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let-756, a *C. elegans fgf* essential for worm development

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In vertebrates, Fibroblast Growth Factors (FGFs) and their receptors are involved in various developmental and pathological processes, including neoplasia. The number of FGFs and their large range of activities have made the understanding of their precise functions difficult. Investigating their biology in other species might be enlightening. A sequence encoding a putative protein presenting 30–40% identity with the conserved core of vertebrate FGFs has been identified by the *C. elegans* sequencing consortium. We show here that this gene is transcribed and encodes a putative protein of 425 amino acids (aa). The gene is expressed at all stages of development beyond late embryogenesis, peaking at the larval stages. Loss-of-function mutants of the *let-756* gene are rescued by the wild type *fgf* gene in germline transformation experiments. Two partial loss-of-function alleles, *s2613* and *s2809*, have a mutation that replaces aa 317 by a stop. The truncated protein retains the FGF core but lacks a C-terminus portion. These worms are small and develop slowly into clear and scrawny, yet viable and fertile adults. A third allele, *s2887*, is inactivated by an inversion that disrupts the first exon. It causes a developmental arrest early in the larval stages. Thus, in contrast to the other nematode *fgf* gene *egl-17*, *let-756/fgf* is essential for worm development.

Keywords: *C. elegans*; FGF; nematode; *let-756*; *egl-17*; worm development

Introduction

Fibroblast growth factors (FGFs) participate in various developmental processes and stimulate the proliferation or differentiation of a variety of cells of mesoderm, ectoderm and endoderm origin. They are prominently involved in mesoderm induction, organ and limb formation, neural development, and branching morphogenesis (for reviews: Mason, 1994; Goldfarb, 1996). In non vertebrates, branchless/FGF is a determinant factor in the tracheal branching pattern of the fruit fly *D. melanogaster* (Sutherland *et al*., 1996) and EGL-17/FGF is required for sex myoblasts migration in the nematode *C. elegans* (Burdine *et al*., 1997, 1998). In the mouse, experimental gene knockouts of *Fgf4* (Rappolee *et al*., 1994; Feldman *et al*., 1995) and *Fgf8* (Meyers *et al*., 1998) induce a lethal phenotype. For several FGFs however, gene knockouts only lead to a mild abnormal phenotype. *Fgf3* null mice are born with tail and ear defects (Mansour, 1994) and *Fgf6* mice with no apparent defect (Fiore *et al*., 1997; Floss *et al*., 1997); targeted disruption of *Fgf5* or *Fgf7* leads to abnormal hair development (Hebert *et al*., 1994; Guo *et al*., 1996), and disruption of *Fgf2* alters vascular tone (Zhou *et al*., 1998; Dono *et al*., 1998). Redundancy may be responsible for the subtlety of these phenotypes, as inactivation of one FGF may be compensated by a different family member. Alternatively, FGFs may execute various functions, some of which are critical for life and others simply luxurious.

FGFs interact both with high and low affinity receptors. Among the first are FGF receptor tyrosine kinases (four in mammals but only one in the nematode *C. elegans*, EGL-15) and cysteine-rich receptors (one in the nematode). The low affinity receptors include heparan sulfate proteoglycans (for reviews see Mason, 1994; Goldfarb, 1996; Green *et al*., 1996). One perlecan gene, *unc-52*, has been identified in *C. elegans* (Rogalski *et al*., 1993). FGF/FGF receptor (FGFR) pathways are involved in pathological processes: germline mutations of *FGFR*s are associated with hereditary skeletal disorders (for reviews see De Moerlooze and Dickson, 1997; Webster and Donoghue, 1997) and somatic alterations of *FGF* and *FGFR* loci are involved in neoplasia (Dickson *et al*., 1984; Chesi *et al*., 1997; Popovici *et al*., 1998; Xiao *et al*., 1998).

To gather information on the various pathways and activities in which FGFs are involved, and learn about the partners required for their action, we chose to work on the nematode *C. elegans*, because of its genetic accessibility and its well described anatomy and development (Wood and the Community of *C. elegans* Researchers, 1988). Only one representative of *fgf* has been identified by a direct blast search (Altschul *et al*., 1990) in the entire *C. elegans* genome (The *C. elegans* Sequencing Consortium, 1998). It is carried by the C05D11 cosmid. The protein shares around 30% identity with the vertebrate FGFs (Coulier *et al*., 1997). Another related FGF, the product of *egl-17*, has been recently characterized by Burdine *et al*. (1997, 1998). Defects in mutants of *egl-17* are reminiscent of those observed in some alleles of *egl-15*, the FGFR encoding gene in *C. elegans* (DeVore *et al*.,

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1995). EGL-17/FGF is required for some cell migrations, but not for the survival of the animal.

