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TOWARDS CLONING THE CLK-3 GENE IN CAENORHABDITIS ELEGANS

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ABSTRACT OF THESES

TOWARDS CLONING THE CLK-3 GENE IN CAENORHABDITIS ELEGANS

Mutations in the clk-1, clk-2, clk-3 and gro-1 genes in Caenorhabditis elegans show alterations in developmental and behavioral timing and lifespan, collectively termed the Clk phenotype. While the clk-1, clk-2, and gro-1 genes have been cloned, clk-3 gene has not been identified. Gene expression changes in clk-3 mutant worms were determined using microarray expression data. I examined genes in the region to which clk-3 gene maps, for strongly reduced expression in the clk-3 mutants and identified thirteen clk-3 candidate genes. RNAi feeding vectors for all these candidate genes were picked and cultured from the RNAi library. Knock-down worm strains were generated by feeding RNAi and analyzed for Clk phenotypes. Of all the candidate genes tested, the Y48E1B.5 gene showed the most similar phenotypic profile to the clk-3 mutants. The Y48E1B.5 gene shows weak homology to a mammalian mitochondrial ribosomal protein. Primers were designed to amplify all 9 exons of the Y48E1B.5 gene. Sequence analysis was carried out on the resulting PCR products from clk-3 mutants. An amino acid change was found in exon 4.

KEY WORDS: Caenorhabditis elegans; clk-3 gene; positional gene mapping; RNAi; phenotype analysis.

Suchita Umesh Desai

August 21, 2008
TOWARDS CLONING THE *CLK-3* GENE IN *CAENORHABDITIS ELEGANS*

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Suchita Umesh Desai

The Graduate School
University of Kentucky
2008
TOWARDS CLONING THE CLK-3 GENE IN *CAENORHABDITIS ELEGANS*

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**THESIS**

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the College of Arts and Sciences at the University of Kentucky

By

Suchita Umesh Desai

Lexington, Kentucky

Co-Directors: Dr. James Lund, Associate Professor
And Dr. Douglas Harrison, Associate Professor

Lexington, Kentucky

2008

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The following thesis, while an individual work, benefited from the insights and direction of several people. In the first place I would like to acknowledge the constant guidance and support of my thesis advisor Dr. James Lund, without whom this thesis would not have materialized. I would also like to thank my committee members Drs. Douglas Harrison and Robin Cooper for reviewing my thesis in a timely manner and offering their helpful suggestions.

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I. BACKGROUND AND SIGNIFICANCE

A. Caenorhabditis elegans

Dr. Sydney Brenner first pioneered the use of the nematode *Caenorhabditis elegans* as a model organism for studying development and nervous system in 1965. Following his major publication on *C. elegans* research in 1974, a new world of research opened up, that helped us better understand cellular and molecular processes in the worm and other higher organisms.

The adult worm is 1 mm long and has an invariant number of somatic cells (959 in the adult hermaphrodite; 1031 in the adult male). All of the developmental stages of the worm can be viewed under a dissecting microscope owing to its transparent body. It feeds on bacteria (*E. coli*) and can be maintained easily in the laboratory on agar plates. Worm strains can also be cryogenically frozen. The hermaphrodite worm has a short lifespan of two weeks and a generation cycle of about three days at 20°C. Males are spontaneously produced at a very low frequency, which can be mated to hermaphrodites. On an average, an adult hermaphrodite produces about 300 progeny by self-fertilization; thus one worm can produce tens of thousands of progeny in a week. During its life cycle spanning three days at 20°C, *C. elegans* progresses through embryogenesis, hatches from the egg and then develops through four larval stages, L1 through L4, each separated by molts. The final L4 larva molts into a sexually mature reproducing adult. A striking feature of the life cycle of the nematode is that under conditions of overcrowding, shortage of food or changes in temperature, the animals produce a pheromone [Golden and Riddle et al., 1984] that induces molting of the L2 larva to the developmentally
arrested, alternative L3 stage dauer larva, which does not feed and is resistant to environmental stress. The animals remain in dauer stage until conditions become favorable, where upon they molt into the L4 stage and continue with the normal developmental stages.

Complete sequencing of the 97-megabase genome for the nematode was completed in 1998 [C. elegans Sequencing Consortium, 1998]. The access to all of the gene sequences accelerated reverse genetics, which is used to determine the function of a gene, starting with a known gene sequence. Complementing the above, the greatest advantage of using worms for biological research is the ready generation of mutants by chemical mutagenesis, irradiation technique, and gene knock-downs by RNAi and creating transgenics by DNA injection [Brenner, 1974; Johnson, 2003].

B. C. elegans as a model for aging

This free-living soil nematode has now become the organism of choice for studying the genetic basis of longevity and aging due to identification of a number of single gene mutations that dramatically increase lifespan in C. elegans. There is growing evidence for the influence of genetic pathways in regulating the lifespan of C. elegans, which have been reviewed in this section.

The Clk genes

In a screen for maternally rescued viable mutants using an EMS mutagenesis protocol in C. elegans, Hekimi et al., 1995, identified 41 mutations that caused
morphological or behavioral defects. Amongst seven different mutant phenotypes categorized in this screen, one of them was the Clk (abnormal function of biological clocks) phenotype; comprised of three genes [\textit{clk-1} (alleles: \textit{e2519, qm11, qm30, qm47, qm51}); \textit{clk-2} (allele: \textit{qm37}) and \textit{clk-3} (alleles: \textit{qm38, qm53})] that affect the developmental and behavioral timing in the animals. \textit{gro-1} (allele: \textit{e2400}) mutants were identified to be slow growing and also showed additional Clk phenotypes and thus was placed in the Clk class of genes later on by Wong et al., 1995. A remarkable feature of the Clk phenotype is that the mutants show a longer mean and maximum lifespan as compared to the wild-type Bristol N2 strains. While the best characterized is the \textit{clk-1} gene, all the Clk mutants display the same Clk phenotype. The Clk mutants display altered timing of the cell cycle; embryonic and post-embryonic development, gametogenesis as well as periodic behaviors like defecation, pharyngeal pumping and swimming cycles. These results suggest the existence of a central physiological clock in the worm [Hekimi et al., 1995].

The \textit{clk-1} gene is a nuclear gene that encodes a homolog of yeast CAT5/COQ7 gene [Ewbank et al., 1997]. In yeast, the \textit{COQ7} gene is a mitochondrial protein involved in the biosynthesis of ubiquinone (UQ9), which is an electron carrier in the mitochondrial respiration process. \textit{COQ7} hydroxylates demethoxyubiquinone (DMQ) to 5-hydroxyubiquinone which is then converted into UQ9 by Coq3p/COQ-3 [Marbois et al., 2005]. The \textit{C. elegans} CLK-1 protein is localized in the mitochondria [Felkai et al., 1999] and is involved in UQ9 synthesis [Jonassen et al., 1998]. CLK-1 affects the level of mitochondrial respiration, the rate of which decreases more rapidly during aging in the \textit{clk-1} mutants as compared to the wild-type mitochondria [Ewbank et al., 1997].
The \textit{clk-2} gene in \textit{C. elegans} encodes a homolog of yeast Tel2p protein [Bénard et al., 2001] and regulates telomere length [Lim et al., 2001] and also functions as a DNA damage checkpoint protein [Ahmed et al., 2001]. In yeast, the Tel2p protein is known to bind to telomeric dsDNA [Kota and Runge, 1998] and regulate yeast telomeric length \textit{in vivo} [Kota and Runge, 1999].

