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The *C. elegans* maternal-effect gene *clk-2* is essential for embryonic development, encodes a protein homologous to yeast Tel2p and affects telomere length

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SUMMARY

The *Caenorhabditis elegans* maternal-effect *clk* genes are involved in the temporal control of development and behavior. We report the genetic and molecular characterization of *clk-2*. A temperature-sensitive mutation in the gene *clk-2* affects embryonic and post-embryonic development, reproduction, and rhythmic behaviors. Yet, virtually all phenotypes are fully maternally rescued. Embryonic development strictly requires the activity of maternal *clk-2* during a narrow time window between oocyte maturation and the two- to four-cell embryonic stage. Positional cloning of *clk-2* reveals that it encodes a protein homologous to *S. cerevisiae* Tel2p. In yeast, the gene *TEL2* regulates telomere length and participates in gene silencing at subtelomeric regions. In *C. elegans*, *clk-2* mutants have elongated telomeres, and *clk-2* overexpression can lead to telomere shortening. Tel2p has been reported to bind to telomeric DNA repeats in vitro. However, we find that a functional CLK-2::GFP fusion protein is cytoplasmic in worms. We discuss how the phenotype of *clk-2* mutants could be the result of altered patterns of gene expression.

Key words: *clk-2*, *TEL2*, *Caenorhabditis elegans*, Maternal effect, Telomere length, Lifespan

INTRODUCTION

The development of sexually reproducing organisms is affected by their parents in two distinct ways: the parents contribute the genetic material, but they also produce the gametes, the living cells whose fusion gives rise to the new organism. How development is regulated by factors provided to the embryo via the gametes is an important question in developmental biology. One way to approach this question is to identify and study genes that can display a parental effect when mutant.

We have previously carried out a genetic screen for viable maternal-effect mutants to identify maternally contributed gene products involved in processes that are not absolutely required for viability (Hekimi et al., 1995). We thus identified new genes required for various aspects of morphogenesis and differentiation (Hekimi et al., 1995; Takagi et al., 1997), as well as genes (*clk* genes) required for the normal timing of development, behavior, reproduction and lifespan (Hekimi et al., 1995; Lakowski and Hekimi, 1996). One interpretation of the maternal effect associated with *clk* genes is that they can induce an epigenetic state during early development that is somehow maintained throughout the life of the animal (Branicky et al., 2000).

Most of the best characterized epigenetic effects are based on regulated chromatin states. For example, the Polycomb-group constitutes an important conserved class of transcriptional repressors well known for their function in stably maintaining the inactive expression patterns of developmental regulators through cell generations and developmental stages (Jacobs and van Lohuizen, 1999). In *C. elegans*, some of the *mes* genes, which regulate silencing and the development of the germline, resemble Polycomb-group genes (Holdeman et al., 1998; Korf et al., 1998). The *mes* genes display a maternal effect similar to that of the *clk* genes, with perfect rescue of the phenotype of mutants of the first homozygous generation (Capowski et al., 1991).

Subtelomeric silencing is one of the best studied examples of epigenetic transcriptional regulation (Lustig, 1998). In some ways, it is similar to position-effect variegation and heterochromatin formation (Henikoff, 1996). The telomeres, complex nucleoproteic structures found at the end of linear chromosomes, are capable of reversibly silencing the expression of genes in immediately adjacent regions of the chromosome. An increasing number of gene products have been found to be involved in the mechanisms of subtelomeric silencing and the regulation of chromatin function at other sites. For example, the products of the *sir* (silent information regulator) genes have been shown to function at the telomeres

as well as at other sites, including the rDNA (Smith and Boeke, 1997). The function of Sir2p, which is a NAD^+-dependent histone deacetylase (Imai et al., 2000), has also been linked to yeast mother cell lifespan, and it has been speculated that its action at the rDNA is a response to variations in the level of mitochondrial metabolism (Guarente, 2000).

We present the phenotypic, genetic, and molecular characterization of the gene *clk-2*. We show that maternal *clk-2* activity is required before the two- to four-cell stage for embryonic development. We report that *clk-2* encodes a predominantly cytoplasmic protein homologous to *S. cerevisiae* Tel2p, which is involved in telomere length regulation and subtelomeric silencing in yeast. *clk-2* mutants have elongated telomeres and overexpression of CLK-2 in transgenic animals shortens telomeres.

MATERIALS AND METHODS

Nematode strains and genetic analysis

Growth conditions were as described (Brenner, 1974), at 20°C unless specified otherwise. Strains and/or alleles used: N2 (wild type), MQ125 *clk-2(qm37)* outcrossed 12 times, *lin-13(n387)*, *lin-39(n1760)*, *mab-5(e1239)*, *sma-3(e491)*, *unc-32(e189)*, *unc-36(e251)*, *glp-4(bn2ts)*, *fem-2(b245ts)* and *fem-3(q20ts)*. Genotypes used and scored for genetic mapping have been submitted to WormBase.

Developmental and behavioral phenotypes at 20°C were scored as described (Wong et al., 1995). For temperature shifts experiments between 20 and 25°C, embryos or worms were transferred onto preincubated plates. Two- to four-cell stage embryos were dissected as described (Wong et al., 1995).

Plasmids and transgenic strains

Cosmid C07H6 (Accession Number, AC006605). pMQ248 is a transcriptional fusion of the promoter region of the *clk-2* operon (bases 37319 to 36932 of C07H6), including only 23 bp of *cux-7* sequence, to *gfp* in pPD95.77 (gift from Dr A. Fire). pMQ254 is a similar transcriptional fusion, with a larger promoter region (bases 40010 to 36932 of C07H6). pMQ251 is a translational fusion of *clk-2* to *gfp* of vector pPD95.77. This fusion construct includes, in addition to the promoter region described for pMQ248 (i.e., bases 37319 to 36932), the entire coding region of *clk-2* (bases 37319-36932) excluding its stop codon and only a small part of the coding region of *cux-7* (bases 36955-36546, and 35077-34999, where bases 36545-35078 have been deleted). Also, the 3′ UTR of *unc-54* present in vector pPD95.77 has been replaced by the 3′ UTR of *clk-2* (bases 31654-30501 of C07H6). pMQ251 rescues the Clk-2 phenotypes, including development and behavior at 20°C, and lethality at 25°C. The three constructs were microinjected into wild-type and *clk-2(qm37)* worms at a concentration of 100 ng/µl along with pRF4 125 ng/µl. ~100 F_1 transgenic worms were examined, and ~30 F_2 transgenics for each of approx. seven independent lines obtained with each construct, by epifluorescence. In addition, wild-type and *clk-2(qm37)* worms were microinjected with blunt linear fragments of these constructs and pRF4, at of 1 ng/µl, along with 100 ng/µl of *Pvu*II-N2 genomic DNA (complex arrays). Approx. 50 F_1 transgenic worms were examined, and ~30 F_2 transgenics for each of ~10 independent lines obtained with each construct. A high level of expression is detected in all somatic tissues of F_1 animals, but by the F_2 generation, most transgenic worms express CLK-2::GFP in the same tissues at very weak levels.

MQ691 *clk-2(qm37); qmEx159* was generated by microinjection of pMQ246 at 50 ng/µl, pRF4 at 125 ng/µl, and salmom sperm DNA at 100 ng. pMQ246 contains bases 37319 to 31528 of C07H6, excluding bases 36544 to 35077, cloned in pBluescript, thus comprising the promoter region of the *clk-2* operon, part of *cux-7* with a large internal deletion that interrupts its reading frame and the entire coding sequence of *clk-2*. A similar clone containing the *qm37* mutation fails to rescue the Clk-2 phenotypes.