Our aim was to characterize the *fgf* gene present on C05D11. We show here that it is essential for worm development since damaging the gene, called *let-756*, induces larval arrest.

Results

Characterization of the fgf *transcript*

No cDNA was available for the C05D11.4 gene (Figure 1) in the collection of Dr Yuji Kohara (*C. elegans* cDNA Project, Mishima, Japan). To test for transcription, we used the J3-J4 Polymerase Chain Reaction (PCR) product covering part of exon 4 (see Figure 3), to probe a Northern blot filter containing RNA extracted from animals at various stages of development. A single, moderately abundant transcript of approximately 1.7 kb was detected. Barely visible in embryos, its expression peaked at the L2 and L3 larval stages. The existence of a major single transcript was confirmed by using the pFGF probe (Figure 2), corresponding to the entire coding region.

RNA was extracted from a mixed stage population and first strand cDNA was synthesized. On the 5′ side, trans-splicing to the spliced leader 1 (SL1) or 2 (SL2) was determined by PCR amplification using primers complementary to either SL1 or SL2 with a primer sequence from exon 4 (J4). Southern blot analysis with the *fgf* probe obtained after PCR amplification with the J3-J4 primers, revealed that a single 0.6 kb fragment is amplified with SL1 (data not shown) but none with SL2. Sequence analysis showed that SL1 is trans-spliced eight bases upstream of the predicted exon 1 of *fgf* (see Figure 3).

A primer pair (J1-J13) spanning the start and stop codons predicted by Genefinder was used to define the translated region. This primer pair failed to amplify the entire coding sequence because the splicing of exons 7 and 8, and thus the open reading frame, differed from the Genefinder prediction. To determine the actual stop codon, we designed primer J15, 3′ of the next putative stop codon. Using the primer pair J1-J15, a fragment of about 1.3 kb was amplified from the mixed stage first strand cDNAs. Sequence analysis revealed that the stop codon is at genomic position 2456 (Figure 3) and that the actual *fgf* coding region is 1278 bp-long (GenBank accession number AJ010553). The PCR-product obtained with the J1-J2 pair, i.e. the entire coding region, was subcloned as pFGF in a pGemT vector.

The size of the transcript observed in Northern blot analysis indicated a short 3′ untranslated region. This was confirmed by PCR amplification with either primers J3 or J5 and an oligo(dT) primer. Hybridization of the PCR product with the probe derived from pFGF revealed two fragments of approximately 1.1 and 1.3 kb, in agreement with an untranslated region of 200–300 bp.

The expected FGF protein sequence (Figure 3) contains several putative structural motifs including an N-glycosylation site (at position 150) and four bipartite nuclear targeting sequences (at 186–202, 281–297, 290–306, and 305–321). Unlike most FGFs, including *C. elegans* EGL-17/FGF (Burdine *et al*., 1997) and *Drosophila* branchless/FGF (Sutherland *et al*., 1996), no putative signal sequence was detected. An unusual finding is a stretch of glutamine residues and a stretch of histidine residues that are not present in mammalian FGFs. The stretch of histidines is found in a region that shows 57% similarity with a portion of the mammalian lamin A molecule (Figure 3).

Conceptual translation of the cDNA reveals a 425 aa product of predicted molecular mass 49 679 Da. The entire coding region (in pFGF) was used for *in vitro* translation using a TNT coupled reticulocyte lysate system. This resulted in the synthesis of a protein

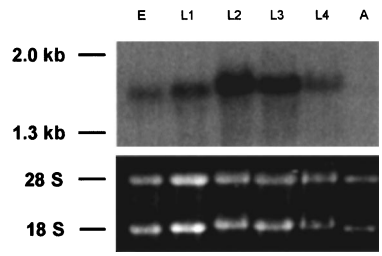

Figure 2 Expression of *fgf* mRNA. Northern blot analysis of RNA extracted from various wild type worm stages (E, all embryo stages; L1 to L4, larval stages isolated after synchronization; A, gravid adults) and hybridized with the pFGF probe