The \textit{gro-1} gene in \textit{C. elegans} encodes a highly conserved cellular enzyme, isopentenylpyrophosphate:tRNA transferase (IPT), which modifies a subset of tRNAs. The \textit{gro-1} transcript harbors a sequence predicted to encode a mitochondrial localization signal within one of the two protein variants, derived from alternate initiator codons. The \textit{gro-1} transcript initiated from the first methionine is localized exclusively to the mitochondria and rescues the mutant phenotype. The \textit{gro-1} transcripts initiated from the second methionine localize diffusely throughout the cell and do not rescue the mutant phenotype. GRO-1 is thus speculated to act in the mitochondria and regulate physiology in the worms by mechanisms yet to be uncovered [Lemieux et al., 2001]. In yeast, this highly conserved enzyme is encoded by the \textit{mod-5} gene which also encodes for both mitochondrial and cytoplasmic proteins [Dihanich et al., 1987; Najarian et al., 1987].

The \textit{clk-3} gene in \textit{C. elegans} was identified by mutation and is now awaiting positional cloning and molecular characterization. \textit{clk-3} mutants have a longer mean and maximum lifespans as compared to wild-type Bristol N2 strains at all tested temperatures; a characteristic of all the Clk genes. There are two known alleles for \textit{clk-3} gene: \textit{qm38} and \textit{qm53}; both of which show phenotypes similar to \textit{clk-1} mutants [Hekimi
et al., 1995]. However, the \textit{clk-3 (qm53)} allele has been lost, and the \textit{clk-3 (qm38)} is the only allele available for the \textit{clk-3} mutants. \textit{clk-3} has been genetically mapped to the right arm of linkage group (LG) II, 1 cM to the left of the cloned gene \textit{unc-52}, in between the \textit{rol-1} and \textit{eat-2} genes, a gene-rich region of 2Mb containing about 500 genes [Hekimi et al., 1995; Figure 1].

Lakowski and Hekimi, 1996 have demonstrated that all the four Clk genes interact genetically in order to determine the length of post embryonic development as well as the adult lifespan of the nematode. Across a range of growth temperatures (15°C, 18°C, 20°C and 25°C), all four mutants showed a longer mean and maximum lifespan than the wild-type strains. The double mutants demonstrated prolonged length of postembryonic development as compared to mutations in the individual genes at all the temperatures tested. The double mutants were found to have much longer mean and maximum lifespans compared to that of single mutants at some temperatures. The \textit{clk-3 (qm38); clk-1 (qm30)} and \textit{clk-3 (qm38); clk-2} double mutants have longer lifespan compared to that of the individual mutants at some temperatures. \textit{clk-3 (qm38); gro-1} double mutants however have a mean lifespan similar to that of the wild-type Bristol N2 strains; although individual mutation increases lifespan. This does suggest that there is a high degree of specificity in the interaction between \textit{clk-3} and the other Clk genes. It would hence be interesting to determine if \textit{clk-3} gene affects the physiological clock of the nematode independently or in conjunction with one or more of the other Clk genes.
The Insulin-like signaling pathway

The best understood of the signaling pathways involved in lifespan control of \textit{C. elegans} is the evolutionarily conserved Insulin-like signaling (IIS) pathway [Gami and Wolkow, 2006; Figure 2]. The insulin/IGF-1 receptor DAF-2 signals a phosphoinositide-3-OH kinase (PI3K) homolog AGE-1 [Dorman et al., 1995; Morris et al., 1996]. Activated PI3K then generates phosphoinositide-3,4,5-P3 (PI-3,4,5-P3) and phosphoinositide-3,4-P2 (PI-3,4-P2) which act as second messengers and activate serine threonine kinases AKT-1, AKT-2, PDK-1 and SGK-1 [Paradis and Ruvkun, 1998; Paradis et al., 1999; Hertweck et al., 2004]. Phosphorylation of FOXO transcription factor DAF-16 by these kinases causes it to be inactivated and sequestered in the cytoplasm [Lee et al., 2001; Lin et al., 2001]. Under favorable environmental conditions, an insulin-like ligand binds to and activates the DAF-2 receptor kinase, which activates the IIS, resulting in phosphorylation of DAF-16. Non-phosphorylated DAF-16 that is nuclear localized represses genes required for reproductive growth and metabolism, but activates genes involved in stress response including super oxide dismutases (SOD) and catalase. Mutation in the genes daf-2 and age-1 lowers or decreases the activity of the IIS pathway, resulting in enhanced stress resistance and extended longevity [Guarente and Kenyon, 2000]. IIS is down regulated by DAF-18, encoded by \textit{daf-18} which is the \textit{C. elegans} ortholog of the human tumor suppressor gene PTEN [Gil et al., 1999].

Interestingly, \textit{daf-2; clk-1} double mutants show a fivefold increase in adult lifespan as compared to wild-type Bristol N2 strains. Also, \textit{daf-16} mutation does not suppress the long-lived phenotype of \textit{clk-1} mutants [Lakowski and Hekimi, 1996]. This
suggests that IIS and *clk-1* might act through different pathways in regulating the lifespan of the nematode. It might be possible that they do affect the same downstream process, but may do so in a manner that sufficiently distinct for the process to be altered more severely when both mutations are present [Hekimi et al., 2001]. Studies for other Clk mutants with IIS mutants have not been reported.

**Caloric Restriction**

It has been demonstrated in *C. elegans* that a reduction of food intake during either the growth, reproductive, or post-reproductive portion of the life cycle is correlated with an increase in the mean life span [Klass, 1977]. Eat mutants with abnormal pharyngeal muscle motion show feeding defects and starved appearance [Avery, 1993]. Lifespan extension in *eat-2* mutants is independent of DAF-16 activity and *eat-2; daf-2* double mutants live even longer than long lived *daf-2* mutants. This implies that caloric restriction regulates lifespan by a mechanism distinct from that of *daf* mutants.

Caloric restriction in *eat-2* mutants does not further increase the lifespan of long-lived *clk-1* mutants, suggesting that caloric restriction and *clk-1* may affect similar processes [Lakowski and Hekimi, 1998]. Caloric restriction experiments with other Clk mutants have not been reported.

**Reactive oxygen species and aging**

The Free Radical Theory of Aging proposes that Reactive Oxygen Species (ROS), produced primarily in the mitochondria as a result of metabolic processes (electron
transfer through the Electron Transport Chain) may have a major physiological role in the accumulation of oxidative damage and control of cell senescence. Other oxidants and free radicals that are derived from superoxide, including hydrogen peroxide, OH• and several other species also inflict oxidative stress on the host cell [Harman, 1956]. The free radicals cause oxidative damage to mitochondria where they are produced and may also leak out and damage other the cellular components, thus decreasing the lifespan of the cell. Defense mechanisms against these free radicals include enzymes like superoxide dismutase (SOD) and catalase that destroy superoxide and hydrogen peroxide, respectively. mev-1 mutant worms that lack succinate dehydrogenase cytochrome b have been found to be hypersensitive to increasing oxygen concentrations [Ishii et al., 1998] and a shortened lifespan [Brand, 2000].

