Suppression of the ELO-2 FA Elongation Activity Results in Alterations of the Fatty Acid Composition and Multiple Physiological Defects, Including Abnormal Ultradian Rhythms, in *Caenorhabditis elegans*

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ABSTRACT

While the general steps of fatty acid (FA) biosynthesis are well understood, the individual enzymes involved in the elongation of long chain saturated and polyunsaturated FA (PUFA) are largely unknown. Recent research indicates that these enzymes might be of considerable physiological importance for human health. We use *Caenorhabditis elegans* to study FA elongation activities and associated abnormal phenotypes. In this article we report that the predicted *C. elegans* F11E6.5/ELO-2 is a functional enzyme with the FA elongation activity. It is responsible for the elongation of palmitic acid and is involved in PUFA biosynthesis. RNAi-mediated suppression of ELO-2 causes an accumulation of palmitate and an associated decrease in the PUFA fraction in triacylglycerides and phospholipid classes. This imbalance in the FA composition results in multiple phenotypic defects such as slow growth, small body size, reproductive defects, and changes in rhythmic behavior. ELO-2 cooperates with the previously reported ELO-1 in 20-carbon PUFA production, and at least one of the enzymes must function to provide normal growth and development in *C. elegans*. The presented data indicate that suppression of a single enzyme of the FA elongation machinery is enough to affect various organs and systems in worms. This effect resembles syndromic disorders in humans.

FATTY acids (FAs) are exceptionally versatile compounds. Most cellular FAs exist as components of various lipids and proteins, although a small quantity is present in a free form. In the form of triacylglycerides (TAG) FAs are involved in energy storage, and as components of phospholipids (PL) they constitute the principal structural material of cells.

FAs have numerous specialized functions, including protein activation (Chen and Manning 2001), signal transduction (Chawla *et al.* 2001; Chinetti *et al.* 2001), and regulation of the inflammatory and immune responses through FA derivatives such as sphingolipids, lysophospholipids, and eicosanoids (Funk 2001; Hannun *et al.* 2001; Hla *et al.* 2001). Moreover, the composition of FAs incorporated into lipids plays an important role. Different combinations of FAs may activate different pathways (Carricaburu and Fournier 2001) and isoenzymes (Madani *et al.* 2001) and determine environmental adaptation (Logue *et al.* 2000) and sexual dimorphism (Hida *et al.* 1998). These examples suggest that the absolute as well as relative amounts of FAs in cells might be under strict genetic control.

Changes in FA compositions accompany various human disorders including diabetes (Bhathena 2000), hypertension (Horrobin 1995), Refsum syndrome (Jansen *et al.* 1997, 2000), atopic disease (Duchen and Bjorksten 2001), and cancer (Agatha *et al.* 2001). Abnormal FA composition was also detected in a number of psychological conditions such as mood disorders (Freeman 2000), depression (Peet *et al.* 1998), and schizophrenia (Komoroski *et al.* 2001).

Our interest in studying FA biosynthesis and homeostasis was induced by our previous genetic work on human macular dystrophy (MD; Kniazeva *et al.* 1999a,b, 2000). We have found that one dominant form of MD is caused by a mutation in ELOVL4, an FA elongation gene (Zhang *et al.* 2001). While the biochemical steps required for ELOVL4 activity are not yet identified, our findings indicate that the disruption of a single enzyme in the FA elongation system can have detrimental effects on the normal functioning of an organism.

Little is known about the genetic and physiological importance of the individual enzymes involved in FA biosynthesis. Several animal enzymes with the FA elongation activities have been biochemically characterized (Beaudoin *et al.* 2000; Leonard *et al.* 2000; Moon *et al.* 2001; Inagaki *et al.* 2002), but none have been implicated in diseases or mutant phenotype. Here we use genetic manipulations on *Caenorhabditis elegans* to address questions about the physiological roles played by FAs and enzymes involved in FA elongation.

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Eight protein sequences homologous to the human ELOVL4 are in the *C. elegans* database. In this article, we report that one of them is a functional protein that we have designated ELO-2. ELO-2 utilizes C16:0 as a substrate in the first step of long chain FA elongation. Directly or indirectly, ELO-2 regulates 18-carbon PUFA elongation and, with condensing enzyme ELO-1 (Beaudoin *et al.* 2000), ELO-2 is required for 20-carbon PUFA biosynthesis. We also show that a loss of the ELO-2 function changes the phenotypes of affected animals. Unlike the *elo-1* knockout, which appears wild type, suppression of *elo-2* causes multiple defects on an organismic level, suggesting a key physiological role for ELO-2. (Note that ELO-2 and *elo-2* stand for the protein and the gene names, respectively.)

MATERIALS AND METHODS

Worm strains: Strains used in this study include Bristol N2, *fat-2(wa17)*, and *fat-3(wa22)* (Watts and Browse 2002), and *elo-1(gk48)* (*C. elegans* Reverse Genetics Core Facility at the University of British Columbia). All worms were cultivated on an HT115 *Escherichia coli* strain transformed with an empty pPD129.36 vector (gift of A. Fire) on isopropyl thiogalactoside (IPTG)/ampicillin agar plates (Kamath *et al.* 2001). N2 was used as a control in all experiments.

Vectors and constructs: The *elo-2::GFP* reporter used in the expression pattern analysis was prepared as follows: A 3.794-kb genomic fragment containing 2.575 kb upstream of the first predicted codon and part of the coding sequence truncated at the 3′ end (this cuts out the presumable ER-retention signal of the protein) was PCR amplified from an N2 worm with the following primers: F, 5′-*Sph*I-AACGGTCCATATAAACTGGAA CATTTTGC-3′, and R, 5′-*Sma*I-TCGAACAAATAACTAGTGT ACATAGGTCCTCC-3′. The fragment was cloned in frame with the green fluorescent protein (GFP) gene into the pPD95.77 vector (gift from A. Fire).

A total of 10 ng/μl of this plasmid DNA was injected into the gonad of N2 worms. Transgenic animals were selected and maintained by monitoring green fluorescence under a dissecting scope.

The *elo-2* overexpression construct was made by cloning a genomic fragment, generated with F, 5′-*Sph*I-AACGGTCCA TATAAACTGGAACATTTTGC-3′, and R, 5′-*Sma*I-TTCACA ATGTTTATCTACTCCTGC-3′ primers, into the pPD95.77 vector (no GFP fusion). The fragment contains 2.575 kb upstream of the first predicted ATG codon and predicted coding sequence, including the stop codon. A total of 10 ng/μl of the plasmid DNA was coinjected with the gut-specific, selective marker KQT1::GFP (M. Kniazeva, unpublished data) into the N2 worms.

