)
Postembryonic development (hr)	49.5 ± 1.7 (25)	74.9 ± 4.0 (25)	47.2 ± 2.0 (25)	64.3 ± 4.1 (25)
Total development (hr)	61.6	86.6	61.6	87.1
Defecation cycle period (sec)	52.7 ± 5.0 (29)	71.6 ± 12.0 (29)	50.2 ± 3.8 (29)	57.3 ± 6.5 (29)

Values are means ± SD with no. of embryos in parentheses. Postembryonic developmental time and defecation cycle period of animals selected on the basis of embryonic developmental time. For each genotype a group of 29 embryos whose developmental times clustered closely around 12 hr (quickly developing embryos) or 14 hr (N2) and 22 hr (*clk-1*) (slowly developing embryos) were selected. The postembryonic developmental time, and later the adult defecation period of these animals was scored. For technical reasons the postembryonic developmental time could not be measured for 4 of the 29 embryos. Total developmental time corresponds to the total of embryonic developmental time plus postembryonic developmental time.

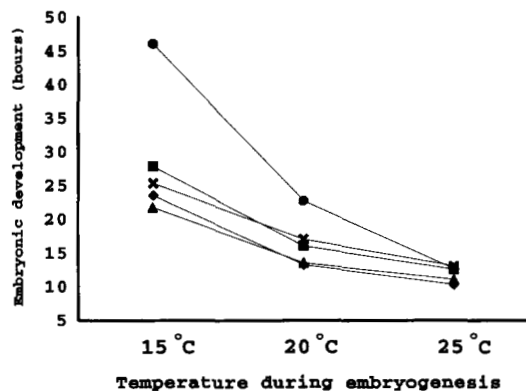


FIGURE 4.—Effects of temperature on the duration of embryonic development. Effects on wild-type (N2), *e2519*, *qm11* and *qm30* and maternally rescued *e2519* animals are shown. Two-celled embryos were dissected from gravid hermaphrodites cultured at 15, 20 and 25° and allowed to develop at the temperature at which their mothers had been raised. They were monitored every 30 min until they hatched. The means are plotted; Wild type, ◆; maternally rescued *e2519* mutants, ▲; *e2519* mutants, ×; *qm11* mutants, ■; and *qm30* mutants, ●. See Table 4 for exact means, sample sizes and standard deviations.

Second, shifting the embryos from 25° to 20° makes the subsequent development of *clk-1* animals more similar to that of wild type treated in the same way, than does continuous development at 25°. Altogether, our results show that *clk-1* embryos are not only defective in sensing or responding developmentally to different environmental temperatures, but also are impaired in sensing or responding to changes in those temperatures.

DISCUSSION

We have isolated three allelic mutations that define the gene *clk-1*. The phenotype resulting from these mutations indicates that the normal function of *clk-1* is necessary for the correct timing of a variety of features of the organism. These features include the cell cycle, embryonic and postembryonic development, gameto-

genesis, death rate and a number of high and low frequency behavioral cycles. In *clk-1* mutants, these features are, on average, slowed down or decreased to an allele-specific degree. In addition, the timing of these features appears to be deregulated, as indicated by a substantial increase in the variability among animals. A particularly striking example of this phenomenon is provided by *clk-1* embryonic development, for which we have found that ~3% of the animals develop faster than even the fastest wild type. These observations suggest that mutations in *clk-1* lead to a partial uncoupling of these functions from a mechanism that normally regulates their rate. Furthermore, temperature-shift experiments suggest that embryos actively sense and adapt to temperature and that *clk-1* mutant embryos appear to be partially uncoupled from this regulatory input (Figure 5). This suggests that *clk-1* is involved in a process that is required for the regulation of developmental rate by temperature.