RT-PCR

RT-PCR experiments were performed from mixed stage N2 total RNA essentially as described (Ewbank et al., 1997). No product could be amplified using primer pairs corresponding to the 3′ region of *cux-7* and the 5′ region of *clk-2*, supporting that the operon encodes two genes that result in separate mRNAs.

RNA interference

Transcription with T3 and T7 polymerases was performed on gel purified linearized clones yk447b4 (*clk-2* cDNA) and yk215f6 (*cux-7* cDNA) using RNA synthesis kit (Promega). Single-stranded RNAs were annealed and injected as described (Fire et al., 1998).

Northern analysis

Worm populations were synchronized at different developmental stages as described by Wood (Wood, 1988). 10 µg of total RNA (Trizol extraction, Gibco) or 0.5 µg of polyA+ selected RNA (Qiagen) were fractionated by electrophoresis in denaturing conditions, transferred to Hybond-N+ membrane (Amersham), and hybridized as per standard methods with a labeled probe (Prime-It II, Stratagene) made from a 1.4 kb central fragment of the *clk-2* cDNA. Identical results were obtained with two independent worm and RNA preparations.

Antibodies and western analysis

A PCR fragment encoding amino acids 401-877 was cloned into the pET16b expression vector (Novagen). Bacterially expressed His_{10}-tagged recombinant protein was purified by chromatography on Ni^{2+}-NTA-Agarose, and injected into two rabbits to obtain polyclonal antibodies. The terminal bleed of each rabbit recognizes the bacterial antigen, in vitro translated CLK-2, and a predominant band at the expected size of ~100 kDa in worm extracts (prepared by grinding in NET buffer). This ~100 kDa band is not detected by pre-immunization sera, and disappears upon preabsorbtion of the antibody with bacterially purified CLK-2, but not upon preabsorbtion with other purified bacterially expressed proteins, including His_{10}-tagged. The same band is detected by blot-affinity purified antibodies. Also, the detected band is drastically reduced in *clk-2(qm37)* extracts as compared to wild type. An additional band at the expected size of ~130 kDa is detected by these antisera and by an anti-GFP antibodies in worm extracts expressing P_{hsp}CLK-2::GFP. Western blotting was performed as per standard methods. The primary antibodies were rabbit anti-CLK-2 antisera diluted 1:1000, and the secondary antibody was HRP-conjugated goat anti-rabbit IgG (Sigma) diluted 1:2000, detected using ECL detection kit (Amersham). Samples concentration was measured by BioRad Assay and equal loading was controlled by Coomassie Blue staining of an identical gel made from the same samples.

Telomeric restriction fragment length analysis

Worms were continuously grown for numerous generations at 20 and 25°C before collection for DNA extraction. Worms were collected as mixed-stage populations for all strains examined. As *clk-2(qm37)* is lethal at 25°C, mixed stage worms from 20°C were transferred to and grown at 25°C for 3-4 days; controls thus treated were also analyzed. At least four independent preparations of worms were analyzed for each condition. Genomic DNA was phenol-chloroform purified and ethanol precipitated. 5 µg of DNA were *Hin*fI digested, separated on a 0.6% agarose gel at 1 Vcm^{-1}, and transferred to Hybond+ under alkaline conditions. Southern blots were hybridized with two probes directed against telomeric repeats, and gave identical results: (1) a

[γ^{32}-P]dATP end-labeled TTAGGCTTAGGCTTAGGCTTAGGCTTAGGCTTAGGCTTAGGCTTAGG oligonucleotide, which was used for the blots presented in Fig. 6A-C, and (2) a probe generated by direct incorporation of [α-^{32}P]dCTP during PCR amplification of telomeric repeats from plasmid cTel55X with primers T7 and SHP1617 (GAATAATGAGAATTTTCAGGC). We also used telomere-specific probes directed against the *Hin*fI terminal restriction fragments, but hybridizing to non-telomeric sequence just adjacent to the terminal telomeric repeats, so that we could detect a particular telomeric terminal fragment. The terminal restriction fragments of the left telomere of chromosome IV (IVL) was detected with a gel-purified 200 bp PCR product from plasmid cTel4X amplified with primers SHP1792 (ATTCCTTCTGTGTACTGTTGCC) and SHP1791 (GATATTGACGACCTAGATGACG) that was [α-^{32}P]dATP randomly labeled. The terminal restriction fragments of chromosome X (XL) was detected with a gel-purified 740 bp PCR product from plasmid cTel7X amplified with primers SHP1797 (TCTGATTTTGACGATATTTCGC) and SHP1794 (AACTGACGGACTTGTGTCCC) that were [α-^{32}P]dATP randomly labeled.

RESULTS

clk-2 is required for normal developmental and behavioral rates

The gene *clk-2* is defined by the recessive temperature-sensitive allele *qm37*. The phenotypes displayed by the *clk-2(qm37)* mutants are fully penetrant at both the permissive and the restrictive temperatures. We examined in detail the rates of development and of rhythmic behaviors of *clk-2(qm37)* mutants grown at 20°C. The average rates of embryonic and post-embryonic development, as well as those of the pumping, defecation and egg-laying behaviors, are dramatically slower in *qm37* than in the wild type (Table 1). In addition, the self-brood size is reduced, but embryonic viability is similar to the wild type (98.4% of the embryos produced are viable and develop into fertile adults).

The developmental and behavioral phenotypes are fully rescued in homozygous mutants derived from a heterozygous mother (Table 1). However, the reproductive phenotypes are only partially rescued (Table 1). A simple interpretation of these results is that the *clk-2(+)* activity provided by the mother is sufficient to rescue events that occur relatively early in the life cycle and/or that require relatively small amounts of CLK-2. However, other events such as egg production might have a higher requirement and cannot be rescued by the amounts provided by the mother. Alternatively, the observed maternal effect could be based on an epigenetic setting that is erased in the germline. This interpretation is supported by the extent of the rescue considering that the animal grows more than 500-fold in volume during post-embryonic development. The dilution of any maternal product would be extreme, yet most of the somatic adult features are fully rescued.

