Mol. Cells, Vol. 10, No. 2, pp. 226–231

Molecules and Cells

Analysis of the *flt-1* Gene Encoding a ECM Protein, Flectin, in *Caenorhabditis elegans*

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(Received on January 6, 2000)

Flectin is a new type of extracellular matrix protein and its function was suggested to provide a micro-environment of great elasticity. The *C. elegans* genome database revealed the presence of a flectin homologue, *flt-1*, which shows approximately 40% similarity (20% identity) to chick flectin. Here we propose a new gene structure for the *flt-1* based on our experiments and the partial cDNA clones obtained from Y. Kohara and further suggest that the previous gene prediction is incorrect. FLT-1 is shown to be expressed in various neurons, hypodermal cells, distal tip cells and vulva epithelial cells. Immunostaining results with anti-FLT-1 antibody, further confirm the FLT-1 expression in vulva epithelial cells. The lipophilic dye, DiI, was used to identify the head neurons expressing GFP and results indicated that none of the head neurons expressing GFP are the 6 chemosensory neurons. In order to determine the function of *flt-1* gene, RNA-mediated interference (RNAi) experiments were conducted.

Keywords: *Caenorhabditis elegans*; Flectin; Green Fluorescent Protein; Lipophilic Dye; RNA Mediated Interference.

Introduction

Extracellular matrices (ECM) regulate cell growth and differentiation, bind to growth factor and provide the scaffolding necessary for tissue morphogenesis (Carson, 1992). In *C. elegans*, there are two types of ECMs which are the cuticle and the basement membranes. The cuticle covers the outside of the animal and the basement membrane covers the pseudocoelomic faces of the hypodermis, pharynx, intestine, gonad and some body wall muscles (Krammer, 1997). The major constituents found in mammalian ECM (collagen, proteoglycan, laminin) have also been identified in *C. elegans* (Rogalski *et al*., 1993) and the roles of these ECM molecules are almost same as that of ECM of other organisms.

Flectin is a new ECM protein, which was identified from the eye extract of chick embryo using a monoclonal antibody (Mieziewska *et al*., 1994a). Flectin has been reported to localize in the developing neural retina, gut and heart of chick and mouse in a specific spatio-temporal manner. Based on these expression patterns, the function of flectin was suggested to be that of providing a microenvironment of great elasticity. For example, flectin may allow the thin myocardial wall to be pushed out during heart development in mouse (Tsuda *et al*., 1996). The deduced amino acid sequences do not show any sequence similarity to known ECM molecules. However, flectin antibody can recognize interphotoreceptor (IPM) proteins in many different vertebrate species ranging from human to frog (Mieziewska *et al*., 1994b). Flectin is also found in the matrix of *Drosophila* compound eye (Mieziewska *et al*., 1994b), suggesting that flectin function is conserved during development.

C. elegans has been a good model system to study the function of specific gene using powerful genetic and cell biological approaches (Riddle *et al*., 1997). In particular, the recent completion of the genome sequencing (the *C. elegans* sequencing consortium, 1998) has made it a more attractive model system to investigate gene function of vertebrate homologues.

Since we were interested in flectin gene function, we attempted to study the characteristics of the flectin gene in *C. elegans.* The *flt-1* gene was identified by searching the *C. elegans* genome database with the amino acid sequence of chick flectin (a protein of 1,802 amino acids). The *flt-*

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Abbreviations: ECM, extracellular matrices; IPM, interphotoreceptor; RNAi, RNA-mediated interference.

1 gene encodes a putative protein of 1,453 amino acids showing approximately 40% similarity (20% identity) to chick flectin, but the conserved domain, spanning the exon 5 region (93 amino acids), shows approximately 63% similarity (44% identity) to chick flectin.

In this report, we investigated the *C. elegans* ortholog of flectin, FLT-1. First, we report the assumed new gene structure including its transcription pattern by northern analysis. Second, we describe the expression pattern of the nematode flectin, FLT-1 in different developmental stages using the green fluorescent protein (GFP) and confirm one of these patterns by immunostaining with FLT-1 antibody. We have also attempted to determine the function of this *flt-1* gene by RNA-mediated interference (RNAi) method.

Materials and Methods

Strains and culture condition The wild type *C. elegans*, the Bristol strain (N2), was obtained from *Caenorhabditis elegans* Genetics Center (CGC) and was grown under conditions described previously (Brenner, 1974; Sulston and Hodgkin, 1988).