Is it surprising that a single function would regulate events of very different nature and on very different time scales? We have shown that the process affected by *clk-1* is required for the normal activity of a number of specific biological clocks devoted to particular features. Behavioral and developmental clocks are known to exist in *C. elegans*. For example, observations on wild type and mutants that alter the defecation cycle suggest that this cycle is regulated by a biological clock (THOMAS 1990; LIU and THOMAS 1994), and, as in other organisms, the regulation of cell-cycle length has been shown to have clock-like properties (e.g., SCHIERENBERG 1984; SCHIERENBERG and WOOD 1985; SCHNABEL and SCHNABEL 1990). *clk-1* could be involved in a function that, by regulating the level of activity of several biological clocks, would ensure the proper temporal coordination of all features of the organism. Such a function might be required to obtain a coordinated response to fluctuations in the environment. Temperature is a good example of an environmental parameter to which one expects organisms to react actively. In-

TABLE 4
Effects of temperature differences and shifts of the duration of embryonic development

Temperature	Embryogenesis when oogenesis and embryogenesis were carried out at the indicated temperature (hr)					Embryogenesis when oogenesis was carried out at the indicated temperature and embryogenesis at 20° (hr)				
	N2	<i>e2519</i> m ⁺ z ⁻ (maternal rescue)	<i>e2519</i>	<i>qm11</i>	<i>qm30</i>	N2	<i>e1519</i> m ⁺ z ⁻ (maternal rescue)	<i>e2519</i>	<i>qm11</i>	<i>qm30</i>
15°	23.6 ± 1.8 (160)	21.8 ± 1.5 (161)	25.4 ± 3.8 (98)	27.9 ± 1.3 (99)	46.1 ± 12.1 (94)	13.6 ± 0.4 (107)	14.8 ± 1.9 (45)	20.7 ± 2.5 (104)	18.5 ± 2.0 (104)	32.4 ± 9.5 (61)
20°	13.3 ± 1.0 (500)	13.6 ± 0.8 (50)	17.1 ± 3.9 (500)	16.1 ± 1.0 (100)	22.8 ± 5.0 (100)	13.3 ± 1.0 (500)	13.6 ± 0.8 (50)	17.1 ± 3.9 (500)	16.1 ± 1.0 (100)	22.8 ± 5.0 (100)
25°	10.3 ± 0.8 (98)	11.1 ± 0.8 (26)	13.1 ± 1.5 (100)	12.6 ± 0.7 (90)	12.7 ± 1.1 (65)	13.1 ± 0.8 (100)	14.5 ± 0.6 (42)	13.7 ± 1.0 (101)	13.7 ± 1.0 (97)	14.2 ± 1.3 (79)

Values are means ± SD with no. of embryos in parentheses. Embryonic developmental time of N2, *e2519*, maternally rescued *e2519*, *qm11* and *qm30* under various incubation conditions. Two-celled embryos were dissected from gravid hermaphrodites cultured at 15, 20 and 25°, following which they were either allowed to develop at the temperature at which their mothers had been raised or placed at 20° for the remainder of embryogenesis. In all cases they were monitored every 30 min until they hatched. The data is summarized graphically in Figures 4 and 5.

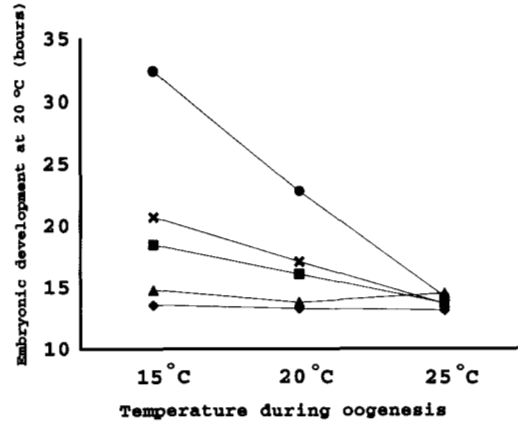


FIGURE 5.—Effects of temperature shifts on the duration of embryonic development. Effects on wild-type (N2), *e2519*, *qm11*, *qm30* and maternally rescued *e2519* animals are shown. Two-celled embryos were dissected from gravid hermaphrodites that had been cultured at 15, 20 and 25°. These embryos were placed singly onto fresh plates and incubated at 20° for the remainder of embryogenesis. They were monitored every 30 min until they hatched. The plotted points correspond to the means; Wild type, ♦; maternally rescued *e2519* mutants, ▲; *e2519* mutants, ×; *qm11* mutants, ●; and *qm30* mutants, ●. See Table 4 for exact means, sample sizes and standard deviations.