Table 1. Phenotypic characterization of *clk-2(qm37)* animals at 20°C

	Wild type (N2)	*clk-2(qm37)*	Maternally rescued *clk-2(qm37)*
Embryonic development (hours)	13.2±0.7 *n*=80	17.0±1.5 *n*=97	13.3±1.6 *n*=40
Post-embryonic development (hours)	53.6±8.7 *n*=184	95.7±1.3 *n*=73	53.9±12.4 *n*=98
Self-brood size (eggs)	302.4±30.5* *n*=20	83.4‡ *n*=10	113.9±30.3 *n*=24
Peak egg-laying rate (eggs per hour)	5.3‡ *n*=10	1.3‡ *n*=10	3.6±0.9 *n*=24
Defecation (seconds)	54.9±0.6§ *n*=70	105.7±15.2 *n*=10	60.3±9.0 *n*=8
Pumping (pumps per minute)	265.3±64.4 *n*=25	180.9±24.8 *n*=25	245.2±24.6 *n*=11

Mean±s.d. and sample size are given.
*Data from Wong et al., 1995.
‡No s.d. shown; the total progeny of a group of 10 animals was scored and divided by 10.
§Data from Felkai et al., 1999.

clk-2 is required for embryonic development

Although *clk-2(qm37)* embryos all develop and grow up to become long-lived adults at permissive temperatures (15-20°C), *clk-2(qm37)* leads to embryonic lethality at 25°C. *qm37* hermaphrodites grown at a permissive temperature and transferred to 25°C before egg laying begins, produce progeny that all die during embryogenesis. Although *qm37* embryos appear normal from the one-cell stage to the beginning of gastrulation, they all arrest development later on in embryogenesis (Fig. 1). Embryonic arrests occur at various stages of development, ranging from arrests of disorganized gastrulating embryos of ~100 cells to arrests of deformed embryos at the threefold stage with differentiated tissues such as muscle, pharynx, gut and neurons. The lethal embryonic defect is irreversible, as embryos that are downshifted to 20°C at various stages of embryogenesis still die as embryos.

clk-2 acts very early in development

To establish how early *clk-2* acts in development, we dissected embryos at the two- to four-cell stage from wild-type and mutant hermaphrodites, grown at either permissive or non-permissive temperature, transferred the embryos to the other temperature and examined whether they could complete development (Table 2). When development proceeds up to the two- to four-cell stages at the permissive temperature (20°C), almost all *qm37* embryos carry out further embryonic and post-

Table 2. Hatching of embryos that were dissected at the two- to four-cell stage from mothers raised at 20 or 25°C, and then transferred to the other temperature

Maternal genotype and culture temperature	% of eggs that hatched	
	Development at 20°C	Development at 25°C
N2 at 20°C	100 *n*=35	97 *n*=36
clk-2 at 20°C	87 *n*=91	91* *n*=93
N2 at 25°C	98 *n*=45	96 *n*=45
clk-2 at 25°C	12* *n*=136	0

*It should be noted that the 12% of *clk-2* eggs transferred from 25 to 20°C that succeed in hatching fail to complete post-embryonic development, while the majority of the 91% *clk-2* eggs that hatch when transferred from 20 to 25°C do reach adulthood.

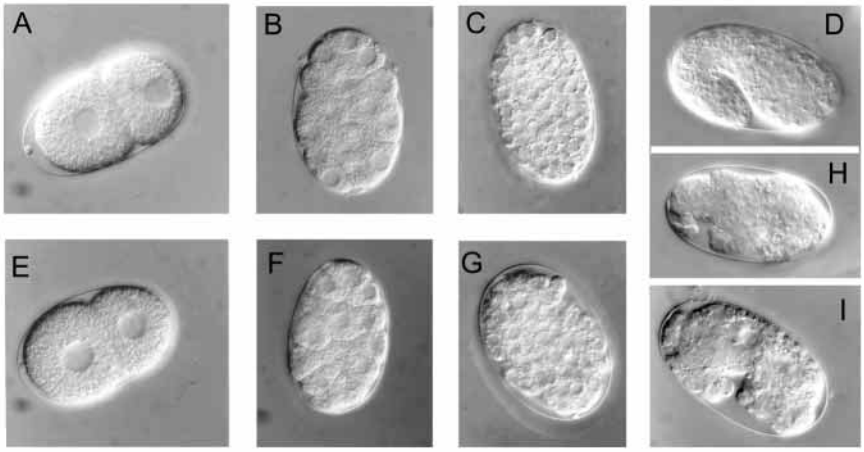


Fig. 1. The lethal embryonic phenotype of *clk-2(qm37)* at 25°C. (A-D) Wild-type embryos; (E-I) *qm37* mutant embryos. The very early embryonic development of the mutant (E,F) is indistinguishable from wild-type development (A,B); however, *clk-2* embryos invariably arrest at different stages of embryogenesis and die subsequently with obvious morphological abnormalities (G-I), compared with the wild type (C,D).

embryonic development at 20 or 25°C. By contrast, when *qm37* embryos develop at 25°C up to the two- to four-cell stage and are then transferred to 20°C, very few hatch and succeed in completing development. Thus, *clk-2* is required for embryonic viability before the two- to four-cell stages.

In addition, we examined the number of embryos present in the uteri of *qm37* hermaphrodites kept at 25°C for 26 hours after adulthood. In these mutant hermaphrodites, there is an average of 9.9 embryos (which we identify by their eggshell) per worm (n=125 worms, three independent experiments). We compared this figure with the total number of dead embryos produced by mutant hermaphrodites, similarly kept at 25°C for 26 hours after adulthood, but then shifted to the permissive temperature. These animals produce an average of 10.7 dead embryos per hermaphrodite before producing only live eggs (n=133 worms, three independent experiments). These observations indicate that after transfer away from the lethal temperature only one embryo dies, on average, in addition to those that have already formed an eggshell. This suggests that *clk-2(qm37)* produces irreversible damage, leading to subsequent lethality, in a narrow window between the very end of oogenesis and the initiation of embryonic development. This corresponds to the time at which oocyte maturation, fertilization, completion of meiosis, pronuclear formation and eggshell formation occur.

We examined late oogenesis and early embryonic development using DIC microscopy and could not detect any obvious abnormality in the events that precede or follow fertilization (data not shown). Early embryos appear invariably normal and healthy, with cells and nuclei of normal size and shape (Fig. 1E,F). Two polar bodies are extruded upon completion of oocyte meiosis in early *qm37* embryos (Fig. 1E). We also visualized chromosomes in early DAPI stained embryos and could not detect abnormal patterns of chromosome morphology or segregation, or any other defects.

clk-2 is not required for gonad and germline development

clk-2(qm37) hermaphrodites shifted to 25°C during post-embryonic development produce reduced broods of dead embryos. The reduced brood size and the death of the embryos at 25°C does not result from a failure of the gonad or germline to develop properly, because it is fully reversible. Indeed, despite developing entirely at 25°C, the gonad and germline of these *qm37* mutants is functional after downshift to the permissive temperature as they resume production of numerous live embryos. This also indicates that sperm and oocytes that have been produced at 25°C are functional after downshift. Similarly, the development and the function of male gametes do not appear to be affected in *qm37* mutants: homozygous males that develop from the early L4 stage at 25°C can sire abundant progeny as adults when mated to wild-type hermaphrodites at 25°C.

Examination by DIC microscopy of the gonads and germlines of *qm37* adult hermaphrodites transferred to 25°C at different stages during their larval development, and of adults that have spent ~25 hours as adults at 25°C, revealed no obvious morphological or cellular defects. The gonads have normal shape and size, containing numerous mitotic and meiotic germ nuclei and abundant sperm of normal appearance. The oocytes develop normally and display size increase and nucleus enlargement as well as nucleolus disappearance and asymmetric nucleus location in late oogenesis (McCarter et al., 1999). Also, the DAPI stained chromosomes of the mitotic and meiotic nuclei and of the oocytes have normal morphologies.