The RNAi feeding vectors were based on pPD129.36 (gift of A. Fire). RNAi primers were as follows:

F11E6.5: F, atggcagcagcacaaacaagtccag; R, actgatgtacatgagtcc tccgatcga
F56H11.4: F, atggctcagcatccgctcgttcaac; R, cgacaattcctttgttggc aatggt
F56H11.3: F, atgtatttgaattatttcgcgacgg; R, tcaaagcaaatgtagagt gtagga
D2024.3: F, atggcaaaatacgactacaatccga; R, cagacgaagagtggtgtg tccagtt
F41H10.7: F, atgtcatcggacgatcgtggcgtga; R, cagtgcatgaaaataa gtggtctct
F41H10.8: F, atgccacagggagaagtctca; R, ccacggctgaagaactcgtg ga
C40H1.4: F, atggagcttgccgagttctgga; R, cgcaatatatcacgcctgggc
Y53F4B.2: F, atgtcggccgaagtgtccgaacgatt; R, cagccaggcagatcga atcgatg

RNAi experiments: RNAi experiments were performed by feeding N2 worms the HT115 *E. coli* strain transformed with the RNAi vector or the control vector pPD129.36. The RNAi feeding conditions were as described in protocol 1 (optimal; Kamath *et al.* 2001). The efficiency of RNA interference was monitored by observing a quenching green fluorescence in worms carrying the *elo-2:GFP* reporter.

Evaluation of *elo-2(RNAi)* phenotypes: *elo-2(RNAi)* animals were maintained on the RNAi plates at 20°. F_2-F_3 generations were used for comparative estimations of the growth rates and the number of eggs. Adult N2 and *elo-2(RNAi)* worms were left to lay eggs for 2–3 hr and then were removed from the plates. These progeny were then followed up until they started to lay eggs themselves. Young adults of each type were scored for the number of eggs in their uterus.

Rhythmic behavior analysis: In each experiment, at least 10 adult hermaphrodites were scored for at least 10 posterior body wall muscle contraction (pBoc) cycles. All counts were carried out at room temperature under the dissecting scope. These data were presented as an average of intervals with standard deviations or as a relation of the intervals' frequencies to the length of the intervals (in seconds). We scored the F_2-F_3 generations of the supplemented and RNAi-fed worms.

Fatty acid supplements: Palmitic and stearic acid sodium salts (Supelco, Bellefonte, PA) were dissolved in 1% NP40 at 80° and added to agar plates containing IPTG and ampicillin (Kamath *et al.* 2001) so that the final concentration was 0.5 mm. The progeny of plated adult animals were scored for pBoc and collected for FA composition analysis. An N2 (Bristol) strain growing on 1% NP40 was used as a control. C20:0 and C22:0 were dissolved in ethanol and 300 μl of 0.5 mm solution was dropped onto agar plates. After the ethanol evaporation a bacterial lawn was formed as described above.

Gas chromatography analysis: A mixed population of well-fed worms were washed off plates with water, rinsed three or four times, and, after complete water aspiration, were frozen at −80°. Fatty acid methyl esters and lipid extraction were performed as described (Miquel and Browse 1992). Gas chromatography (GC) was performed on the HP6890N (Agilent) equipped with a DB-23 column (30 m × 250 μm × 0.25 μm). A constant pressure of 17.65 psi was applied to the column. The oven temperature at the time of injection was 100°, and then it was increased to 180° at a rate of 10°/min, where it was held for 5 min and then increased to 240° at a rate of 5°/min. The flame ionization detector temperature was 300°. FA species were identified by comparison with FA standards (C4-C24, C8-C24, GLC10-GLC90, C20:1-C20:5, and individual FA standards, Supelco) and mass spectroscopy analysis. For each sample, peak areas of all major peaks (C14:0, C14:1, C15:0, C16:0, C16:1, C17ISO, C17:0, C18DMA, C17Delta, C18:0, C18:1n9, C18:1n7, C18:2n3, C19:0, C18:3n3, C19Delta, C18:3n6, C20:0, C20:2n6, C20:3n3, C20:4n3, C20:4n6, C20:5n3) were summed, and for each compound the percentage of the total was calculated. Each experiment was carried out at least in triplicate. Average values and standard deviations were then calculated for each of the compounds in the experiment.

Lipid separation: At least 100 mg of worms were killed rapidly by immersion in liquid nitrogen, transferred to a screw-capped glass centrifuge tube with 5 ml of ice-cold chloroform/methanol/formic acid (10:10:1, by volume), vortexed for 2 min, and stored 4 hr to overnight at −20°. A total of 2.2 ml of H_3PO_4,

1 M KCl (HAJRA 1974) was added and lipids were recovered in the chloroform phase, dried under N_2, and dissolved in 0.2 ml chloroform with 0.01% butylated hydroxytoluene. Triacylglyceride and phospholipid fractions were purified from the extracts by one-dimensional thin layer chromatography on Silica H plates. The plates were activated for 75 min at 110° before loading lipid extracts and standards. Plates were developed in hexane-diethylether-acetic acid (80:20:2, by volume). Lipids were located by brief staining with I_2. To determine the fatty acid composition and the relative amounts of individual lipids, the silica gel from each lipid spot was transferred to a screw-capped tube, 5 μg of 15:0 fatty acid was added as an internal standard, and fatty acid methyl esters were prepared and analyzed as described above.

RESULTS

A family of the FA elongation enzymes in *C. elegans*: Eight predicted *C. elegans* protein sequences were identified by a BLAST search as members of the GNS1/SUR4 family of FA elongation enzymes. Interestingly, no significant homology was detected between the corresponding genes when a BLASTN search was performed using either genomic or cDNA sequences. This is especially striking because two pairs of the predicted genes, F56H11.3 and F56H11.4 as well as F41H10.7 and F41H10.8, have paired chromosomal locations, suggesting an ancestral duplication event. If there was a common phylogenic root, these genes no longer share significant structural similarity. This suggests that the conserved blocks seen on the protein level indicate functionally essential domains in the FA elongation enzymes.

We anticipated that the loss of an FA elongation activity might lead to the accumulation of its unused substrate. This could then be detected through GC analysis and provide information on the biochemical activity of the corresponding proteins. We performed RNAi-mediated suppression on each of the genes and evaluated the resulting FA composition and other phenotypes. No RNAi-associated phenotypes were detected for the F56H11.3, F41H10.8, D2024.3, Y53F4B.2, and C40H1.4 genes. The suppression of F56H11.4 resulted in an abnormal FA composition with no other apparent phenotypic changes. The suppression of the F11E6.5 and F41H10.7 genes caused significant alterations in FA composition and multiple growth defects. The F56H11.4 and F11E6.5 loss-of-function phenotypes are discussed in detail in this article.

It has been previously shown by expression in yeast that F56H11.4 (*elo-1*) encodes a condensing enzyme involved in the elongation of the n6 (and to some extent the n3) series C18 PUFA as well as in the elongation of palmitoleic acid (BEAUDOIN *et al.* 2000). Being interested in the evaluation of FA elongation activity in live worms, we analyzed the FA composition in the *elo-1* deletion strain, *elo-1(gk48)*, obtained from the genome knockout consortium. This strain contains a 524-bp deletion, which removes 37 bp of the 5′-untranslated region, the first exon, and two-thirds of the first intron. It eliminates 49 N-terminal amino acids of the encoded protein and likely affects proper splicing of the gene. It seems unlikely that another *elo*-gene located on F56H11.3 cosmid is affected by the deletion and contributes to the *elo-1(gk48)* phenotype since it is not in an operon and is ~4 kb from F56H11.4. As mentioned earlier, F56H11.3 (RNAi) does not change the FA composition and no expressed sequence tags are reported for this gene, suggesting that it may not be actively transcribed.