deed, we have shown that the response to changes in temperature is profoundly altered in *clk-1* mutants. In the future, we plan to test if *clk-1* also could be required for the sensing of or the reaction to changes in other environmental parameters (e.g., nutrient availability).

The biological clocks that are altered in *clk-1* mutants are very distinct from each other, expressed in very different tissues at different times and with very different periods. For example, the cell cycle and the defecation cycle have nothing obvious in common. What kind of mechanisms could affect such disparate processes? Variation in intracellular Ca²⁺ concentration is a cellular mechanism with regulatory properties that is sufficiently universal that its alteration could have highly pleiotropic effects. A vast literature describes the intracellular calcium oscillations which are observed in numerous cell types (for reviews see BERRIDGE 1990, 1993). Variations in Ca²⁺ concentration and other second messengers interacting with Ca²⁺ have been linked to almost all aspects of cell function. Furthermore, theoretical consideration suggest that regulatory control loops involving second messenger system could form the basis of high-frequency biological rhythms (e.g., RAPP and BERRIDGE 1977).

An alteration in the availability of ATP is also a condition that would impinge on all functions of the organism, including biological clocks. It is possible that *clk-1* mutations alter some part of normal cellular energy procurement. Indeed, glycolytic oscillations, primarily controlled by phosphofructokinase, have been demonstrated in several systems (HESS and PLESSER 1979; TOR-

NHEIM 1980; ANDRES *et al.* 1990). Given the ubiquitous distribution of glycolytic enzymes, it has often been proposed that metabolic oscillations could drive other cyclic physiological processes, including rhythmic smooth muscle contraction (CONNOR 1979), electric activity of neurons (MEECH 1979) and heart muscle cells (O'ROURKE *et al.* 1994) and hormone secretion (CORKEY *et al.* 1988). It would be illuminating if future investigations would show a link between the *clk-1* gene and metabolic oscillations, as it would provide a rationale to understand the pleiotropic effects of *clk-1* mutations.

A different question is to ask whether the *clk-1* mutant phenotype could be the result of a simple decrease of ATP production or availability. The following arguments suggest that this is unlikely to be the case. *clk-1* mutants do not appear sick or anatomically abnormal, whereas metabolically restricted worms, for example because of insufficient food intake, have a typical "starved" appearance (AVERY 1993). The maternal effect on the *clk-1* phenotype is profound and extends to adult phenotypes. This makes it unlikely that *clk-1* could encode a major structural component (*e.g.*, a glycolytic enzyme) of the pathway which extracts energy from food. The results of our genetic screen indicate that probably only few genes exist that can mutate readily toward a *clk-1*-like phenotype. If the *clk-1* phenotype were a secondary consequence of a nonspecific reduction in metabolism, one would imagine that a larger number of genes could mutate to bring about such an effect. Finally, of all the features of the *clk-1* mutant phenotype, only the decreased mean growth rate would have been predicted for mutations that decrease metabolism. In fact, *clk-1* mutations uncover processes such as, for example, the complex temperature dependence of developmental rates and the complex correlation between developmental and behavioral rates, that could not have been extrapolated from the idea of a metabolic impairment.

In the future, molecular analysis of the *clk-1* gene and analysis of other genes involved in the timing phenomena revealed by the mutations in *clk-1* will allow us to test further the hypothesis that *clk-1* regulates biological clocks.

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