Thus, the mutant hermaphrodites produce reduced broods in spite of containing numerous functional gametes. One possibility is that an abnormal function of the somatic gonad of *qm37* mutants at the restrictive temperature contributes to the small brood size. In fact, most somatic structures of *clk-2* mutants appear affected by the non-permissive temperature, as mutant hermaphrodites that are transferred to 25°C as adults take on a sick appearance, move sluggishly, and their egg-laying rate eventually drops to zero before they have exhausted all their sperm.

clk-2(qm37) displays maternal effects at the restrictive temperature

We examined whether the phenotypes of the *qm37* mutants could be maternally rescued at 25°C. All progeny produced at 25°C from heterozygous mothers raised at 25°C are alive and develop into phenotypically wild-type adults. These maternally rescued adults appear undistinguishable from the wild type, but produce only a small brood of dead embryos. Thus, the maternal-rescue effect at 25°C is complete for development and behavior, as it is at 20°C, but fails to rescue fertility.

The maternal contribution of *clk-2* to development is further

revealed by the observation that the lethality of embryos produced at 25°C is strictly dependent on the maternal genotype. When *clk-2* hermaphrodites are mated with wild-type males at 25°C they nonetheless produce only dead embryos. When transferred to the permissive temperature at various times after mating, these hermaphrodites produce live progeny, including ~50% live males, indicating that the mating was successful. Thus, the presence of a wild-type allele in the embryo is insufficient for normal embryonic development to occur if the embryo is produced in a homozygous mutant mother. This strict maternal-effect lethality suggests a very early focus of action for *clk-2*, before activation of the zygotic genome, which is consistent with our finding of a requirement for *clk-2(+)* before the two- to four-cell stage (see above).

clk-2 encodes a protein homologous to *Saccharomyces cerevisiae* Tel2p

clk-2 was previously mapped to LGIII between *sma-4* and *mab-5* (Hekimi et al., 1995). We refined this position using additional genetic markers and found *clk-2* to be inseparable from *lin-39* (Fig. 2A). Cosmids from the corresponding genomic region were injected into *clk-2(qm37)* animals to assay for rescue of the mutant phenotypes (Fig. 2B). A 3.6 kb region of the rescuing cosmid C07H6 (Fig. 2C) is sufficient to rescue the developmental and behavioral phenotypes at 20°C, as well as the lethality at 25°C.

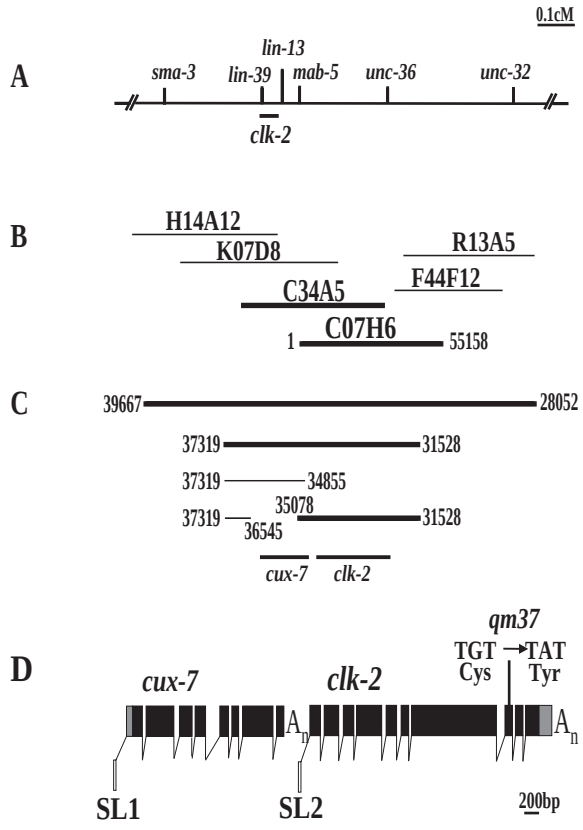


Fig. 2. Positional cloning of the gene *clk-2*. (A) *clk-2* is inseparable from *lin-39* by genetic mapping. (B) Cosmids in bold rescue *clk-2(qm37)*. (C) Fragments and subclones of C07H6; rescuing clones are in bold. Coordinates refer to bases on C07H6. (D) Genomic structure of *clk-2* and *cux*-7, which is the upstream gene in the operon of *clk-2*. cDNA clone yk215f6 does not contain the entire 3′ end of *cux-7*, stopping at 35040; we use the in-frame stop codon, at 34999, as the 3′ end in the drawing. (Accession Numbers: *clk-2*, AF400665; *cux-7*, AF400666.) The *qm37* mutation is indicated.

To characterize the gene structure of *clk-2*, the cDNA clone yk447b4, mapped to this region by Dr Y. Kohara, was sequenced (Accession Number, AF400665) and found that it differs from the Genefinder prediction (Fig. 2D). Six bases at the 5′ end of yk447b4 correspond to transpliced sequence from SL2. RT-PCR experiments yield a single band of the expected size when amplifying with a primer internal to *clk-2* and with an SL2-, but not with a SL1-specific primer (not shown), indicating that a single SL2-transpliced *clk-2* transcript is produced in vivo. A single band of the expected size is detected by northern analysis. The cDNA clone yk215f6, provided by Dr Y. Kohara, which corresponds to the gene immediately upstream of *clk-2*, which we call *cux-7* (upstream in *clk-2* operon and homologous to the human gene XE7; Accession Number, I54325), was also sequenced (Accession Number, AF400666). We also amplified the ends of *cux-7* in RT-PCR experiments. The 5′ end is amplified with SL1- but not with SL2-specific primers (not shown), indicating that *cux-7* is SL1-transpliced. Given their proximity and transplicing pattern, *clk-2* and *cux-7* appear to be in an operon (Spieth et al., 1993; Zorio et al., 1994).

clk-2 encodes a predicted protein of 877 amino acids. Database searches for sequences homologous to *C. elegans* CLK-2 revealed that it is similar to *Saccharomyces cerevisiae* Tel2p, as well as to predicted proteins in vertebrates and plants (Fig. 3). *S. cerevisiae TEL2* is an essential gene required for telomere length regulation (Runge and Zakian, 1996). After sporulation of a diploid heterozygote, *tel2* knockout cells divide no more than two or three times and arrest with an abnormal cellular morphology. A missense temperature-sensitive mutation (*tel2-1*), however, leads to slow growth and shortened telomeres. Furthermore, Tel2p has been found to bind single-stranded and double-stranded yeast telomeric repeats in vitro, as well as RNA (Kota and Runge, 1998; Kota and Runge, 1999). The existence of homologs of Tel2p in multicellular organisms has not been reported before, presumably because only alignments with multiple sequences are capable of revealing their relatively weak similarity (Fig. 3). Extensive searches have uncovered only one protein of the Tel2p/CLK-2 family in every eukaryote for which a complete or almost complete genome sequence is available, suggesting that these genes are orthologs. Functional conservation between yeast and worm has also been observed in the case of *mrt-2*/*rad1*$^{+}$*/RAD17*, which affect telomere biology, in spite of their low sequence similarity (Ahmed and Hodgkin, 2000).

qm37 is a partial loss-of-function allele at 20°C

In order to further confirm the identity of the gene and determine the null phenotype of *clk-2*, we depleted *clk-2* transcripts by RNA interference (Fire et al., 1998). While injection of wild-type worms with *cux-7* dsRNA produced no phenotype, injection of *clk-2* dsRNA elicited a robust phenocopy of the *clk-2* phenotypes in the F1 progeny of injected hermaphrodites. At first, all wild-type and *clk-2(qm37)* animals injected with *clk-2* dsRNA produce embryos that hatch and develop into slow growing larvae that become slow behaving and sterile adults. Approximately 24 hours after