***flt-1*::*gfp* fusion construct and analysis of GFP expression** Cosmid ZK783 encoding FLT-1 was obtained from A. Coulson (Sanger Center, U.K). The plasmid pSJ301 was constructed by subcloning a genomic 3,570 bp fragment of the cosmid into the pPD95.75 vector (Fire *et al*., 1998a), creating a translational fusion at the *Bam*HI site within the third exon of *flt-1* with the green fluorescent protein (GFP) (Chalfie *et al*., 1994) coding region. Microinjection was performed as described by Mello (1995). A mixture of plasmids [60 ng of *gfp* fusion construct and 80 ng of pRF4 (Krammer *et al*., 1990) as a transformation marker] were co-injected into the hermaphrodite gonad. Plasmid pRF4 contains a dominant mutant allele of the *rol-6* gene and this will allow transformed animals to be identified by their rolling phenotype. Animals of the second (F2) and subsequent generations from the injected worms showing the roller phenotype were picked and transferred onto a 2% agarose pad (Cho *et al*., 1999). After treatment with 1% levamisole solution to immobilize the animals, the expression patterns of green fluorescent signals were examined by fluorescence microscopy (Olympus BX50). Nomarski optics (Nomarski, 1955) was also used to observe wild type and transgenic animals.

Dye-filling Transgenic worms of the *flt-1*::*gfp* line were placed in 4 µl of DiI solution (Molecular Probes, catalog # D-282, 2 mg/ml DiI dissolved in dimethyl formamide) diluted in 800 µl of M9 buffer, incubated for 2–3 h at room temperature, and allowed to recover by transferring the worms to a fresh bacterial seeded plate, and let them crawl on the bacterial lawn for 1 h. Recovery step was also needed for destaining (Koelle, 1994). Following recovery, the worms were picked and transferred onto a 2% agarose pad. After treatment with 1% levamisole solution to immobilize the animals, cells stained with the lipophilic dye were examined under a fluorescence microscope (Olympus BX50). The dye allowed the stained cells to be visualized as red fluorescence (Hedgecock *et al*., 1985). By changing the filter equipped with the microscope, the GFP signals and the dye signals were compared and photographed separately. Following scanning of the films (Asa 400, Kodak), the two images were merged using the Adobe Photoshop graphics program.

FLT-1 antisera and immunostaining To raise FLT-1 polyclonal antibodies in rat, cDNA fragments encoding amino acids 1,380-1,565 (exon 8) of *flt-1* were fused to the expression plasmid pGEX–4T1 (Phamacia Biotech) and overexpressed as GST (glutathione-S-transferase) fusion proteins in *E. coli*. The fusion protein was subjected to SDS-PAGE, purified by electroelution from the excised gels and used to immunize rats (approximately 90 µg/injection). After the fifth injection the antiserum was used for immunostaining as described by Ahnn and Fire (1994). Mixed stage worms on the plates were washed with M9 buffer solution (5 g NaCl, 6 g Na_2HPO_4, 3 g KH_2PO_4, 1 ml 1 M $MgSO_4$ in 1 L H_2O), centrifuged and transferred to 0.03% poly-L-lysine coated slides. Worms were permeabilized by freeze cracking and fixed in cold methanol for 3 min. After stepwise rehydration in methanol: H_2O mixtures [75, 50, 25% (v/v) methanol, each for 2 min] and washing with TBS-T [50 mM Tris·Cl (pH 7.8), 150 mM NaCl, 0.1% Tween 20] samples were incubated for 5–6 h with primary antibody diluted in TBS-T. Extensive washing with TBS-T was carried out before incubation with secondary antibody. After incubation with fluorescein conjugated goat anti-rat secondary antibody (Santa Cruz) diluted in TBS-T, slides were extensively washed with TBS-T, mounted with mounting medium (80% glycerol, 1% N-propyl gallate) and observed under fluorescence microscope (Olympus BX50).