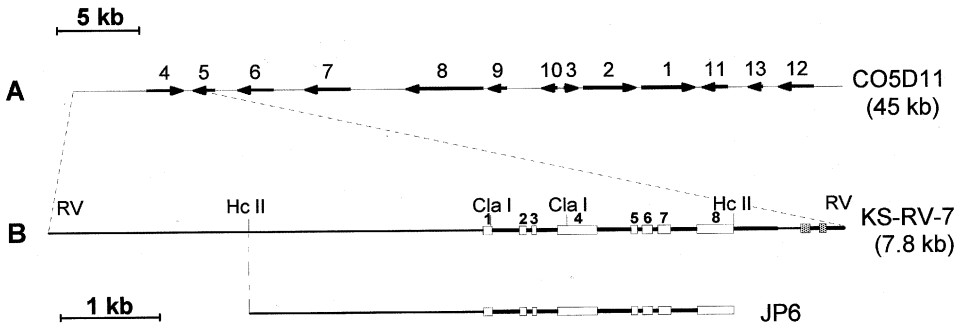

Figure 1 (**a**) Schematic representation of the *fgf* locus and constructs. Cosmid C05D11 is shown. A total of 13 potential genes (1–13) have been identified by Genefinder on this cosmid (GenBank accession number U00048). The *fgf* locus is number 4. (**b**) Constructs derived from the *fgf* genomic DNA (RV: *Eco*RV, HcII: *Hinc*II)

with an apparent molecular mass of 47 kDa when using the Sp6 promoter (not shown).

Comparison of C. elegans *FGFs with FGFs from other species*

Two FGF proteins have been recognized in the worm: EGL-17/FGF (Burdine *et al*., 1997, 1998), and LET-756/FGF, described here. Plain alignments (not shown) indicate that LET-756/FGF has 23–39% aa sequence identity in the FGF core with mammalian FGFs, while EGL-17/FGF shows 12–22% identity. For LET-756/FGF, the best scores were observed with FGF9 and FGF16 (Figure 4). To determine the place of the two *C. elegans* FGFs within the FGF family, we constructed a phylogenetic tree. This analysis allowed to recognize subfamilies of FGF members, e.g. FGF11–14, or FGF8, FGF17 and FGF18 (not shown). However, low bootstrap values prevented to assign the two *C. elegans* FGFs to any particular subfamily. The two *C. elegans* FGFs did not group together either. From these data, it is not possible to determine whether one or two *fgf* genes existed in the vertebrate and worm's last common ancestor.