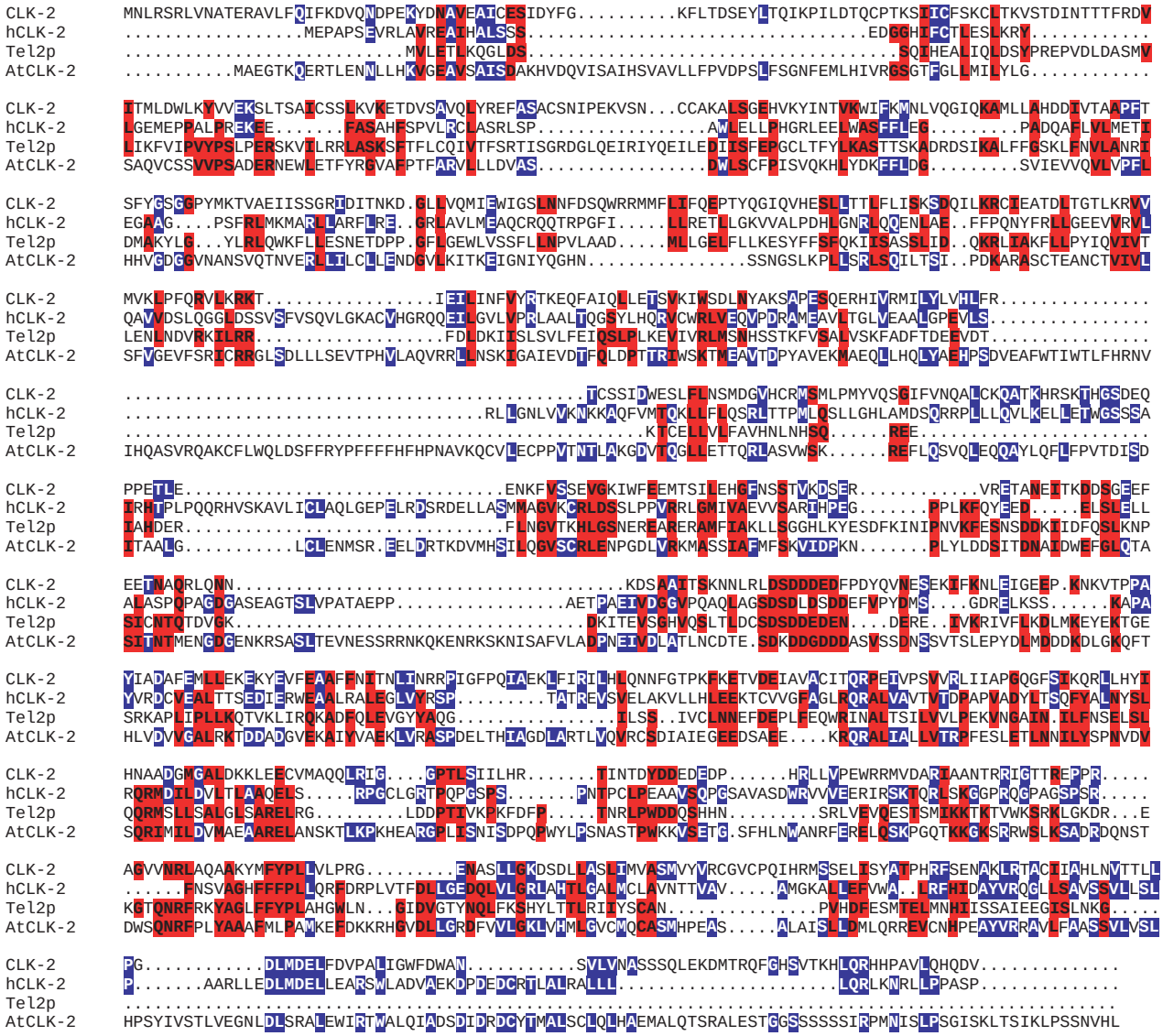


Fig. 3. An alignment of the predicted amino acid sequence of CLK-2 with its homologues from *S. cerevisiae* (Tel2p), *A. thaliana* (AtCLK-2) and humans (hCLK-2). Sequences were aligned using DNAMAN 4.1 and adjusted by hand. Similarities are shown in red when they include a residue in Tel2p, and in blue when the similarity is among sequences from multicellular organisms only. When CLK-2 is used to initiate a search by Psi-blast (http://www.ncbi.nlm.nih.gov/blast/psiblast.cgi), the algorithm finds five predicted proteins to be similar to CLK-2 and to each other (E-values are given in brackets): the *Drosophila* protein CG13854 (e-177); the human protein KIAA0683 (e-169); the *Arabidopsis* protein CAB88328 (>e-177); the *S. pombe* protein CAB93845 (e-106); and the *S. cerevisiae* Tel2p (e-105).

dsRNA injection, the injected animals lay only dead embryos at all temperatures. These results indicate that *clk-2* is required for embryonic development at all temperatures and that *qm37* is a partial loss-of-function mutation that, at 25°C, displays a much stronger loss-of-function phenotype, which might be the null phenotype. Sequencing of the *clk-2* genomic region in *qm37* reveals the mutation to be a G to A transition at base 32069 of C07H6 (Fig. 2C), resulting in a cysteine to tyrosine substitution at residue 772 of the predicted protein.

The level of the CLK-2 protein is similar at all temperatures (Fig. 4C), in both the wild type and in *qm37* mutants. The *qm37* mutation greatly reduces the level of CLK-2 at all temperatures (Fig. 4C). This indicates that the stronger phenotype of *qm37* at 25°C is not caused by increased instability of the mutant CLK-2 protein at this temperature. It is unclear therefore, whether CLK-2 is required at a high level at 25°C or whether the mutant protein is less functional at 25°C in a way that does not affect stability.

Abundant *clk-2* transcript is stored in the hermaphrodite germline

We examined the abundance of the *clk-2* transcript throughout development in wild-type worm populations at different developmental stages, by northern analysis (see Materials and Methods). While a modest level of the *clk-2* transcript is detected from the embryonic stage through the larval stages, the highest level is detected in young adults (Fig. 4A). Wild-type young adults contain a large germline with mitotic and

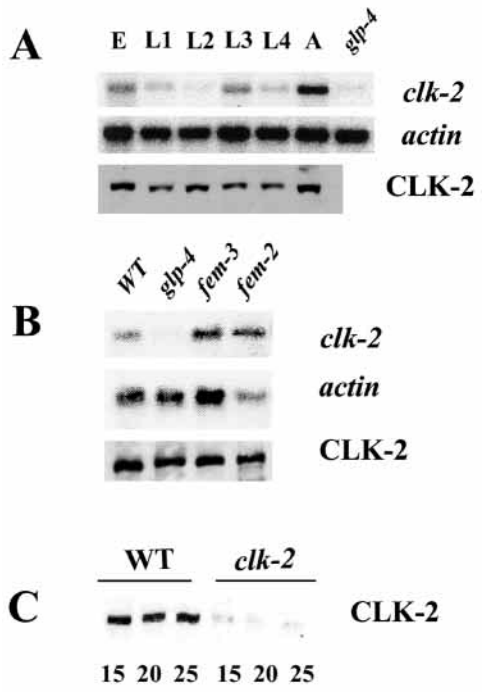


Fig. 4. The expression pattern of *clk-2*. (A) Northern and western analyses of *clk-2* at all developmental stages (E, embryos; L1-L4, larval stages; A, young adults; *glp-4(bn2ts)* adults). (B) *clk-2* mRNA and CLK-2 protein levels in mutant backgrounds *glp-4(bn2ts), fem-2(b245ts)* and *fem-3(q20ts)* at 25°C. (C) CLK-2 protein levels in the wild type and *clk-2(qm37)* mutants at three temperatures. For northerns, actin is used as a loading control; for westerns, equal loading was confirmed by Coomassie Blue staining of identical gels (not shown). As expected, the *clk-2* band detected in the northerns is at 2.8 kb, and the CLK-2 band detected in westerns is at ~100 kDa.