Clk mutants display slowing down of many temporal processes as well as increased lifespan. It was thus hypothesized that if the metabolic rate of the cell is lowered, the accumulation of ROS can be decreased and hence extend the lifespan of the cell. However, in a set of elaborate experiments carried out by Braeckman et al., 2002; it was observed that there is no reduction of energy metabolism of the cell, as suggested by no changes in the oxygen consumption, heat output and ATP levels. The reason why mutations in the Clk genes lead to the Clk phenotype thus remains unclear. SOD-2, a superoxide dismutase detoxifies superoxide in the cytoplasm and mitochondria. Yang et al., 2007 showed that sod-2(RNAi) increased the lifespan of clk-1 mutants with unchanged oxidative damage levels as compared to wild-type worms. This suggests that the increased longevity may not be due to low oxidative damage.
C. RNA interference (RNAi) in *C. elegans*:

Over the past decade, RNAi has proved to be a valuable tool for investigating gene function using a reverse genetics approach. RNAi was first understood in *C. elegans*, where it was observed that introduction of double stranded RNA in *C. elegans* by injection causes a potent and specific interference in gene expression in both the injected animals and their progeny [Fire et al., 1998]. Also interesting is the fact that the RNAi phenomenon spreads across cell boundaries and is effective in other regions of the animal rather than just being focused at the point of injection [Timmons and Fire, 1998]. dsRNA (double stranded RNA) can be introduced experimentally in the nematode by soaking them in dsRNA solution [Tabara et al., 1998]; feeding them dsRNA-expressing bacteria [Timmons and Fire, 1998] or by generating heritable inverted repeat (IR) [Tavernarakis et al., 2000].

The Ahringer lab generated a full genome RNAi library. PCR products (~1-1.5kb long) were amplified from the *C. elegans* genomic DNA and were ligated into L4440 expression vectors, where the insert is flanked by copies of bacteriophage T7 promoter for bidirectional transcription by T7 polymerase. The ligated L4440 expression vectors were then transformed into HT115(DE3), an *E. coli* strain deficient in RNaseIII [Kamath and Ahringer, 2003; Timmons and Fire, 1998; Timmons and Fire, 2001; Figure 3]. This RNAi feeding library consists of 16,757 such bacterial strains (each identified by a specific GenePair name) that can produce dsRNA to target ~86% of the *C. elegans* genes for loss-of-function analysis. They are publicly available through Geneservice Ltd. at [http://www.geneservice.co.uk/products/rnai/Celegans.jsp](http://www.geneservice.co.uk/products/rnai/Celegans.jsp).
This RNAi library has been used effectively for rapid, genome wide RNAi screening in *C. elegans*. However, not all genes are susceptible to inhibition by RNAi, complicating RNAi assays and genomic screens [Fraser et al., 2000; Kamath et al., 2003; Simmer et al., 2003]. The RNAi supersensitive *rrf-3* strain lacks an inhibitory RNA-directed RNA polymerase (RdRP) and is effective in generating stronger RNAi phenotypes for genes that have weak or no phenotype in wild-type Bristol N2 backgrounds [Simmer et al., 2002].
Figure 1. Genetic map position for *clk-3* gene [From Hekimi et al., 1995]. The *clk-3* gene has been genetically mapped to the right arm of linkage group (LG) II: $22.09 \pm 0.221$ cM between *rol-1* gene (12.2 Mb) and *eat-2* gene (14.2 Mb).
Figure 2. Overview of the Insulin Signaling pathway in *C. elegans* [From Gami and Wolkow, 2006]. Insulin-like ligands activate the DAF-2/InsR receptor that recruits AGE-1/PI3K to the cell membrane. AGE-1/PI3K generates phospholipid signals, PIP’s. PIP’s include phosphoinositide-3,4,5-P3 (PI-3,4,5-P3) and phosphoinositide-3,4-P2 (PI-3,4-P2). These PIP’s activate serine/threonine kinases AKT-1, -2, PDK-1 and SGK-1 which phosphorylate DAF-16/FOXO preventing its nuclear translocation. DAF-18/PTEN negatively regulates AGE-1 signaling by dephosphorylating PIPs. Orange boxes designate genes whose loss-of-function phenotype is increased lifespan and blue boxes designate genes whose loss-of-function phenotype is reduced lifespan.
Figure 3. HT115(DE3), RNaseIII deficient E. coli strain hosting the L4440, double-T7 expression vector. A fragment of the gene of interest is amplified by means of PCR and cloned into the multiple cloning site (MCS) of the L4440 expression vector, flanked by the bacteriophage T7 promoter on either side for bidirectional transcription by T7 polymerase. Ampicillin resistance gene confers antibiotic resistance to HT115(DE3) to enable selection on an antibiotic medium [adapted from Timmons and Fire, 1998; Kamath et al., 2000].
II. MATERIALS AND METHODS

A. General methods and C. elegans strains

*C. elegans* strains were maintained in the lab following basic procedures as outlined in Brenner, 1974. Strains were maintained at 20°C, unless specified otherwise. Strains and/or alleles used include: wild-type Bristol N2 strain, *clk-3 (qm38)* MQ131 strain and *rrf-3* mutant strain. RNAi feeding experiments were carried out using the *rrf-3* strain that is hypersensitive to RNAi [Simmer et al., 2002].

B. Growing up bacterial strains and their induction

The control bacterial strain used was HT115(DE3), an RNaseIII deficient *E. coli* strain which is beneficial for RNAi feeding experiments using *C. elegans*. HT115(DE3) bacteria containing cloned L4440 plasmids from the RNAi library were used as experimental strains. A loop full of HT115(DE3) bacteria containing cloned L4440 plasmids from the RNAi library for each GenePair was plated individually onto an LB plate containing 50 μg/ml ampicillin and 10 μg/ml tetracycline and allowed to grow overnight at 37°C. On day 2, a colony was picked up from each individual plate and cultured overnight in LB medium containing 50 μg/ml ampicillin at 37°C with continuous shaking. To carry out expression of dsRNA, the bacteria were seeded directly onto standard NGM agar plates with 1 mM IPTG and 25 μg/ml carbenicillin. Seeded plates were allowed to dry at room temperature and induction was allowed to continue at room temperature overnight [Fraser et al., 2000; Kamath et al., 2000].
C. Feeding protocol using *rrf-3* mutant strains

About 75 L1 stage synchronized worms were placed in fresh RNAi plates for each gene and left for approx. 72 hours at 20°C. Adult worms were then removed by means of a platinum pick and they were scored for developmental and behavioral phenotypes at 20°C [Wong et al., 1995].

D. Assays for developmental phenotypes

**Lifespan assay:** Embryos were isolated by bleaching adults on NGM plates. These embryos were allowed to synchronize to L1 stage in liquid M9 buffer at 20°C for 16 hours and then placed on NGM/ Carbenicillin plates seeded with desired RNAi bacteria: HT-115(DE3) bacterial strain for control worm strains and RNAi bacterial strains for the experimental worm strains. The animals were transferred daily while producing eggs to separate them from their progeny and every alternate day thereafter. The animals were scored daily or every other day for live/dead/missing worms. Animals were scored as dead if the pharynx stopped pumping and they did not respond to prodding with an eyelash pick. They were removed off the plate using a platinum pick. Scoring was carried out until all animals were dead (N= 90). Kaplan-Meier survival analysis and Mantel-Haenszel logrank tests were used to compare the controls to the experimental samples. A p-value less than 0.05 was considered as significant.