RNA blots Synchronous and mixed-stage populations of the wild type hermaphrodites were obtained by standard methods (Sulston and Hodgkin, 1988). Mixed and stage specific RNAs were prepared using Trizol reagent (MRC) according to the manufacturer's protocol. Total RNA (about 20 µg) was fractionated by electrophoresis under denaturing conditions (Sambrook *et al*., 1989), transferred to NY13 N NYTRAN membrane (Schleicher & Schuell), and hybridized in 0.25 M Na_2HPO_4 (pH 7.2), 0.1% H_3PO_4, 0.25 M NaCl, 1 mM EDTA, 7% SDS, 50% Formamide, 5% Dextran sulfate, 100 µg/ml denatured salmon sperm DNA, with random-primed ^{32}P-labeled probes. The probe template was 820 bp long, covering the exon 5 region of *flt-1*. Exposure of the blot following low stringent washing was performed in BAS-1500 (FUJI FILM).

RNA-mediated interference of *flt-1* expression Double stranded RNA (dsRNA) corresponding to the most conserved exon 5 region (820 bp) of *flt-1* was prepared using an *in vitro* transcription kit (Promega) as described previously (Fire *et al*., 1998b; Montgomery *et al*., 1998; Timmons and Fire, 1998). Double stranded RNA was injected into the syncytial gonad of young N2 hermaphrodite adults, which were allowed to recover at 20°C for 12 h. Animals that died or sustained obvious damage from the injection were not included in the subsequent analysis. Injected animals were transferred to bacterial-seeded plates (1 worm per plate) and allowed to lay eggs for another 12 h. The F1 progenies were observed for hatching ability and phenotypic variances.

Results and Discussion

Gene structure of *C. elegans* flectin homologue, *flt-1* The *C. elegans* genome sequencing project has made whole gene sequences available and has also provided the predicted gene structures based on these sequence information. The *C. elegans* flectin homologue, *flt-1* is contained in the cosmid ZK783 and has been physically mapped to the LG III gene cluster, which corresponds to the region between the *lin-39* and *lin-13* loci on the genetic map (Fig. 1A). The *flt-1* was predicted to consist of 10 exons, which encoded a putative protein of 1,453 amino acids (Fig. 1B). However, based on our findings the prediction seemed to be ambiguous. Firstly the partial cDNA clones (yk294e2, yk177d6, yk296d1, and yk53e2), obtained from Y. Kohara, ended at the intron region between the predicted exon 9 and exon 10. Additionally, we identified a stop codon, UAA, immediately after exon 9 indicating that the gene terminates prematurely. Hence, from this information, we suggested that *flt-1* contains up to 9 exons instead of the predicted 10 exons.

To further confirm this suggestion, PCR was conducted from the cDNA library of a mixed-stage worm. Primers were designed based on the genomic sequence of *flt-1* gene to cover the region spanning from exon 8 through exon 10 and the amplified cDNA fragments were analyzed by electrophoresis. To our surprise, a 2.0 kb band (Fig. 1C, lane B) and a 1.9 kb band (Fig. 1C, lane C) were amplified. If the previous prediction of gene structure was correct, then the PCR would have yielded a smaller band or no band on the agarose gel. Hence, PCR results indicated that the sequences, which was predicted as intron, exist in cDNA clones. This was further confirmed by sequencing the PCR products (data not shown). Therefore, we suggest that the *flt-1* gene should consist of 9 exons instead of 10 exons. Moreover, our results also indicate that there must be other cDNA clones which contain longer 3′-UTR region not represented by currently identified yk clones. Additionally, when the entire genomic sequence (including exon 10) of *flt-1* was fused with GFP expression vector and injected into the worms, no expression of GFP was seen, indicating that the premature stop codon at the end of exon 9 interrupted the translational frame with GFP. Taken together, we suggest that the region previously predicted as intron sequences should be corrected to the 3′-UTR region of the *flt-1* gene.