meiotic germ nuclei, sperm, and developing oocytes. Given the maternal contribution of *clk-2* to development and the high transcript level in young adults, we investigated the *clk-2* transcript levels in the germline by comparing wild-type young adults with adults carrying mutations that affect the germline. The *clk-2* transcript level is highly reduced in *glp-4(bn2ts)* mutants, which fail to develop a germline altogether at 25°C (Beanan and Strome, 1992), indicating that most of the *clk-2* mRNA in young adults is in the germline. However, the *clk-2* mRNA level is low in mid-L4 larvae compared with young adults, even though these larvae possess an already large germline. While the L4 germline contains numerous mitotic and early meiotic germ nuclei, it contains few sperm and lacks oocytes. We interpret this result to indicate that most of the *clk-2* mRNA in wild-type young adults is likely to be localized to oocytes.

That *clk-2* mRNA is abundant in the oocytes is consistent with our finding that the level of *clk-2* transcript is high in *fem-2(b245ts)* mutants that make only oocytes at 25°C (Fig. 4B). *clk-2* mRNA appears even more abundant in *fem-2(b245ts)* than in wild-type young adults, which is probably due to the abnormal accumulation of oocytes in these mutants. We also find that the level of *clk-2* transcripts in *fem-3(q20ts)* adults that make only sperm at 25°C is no different from that of wild-type young adults (Fig. 4B), indicating that sperm also contains *clk-2* mRNA. Note that the similarity of the levels of *clk-2* transcript in *fem-3(q20ts)* mutants and the wild type does not indicate that in young adults much of the *clk-2* mRNA comes from sperm, as these mutants contain a quantity of sperm that largely exceeds the amount normally present in wild-type adult hermaphrodites. Taken together, these results indicate that gametes, in particular oocytes, accumulate high levels of *clk-2* mRNA, presumably as a store to be used by the embryo, which is consistent with the maternal-rescue effect. However, as no paternal rescue is observed with *clk-2* mutants, the amount present in sperm appears insufficient to the requirement of the developing embryo.

We further characterized the expression pattern of *clk-2* by determining the levels of protein throughout development and in germline mutants by western analysis (see Materials and Methods). Surprisingly, the content of CLK-2 protein is similar through all developmental stages including in young adults (Fig. 4A). In addition, the concentration of CLK-2 in *glp-4(bn2ts)*, *fem-3(q20ts)* and *fem-2(b245ts)* mutants is no different from the wild type (Fig. 4B). These observations suggest that the transcript levels differences are mostly due to stores of presumably untranslated transcripts in the germline. However, the constant levels of CLK-2 protein throughout development are consistent with a continual requirement for CLK-2 in somatic tissues, as reflected by the temperature-sensitive period that extends throughout life (see above).

A functional CLK-2::GFP fusion is cytoplasmic

We constructed transcriptional and translational fusions of *clk-2* with the gene that encodes green fluorescent protein, and examined transgenic worms carrying these reporter genes (see Materials and Methods). Expression from the transgene is ubiquitous in somatic tissues including hypodermis, muscles, neurons, pharynx, gut, excretory canal, somatic gonad, vulva and presumably all cells (Fig. 5; data not shown). However, even using complex arrays to help prevent transgene silencing in the germline (Kelly et al., 1997), no CLK-2::GFP expression could be detected in the germline.

A full-length CLK-2::GFP fusion protein that complements the mutant phenotype for development, behavior and viability at 25°C, is localized virtually exclusively to the cytoplasm (Fig. 5), which is consistent with the absence of an obvious nuclear localization signal in the predicted protein. The pattern we observe is unlikely to be a consequence of overexpression as we have used very small transgene concentrations in complex arrays (Kelly et al., 1997). However, although the nucleus appears dark in the fluorescent images, we cannot exclude the possibility that it contains very small amounts of the fusion protein. Whether the CLK-2::GFP fusion exactly reflects the distribution of native CLK-2 will need to be addressed in further studies.

clk-2 is required for the regulation of telomere length in *C. elegans*

We examined the length of telomeres in *clk-2(qm37)* worms raised for numerous generations at 20°C and 25°C by Southern blotting (see Materials and Methods). In *C. elegans*, tracks of numerous TTAGGC telomeric repeats are present at the ends of the six chromosomes (Wicky et al., 1996). In addition, numerous interstitial blocks of perfect and degenerate telomeric repeats are located more internally to the chromosomes (Riddle, 1997). Analysis of genomic DNA after