**Egg production rate assay:** Ten 24 hour-old adult hermaphrodites were picked and placed on fresh RNAi plates for 4 hours at 20°C. All the hermaphrodites were then
removed by means of a platinum pick and the total numbers of eggs laid by each strain were counted.

**Self-brood size assay:** Ten larvae were placed onto fresh RNAi plates and incubated at 20°C until they matured and laid the first few eggs. The hermaphrodites were then transferred onto fresh RNAi plates daily to prevent overcrowding until egg-laying ceased. The progeny were counted 3 days after removal of the parents by means of a platinum pick.

**E. Assays for behavioral phenotypes**

**Worm growth and egg laying phenotype:** RNAi treated worm strains were visibly estimated under the light microscope to check for their growth in terms of their body size as compared to *clk-3* mutants (positive control) as well as *rrf-3* mutants on control HT115(DE3) (negative control). Time course for their egg laying behavior was also noted (N=25).

**Thrash assay:** Fifteen 24 hour-old adult hermaphrodites were picked individually onto a drop (20 µl) of liquid M9 buffer. Rhythmic thrashing of their tails was scored for a minute and each animal was scored twice (N=15). A p-value less than 0.01 was considered as significant.
F. C. elegans genomic DNA isolation

Fresh large agar plates were prepared and seeded with E. coli. Plates were incubated at 37°C for 24 hours to produce a lawn of E. coli, and then approximately 500 adult worms were placed on them. Five plates were used to obtain a sufficient quantity of worms to isolate one genomic DNA sample. After 3 days of incubation at 20°C, the plates were covered with adult worms. The worms were washed off the plate using 10 ml of 100mM NaCl solution using a Pasteur pipette and transferred into 15 ml centrifuge tubes. Worms were pooled from three seeded plates, centrifuged at 1,000g for 3 minutes at room temperature, washed once with saline, centrifuged again and finally resuspended in 3 ml of saline. 3 ml of an ice-cold 60% sucrose solution was mixed with the worm suspension and allowed to cool on ice for 10 minutes. The worms were centrifuged at 2,000g for 5 minutes at 4°C in the sucrose gradient. The worms formed a visible layer approximately halfway down the liquid interface; bacteria and debris pelleted to the bottom of the centrifuge tube. Worms were harvested and washed three times with saline to remove the sucrose, which is toxic to the worms.

A microliter of the worm suspension was dropped onto a glass slide and counted under the light microscope to determine the number of worms per microliter. Approximately 50,000 worms (mostly adults) were used for each genomic DNA isolation. Worms were pelleted in the 15 ml centrifuge tube and supernatant removed. The worms were resuspended in about 4ml of TEN and frozen over night at -80°C. This step is useful since frozen worms seem to be easier to digest and the worms can also be stored at -80°C indefinitely.
Following three rounds of freezing at -80°C and thawing at room temperature, 4 ml of the suspension was distributed evenly in four 1.5 ml microfuge tubes. 25 µl 10% SDS and 2.5 µl 20 mg/ml Proteinase K was added to each tube and incubated 60°C for one hour. The pellet was resuspended at 10 min., 20 min., and 30 min. Another 2.5µl of Proteinase K was added to each tube and incubated further for another hour. The solutions were then extracted with 0.5 ml phenol and mixed well by inversion. The solutions were then extracted with equal amounts of Phenol/CIA solution (CIA= 24:1 Chloroform: isoamyl alcohol). The solutions were then extracted with equal amounts of CIA. 45 µl of 3M NaOAc and 0.8 ml 100% ethanol was added to each tube and mixed gently at room temperature, causing the DNA to precipitate almost immediately. The tubes were spun very lightly (2-5 sec.) in a microfuge to precipitate the DNA. The ethanol was drained off and the DNA pellet was washed in ice cold 70% ethanol. The 70% ethanol was then drained and tubes were allowed to dry completely. Any drops of ethanol were aspirated off with a pulled-out Pasteur pipette. The pellets were resusupended in 0.5 ml TE and 2 µl of 10 mg/ml RNase A was added to each tube and incubated at 37°C for one hour. The DNA concentration in each tube was measured using NanoDrop 1000 by Thermo Scientific. Concentration in each tube was adjusted to 1 µg/µl using additional TE or vacuum pump as required [Modified from Wormbook and Herman lab protocols [Available at http://www.k-state.edu/hermanlab/protocols/genomic_dna_prep.htm].

G. Generation and cloning of PCR products

Primers (listed in Table 2) were designed for all exons of the candidate gene Y48E1B.5 using an online tool: Primer3 [http://fokker.wi.mit.edu/primer3/input.htm].
PCR products were synthesized using Extension Taq Polymerase in a reaction that contained 1µg/µl of clk-3 (qm38) genomic DNA template, 100 pmol of forward and reverse primer pairs each, 100 µM of dNTPs and 30 cycles of PCR using the following temperature cycle: 94°C, 3’; 94°C, 1’; 57°C, 30s; 72°C, 55s and a final extension of 72°C, 5’ [Fraser et al., 2000]. Control PCR was run under the same conditions using wild-type Bristol N2 strain genomic DNA template. Sizes of the resulting PCR products were ascertained by gel electrophoresis.

H. DNA Sequencing

PCR products were cleaned up using QIAGEN QIAquick® PCR Purification Kit. PCR samples along with their respective primers were delivered to The University of Kentucky Advanced Genetic Technologies Center (AGTC). AGTC runs the sequencing reaction on ABI 3100. Sequencing data was analyzed using CodonCode Aligner program [http://www.codoncode.com/aligner/]. Results are presented in Table 3.
Figure 4. Schematic flowchart showing the *C. elegans* RNAi assay. A loop full of HT115(DE3) bacteria containing cloned L4440 plasmids from the RNAi library for each GenePair was plated individually onto an LB plate containing 50 μg/ml ampicillin and 10 μg/ml tetracycline and allowed to grow overnight at 37°C. On day 2, a colony was picked up from each individual plate and cultured overnight in LB medium containing 50 μg/ml ampicillin at 37°C with continuous shaking. About 75 L1 stage synchronized worms were placed in fresh RNAi plates for each candidate gene and left for approx. 72 hours at 20°C. Adult worms were then removed by means of a platinum pick and they were scored for developmental & behavioral phenotypes at 20°C [Wong et al., 1995].
III. RESULTS

A. Candidate gene approach to cloning \textit{clk-3} gene

In our approach to identify possible \textit{clk-3} candidate genes, we utilized the microarray technique as a screening method to identify and investigate down-regulated genes in the \textit{clk-3} mutants in a \textit{glp-4} background. The \textit{glp-4(bn2ts)} mutation interferes with the proliferation of the germ line of \textit{C. elegans} and thus can be used to generate worm populations that are severely depleted in germ cells. Using the \textit{glp-4} background for microarray experiments can thus facilitate determination of whether any gene of interest is expressed in the germ line or soma or both [Beanan and Strome, 1992]. The microarray experiments were designed and carried out by Scott Frasure, Scientist I, in our lab. While not all mutations that give rise to phenotypes reduce transcript levels, many classes of mutations do have this effect. If the molecular mutation in \textit{clk-3} gene reduces transcript levels, then an examination of the expression of genes in the region that the \textit{clk-3} gene is mapped to may identify it. We reasoned that a candidate gene with Clk RNAi knock-down phenotypes comparable to that of \textit{clk-3} mutant phenotype would be a good candidate and could be checked for mutations by PCR amplification, sequencing and comparison to wild-type Bristol N2 strain genomic sequence.