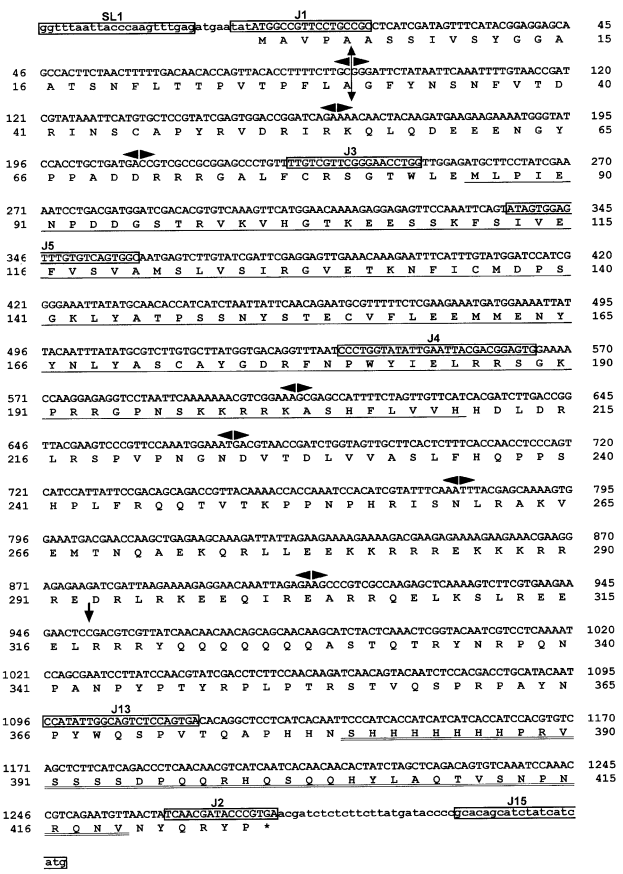

Figure 3 *let-756/fgf* nucleotide and deduced amino acid sequence. Nucleotide sequence obtained from the RT–PCR product using the primer pair SL1-J4 and J5-J2. Exon limits are indicated by double head arrows. Boxes delineate primer sequences. The predicted 425 amino acid sequence of LET-756/FGF is shown. The core FGF region is underlined. The region of lamin similarity is doubly underlined. The location of the C to T mutation, leading to the replacement of a R residue by a stop, present in both *s2613* and *s2809* alleles, is indicated with an arrow as is the inversion breakpoint (in intron 1) of the *s2887* allele

Identification of the fgf *gene by mapping candidate genes and by transformation rescue*

The *fgf*-containing cosmid mapped between the two cloned genes *sma-4* (*III, −1.43* and *sma-3* (*III, −0.91*), a region of 1.06 megabases of DNA possibly containing 43 genes identified by mutation and yet uncloned. Twenty-six of these genes are lethal or sterile. The number of candidates was reduced by refining the genetic map (raw data have been deposited at the Caenorhabditis Genetics Center). The presence of the *fgf* sequence was assessed in five deletions removing sections of chromosome III near the gene (*nDf17*, *nDf20*, *nDf16*, *sDf127* and *sDf125*) by PCR amplification of homozygous Df/Df embryos, individually picked (Williams *et al*., 1992). The *fgf* gene was present in *nDf16* and *nDf20* and absent from *nDf17*, *sDf125* and *sDf127* leaving the 13 lethal or sterile genes mapped in *sDf125* and out of *nDf16* as candidate genes. Complementation tests (Mello *et al*., 1991) with a transformant containing cosmid C05D11 allowed to limit to five the number of candidates (Janke *et al*., 1997), i.e. *let-713*, *let-721*, *let-725*, *let-756* and *mel-32*.

We generated stable germline transformants by coinjecting cloned genomic fragments containing the wild type *fgf* gene and the dominant marker *rol-6(su1006)*. A plasmid (pKS-RV-7, see Figure 1) encoding the complete *fgf* gene but no other predicted gene generated four stable transformant lines, *fEx50* to *53*. Alleles of each candidate gene were tested; only *let-756(s2613)* (Table 1) was rescued. All four arrays were efficient, and the frequency of rescued *let-756+* adults in each *let-756; fEx* strain matched the frequency of Rollers in the original +/+; *fEx* strain, indicating cosegregation of [Let-756+] and *fgf*. These results provide a strong indication that the *fgf* gene corresponds to *let-756*.

let-756 is the fgf *gene*

The genomic DNA of the two EMS alleles (*s2613* and *s2809*) and the wild type was amplified and sequenced. Sequencing each allele three times revealed a single difference with the wild type. The mutation, a C to T transition, was identical in both alleles. It is located in exon 8 (position 2130 of the genomic DNA, or 952 of

	LET-756	EGL-17	dm-FGF	hs-FGF-18	hs-FGF-17	hs-FGF-16	mm-FGF-15	hs-FGF-14	hs-FGF-13	hs-FGF-12	hs-FGF-11	hs-FGF-10	hs-FGF-9	hs-FGF-8	hs-FGF-7	hs-FGF-6	hs-FGF-5	hs-FGF-4	hs-FGF-3	hs-FGF-2	hs-FGF-1
hs-FGF-1	30	12	33	31	23	43	31	39	36	35	35	35	42	23	40	34	40	39	38	57	100
hs-FGF-2	29	15	31	30	24	42	29	36	34	34	33	37	41	30	40	44	43	45	43	100	
hs-FGF-3	36	18	34	35	29	48	27	37	39	38	36	54	47	33	46	42	50	36	100		
hs-FGF-4	31	15	29	34	31	42	27	44	42	43	43	33	43	31	36	69	51	100			
hs-FGF-5	34	19	33	28	29	48	29	39	41	37	38	44	47	29	44	51	100				
hs-FGF-6	33	16	30	36	32	41	24	38	40	39	37	40	43	35	38	100					
hs-FGF-7	36	15	30	35	33	45	32	37	36	34	35	55	43	31	100						
hs-FGF-8	27	21	25	68	76	34	16	26	27	22	27	29	36	100							
hs-FGF-9	37	17	35	37	32	90	26	42	43	39	40	43	100								
hs-FGF-10	31	14	34	30	27	43	26	31	37	36	31	100									
hs-FGF-11	32	19	28	29	28	39	21	69	64	66	100										
hs-FGF-12	29	18	32	27	22	37	22	82	78	100											
hs-FGF-13	33	19	32	32	25	40	24	79	100												
hs-FGF-14	31	17	29	28	25	40	24	100													
mm-FGF-15	23	15	27	19	21	27	100														
hs-FGF-16	39	18	38	34	32	100															
hs-FGF-17	28	21	24	65	100																
hs-FGF-18	29	22	24	100																	
dm-FGF	31	18	100																		
EGL-17	13	100																			
LET-756	100																				