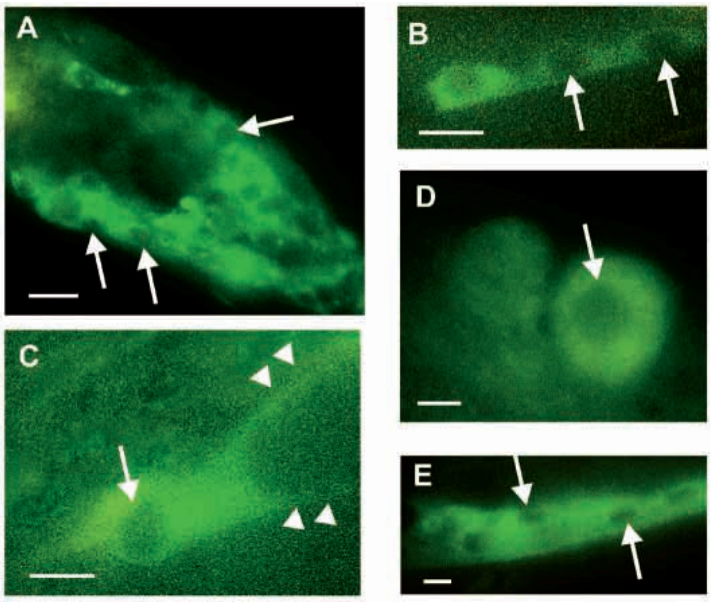


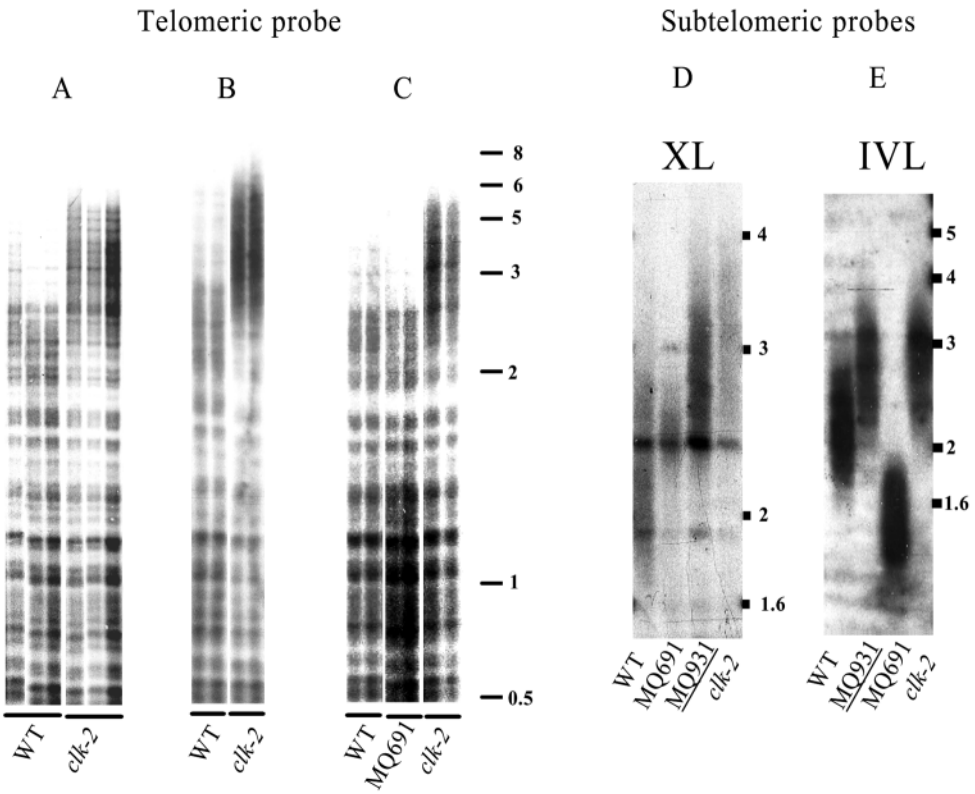
Fig. 5. Subcellular localization of a functional CLK-2::GFP fusion. Bright fluorescence is found in the cytoplasm of a variety of cells, and appears excluded from their nuclei (arrows). (A) Neurons in ganglia of the head. (B) Three neurons in the pre-anal ganglion. (C) A distal tip cell and its projections (arrowheads). (D) A two-cell embryo. Only one nucleus is in the plane of focus, but fluorescence was observed to be extranuclear in the second cell as well. (E) A region of the hypodermis. Scale bars: ~10 μm.

restriction digestion with a frequent cutter that does not cleave within the telomeric repeats (*Hin*fI), electrophoresis, and hybridization to telomeric probes, reveals the telomere-carrying end fragments of the chromosomes (Wicky et al., 1996). Telomeres, and thus the restriction fragments containing them, are heterogeneous in size and appear as smears. However, restriction fragments carrying tracts of internal telomeric repeats are of fixed size and appear as discrete bands in the 0.5-3 kb range (Ahmed and Hodgkin, 2000; Wicky et al., 1996).

In *qm37* mutants, telomeres are up to two times longer on average than in the wild type (Fig. 6A-C). We detect the smear corresponding to the terminal telomeric fragments in the range of 2.5-6 kb in *clk-2(qm37)*, which in the wild-type animals is in the range of 2-4 kb (Fig. 6A-C). The increased telomeric length of *qm37* mutants is a stable feature of the phenotype that we have repeatedly observed in more than four independent worm cultures for each genotype, at a variety of temperatures, and in at least two independent DNA preparations and analyses for each of the worm cultures. Lengthening of telomeric repeats in *qm37* mutants appears to occur only at the terminal telomeric fragments and not at internal sites, as the bands that correspond to them are at identical positions in *clk-2(qm37)* and the wild type (Fig. 6A-C).

We analyzed the length of terminal telomeric fragments in the animals of the strain MQ691, which carries an extrachromosomal array containing functional wild-type CLK-2 that rescues development and behavior at 25°C in a *clk-2(qm37)* chromosomal background. In these animals, the length of terminal telomeric fragments appear very

Fig. 6. The telomere-lengthening phenotype of *clk-2(qm37)* mutants. (A-C) In *C. elegans*, genomic DNA hybridization to telomeric probes after restriction digestion with *Hin*fI reveals smears that correspond to the terminal fragments of the chromosomes containing the telomeres, and discrete bands that correspond to fragments containing internal tracts of telomeric repeats (see text). (D,E) Hybridization with telomere-specific probes reveals a smear. For XL, discrete bands at 1.6, 1.9, and 2.4 kb are also detected by the subtelomeric probe and correspond to internal genomic fragments containing non-telomeric repetitive DNA located on other chromosomes that cross-hybridize with the probe. Worms were grown at 20°C (A,B) and at 25°C (C-E). Each panel represents an independent experiment (distinct worm cultures, DNA extractions and enzymatic digestions). MQ691 is a strain carrying a *clk-2(+)* transgene in a *clk-2(qm37)* background. MQ931 was derived from MQ691 by loss of the extrachromosomal array, and thus lacks *clk-2(+)*. The sizes indicated are in kb.



similar to the wild type, and even slightly shorter, indicating that the lengthened telomere phenotype of *qm37* mutants is rescued by the expression of *clk-2(+)* (Fig. 6). We further examined the telomere length of non-transgenic animals of the strain MQ931, derived from MQ691, which have lost the extrachromosomal array and thus again lack *clk-2(+)*. The terminal telomeric repeats in this strain are long again (data not shown). Thus, the lengthened telomere phenotype of *clk-2(qm37)* can be rescued by *clk-2(+)* and reverses back to mutant length after the loss of the transgene.

The quality of visualization of the length of telomeres in *C. elegans* with a hybridization probe that detects telomeric repeats is marred by the numerous internal repeats that also hybridize to the probe. In particular, they can mask the detection of the telomeres of chromosomes that have small *Hin*fI terminal telomeric fragments. To further describe the telomere phenotype of *clk-2(qm37)* mutants, we have characterized the length of individual telomeres. The subtelomeric regions just adjacent to the terminal telomeric repeats share no sequence homology among telomeres (Wicky et al., 1996). Taking advantage of this sequence diversity, we designed probes specific to particular telomeres. The size of a given *Hin*fI terminal fragment is related to the fixed distance between the most exterior *Hin*fI site of the chromosome and the beginning of the telomeric repeats, and by the variable number of terminal telomeric repeats. Upon genomic DNA digestion with *Hin*fI and Southern blotting with a probe specific to a particular telomere, the terminal fragments, which are heterogeneous in size, again appear as a smear. We present the results obtained for two individual telomeres in Fig. 6D,E.

The length of the terminal fragment of the left telomere of chromosome X is ~1 kb longer in *qm37* than in the wild type, which ranging from 2.4 to 4.2 kb and from 1.7 to 2.8 kb, respectively (Fig. 6D). This telomere is of wild-type length in MQ691, which carries the rescuing transgene, and lengthens again to the *clk-2(qm37)* values in the non-rescued MQ931 strain (Fig. 6D). The length of another terminal fragment (left telomere of chromome IV) is also ~1 kb longer in *qm37* than in the wild type, ranging from 2.2 to 3.9 kb and from 1.8 to 2.8 kb, respectively (Fig. 6E). This telomere becomes shorter than the wild type in MQ691, ranging from 1.3 to 2 kb only (Fig. 6E). This telomere acquires the mutant length again after loss of the transgene in MQ931 (Fig. 6E). Thus, the overexpression of *clk-2* can shorten the tracks of telomeric repeats, but not at each telomere.