The *elo-1(gk48)* worms have an abnormal FA composition that is identical to the above-mentioned F56H11.4 (RNAi) animals and to the *elo-1(wa7)* mutant, which lacks the final 50 C-terminal amino acids (WATTS and BROWSE 2002). It is characterized by a significant decrease in the n6 and to a lesser extent in the n3 series of 20-carbon PUFA.

Interestingly, despite significant alterations in FA composition, all *elo-1* mutants display no obvious phenotypic changes, indicating a tolerance to the low C20 PUFA amounts.

In the rest of this article, we describe our results obtained for the F11E6.5 protein, designated ELO-2. ELO-2 shares a significant homology with all characterized condensing enzymes. It is 39% identical to the *C. elegans* long chain polyunsaturated fatty acid elongation enzyme ELO-1 (GenBank accession no. AF244356; BEAUDOIN *et al.* 2000), 38% identical to the murine long chain elongase LCE (MOON *et al.* 2001), and 26% identical to the human very long chain elongase HELO1 (LEONARD *et al.* 2000) (Figure 1).

RNAi-mediated suppression of ELO-2, a predicted *C. elegans* enzyme with FA elongation activity, results in significant changes in FA composition in living worms: GC analysis of the total lipids revealed that the RNAi-mediated suppression of *elo-2* resulted in alterations in FA composition. The major change was a sixfold increase in palmitic acid (C16:0) as compared to the wild-type N2 Bristol strain (Figure 2). Apparently, palmitate could not be properly metabolized in the absence of ELO-2 activity. The large C16:0 increase suggests that palmitate is likely to be a main substrate for ELO-2. This increase, however, is not accompanied by a proportional decrease in C18:0, a product of the C16:0 elongation. Several factors may contribute to the presence of stearic acid in *elo-2(RNAi)* worms. First, the bacterial food may be a sufficient source of stearate even in the absence of C16:0 to C18:0 elongation activity. Indeed, HT115 *E. coli* strain used to feed worms in RNAi experiments contains C18:0 in addition to C16:0, C17D, C14:0, C19D, C16:1 n7, and C18:1 n7 FA species. Second, it is possible that the ELO-2 suppression by RNAi is incomplete. Third, there could be a functional redundancy in this elongation process.

In addition to changes in the saturated FA fraction, two- and threefold decreases were observed in the C18:1 n9 and C20:2 n6 fractions, respectively. This implies that a loss of the ELO-2 function may also affect biosynthesis of mono- and polyunsaturated FAs.

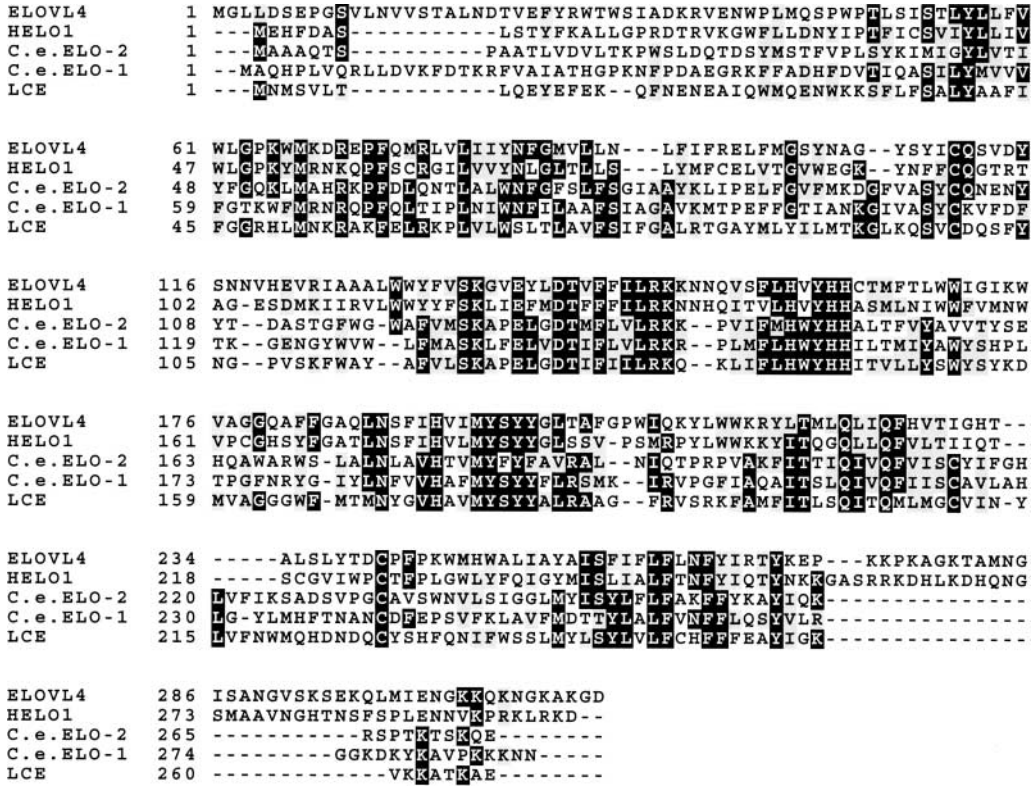

FIGURE 1.—Multiple alignment for the selected proteins of the GNS1/SUR4 family obtained with the ClustalW, v1.8 and BOXSHADE programs: ELO-2 (T20786) homology to the human ELOVL4 (AAG47669), possibly involved in the very long chain FA elongation (ZHANG *et al.* 2001), and condensing enzymes; ELO-1 (AF244356; BEAUDOIN *et al.* 2000); LCE (AAL14239), the murine palmitic acid elongase (MOON *et al.* 2001); and HELO1 (NP_068586), human homolog of the yeast long chain polyunsaturated fatty acid elongation enzyme 2 (T20786; LEONARD *et al.* 2000). The ELO-1 and ELO-2 protein sequences derived from the translation of corresponding cDNAs predicted by Genefinder.

GC and statistical analysis showed a similar increase in palmitate in both triacylglycerols (TAG) and phospholipids (PL) (Figure 3). This was accompanied by significant changes in the relative amounts of C16:1 n7, C18:1 n9, C18:2 n6, C20:4 n6, and C20:5 n3 FA species, indicating an imbalance in the FA composition. The data predict that the disruption of ELO-2 could result in multiple metabolic and physiological defects involving energy storage and membrane properties. As we discuss below, *elo-2(RNAi)* animals display various abnormalities.