Figure 4 Amino acid identity scores between FGF sequences. Scores are given in percentages. Alignments were done using the core sequence of FGFs as defined in Coulier *et al*. (1997)

the cDNA) (see Figure 3). This nonsense mutation occurs in a putative nuclear localization signal and leads to the replacement of arginine 318 by a stop. It truncates the FGF protein of its C-terminal one-fourth, and removes the region that shows similarity with lamin A. A Southern blot analysis of the *s2613* containing strain, probed with a 4 kb fragment derived from the upstream region of the *fgf* gene, showed no major alteration of this region (not shown).

The third allele, *s2887*, was generated by UV irradiation, which induces chromosomal rearrangements. Southern blot and PCR analyses showed that the 5′ of the *fgf* gene is rearranged in *s2887*. The *s2887* allele contains an inversion separating the 5′ region of the *fgf* locus from the rest of the gene, and represents a null allele. The *fgf* encoding plasmid (in *fEx51*) rescues all phenotypes of *s2887*.

The plasmids pJP6 and Roller were injected in a strain containing the *dpy-17 let-756(s2613) unc-32* chromosome. Eight independent Dpy Unc rescued progeny transmitted. The transformed lines *dpy-17 let-756 unc-32; fEx54 to 63* were used to assess cosegregation of the Roller and rescued *Let-756*$^+$ phenotypes, and evaluate the phenotype of *fEx(fgf+)* in a wild type background. These animals were mated to wild type males, and 24 of their Rol progeny segregated both Rol and rescued Dpy Unc, while 26 of their Rol+ progeny segregated neither Roller nor rescued. Numeration of the complete progeny of 14 *dpy-17 let-756 unc-32/+++; fEx* hermaphrodites yielded 1405 wild type, 25 Dpy and 22 Unc recombinants, 644 Rol (31% average Roller transmission) and 300 rescued Dpy Unc (43% average rescue transmission). The Roller animals were short, due to the presence of the *fgf* gene. A few unusual Roller animals were of regular size, 4/4 did not transmit the Rol or the rescue, indicating that they were germline mosaics and that the small phenotype is likely associated to expression of FGF in the progeny of the D (and may be C) blast embryonic cells, which are mostly muscle cells.

Phenotypes associated with fgf *gene disruption*

The disrupted *s2887* allele represents the loss-of-function phenotype, and is a recessive zygotic larval lethal. When born from *let-756 (s2887) unc-32 (e189)/++* mothers, the *let-756 unc-32* larvae grew slowly and arrested their development as transparent and scrawny looking L2 (87%), L3 (11%) or L4 (2%) animals.

Homozygotes for the truncated alleles *s2613* or *s2809* have a similar but much milder phenotype than *s2887*. *let-756 (s2613)* homozygotes all slowly develop into fertile adults, allowing a homozygous stock to be maintained. This shows that the truncated FGF, deprived of the C-terminus, but retaining the FGF core element, has retained the FGF function required for survival. Though viable, the *s2613* animals look transparent, small and starved at all stages, and a very reduced amount of vitellogenin is produced in adults (Figure 5). Also, all the vital processes, such as development and growth, movement, feeding and defecation, or egg production and laying, happen at a slow rate. For example, development from fertilized embryo to egg-laying adult requires an average of 5 days at 20°C ($n=150$; $t=64-180$ h), *versus* less than 3 days for the wild type ($n=3331$, $t=55-60$ h). The delay is partitioned over all larval stages. Movement appears uncoordinated, especially in young larvae. Fertility is reduced to an average of 36 progeny per hermaphrodite over a 7-day period ($n=17$, variable from 0–84 eggs per mother), *versus* 303 ± 40 over 3 days for the wild type ($n=11$).