Evidence indicates that short telomeres are sensed as double-stranded breaks in the cell and elicit a DNA repair response that can lead to end-to-end chromosome fusions and genomic instability (Gasser, 2000). However, in contrast to what happens in *S. cerevisiae tel2-1* mutants, telomeres are lengthened in *qm37* mutants and not shortened. We nonetheless examined a number of markers of chromosome instability. However, we found no high frequency of males, no embryonic lethality and no decrease in the reproductive capacity over time in *qm37* mutants at 20°C (data not shown). In addition, DAPI stained chromosomes of oocytes arrested in diakinesis of meiosis I in *qm37* hermaphrodites after ~25 hours of adulthood at 25°C, and at 20°C, display normal morphology and relative disposition (n>300 diakinesis oocytes in ~60 gonad arms at each temperature). Finally, while γ-radiation, a double-stranded break generator, of *qm37* L4 hermaphrodites grown at 20°C significantly affects the mutants' brood size, the survival of the progeny produced is not affected (data not shown).

DISCUSSION

clk-2(qm37) mutants develop, behave and reproduce slowly at the permissive temperatures (15-20°C). Yet, these pleiotropic phenotypes are not associated with increased lethality. In addition, except for reproduction, these phenotypes can be maternally rescued in spite of the dramatic increase in size of the animal. However, *clk-2(qm37)* embryos produced at the restrictive temperature (25°C) invariably arrest embryonic development. As *clk-2(RNAi)* leads to similar phenotypic consequences at all temperatures, embryonic arrest is probably the consequence of a complete loss of function of the gene. Our analysis also reveals that maternal *clk-2* activity is absolutely required for embryogenesis during a narrow time window between oocyte maturation and the two- to four-cell stage of the embryo.

clk-2 encodes a protein that is homologous to yeast Tel2p and, like *TEL2* in yeast, affects the length of telomeres in worms. The biological function of CLK-2/Tel2p that affects telomeres has thus been conserved from yeast to worms in spite of a relatively low level of sequence conservation. *clk-2* and *tel2* mutations result in opposite effects on the telomeres, lengthening them for *clk-2* and shortening them for *tel2*. However, both *tel2-1* and *clk-2(qm37)* are partial loss-of-function missense mutations in different regions of the predicted proteins (N- and C-terminal, respectively). Presumably, these mutations affect the function of the protein in different ways, yet both affect telomere length.

One of the factors that affect telomere length is the access of telomerase to the telomeres (Evans and Lundblad, 2000). Yeast Tel2p has been found to bind telomeric repeats in vitro. Therefore, one possibility is that CLK-2 also binds telomeres directly and that this binding limits the access of telomerase. We find, however, that CLK-2::GFP is excluded from the nucleus, suggesting that CLK-2 and Tel2p do not in fact bind telomeres in vivo, but act indirectly like other factors. It is worth noting here that the subcellular localization of Tel2p is unknown, and that Tel2p has been shown to bind RNA in vitro (Kota and Runge, 1999). Interestingly, subtelomeric silencing and telomere length regulation can also be affected by events in the cytosol. For example, Hst2p, a cytosolic NAD^+-dependent deacetylase homologous to Sir2p, can modulate nucleolar and telomeric silencing in yeast (Perrod et al., 2001), and the nonsense-mediated mRNA decay pathway appears to affect both telomeric silencing and telomere length regulation (Lew et al., 1998). Other proteins that affect telomere length, like tankyrase (Smith and de Lange, 2000), are mostly extranuclear (Chi and Lodish, 2000; Smith and de Lange, 1999), with only a very small amount of protein localized to the telomeres (Smith et al., 1998).

In addition to affecting the length of telomeres, Tel2p has been shown to be involved in telomere position effect (TPE) and thus contributes to silencing of sub-telomeric regions (Runge and Zakian, 1996). Mutations in other genes, such as *tel1*, that also affect telomere length, do not result in abnormal TPE, indicating that the TPE defect in *tel2* mutants is not a

simple consequence of altered telomere length. Furthermore, the rapid death and abnormal cellular morphology of cells that fully lack Tel2p suggests that Tel2p has telomere-independent functions (Runge and Zakian, 1996). *clk-2* mutant embryos die invariably at the restrictive temperature because of an absence of *clk-2* activity between oocyte maturation and the two- to four-cell stage, yet their chromosomal and cellular morphologies are normal. This suggests that the crucial function for CLK-2 during early embryogenesis is in regulating gene expression.

CLK-2 is likely required throughout life as indicated by its broad expression and given that the temperature-sensitive period of the mutant extends throughout its life cycle. Yet most of the phenotypes can be maternally rescued. Postulating that CLK-2/Tel2p is involved in the regulation of chromatin function at telomeric and non-telomeric sites provides a model to understand the basis of this maternal effect. During post-embryonic development, the worm grows >500 times in volume. However, most of somatic post-embryonic development consists in an increase in size without cellular multiplication (Sulston and Horvitz, 1977). Therefore, if abundant maternally provided *clk-2* activity is present in the embryo, as suggested by the high level of *clk-2* mRNA in the germline, it might suffice to establish a chromatin state that can persist in the absence of further cell division and differentiation (at least under the stable and favorable conditions of the laboratory). This model is also consistent with the observation that the reproductive phenotypes are only partially rescued, as the germline is mitotic throughout post-embryonic development, and that epigenetic states are generally erased or never implemented in the germline.

How does *clk-2* function affect physiological rates? As mentioned above, one possibility is that *clk-2* regulates genes whose level of expression can alter cellular function. The imbalance resulting from the misregulation of such genes could then lead to slower cellular physiology. The effect of *clk-2* on gene expression could be direct, by participating in the regulation of chromatin structure at particular loci, or indirect, by affecting the length of telomeres. Increased telomere length can affect gene expression at nearby subtelomeric sites by increasing the level of silencing at these sites (Kyrion et al., 1993; Li and Lustig, 1996; Park and Lustig, 2000). Long telomeres could also alter gene expression by functioning as a sink for telomere binding factors that also function at other sites. For example, Sir proteins mediate silencing at the telomeres and the HM loci (Palladino et al., 1993; Rine and Herskowitz, 1987). However, when displaced from the telomeres by mutation (Kennedy et al., 1997), part of the Sir complex can move to the nucleolus where its action appears to silence rDNA and prolong replicative lifespan. These and other studies (Marcand et al., 1996) suggest that telomeres are a reserve compartment for silencing factors and participate in regulating silencing in other parts of the genome.

Another possibility is that the altered telomeres in *clk-2* mutants generate a stress signal that affects cellular physiology. Many protein factors that are important for telomere maintenance and function are also involved in the sensing and repairing of double strand breaks and even more generally in checkpoint controls (Gasser, 2000), including in *C. elegans* (Ahmed and Hodgkin, 2000). Thus, an altered structure of telomeres in *clk-2* mutants could be the source of a stress signal that could, in turn, affect energy metabolism, such that less energy and biosynthetic power is devoted to growth and other energy-consuming processes, in anticipation of the metabolic demands of repair processes.

clk-2 mutants have an extended lifespan (Lakowski and Hekimi, 1996). It is known that lifespan depends on metabolic rates (Lakowski and Hekimi, 1996; Sohal et al., 2000), and thus, most of the mechanisms discussed above to explain the slow physiological rates of *clk-2* mutants could also help to explain their increased lifespan. On the other hand, if *clk-2* mutations produce a cellular stress response, this might result in an increased lifespan through increased stress resistance (Lithgow, 2000).

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