Disruption of both ELO-2 and ELO-1 functions blocks the synthesis of 20-carbon PUFA and causes severe phenotypes: To further address ELO-2's possible involvement in PUFA biosynthesis, we asked if a suppression *elo-2* would further decrease the amounts of PUFA in the *elo-1(gk48)* strain. For this experiment *elo-1(gk48)* worms were grown on *elo-2(RNAi)* plates. The following GC analysis of the total lipids revealed that the 20-carbon PUFA fractions are nearly eliminated in *elo-1(gk48);elo-2(RNAi)* animals (Figure 4). While a redundancy of the ELO-1 and ELO-2 functions would explain the observed shortage of C20 PUFA in these double "mutants," in the absence of biochemical proofs, other possibilities may be considered. As it was shown in another system and is discussed later, large amounts of palmitate could influence lipogenesis (DOBROSOTSKAYA *et al.* 2002; SEEGMILLER *et al.* 2002) possibly reducing FA biosynthesis. This would represent an indirect effect of the ELO-2 suppression on FA composition. Another possibility is that a decrease in C20:2 n6, resulting from the ELO-2 suppression, could be critical for C20 PUFA biosynthesis on the *elo-1(gk48)* background. In any case, the data suggest that the ELO-2 function is important for PUFA biosynthesis when ELO-1 is suppressed.

The double suppression of the ELO-1 and ELO-2 activities results in the very sick appearance of the affected worms. The worms in the first generation, F_1, have small scrawny bodies and various structural abnormalities (Figure 4). A brood size of P_0 *elo-1(gk48);elo-2(RNAi)* is reduced to 30–40 worms. While early F_1 animals lay eggs that hatch, the late F_1 worms are sterile because of severe gonad defects. A small number of produced L1 of the second generation do not develop past this larval stage and eventually die. Therefore, the double phenotype drastically differs from both the *elo-1(gk48)*, which looks wild type, and the *elo-2(RNAi)*, which has mild phenotype (discussed in detail below). Interestingly, the double mutants also differ from *fat-3(wa22)*, a slow-growing but viable and fertile mutant characterized by a lack of C20 PUFA (WATTS and BROWSE 2002). The severity of the *elo-1(gk48);elo-2(RNAi)* phenotype as compared to *fat-3(wa22)* implies that other factors are at work in addition to a shortage in C20 PUFA. For example, the *elo-2(RNAi)*-derived accumulation of saturated FA, C16:0, may be poisonous in the low PUFA background.

Taken together, the data suggest that the ELO-1 and ELO-2 combined function is essential, and at least one of the enzymes must be active to provide normal growth and development in *C. elegans*.

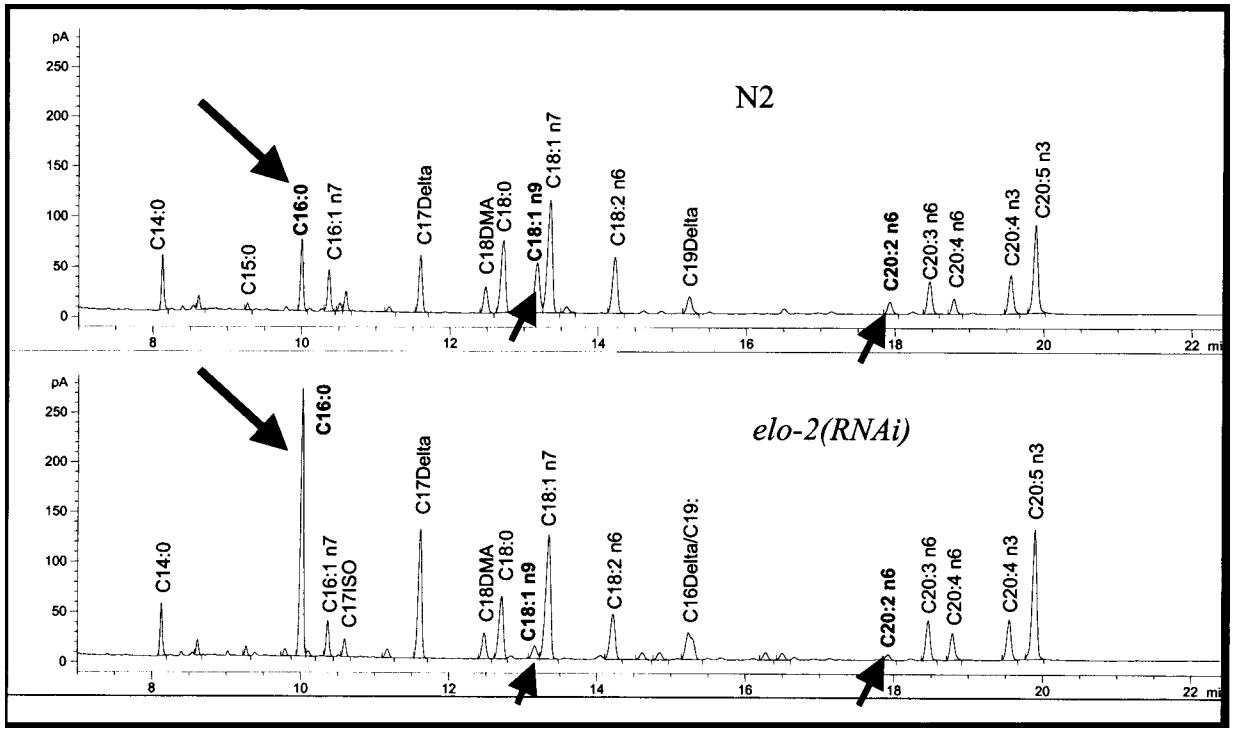

FIGURE 2.—FA composition in *elo-2(RNAi)* is compared to the wild-type N2 Bristol animals. GC profiles: *x*-axis, retention times; *y*-axis, intensity of signals. Long arrow points to the increased peak corresponding to C16:0 in *elo-2(RNAi)*. Short arrows point to the decrease in oleic and auricolic FAs. The observation was supported by statistical analysis (average and standard deviations) using >10 samples of each kind taken at different days and different temperatures.

***elo-2* is predominantly expressed in intestinal cells in *C. elegans*:** The ELO-2::GFP reporter driven by the putative *elo-2* promoter (a sequence ~3 kb upstream of the first ATG codon) was used to evaluate the tissue expression pattern of *elo-2*. The open reading frame of the construct encodes ELO-2 truncated at the C terminus and fused with GFP. While being detected in various tissues and parts of the body, including the ventral cord, pharyngeal muscles, uterus, and the tail, the reporter is most strongly expressed in intestinal cells (Figure 5). In the intestinal cells, GFP fluorescence could be detected from the late embryonic stage onward. In very old (30 days) and even dead worms the intestinal fluorescence stays bright, suggesting that the ELO-2::GFP protein is stable. The gut cells in *C. elegans* are responsible for digestion, energy storage, and the distribution of nutrients, all functions that are agreeable with active FA biosynthesis.