C. elegans proteins related to the FGF pathway include EGL-17/FGF (Burdine *et al*., 1997, 1998) and the FGF tyrosine kinase receptor encoded by *egl-15* (DeVore *et al*., 1995). Interestingly, the complete loss-of-function of EGL-15/FGFR leads to larval arrest, much as does the loss of LET-756/FGF. This contrasts with mutants with complete loss-of-function of *egl-17/fgf*, that are fully viable, but display egg-laying problems resulting from abnormal cell migration of the sex myoblasts. Partial loss-of-function alleles of the receptor encoded by *egl-15* display a similar phenotype. In contrast, *let-756 (s2613)* animals lay their few embryos normally, and do not turn into 'bags of worms'. The gonads of these adults are properly shaped and organized, the vulva is formed normally, though sometimes protruding and fragile looking. These observations suggest that the five cell types that undergo long range migrations to build functional

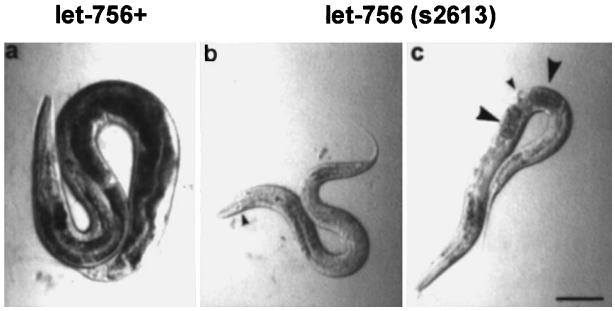

Figure 5 Phenotype of the partial loss-of-function allele *s2613*. Nomarski micrographs showing animals with a mutant or wild type *let-756* gene. (**a**) control *let-756+ unc-32(e189)* adults, 84 h at 20°C post fertilization. (**b**) and (**c**) the most developed animals *let-756(s2613) unc-32(e189)* at the same time after fertilization. Note the difference in size. Adults of this weak allele *s2613* are however viable, and they lay a few eggs that develop into small and starved looking adults. (**b**) shows a young *s2613* adult, the arrow points to a degenerating cell in the head. (**c**) shows an adult with a slightly protruding vulva (small arrow) and two embryos (large arrows). The same magnification was used for the three pictures. The bar equals 100 microns

Table 1 Rescue experiments with candidate genes located on the C05D11 cosmid

	Transmission of roller	*let-756*		*Complementation rescues*			
Genotype	*+/+*	*(s2613)*	*(s2887)*	*let-713 (s2449)*	*let-721 (s2447)*	*let-725 (s2454)*	*mel-32 (s2518)*
+; fEx51	51% ($n=373$)	54% ($n=241$)	57% ($n=172$)	0% ($n>500$)	0% ($n>500$)	0% ($n>500$)	0% ($n>500$)

somatic gonads and the vulva do so in *let-756/fgf* mutant animals.

We tested whether the two *C. elegans* FGFs may have functional relationships. No interaction or mutual enhancement was observed in the combinations of *let-756 (s2613)* with a presumably loss-of-function allele of *egl-17*, *e1313*. We then tested if functional replacement of *egl-17* by *let-756* could occur, taking advantage of the observation that some transgenic strains described above express 5–20 times more transcript than the wild type, as seen from mixed stage Northern analysis. Part of this RNA may translate into excess protein since the transformants display a recognizable phenotype: they are distinguishably shorter and less fecund than the wild type. We introduced by mating two such transgenes overexpressing *let-756/fgf* into *egl-17* (*e1313*) animals. These failed to rescue the Egl phenotype of *e1313* animals, indicating that an excess of LET-756/FGF produced under its own promoter cannot substitute for the lack of EGL-17/FGF. On the other hand, the lack of *egl-17* appears to enhance the effects associated with the transgene, as if they were interacting with a common partner. *egl-17; fEx54* animals are often very small, retarded and have a reduced fertility. These phenotypes, possibly resulting from overexpression of the gene, are reminiscent of those associated with a lack of the gene.