Following the approach outlined above, microarray expression data from \textit{clk-3} mutants generated previously in the lab was analyzed for strongly down-regulated genes in the region \textit{clk-3} mapped to on chromosome II [Figure 5]. Affymetrix microarray hybridizations were carried on six biological samples of six day old \textit{clk-3;glp-4} worms and compared to age matched \textit{glp-4} controls. The rationale for using six day old adults
was that they have a declining reproduction rate. The microarray expression data was screened for genes that had transcript levels $\geq 1.5$ fold lower in five of the six $clk-3;glp-4$ samples compared to the mean of the control samples.

Thirteen genes meeting these criteria were identified and are listed in Table 1; along with their GenePairs name, locus and description. Two of the candidate genes have two RNAi clones each. Appropriate RNAi strains were located in the Ahringer RNAi library and the candidate gene bacterial strains were cultured. $rrf-3$ strains of $C. elegans$ were fed individually with the candidate RNAi bacterial strains to knock-down their expression and the knock-down phenotypes were analyzed for the Clk phenotypes as described by Wong et al., 1995. The assay results are described below.

**B. Assays for developmental phenotypes**

*Lifespan assay:* One of the most interesting aspects of the Clk mutants is that they have a longer mean and average lifespan [Wong et al., 1995, Hekimi et al., 1995]. Lifespan assays were carried out on $rrf-3$ mutant strains fed on candidate gene RNAi bacteria. We found that none of the candidate genes had extended lifespans. Eleven of the candidate gene RNAi knock-down strains had significantly shorter lifespans. Y48E1B.5; ZK131.7; Y54G11A.4 and Y46G5A.31 had a lifespan indistinguishable from the control $rrf-3$ mutant strains [Figures 6a; 6b].

*Egg production assay:* The egg production rate is one of the most strongly affected features in the Clk mutants [Wong et al., 1995, Hekimi et al., 1995]. Among the
candidate genes, the ones that were most affected include C50E10.4 (one of two RNAi clones for this gene), F29C12.4 with extremely low egg production rate, and Y48B6A.3 with no egg production at all. ZK131.7 and clk-3 mutants demonstrate a typical Clk phenotype of a delayed start in egg production; at ~ 90 hours of life as compared to wild-type animals that start laying eggs at ~ 72 hours of life at 20°C. The rest of the candidate genes showed an egg production rate similar to the wild-type Bristol N2 and rrf-3 control worm strains [Figure 7].

**Self-brood size assay:** Self-brood size assay is also strongly affected in the Clk mutants [Wong et al., 1995, Hekimi et al., 1995]. Among the candidate genes, the ones that were most affected include ZK131.7; Y48E1B.5 and F29C12.4. Y48B6A.3 was sterile with no egg production. ZK131.7 and clk-3 mutant strains were assayed a day later than all the other strains owing to their delay in egg production. The rest of the candidate genes showed a self-brood size similar to the wild-type Bristol N2 and rrf-3 control worm strains [Figure 8].

C. Assays for behavioral phenotypes

**Worm growth and egg laying phenotype:** RNAi treated worm strains were examined for their size and their egg laying phenotype as compared to clk-3 mutant strains (positive control) as well as rrf-3 mutant strains on control HT115(DE3) bacterial strain (negative control). ZK131.7 and Y48B6A.3 were smaller as compared to all the controls. C50E10.4 (one of two RNAi clones for this gene) strain showed sparse eggs on
the feeding plate on day 3 of life. ZK131.7, Y48B6A.3, and clk-3 mutants showed no eggs on day 3 of life [Figure 9].

**Thrash assay:** Worms move forward and backward in a sinusoid manner on agar surface which is difficult to monitor. Thrash assay was developed by Wong et. al., 1995 to measure their locomotory performance and found that all three alleles of *clk-1* examined (*qm11, qm30* and *e2519*) showed slowing of swimming frequency when placed in a drop of liquid M9 buffer. Of all the candidate genes assessed by thrash assay, Y48E1B.5 strain showed the lowest thrash rate of 60.6 ± 9.94 per minute (p-value = 7.54 x 10^{-22}) as compared to *rrf-3* mutant strains with a thrash rate of 104.87 ± 12.51 per minute. The control *clk-3* mutant strains had a thrash rate of 104.9 ± 6.24 per minute which was significantly different from the wild-type Bristol N2 strain with 110 ± 6.92 per minute (p-value = 0.004) [Figure 10].

**D. Y48E1B.5 is the most promising candidate for clk-3 gene**

Considering the above behavioral and developmental phenotype assays, the RNAi knock-down animals for the ZK131.7, Y48B6A.3 and Y48E1B.5 genes were chosen as the most probable candidates. Knock-down strains for the Y48E1B.5 gene showed a significantly lower thrash rate per minute which is characteristic of the Clk phenotype and unlike the other two candidates it was not short lived. The egg production rate and the self brood size were not lowered as seen in Clk phenotype. However, RNAi is not always completely effective, complicating RNAi assays and genomic screens as
mentioned earlier. RNAi was started in these animals after hatching so it is not surprising that these worms show only a subset of their loss-of-function phenotypes.

RNAi phenotypes for the Y48E1B.5 gene listed on wormbase include: slow growth; sterile (Ste); embryonic lethal (Emb); larval arrest (Lva); reduced brood size; clear (Clr) and protruding vulva (Pvl). *clk-3* mutants show slow growth and lay eggs slower giving it a reduced brood size in RNAi screens. At 25°C, *clk-3* mutation is embryonic lethal and sterile. Considering the set of assays, the Y48E1B.5 gene was the closest phenotypic match for the *clk-3* gene among the thirteen candidate genes examined.

The complete unspliced Y48E1B.5 gene is located on chromosome II at 13.6 Mb. and extends 6,950 bps. The complete mature mRNA transcript is 1,488 bp long with 9 exons [Figure 11]. Y48E1B.5 gene is orthologous to human 39S ribosomal protein L37, mitochondrial precursor (L37mt).

**E. Candidate gene sequencing**

Primers were designed against the basal promoter, exons 1 through 9, and the 3’UTR for the Y48E1B.5 gene; primers are listed in Table 2. PCR products amplified from *clk-3* mutant worms were sequenced individually from the forward as well as the reverse strands and compared to the genomic sequence of the gene in WormBase. Sequencing data from both strands for the PCR products were aligned with the genomic sequence for the Y48E1B.5 gene using CodonCode Aligner software. Exons 1, 2, 3, 5, 7
and 9 showed no mutations. Exon 4 had a change at base pair number 210 from A to G, which however did not alter the amino acid. Exon 4 also had a change at base pair number 214 from T to A; and at base pair number 215 which is in the same codon as base pair number 214 from C to A. This changed the amino acid residue number 191 from Lysine to Arginine. Exon 6 had a base change at amino acid residue number 102, which did not alter the amino acid. Exon 8 had a base change at amino acid residue number 491, which did not alter the amino acid. The results are tabulated in Table 4.
Figure 5. Microarray expression data for the thirteen candidate genes. On the left panel, each column is the average of six to eight microarray chips. The Clk and eat-2 expression levels are shown relative to the glk-4 control samples while the axenic samples have a separate control. The candidate genes show $\geq 1.5$ fold lower expression in five of the six clk-3 samples. Some of them are also down regulated in caloric restricted worms, the eat-2 and axenic samples. The right panel shows the six individual microarray chip expression data for the thirteen candidate genes chosen for this study.
Table 1. List of \textit{clk-3} candidate genes