***elo-2(RNAi)* results in multiple phenotypic changes:** The *elo-2(RNAi)* worms develop 1.5 times slower as measured by the time required for a laid egg to transform into an egg-laying adult, although its life span is not affected. A grown adult hermaphrodite is ~20% smaller than an adult N2. In addition, *elo-2(RNAi)* worms have a prominent pale coloring to their intestine, probably due to some different refractive property (Figure 6).

Although the RNAi-treated animals are vital and fertile, they have apparent reproductive defects, which result in a smaller number of progeny. A brood of one *elo-2(RNAi)* hermaphrodite consists of 30 ± 10 worms *vs.* 188 ± 10 in the wild type (animals were scored on the fourth day of plating synchronized L4; progeny of 10 animals of each type were counted; ± represents standard error of mean). There is no increase in the number of dead eggs or in the frequency of early embryonic lethality as compared to the wild type; however, there is a difference in the average number of eggs in the uterus of young adults: 3.2 in *elo-2(RNAi) vs.* 8.6 in the wild type (30 synchronized worms of each type were scored).

***elo-2(RNAi)*-treated worms change their rhythmic behavior:** To test if FA composition affects physiological rhythms, we examined the intervals of pBoc, components of the defecation cycle (LIU and THOMAS 1994). These rhythmic contractions represent an ultradian cycle in worms that repeats every 47–50 sec and is controlled through inositol trisphosphate receptors in the posterior gut cells (DAL SANTO *et al.* 1999). We found that RNAi-mediated suppression of ELO-2 activity results in short, but still orderly, pBoc intervals (Table 1).

To test the effect of ELO-2 overexpression, we injected worms with an *elo-2* genomic fragment (3 kb of putative promoter and a coding region truncated after the stop codon) cloned into the pPD95.77 vector (gift of A. Fire). Since the *elo-2* promoter is functional and strong in the ELO-2::GFP reporter mentioned above,

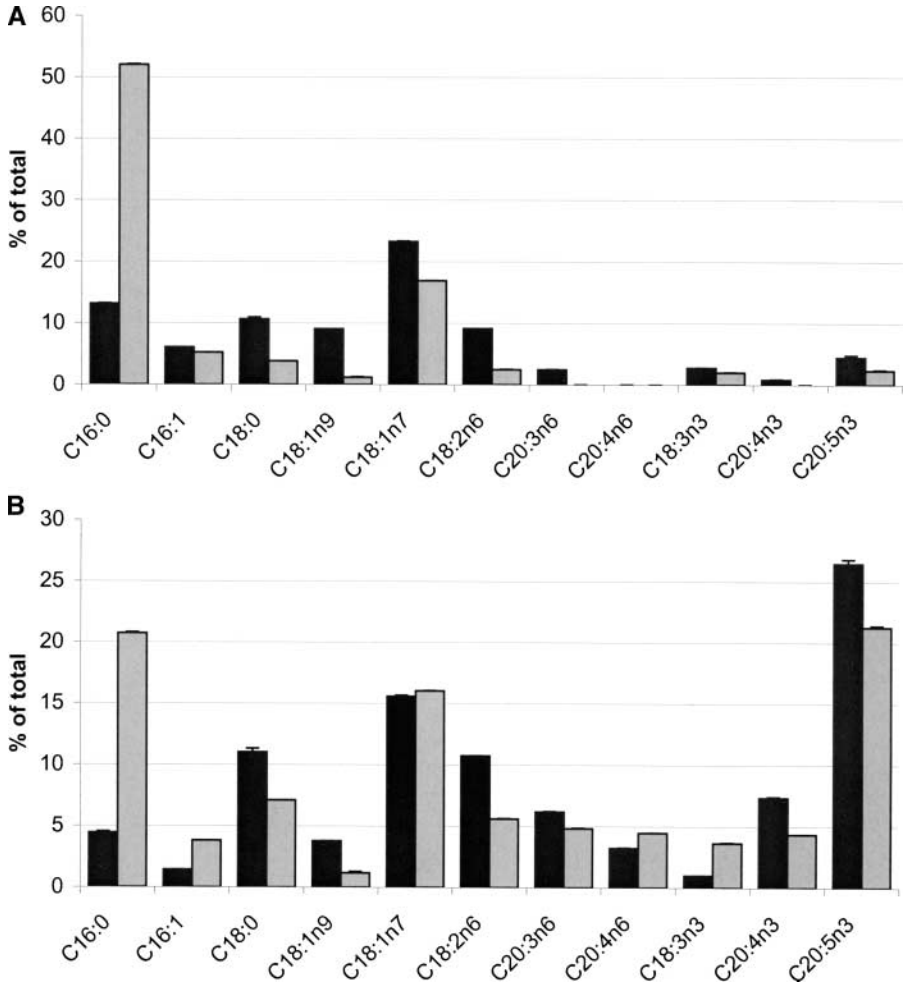

FIGURE 3.—FA compositions determined for two lipid classes (A) triacylglycerides (TAG) and (B) phospholipids (PL). The bars show a portion of the individual FA as a percentage of the total. Shaded bars represent *elo-2(RNAi)*; solid bars represent N2. Results are based on data done in triplicates.

we expected it to be efficient in the production of ELO-2. To trace the *elo-2* expression, a gut-specific GFP reporter vector, *ku142Ex[kqt-1::GFP]*, was coinjected along with *ku141Ex[elo-2(+)]*. Thus, the extrachromosomal array contained two genes coding for ELO-2 and GFP. The promoters of both genes are active in intestinal cells; therefore the presence of GFP indicates a presence of ELO-2 in the same cell. The worms injected with the GFP reporter alone were used as a control. When *kuEx141[elo-2 (+)];kuEx142[kqt1::GFP]* was introduced into the worms, we noticed that in 35 analyzed animals 21 had long and 14 had short pBoc intervals. The array was not integrated and we suspected that the inconsistency may result from mosaic distribution of the array. Since the most posterior gut cells are pacemaker cells for pBoc rhythms (DAL SANTO *et al.* 1999), we specifically examined worms in which *ex[elo-2 (+);kqt1::GFP]* (green fluorescence) had been missing in the posterior gut cells but present in the other intestinal cells. In 19 out of 20 such mosaic worms, the average pBoc interval was 45.5 ± 3.6 sec, while in the worms with the posterior gut cell expression, the average pBoc interval was 67.3 ± 12.2 sec where ± is the standard deviation (Table 1). The expression of the GFP reporter alone in the posterior cells did not cause changes in pBoc frequency as compared to N2. These data indicate a correlation between *elo-2* dosage, or ELO-2 activity, in the gut pacemaker cells and pBoc rhythmicity.