Discussion

It was shown previously that the product of a predicted gene from chromosome III of *C. elegans* had 30 to 40% similarity with FGFs from other species (Wilson *et al*., 1994; Coulier *et al*., 1997). A screening of the nematode sequences (The *C. elegans* Sequencing Consortium, 1998), revealed just one other *C. elegans fgf*, on chromosome X, already identified as *egl-17* (Burdine *et al*., 1997, 1998). Phylogenetic analysis indicated that none of the two *C. elegans fgf*s could be considered as a direct ortholog of a particular vertebrate *FGF*. They are related to all vertebrate members with no particular association.

We selected mutants from a large collection of lethal, sterile or retarded animals (Stewart *et al*., 1998), and isolated a null allele which is larval lethal and corresponds to a disruption of the gene and two other alleles with a milder phenotype, the latter are viable but small, clear, slow growing and have a reduced fertility. The partial alleles encode an FGF retaining a region with similarity to the FGF core, but truncated of the last 108 amino acids including a nuclear localization signal and a region with similarity to lamin A. The shorter FGF has retained enough function to ensure viability, yet it is associated to numerous phenotypical defects. It is possible that in some situations the region of FGF with lamin similarity influences the localization or stability of the protein and that its lack explains the mutant phenotype of the partial loss-of-function alleles.

LET-756/FGF has no consensus signal sequence, but it does not preclude from having a role outside the cell. Some mammalian FGF lack classical leader sequences for secretion and are released from the cell by other, as yet unidentified, secretory mechanisms. FGF1 and 2 are synthesized in the cytosol and are released by a mechanism independent of the ER-Golgi secretory pathway (Mignatti *et al*., 1992). Although FGF9 lacks a cleavable amino terminal signal, it is glycosylated and efficiently secreted from various cell lines, presumably via the ER-Golgi pathway (Miyamoto *et al*., 1993). In contrast, FGF3 is retained in the Golgi complex of Cos cells even though it has some kind of signal sequence, whereas it is efficiently secreted by certain fibroblast lines (Kiefer *et al*. 1983).

LET-756/FGF has several putative nuclear localization signals, supporting the idea that this FGF might play a role in the nucleus. Some FGFs added exogenously are translocated to the nucleus *in vitro* (Baldin *et al*., 1990), and isoforms of FGF2 and 3 are found in the nucleus of cells in which they are synthesized. These findings have raised the possibility of specific nuclear functions for these molecules, in addition to their signaling role at the cell surface. Differential subcellular targeting may be important for the function of *C. elegans* FGFs.

let-756/fgf is essential to worm development. Contrary to the *egl-17/fgf* gene, *let-756/fgf* belongs to the estimated 25% of genes that are essential to the nematode. This contrasts with some of the mammalian *FGF* genes or the *Drosophila Fgf* gene. Mutants with an altered *let-756/fgf* gene are deficient in larval development. The larval lethal phenotype matches the timing of expression of the *let-756/fgf* gene. Although *let-756/fgf* is a lethal locus in the worm, it does not appear to be required during embryogenesis, unless there is a maternal contribution masking its defect. A partial loss-of-function allele, which is viable and can be kept as a homozygous stock, may be informative in trying to analyse the function of FGF in the worm.

Disruption of the two known *C. elegans fgf*s, *egl-17* and *let-756*, leads to distinct phenotypes. Functional redundancy between them is not obvious, as a lack of EGL-17/FGF is not rescued by overexpressing *let-756*.

The same receptor, EGL-15/FGFR, possibly responds to both FGF products. In favor of an interaction between LET-756 and EGL-15 is the similarity of the phenotypes observed, in particular the stage of lethality and the scrawny aspect (De Vore *et al*., 1995). LET-756 could also interact with the cysteine-rich receptor encoded by the F14E5.2 gene, or with proteoglycans, for example unc-52/perlecan (Rogalski *et al*., 1993) or it could use yet another mode of action.