<table>
<thead>
<tr>
<th>GenePairs</th>
<th>Locus</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>C50E10.4</td>
<td>sop-2</td>
<td>SAM domain-containing protein, Suppressor of Pal-1</td>
</tr>
<tr>
<td>E01G4.2</td>
<td>pqn-27</td>
<td>Prion-like-(Q/N-rich)-domain-bearing protein</td>
</tr>
<tr>
<td>Y57A10B.1</td>
<td></td>
<td>Protein involved in mRNA turnover and stability</td>
</tr>
<tr>
<td>F58G1.4</td>
<td>dct-18</td>
<td>DAF-16/FOXO Controlled, germline Tumor affecting, unnamed protein</td>
</tr>
<tr>
<td>Y48E1B.5</td>
<td></td>
<td>Weak homology to 39S ribosomal protein L37, mitochondrial precursor (L37mt)</td>
</tr>
<tr>
<td>C50E10.4 (ii)</td>
<td>sop-2</td>
<td>SAM domain-containing protein, Suppressor of Pal-1</td>
</tr>
<tr>
<td>ZK131.1</td>
<td>his-26</td>
<td>Encodes an H4 histone</td>
</tr>
<tr>
<td>F29C12.4</td>
<td></td>
<td>Mitochondrial elongation factor</td>
</tr>
<tr>
<td>ZK131.7</td>
<td>his-13</td>
<td>Encodes an H3 histone</td>
</tr>
<tr>
<td>Y48B6A.3</td>
<td>xrn-2</td>
<td>XRN (mouse/S. cerevisiae) ribonuclease related</td>
</tr>
<tr>
<td>Y54G11A.4</td>
<td></td>
<td>Uncharacterized conserved protein</td>
</tr>
<tr>
<td>Y54G11A.7</td>
<td></td>
<td>Uncharacterized conserved protein</td>
</tr>
<tr>
<td>R06A4.8</td>
<td></td>
<td>Orthologous to Human Glycogen debranching enzyme isoform 6</td>
</tr>
<tr>
<td>Y46G5A.31</td>
<td>gsy-1</td>
<td>Orthologous to Human Glycogen synthase-1</td>
</tr>
<tr>
<td>Y46G5A.31 (ii)</td>
<td>gsy-1</td>
<td>Orthologous to Human Glycogen synthase-1</td>
</tr>
</tbody>
</table>

Affymetrix microarray hybridizations were carried on six biological samples of six day old \textit{clk-3}:\textit{glp-4} worms and compared to age matched \textit{glp-4} controls. The microarray expression data was screened for genes that had transcript levels $\geq 1.5$ fold lower in five of the six \textit{clk-3}:\textit{glp-4} samples compared to the mean of the control samples. Thirteen genes meeting these criteria were identified and are listed in the table above; along with their GenePairs name, locus and description. Two of the candidate genes have two RNAi clones each.
Figure 6a. RNAi lifespan assays of candidate genes: C50E10.4; E10G4.2; Y57A10B.1; F58G1.4; Y48E1B.5; C50E10.4 (ii); ZK131.1; F29C12.4 compared to control rrf-3 mutant strain; and clk-3 mutant strain compared to wild-type Bristol N2 strain. Synchronized L1 stage animals were placed on NGM/ Carbenicillin plates seeded with desired RNAi bacteria: HT115(DE3) bacterial strain for control worm strains and RNAi bacterial strains for the experimental worm strains. The animals were scored daily or every other day for live/dead/missing worms. Scoring was carried out until all animals were dead (N= 90). Kaplan-Meier survival analysis and Mantel-Haenszel logrank tests were used to compare the controls to the experimental samples. A p-value less than 0.05 was considered as significant.
Figure 6b. RNAi lifespan assays of candidate genes: ZK131.7; Y48B6A.3; Y54G11A.4; Y54G11A.7; R06A4.8; Y46G5A.31; Y46G5A.31 (ii) compared to control *rrf-3* mutant strain; and *clk-3* mutant strain compared to wild-type Bristol N2 strain. Synchronized L1 stage animals were placed on NGM/ Carbenicillin plates seeded with desired RNAi bacteria: HT115(DE3) bacterial strain for control worm strains and RNAi bacterial strains for the experimental worm strains. The animals were scored daily or every other day for live/dead/missing worms. Scoring was carried out until all animals were dead (N= 90). Kaplan-Meier survival analysis and Mantel-Haenszel logrank tests were used to compare the controls to the experimental samples. A p-value less than 0.05 was considered as significant.
Table 2. Results from RNAi lifespan assays of candidate genes.

<table>
<thead>
<tr>
<th>GenePairs</th>
<th>Mean lifespan ± Standard Error</th>
<th>p-value</th>
<th>% control lifespan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control rrf-3</td>
<td>12.4 ± 0.3</td>
<td>100.0%</td>
<td></td>
</tr>
<tr>
<td>C50E10.4</td>
<td>10.3 ± 0.2</td>
<td>2.92e-11</td>
<td>83.1%</td>
</tr>
<tr>
<td>E01G4.2</td>
<td>11.7 ± 0.2</td>
<td>1.79e-02</td>
<td>94.4%</td>
</tr>
<tr>
<td>Y57A10B.1</td>
<td>11.1 ± 0.2</td>
<td>2.94e-04</td>
<td>89.5%</td>
</tr>
<tr>
<td>F58G1.4</td>
<td>11.6 ± 0.2</td>
<td>1.84e-02</td>
<td>93.5%</td>
</tr>
<tr>
<td>Y48E1B.5</td>
<td>12.1 ± 0.3</td>
<td>2.43e-01</td>
<td>97.6%</td>
</tr>
<tr>
<td>C50E10.4 (ii)</td>
<td>10.1 ± 0.1</td>
<td>3.53e-12</td>
<td>81.5%</td>
</tr>
<tr>
<td>ZK131.1</td>
<td>11.6 ± 0.2</td>
<td>9.75e-04</td>
<td>93.5%</td>
</tr>
<tr>
<td>F29C12.4</td>
<td>10.3 ± 0.3</td>
<td>2.83e-07</td>
<td>83.1%</td>
</tr>
<tr>
<td>Control N2</td>
<td>12.8 ± 0.3</td>
<td></td>
<td>100.0%</td>
</tr>
<tr>
<td>Control clk-3</td>
<td>13.2 ± 0.3</td>
<td>2.61e-01</td>
<td>103.1%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>GenePairs</th>
<th>Mean lifespan ± Standard Error</th>
<th>p-value</th>
<th>% control lifespan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control rrf-3</td>
<td>9.11 ± 0.2</td>
<td>100.0%</td>
<td></td>
</tr>
<tr>
<td>ZK131.7</td>
<td>8.82 ± 0.3</td>
<td>8.66e-01</td>
<td>96.8%</td>
</tr>
<tr>
<td>Y48B6A.3</td>
<td>7.74 ± 0.3</td>
<td>3.86e-03</td>
<td>85.0%</td>
</tr>
<tr>
<td>Y54G11A.4</td>
<td>9.02 ± 0.3</td>
<td>7.27e-01</td>
<td>99.0%</td>
</tr>
<tr>
<td>Y54G11A.7</td>
<td>7.93 ± 0.2</td>
<td>7.81e-06</td>
<td>87.0%</td>
</tr>
<tr>
<td>R06A4.8</td>
<td>7.85 ± 0.2</td>
<td>6.55e-06</td>
<td>86.2%</td>
</tr>
<tr>
<td>Y46G5A.31</td>
<td>7.88 ± 0.2</td>
<td>1.09e-04</td>
<td>86.5%</td>
</tr>
<tr>
<td>Y46G5A.31 (ii)</td>
<td>8.59 ± 0.2</td>
<td>1.90e-01</td>
<td>94.3%</td>
</tr>
<tr>
<td>Control N2</td>
<td>9.12 ± 0.2</td>
<td></td>
<td>100.0%</td>
</tr>
<tr>
<td>Control clk-3</td>
<td>9.98 ± 0.2</td>
<td>3.57e-03</td>
<td>109.4%</td>
</tr>
</tbody>
</table>