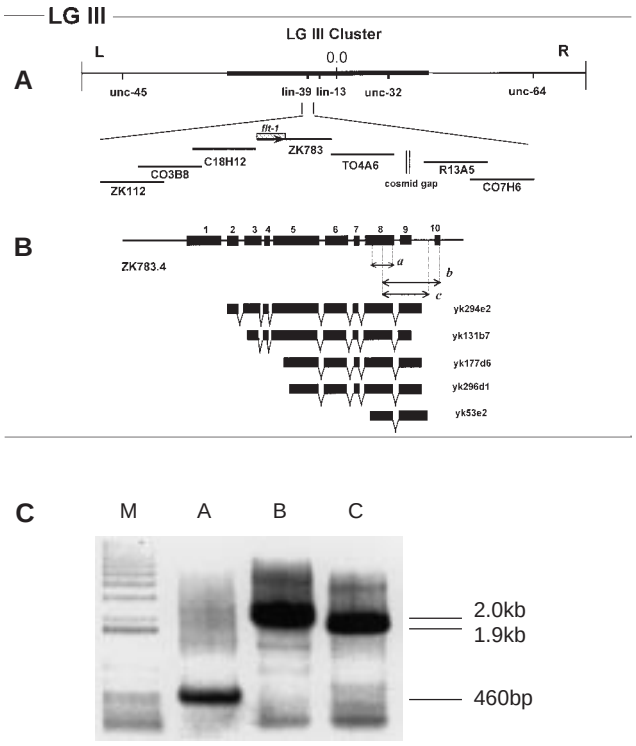

Fig. 1. Molecular nature of *flt-1* (*ZK783.4)* **A.** Genetic and Physical map of *flt-1* region. The position of *flt-1* relative to nearby genetic markers on the center region of LGIII is shown. *flt-1* (striped box), which is mapped to the cosmid ZK783 and the neighboring cosmids are indicated. **B.** The predicted *flt-1* gene of the cosmid ZK783 is predicted to compose of 10 exons (black box) separated by 9 introns. Four out of five partial cDNA clones (yk294e2, yk177d6, yk296d1, and yk53e2) obtained from Y. Kohara, which ended in the intron region between exon 9 and exon 10 covering approximately 250 bases. **C.** cDNA library PCR with primers covering different parts of the genomic sequence of *flt-1*. The individual primer pairs used for lanes A, B, and C are indicated in Fig. 1B as *a*, *b*, and *c* respectively.

Northern blot analysis To determine the temporal expression patterns of *C. elegans* flectin homologue, *flt-1*, we performed northern blot analysis using total RNAs from wild type animals of mixed stages and as well as of different developmental stages (L2, L3, L4 larvae, and adults). To obtain total RNA from adult stages purely, we used a mutant strain, *fem-2* (*b245*), defective in germ-line development. The *fem-2* (*b245*) mutant line was subjected to heat shock at 25°C at early larval stages to obtain adults devoid of embryos. As shown in Fig. 2, two transcripts of approximately 4.9 kb and 3.3 kb were detectable at all developmental stages. The lower band is probably an alternatively spliced form of *flt-1* since database information reveals no possibility of cross-reaction. However, we have not yet identified any cDNA clones containing alternatively spliced form. Further characterization will be required to elucidate the nature of alternative splicing in *flt-1* gene.

Expression pattern of FLT-1 To examine the expression and subcellular localization of *C. elegans* flectin, FLT-1, we constructed several GFP tagged DNA plasmids containing various regions of promoters and putative coding sequences (Shin *et al*., 1998). The plasmids were microinjected to produce germline-transformed, transgenic

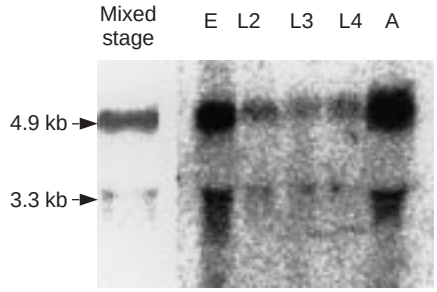

Fig. 2. Northern blot analysis of *flt-1*. RNA was harvested from wild type animals of mixed stages and larval stages (L2, L3, L4). To obtain RNA from adult stage purely, the *fem-2* (*b245*) mutant strain was used (**A**). The 820 base long cDNA fragment of the most conserved exon 5 region, was amplified by PCR and used as a probe. Two transcripts of approximately 4.9 kb and 3.3 kb were detected at all developmental stages. The strong signals in embryo and adult stages are due to the higher level of RNA loaded in these lanes.