The increased amount of palmitate, but not the decrease in PUFA, seems to be relevant to the observed acceleration of pBoc rhythms in *elo-2(RNAi)* worms: We asked if the reduced relative amounts of PUFA observed in *elo-2(RNAi)* animals contributes to the pBoc phenotype. Changes in PUFA composition may cause differences in lipid-protein interactions in cell membranes and affect various physiological functions (JUMP 2002). To examine if amounts of PUFA correlate with the frequency of pBoc, we analyzed mutant *C. elegans* strains with altered PUFA biosynthesis: *fat-2(wa17)*, characterized by a reduced amount of total PUFA, and *fat-3(wa22)* and *elo-1(wa7)* mutants, characterized by reduced C20 PUFA (BEAUDOIN *et al.* 2000; WATTS and BROWSE 2002). In contrast to *elo-2(RNAi)*, the pBoc intervals in all these animals were long and irregular (Table 1). A significant shortening of the intervals was observed in *fat-2(wa17); elo-2(RNAi)* and *fat-3(wa22);elo-2(RNAi)* mutants. This suggests that not decrease in PUFA but possibly increase in saturated FAs influences the pBoc phenotype.

Since the accumulation of palmitate is the most remarkable characteristic of the FA composition in *elo-*

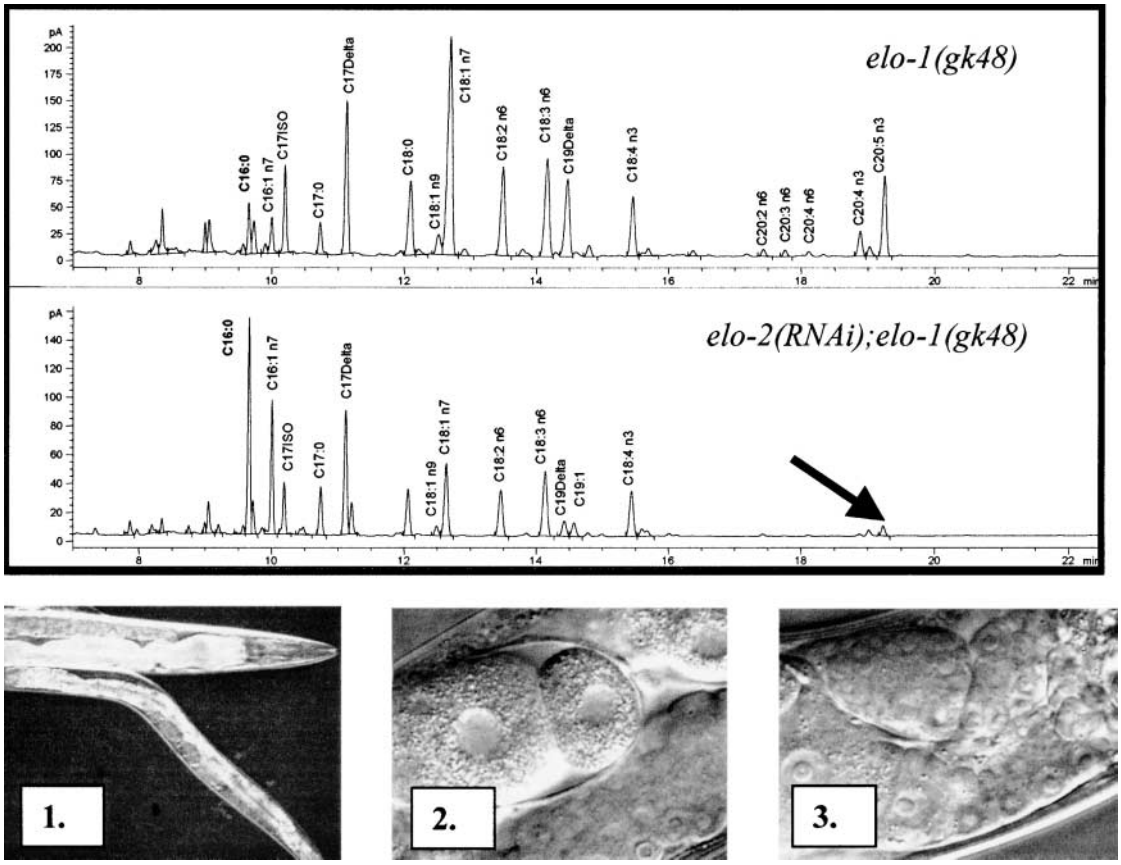

FIGURE 4.—C20 PUFA is almost eliminated in worms after double suppression of *elo-1* and *elo-2*. Gas chromatogram shows a typical FA composition in an *elo-1(gk48)* strain with decreased amounts of the C20 PUFA n6 series and a corresponding increase in C18:4 n6. In *elo-1(gk48);elo-2(RNAi)* animals C20 PUFA is nearly eliminated. Arrow points to the reduced peak corresponding to C20:5 n3. This observation was supported by additional analysis of 10 independent samples of each kind (average values and standard deviations were calculated, data not shown). A lack of C20 PUFA is accompanied by various structural and morphological changes in live worms. *elo-1(gk48);elo-2(RNAi)* adults are small compared to N2 (1) and thin (2). Gonads are often disordered and squeezed (3). Magnifications for 1, 2, and 3 are ×10, ×100, and ×100, respectively.

2(RNAi) animals, we tested if palmitate alone could be responsible for the pBoc interval shortening. In this experiment, wild-type worms were grown on plates supplemented with palmitate. The pBoc intervals were compared with those in wild-type control worms grown on plates with 1% NP40 without FA supplements. The results shown in Figure 7 indicate that supplementation with palmitate increases the pBoc frequency (*t*-test = 7.36452E-48, two-tailed distribution, unequal variance analysis). To test if the observed pBoc interval shortening is associated with a general increase in lipid saturation, we performed a similar experiment using stearate (C18:0) as a supplement and did not detect significant changes as compared to the controls (Figure 7). Therefore, the increased amount of palmitate, but not the decrease in PUFA, seems to be relevant to the observed acceleration of pBoc rhythms in *elo-2(RNAi)* worms.

Long chain saturated FAs C20:0 and C22:0 are unlikely to be involved in the *elo-2(RNAi)* phenotype: We considered the possibility that ELO-2 may also be engaged in the elongation of other saturated FAs, such as C20:0 and C22:0, whose amounts are normally low and therefore difficult to detect in total FAs through GC. Very long chain FAs are important components of sphingolipids and, through sphingolipid signaling, may contribute to the pBoc regulation, in particular. We tested the hypothesis that a possible decrease in C20:0 and C22:0 in *elo-2(RNAi)* may have caused the observed acceleration of pBoc intervals. To do this, we fed *elo-2(RNAi)* animals with each of these FAs and then counted pBoc intervals and evaluated other aspects of the phenotype. In these experiments we did not detect any differences between *elo-2(RNAi)* and *elo-2(RNAi)* worms fed with arachidic and behenic FAs in terms of pBoc rhythms, number of progeny, or growth rate. We also did not see any changes in the phenotypes of N2 worms supplemented with these same FAs. Therefore it is unlikely that the amounts of C20:0 or C22:0 mediate the *elo-2(RNAi)* phenotype.