For the RNAi lifespan assays of candidate genes, Kaplan-Meier survival analysis and Mantel-Haenszel logrank tests were used to compare the controls to the experimental samples. The table indicates the mean lifespan of each strain with standard error, p-values and % control lifespan with respect to their respective controls. A p-value less than 0.05 was considered as significant.
Figure 7. Egg production assay. Ten 24 hour-old adult hermaphrodites were picked and placed on fresh plates for 4 hr at 20°C. All the hermaphrodites were then removed and the numbers of eggs laid were counted. ZK131.7 strains appeared to be sick; slow moving and showed much lowered egg production rate. Y48B6A.3 strains did not reach adulthood and hence showed no egg production at all.
Figure 8. Self-brood size. Ten larvae were placed onto fresh RNAi plates and incubated at 20°C until they mature and lay the first few eggs. The hermaphrodites were then transferred onto fresh RNAi plates daily to prevent overcrowding until egg-laying ceased. The progeny was counted 3 days after removal of the parents. ZK131.7 strains appeared to be sick; slow moving and showed much lowered egg self-brood size. Y48B6A.3 strains did not reach adulthood and hence showed no egg production at all.
rrf-3 mutants

clk-3 mutants

Wild-type Bristol N2

mutant clk-3 mutants

mutan

rrf-3 mutants

mutants Wild-type Bristol N2

(ii) R06A4.8 Y46G5A.31

Y46G5A.31 (ii)

Y48G11A.7

Y54G11A.4

Y48B6A.3

ZK131.7

F29C12.4

ZK131.1

C50E10.4 (ii)

Y48E1B.5

F58G1.4

(ii) E01G4.2

Y57A10B.1

C50E10.4
Figure 9. Worm growth and egg laying phenotype. ZK131.7 and Y48B6A.3 strains were smaller as compared to the wild-type Bristol N2 strain, \textit{clk-3} and \textit{rrf-3} controls. C50E10.4 (ii) strain showed sparse eggs on the feeding plate on day 3 of life. ZK131.7, Y48B6A.3, and \textit{clk-3} mutants showed no eggs on day 3 of life. All other strains showed egg production on day 3 of life, as indicated by black arrows.
Figure 10. Thrash Assay. 24 hour-old adult hermaphrodites were placed individually in a drop (20 µl) of M9 buffer. Rhythmic thrashing of their tails was scored for a minute and each animal was scored twice (N=15). Significant differences between the RNAi knock-down strains for the candidate genes compared to control *rrf-3* mutant strain; and *clk-3* mutant strain compared to wild-type Bristol strain are shown as * p<0.01 as determined by Student’s t-test.
Indicates primer position

**Figure 11. The Y48E1B.5 gene.** According to wormbase, the genomic location for Y48E1B.5 gene is chromosome II at 13.56 Mb. Primer pairs were designed to amplify promoter region with exons 1 through 4; exons 5 and 6; exon 7; exon 8 and exon 9 with 3’ UTR. Primer sequences with PCR product sizes are listed in Table 2.
Table 3. List of primer pairs for amplifying promoter, exons and 3’UTR of Y48E1B.5 gene.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Number of Bases</th>
<th>Sequence (5’→ 3’)</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y48E1B.5 Promoter, Exons 1 through 4, Forward Primer</td>
<td>20</td>
<td>TTT CCA TCG ATT TTG CCA AT</td>
<td>1228 bp</td>
</tr>
<tr>
<td>Y48E1B.5 Promoter, Exons 1 through 4, Reverse Primer</td>
<td>20</td>
<td>TTT CGC CTC GAG ACC TCT AC</td>
<td></td>
</tr>
<tr>
<td>Y48E1B.5 Exons 5 and 6, Forward Primer</td>
<td>21</td>
<td>GCA TTT TAA TGG GAT TTT TGG</td>
<td></td>
</tr>
<tr>
<td>Y48E1B.5 Exons 5 and 6, Reverse Primer</td>
<td>23</td>
<td>TTT CAG CAG TTT TCA AGT TTT CC</td>
<td>475 bp</td>
</tr>
<tr>
<td>Y48E1B.5 Exon 7, Forward Primer</td>
<td>21</td>
<td>TCA GAC ATT TTT CGC CAT TTT</td>
<td>535 bp</td>
</tr>
<tr>
<td>Y48E1B.5 Exon 7, Reverse Primer</td>
<td>21</td>
<td>CGT GGA ATT TTG TTG AAG GAA</td>
<td></td>
</tr>
<tr>
<td>Y48E1B.5 Exon 8, Forward Primer</td>
<td>20</td>
<td>GGA GAA AAT TTC CAC GGA TG</td>
<td>296 bp</td>
</tr>
<tr>
<td>Y48E1B.5 Exon 8, Reverse Primer</td>
<td>22</td>
<td>AAA ACC CAC ACA TTT CTG ACA A</td>
<td></td>
</tr>
<tr>
<td>Y48E1B.5 Exon 9 and 3’UTR, Forward Primer</td>
<td>20</td>
<td>GGG AAA AGC TGG GAA AAA CT</td>
<td></td>
</tr>
<tr>
<td>Y48E1B.5 Exon 9 and 3’UTR, Reverse Primer</td>
<td>20</td>
<td>TTC TCC CCA AAA TTC CCT CT</td>
<td>441 bp</td>
</tr>
</tbody>
</table>

Primer pairs were designed to amplify promoter region for the candidate gene Y48E1B.5 with exons 1 through 4; exons 5 and 6; exon 7; exon 8 and exon 9 with 3’ UTR. Primer sequences with number of bases and PCR product sizes are listed in the table above.
Table 4. Sequence analysis for Y48E1B.5 gene.