worms and GFP expression patterns were observed under a fluorescence microscope (see Materials and Methods). Transgenics carrying the construct containing 5′-upstream 2.5 kb and 340 bases of coding sequences showed strong expressions of GFP in neuronal cells (Figs. 3A and 3B). GFP expression was previously detected from the beginning of the comma stage through larval and adult stages (Shin *et al.*, 1998). During embryonic stages, GFP expression was restricted to AB lineage cells which become hypodermis, head neuron ganglia and ventral cord neurons and this expression pattern persisted in embryonic and larval stages (Shin *et al.*, 1998). During post-embryonic development, GFP was highly expressed in ventral cord neurons (Fig. 3A) and head neurons (Fig. 4A). In addition, GFP signals were detected in hermaphrodite specific neurons (HSN) and vulva (Figs. 3A and 3B). Hermaphrodite specific neurons, which are located on both sides of the vulva, are known to modulate the vulva muscle contraction (Chalfie *et al.*, 1985) and vulva is the opening through which eggs are laid by the hermaphrodites. Other cells expressing GFP include PVM neuron having mechanosensory function (Fig. 3B), distal tip cells (DTC) which are located at distal region of the gonad and tail ganglia (Shin *et al.*, 1998). Lateral hypodermal cells (seam cells) also expressed GFP throughout the developmental stages.

To localize FLT-1 *in situ*, we performed whole mount immunostaining using anti-FLT-1 antibodies (see Materials and Methods). A weak but distinct signal was detected around the vulva region (Fig. 3D) which was consistent with our GFP expression pattern.

GFP expression in the head neurons which are not chemosensory neurons GFP expression pattern of FLT-1 in neuronal cell bodies (Fig. 4A) and in neuronal

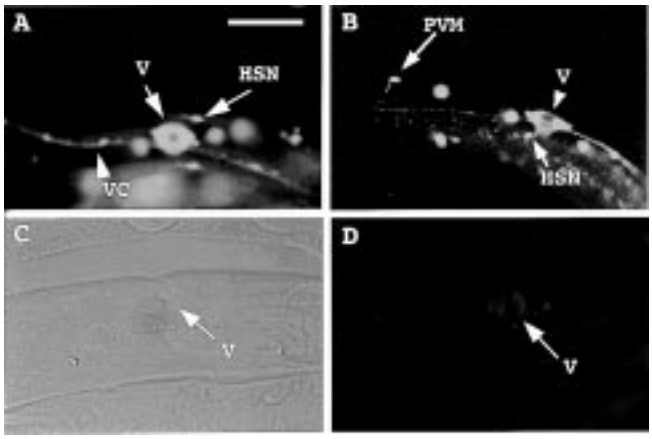

Fig. 3. Expression patterns of FLT-1 (**A** and **B**) Green fluorescent protein (GFP) expression is under the control of the *flt-1* promoter. Live transgenic animals were observed under a fluorescence microscope. **A.** Ventral view of L4 stage worm showing GFP expression in vulva epithelial cells (V), ventral cord (VC) and hermaphrodite specific neuron (HSN). **B.** Lateral view of mid-body trunk showing GFP expression in vulva epithelial cells (V), hermaphrodite specific neuron (HSN) and PVM neuron. **C.** Normarski image of Fig. 3D indicating vulva epithelial cells (V). **D.** Immunofluorescence photomicrograph of the vulva region (V) of a wild type hermaphrodite stained with anti-FLT-1 antibody. Bar, 50 μm.

projections (not shown in the figure) suggest that the gene is probably expressed in sensory neurons. To examine this fact, we used the lipophilic dye, DiI which is readily absorbed into exposed cell membranes to stain cell processes completely. DiI stains 6 head chemosensory neurons (ASK, ADL, ASI, ADF, ASH, and ASJ) and 2 tail neurons (PHA and PHB) (Hedgecock *et al.*, 1985). Worms stained with DiI (see Materials and Methods) showed staining in 6 chemosensory neurons which were shown as prominent red signals (Fig. 4B). When GFP signals were compared with DiI staining signals, none of the head neurons expressing GFP belonged to the 6 chemosensory

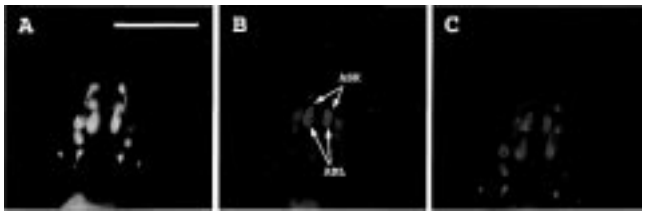

Fig. 4. Dye-filling with lipophilic dye DiI. **A.** The GFP expression in head neurons of a wild type (N2) animal. **B.** DiI stains six pairs of amphid neurons: ASK, ADL, AWB, ASH, ASJ, ASI (pairs of ASK, ADL are indicated). **C.** Superimposed view of GFP and DiI image in Adobe Photoshop 4.0 showing that the cells expressing FLT-1 are not overlapping with the 6 chemosensory neurons stained with DiI. Bar, 50 μm.