DISCUSSION

In at least five elongation and nine desaturation reactions, *C. elegans* is able to synthesize straight saturated, and mono- and polyunsaturated FAs (Figure 8). In con-

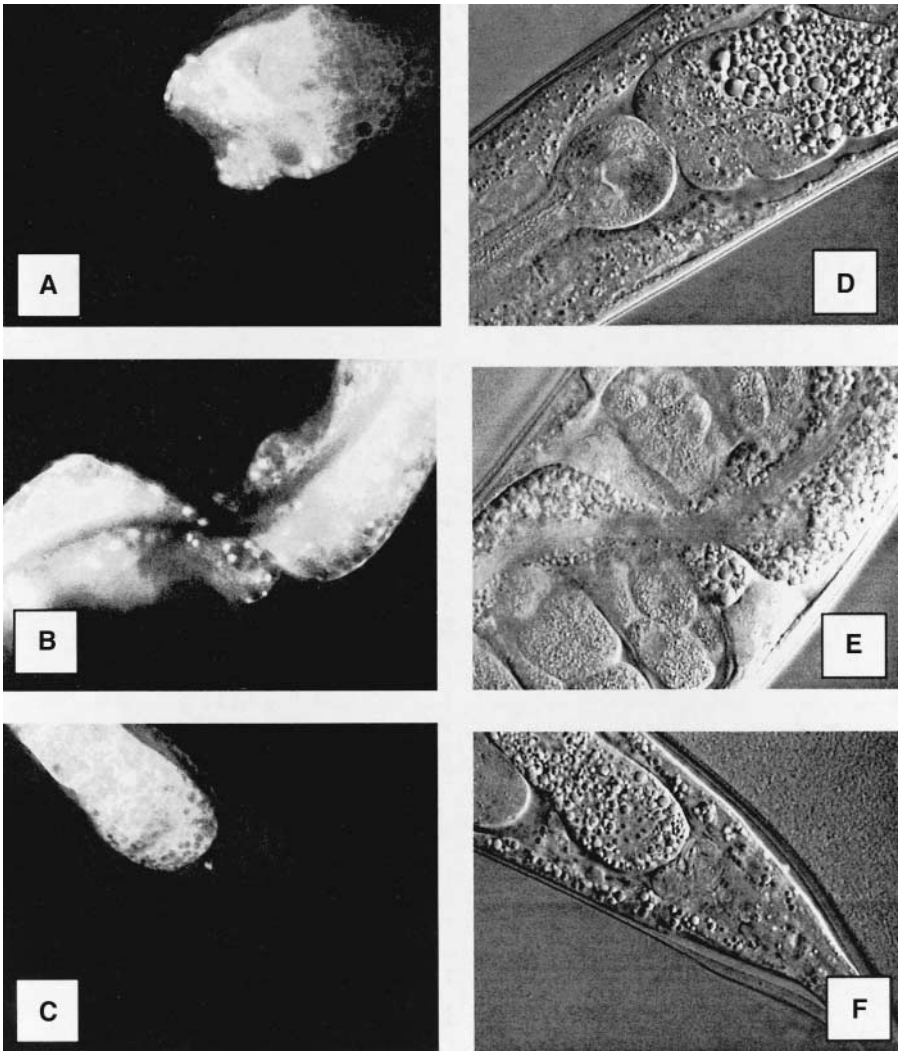

FIGURE 5.—*elo-2* is expressed in the gut cells as determined by ELO-2::GFP translational fusion. Micrographs show different parts of intestine under UV light (A–C) and matching Nomarski images (D–F). Magnification, ×100.

trast to the well-characterized FA desaturation system (WATTS and BROWSE 1999, 2002; PEYOU-NDI *et al.* 2000), only one enzyme involved in the long chain FA elongation in worms, ELO-1, has been described (BEAUDOIN *et al.* 2000; WATTS and BROWSE 2002).

Being interested in the analysis of the physiological changes that could arise from mutations in FA elongation genes, we performed RNAi-mediated suppression of eight predicted *C. elegans* proteins recognized as members of the GNS1/SUR4 family of the long chain fatty acid elongation enzymes. This experiment resulted in the identification of two functional enzymes, F11E6.5 and F41H10.7, whose loss of function leads to prominent phenotypic changes. In this article we report the biochemical and physiological characteristics of the *C. elegans* F11E6.5 protein, designated ELO-2, as deduced from the genetic analysis of live worms. The biochemical conclusions reported here are based on the GC data obtained on a whole animal.

RNAi-mediated suppression of *elo-2* results in the significant accumulation of palmitate, suggesting that C16:0 could be a major substrate for ELO-2. The accumulation of palmitate causes an imbalance in total FA composition that can be critical for many cellular functions. Indeed, portions of the palmitate fraction in PL and TAG lipid classes rose from 4 to 22% and from 12 to 51%, respec-

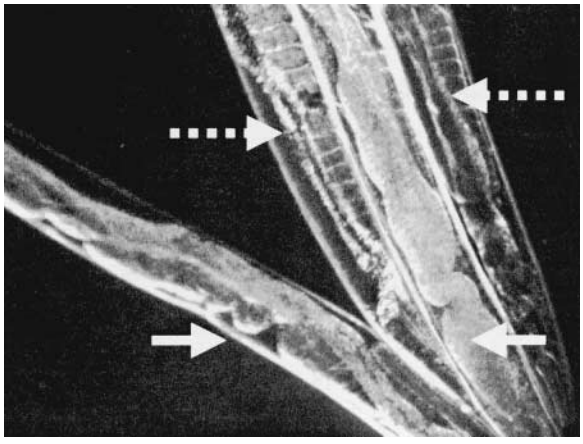

FIGURE 6.—*elo-2(RNAi)* worms have a pale intestine (dashed arrows) compared to N2 (solid arrows). Magnification, ×40.

TABLE 1

Posterior body wall muscle contractions, pBoc, in worms with abnormal FA composition

Strain	pBoc intervals, sec
N2, Bristol	47.6 ± 4.2
elo-2(RNAi)	35.9 ± 4.3
elo-2(+)[Ex] not in posterior gut cells	67.3 ± 12
elo-2(+)[Ex] in posterior cells	45.5 ± 3.6
GFP control	46.9 ± 3.7
fat-2	108.4 ± 29.3
fat-2;elo-2(RNAi)	62.3 ± 16
fat-3	95.5 ± 31.2
fat-3;elo-2(RNAi)	60.6 ± 11.7
elo-1	68.3 ± 18
elo-1;elo-2(RNAi)	64.1 ± 14.5

pBoc intervals are shown in seconds with standard deviations. At least 10 intervals in at least 10 worms of each type were counted. The alleles used were *fat-2(wa17)*, *fat-3(wa22)*, and *elo-1(gk48)*.