<table>
<thead>
<tr>
<th>Exon</th>
<th>Mutation</th>
<th>Protein changed?</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>CGA→CGG; R192→R192; CTA→AAA; L191→K191</td>
<td>Yes</td>
</tr>
<tr>
<td>5</td>
<td>None</td>
<td>No</td>
</tr>
<tr>
<td>6</td>
<td>G102→G102</td>
<td>No</td>
</tr>
<tr>
<td>7</td>
<td>None</td>
<td>No</td>
</tr>
<tr>
<td>8</td>
<td>GCT→GCA; A491→A491</td>
<td>No</td>
</tr>
<tr>
<td>9</td>
<td>None</td>
<td>No</td>
</tr>
</tbody>
</table>

PCR products amplified from *clk-3* mutant worms were sequenced individually from the forward as well as the reverse strands and compared to the genomic sequence of the gene in WormBase. Sequencing data from both strands for the PCR products were aligned with the genomic sequence for the Y48E1B.5 gene using CodonCode Aligner software. While exons 1, 2, 3, 5, 7 and 9 showed no mutations, exons 4, 6 and 8 had base changes that did not alter the amino acid, while one of the base pair changes in exon 4 had an amino acid change from L191 to K191.
IV. DISCUSSION

In summary, I describe here an effort to clone the *clk-3* gene using a simple RNAi screen to look for Clk phenotypes to enable positional cloning of the *clk-3* gene. Microarray data for *clk-3;glp-4* double mutants was screened examining genes that were in the region to which *clk-3* gene has been mapped on chromosome II for transcript levels $\geq 1.5$ fold lower in five of the six *clk-3;glp-4* samples compared to the mean of the control samples. Y48E1B.5 was identified as the best candidate for *clk-3* gene by analyzing all the candidate genes for the characteristic Clk phenotypes using the RNAi. No differences predicted to alter the Y48E1B.5 protein sequence have been identified between *clk-3* mutant and wild-type Bristol N2 strains but only four of the nine exons have been sequenced at this time. Y48E1B.5 remains a strong candidate for *clk-3* gene.

Clk phenotype assays are relatively easy to carry out under regular lab conditions, using a light microscope. Lifespan assays are reliable in determining extension vs. non-extension of lifespans. Egg production rate and self-brood size assays require precision. In spite of the Clk mutants being documented as slow moving animals compared to the wild-type strains; it was difficult to visibly estimate slow vs. fast movement of the worms under light microscope. This led to the development of thrash assay by Wong et al., 1995 wherein the rhythmic sinusoid movement of the animals on a solid medium can be replicated in a liquid medium using M9 buffer. In our hands the thrash rate for the *clk-3* mutants that was comparable to the wild-type Bristol N2 as well as the *rrf-3* mutant strains. A reason for this difference may be that sometimes bacteria from the plate are carried over into the liquid M9 buffer drop, which may cause the animal to move steadily.
at a faster rate as compared to animals completely devoid of bacteria. This is because worms show movement in the direction of bacteria, if present. Also, the thrash assay was carried out on 24 hour-old adults. ZK131.7, Y48B6A.3, and \textit{clk-3} mutants reached adulthood ~24 hours later as compared to other controls and RNAi knock-down animals. We believe that it might be useful to perform the thrash assay on day 5 for all strains when they are all mid-olds. Another assay that might be useful to perform is the pharyngeal pumping rate assay which is shown to be lowered in case of \textit{clk-1} mutants (Wong et al., 1995). As Klass, 1977 has shown that food intake is linearly related to pumping rate, pumping rate is a reliable measure of nutritional status.

Since a mutation is identified in exon 4 in Y48E1B.5 gene that causes an amino acid change from L191 to K191, it will be necessary to determine if this mutation causes the \textit{clk-3} phenotypes. One approach to confirm Y48E1B.5 as the \textit{clk-3} gene would be to carry out microinjection transformation rescue. Primers can be designed against the entire 6.9 kb gene and the PCR product can be amplified from the wild-type genomic DNA. \textit{clk-3} mutant worms can be injected with this amplified PCR product along with a \textit{myo-2}::GFP as a co-transformation marker. Transformed strains are prone to pick up secondary phenotypes that are unrelated to the primary phenotype being investigated. The transgenic strains hence would be required to be out crossed with wild-type Bristol N2 strains to eliminate sick strain problems and choose for strains that demonstrate a positive change. The progeny can be analyzed for transformation. If \textit{myo-2}::GFP expressing progeny also show a rescue of the Clk phenotype, it would confirm that Y48E1B.5 is the \textit{clk-3} gene. Per the assays that were carried out in our lab, I believe that the lifespan
assay as well as the egg laying behavior are reliable means of establishing the same. I would expect complete rescue of the mutant strains because \textit{clk-3} mutation is recessive.

If Y48E1B.5 does not prove to be the correct gene, other approaches would be required. One would be to test the other two candidates from the current batch: ZK131.7 and Y48B6A.3 by sequencing their exons in a manner similar to that of Y48E1B.5 as described above. Alternatively, more candidate genes with down-regulated expression can be identified from the microarray expression data using less stringent filtering criteria. It is possible that the expression of \textit{clk-3} gene changes with age. So, in the adult \textit{clk-3; glp-4} animals used for microarrays, the \textit{clk-3} gene might have a reduced expression level. Another possibility is that molecular mutation in \textit{clk-3} may reduce its’ transcript levels, but to a lesser extent as compared to other down-regulated genes in \textit{clk-3} worms. There are several ways to loosen criteria for selecting candidate genes from the microarray data. Genes that have transcript levels $\geq 1.5$ fold lower in four of the six \textit{clk-3;glp-4} samples compared to the mean of the control samples could be examined for Clk phenotypes in a similar fashion. Or genes consistently down in all the samples but with a lower fold-change could be selected. Identifying candidate genes would also be helped by reducing the number of possible genes by finer mapping of \textit{clk-3}.

To further refine the position of the \textit{clk-3} mutation on chromosome II, genetic markers known as Single Nucleotide Polymorphisms (SNPs) can also be used in conjunction with interval mapping. SNPs that alter a restriction site, also known as snip-SNPs are used in this approach. Primers can be designed using the snip-SNPs genomic
sequence, amplified by PCR, digested by the specific restriction enzyme and ascertained by means of gel electrophoresis [Davis et al., 2005]. SNPs occur at a frequency of 1 in 1000 base pairs between wild-type Bristol N2 and the Hawaiian CB4856 strain, and convenient snip-SNPs are common enough to allow mapping down to a region of a few genes in most cases. These experiments are underway and will provide additional support for Y48E1B.5 being clk-3 or exclude it.

Clk phenotypes show a general reduction in the rate of various temporal processes as well as a mean increase in lifespan, however, there is no decrease in metabolic rates in Clk mutants as suggested by oxygen consumption, heat output and ATP levels. [Braeckman et al., 2002]. These proteins have also been found to be localized in different compartments of a cell. CLK-1 is a nuclear gene encoded protein found in the mitochondria, while CLK-2 is distributed in the cytoplasm. GRO-1 is also a nuclear gene encoded protein localized in the mitochondria, cytoplasm or nucleolus, depending on the presence or absence of the mitochondrial targeting sequence. It will be interesting to uncover the cellular localization of the CLK-3 protein as well as its’ expression pattern, whether similar to other Clk genes or different. This can be carried out by examining the subcellular distribution of the CLK-3::GFP protein.

Till date it is unclear how these proteins may act to contribute to the changes that occur during aging. Hence, studies aimed at determining the cellular function and unraveling the metabolic effects of CLK-3 will be helpful. It will be interesting to find out whether like CLK-1 and GRO-1, it is mitochondria localized protein involved in the
respiratory process or scavenging free radicals, or if it is a cytoplasmic protein with a regulatory effect in the cell like CLK-2. This information will be useful in obtaining a better understanding of the biochemical processes that the four Clk genes may act in to coordinate the maintenance of the physiological clock of the nematode and control its senescence.
REFERENCES


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