neurons (Fig. 4C). Therefore, it is more likely that the neurons expressing GFP are the other types of chemosensory neurons in the head (AWA, AWB, AWC, AFD, ASE, and ASG) (Bargmann and Mori, 1997). However, a more detailed study will be required to identify these neurons expressing GFP.

Possible function of FLT-1 in *C. elegans* To speculate the function of FLT-1 in *C. elegans*, we performed RNAi (RNA-mediated interference) which describes the use of exogenous RNA to interfere with the function of an endogenous gene. Double-stranded RNA (dsRNA) molecules transcribed *in vitro* and injected into *C. elegans* have been shown to interfere with the expression of the target gene specifically via unknown mechanisms (Fire *et al*., 1998b; Montgomery and Fire, 1998; Sharp, 1999; Tabara *et al*., 1998; Timmons and Fire, 1998). The cDNA clones for *flt-1* and calcineurin B (a calcium binding regulatory subunit of a serine-threonine protein phosphatase which is dominantly expressed in neuronal cells) were used to prepare dsRNAs. FLT-1 and calcineurin B exhibited similar neuronal expression patterns and hence RNAi with the latter was used to compare the phenotypes of calcineurin B RNAi with those of *flt-1* RNAi. The progeny of injected wild type animals showed about 11.92% and 6.0% dead embryo for *flt-1* and calcineurin B RNAi, respectively (data not shown). This effect did not seem to be a *flt-1* specific effect. Additionally, it was found that *flt-1* RNAi failed to work in some neurons which was revealed by injection of gfp dsRNAs into the FLT-1::GFP stable line. Though drastic reduction of GFP signal was observed in majority of the cells that showed GFP expression, some retention of signals in HSN and some head neurons, which escaped the RNAi, was observed (data not shown). It has already been discussed elsewhere that certain tissues, including some neuronal cells are resistant to RNAi, and the underlying mechanism for such tissue-specific interference is still not known (Fire *et al*., 1998b). Hence we conclude that some degree of dead embryo level is not the result of FLT-1-specific functional block but is due to the result of mechanical stress by injection.

GFP expression in head and tail neurons, the ventral cord, vulva epithelial cells, HSN, hypodermis and DTC may suggest that FLT-1 has a possible role in neurogenesis and early development of hypodermis. From the larval stage, strong reporter gene expression can be monitored throughout the maturation process of ventral cord and vulva formation. Distinct expression patterns in neurons and hypodermis were maintained until late larval stages. In the adult stage, expression patterns in ventral cord, HSN, vulva, hypodermis and DTC gradually weakened whereas head neuronal expression was maintained. Based on our observations it may be suggested that FLT-1 is important for generation and maturational processes of hypodermis and neurons.

Another putative role of FLT-1 may be related to cell migration and neuronal projections. Distal tip cells (DTC) which lead the gonad to a proper direction during gonad development show a distinct expression of GFP. Another example is the expression seen in the hermaphrodite specific motor neurons (HSN). HSN moves from the posterior end of the animal to a midway point along the worm body (Sulston *et al*., 1983), showing strong reporter gene expression. Additionally, the reporter gene expression is apparent in various head neurons which require a proper projection to the tip of anterior part. Based on these expression patterns, we expected migration defects in the migrating cells and embryonic or larval stage arrest by injecting double-stranded RNA of exon 5 into the GFP integrated transgenic line. However we did not observe any drastic changes or defects in migration and development. Further research is still underway to suggest a possible function of FLT-1 in *C. elegans.*

Acknowledgments We would like to thank Dr. H. Y. Yoon for sharing unpublished amino acid sequence of chick flectin. Our thanks also go to Dr. A. Coulson for cosmids and the *C. elegans* Genetics Center for strains. We thank Jaya Bandyopadhyay for her helpful assistance during manuscript preparation. This work was supported by the KOSEF grant (1999-2-2-10-001-3) and the K-JIST Grant.

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