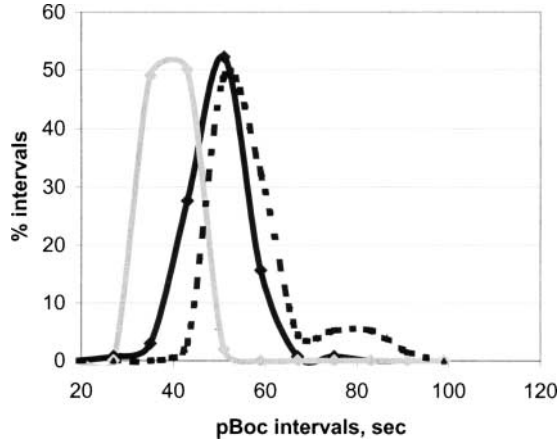

FIGURE 7.—pBoc intervals are shortened in the presence of palmitate but not stearate. Graphs show the distribution of pBoc intervals in N2 controls supplemented with 1% NP40 (n = 13; black line), N2 fed with palmitate (C16:0/1% NP40, n = 15; gray line), and N2 fed with stearate (C18:0/1% NP40, n = 14; dashed line). n, number of evaluated worms, 10 pBoc counts for each.

tively, at the expense of C20 PUFA. These changes in the FA composition may affect the energy storage function as well as the membrane properties and associated signal transduction mechanisms. This would explain why ELO-2 suppression has multiple phenotypic impacts involving growth, reproduction, and rhythmic behavior.

While future experiments are needed to isolate the particular molecules responsible for the phenotypic pleiotropy in *elo-2(RNAi)*, there are indications that C16:0 itself plays an important role. Our supplementation experiments have shown that the addition of palmitate to the food source can change physiological rhythms, as exemplified by the shortening pBoc intervals, and that this effect is not attributed to an increase in lipid saturation or a relative decrease in PUFA amounts. It seems feasible that the increase in palmitate could be linked to the various associated phenotypic changes through the sphingolipid pathway. This is a testable hypothesis and we will address the problem through future biochemical studies.

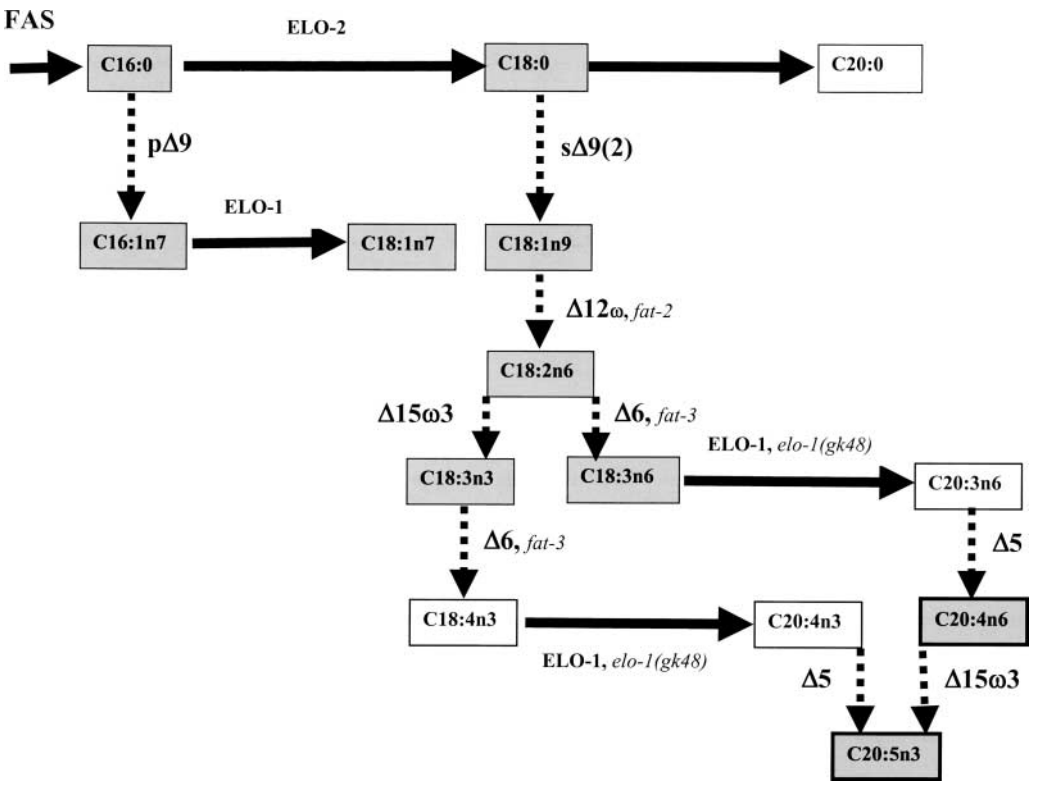

FIGURE 8.—Schematic presentation of the FA elongation (horizontal arrows) and desaturation (vertical dotted arrows) steps in *C. elegans*. If known, corresponding enzymes are shown next to the arrows. The data for desaturases are taken from NAPIER and MICHAELSON (2001) and WATTS and BROWSE (2002). Mutant strains are shown next to the corresponding enzymes. Shaded boxes with FA names represent FA species easily detectable by GC as compared to the less abundant FA in open boxes. FAS, fatty acid synthase.

In addition to the increase in palmitate, a statistically significant decrease in the C18:1 n9, a product of stearoyl-CoA desaturase activity, was observed in *elo-2(RNAi)* (Figure 2). We hypothesize that these changes are also related to the accumulation of palmitic acid. It has been recently shown in Drosophila that palmitate is indirectly involved in the regulation of lipogenesis through the sterol regulatory element binding protein (SREBP) pathway (Dobrosotskaya *et al.* 2002; Seegmiller *et al.* 2002). In regard to these findings, it is important to understand if a similar mechanism exists in *C. elegans* and whether or not ELO-2 is an active part of it, possibly regulating internal amounts of palmitate. We consider *elo-2(RNAi)* animals to be a useful model for the genetic analysis of FA biosynthetic gene expression and the SREBP pathway.

ELO-1 and ELO-2 function in concordance, providing worms with the necessary amounts of PUFA. The double suppressed *elo-1(gk48);elo-2(RNAi)* animals are unable to synthesize C20 PUFA and are barely viable. Taken separately, *elo-2(RNAi)* and *elo-1(gk48)* phenotypes are noticeably different. Thus, despite possibly overlapping biochemical functions, ELO-1 and ELO-2 may have different impacts on physiological integrity in *C. elegans*.

In conclusion, we have shown that the predicted *C. elegans* protein, designated ELO-2, is an active enzyme involved in FA elongation. ELO-2 functions together with ELO-1 in PUFA biosynthesis. The activity of at least one of these enzymes is necessary for C20 PUFA production. A loss of ELO-2 function results in a significant accumulation of palmitate that directly or indirectly causes multiple phenotypic defects in growth, reproduction, and physiological rhythms. More biochemical studies are necessary to explain the observed phenotypes and to isolate the particular molecules that are involved. *elo-2(RNAi)* animals provide a useful tool for studying the genetic and physiological links between FA biosynthesis and signal transduction, which are essential for understanding human metabolic and rhythmic disorders.

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