Materials and methods

Growth and handling of C. elegans

Standard genetic methods were used (Brenner, 1974). New deletions and mutants in chromosome III were screened and mapped as described (Stewart *et al*., 1998). Strains used in the study included BC4159 *dpy-17 (e164) let-713 (s2449) unc-32 (e189) III, sDp3(III; f [dpy-17+let-713+unc-32 (e189)]*; BC4164 *dpy-17(e164)let-725 (s2454) unc-32 (e189) III, sDp3*; BC4253 *dpy-17 (e164) let-756 (s2613ems) unc-32 (e189) III, sDp3*; BC4839 *dpy-17 (e164) let-756 (s2809ems) ncl-1 (e1865) unc-32 (e189) III, sDp3*; BC4931 *sEx148 III [C05D11 and pCes1943 (rol-6(su1006) 20:80]*; BC5078 *dpy-17 (e164) mel-32 (s2518) unc-32 (e189), sDp3*; BC5147 *dpy-17 (e164) let-756 (s2887uv) unc-32 (e189) III, sDp3*; CX2914 *nDf16/dpy-17 (e164) unc-32 (e189) III*; MT1565 *egl-17*

(e1313) Ion-2 (e678) X. New complementation and mapping data generated for this study were sent to the CGC. Transgenic strains generated are: FF527 to FF531 *fEx 50 to 53 [pKS-RV-7 and pRF4 (rol-6 dm) 10:150]*; FF550, 619, 567, 551, 549, 569, 552, 553 *dpy-17 (e164) let-756 (s2613) unc-32 (e189) III; fEx 54, 56, 57, 58, 59, 61, 62, 63 [pJP6 and pRF4 30:110]*. The *let-756* alleles were outcrossed four times to the wild type N2 strain, and separated from the markers by genetic recombination.

General methods

Synchronous and mixed stage populations of wild type hermaphrodites were obtained by standard methods (Wood and the Community of *C. elegans* Researchers, 1988). RNA isolation, Northern and Southern blot hybridization, subcloning and general DNA manipulations were done as described in Sambrook *et al*. (1989). Sequences from PCR products or from plasmid DNA were determined by Genome Express (Grenoble, France) using the dye terminator method. Coordinates are given relative to the initiating ATG in the genomic DNA, or in the cDNA when stated.

Phylogenetic analysis

Protein sequences for 18 mammalian FGF paralogs and the fruit fly and nematode FGFs were obtained directly from EMBL, NCBI or Swissprot databases. Accession numbers for FGF sequences are: hs-FGF-1:P05230; hs-FGF-2:P09038; hs-FGF-3:P11487; hs-FGF-4:P08620; hs-FGF-5:P12034; hs-FGF-6:P10767; hs-FGF-7:P21781; hs-FGF-8:P55075; hs-FGF-9:P31371, hs-FGF-10:AB002097; hs-FGF-11:Q92914; hs-FGF-12:Q92912; hs-FGF-13:Q92913; hs-FGF-14:Q92915; mm-FGF-15:AF007268; hs-FGF-16:AB009391; hs-FGF-17:AB009249; hs-FGF-18:AB007422; dm-FGF:U82273; egl-17:U85766; let-756:AJ010553. Species abbreviation are as follows: dm: *Drosophila melanogaster*; hs: *Homo sapiens*; mm: *Mus musculus*.

FGF 'core' sequences corresponding to hs-FGF1 aa 28 to 151, [after removing aa 120 and 121 of hs-FGF1 (Coulier *et al*., 1997)], were used for sequence alignment and phylogenetic analysis using the Clustal X program (Thompson *et al*., 1997). Human Interleukin 1b (hs-IL1b, accession number P01584) was used as the outgroup in the construction of the phylogenetic tree. A total of 1000 bootstrapped datasets were used to test the robustness of the branching (Felsenstein, 1985).

Characterization of the fgf *transcript*

Total RNA was prepared from mixed stage N2 worms by Lithium Chloride precipitation. First strand cDNAs were synthesized using random hexamers and Superscript reverse transcriptase (Stratagene). These first strand cDNAs were used as template for PCR amplification. When appropriate, identification of PCR products was done by Southern blot hybridization and then sequencing.

In vitro translation was done on pFGF using the TNT rabbit reticulocyte lysate coupled transcription/translation reactions (Promega).

Germline transformation

Cosmid C05D11 (GenBank accession number U00048, graciously provided by A Coulson, Sanger Centre, Hinxton, UK) was digested with *Eco*RV (see Figure 1). A 7.8 kb fragment containing 4.3 kb of non coding sequence upstream of the *fgf* gene, the 2.4 kb of the *fgf* gene itself, and 1.1 kb downstream of the *fgf* gene was cloned in Bluescript and is referred to as pKS-RV-7. A *Hinc*II fragment from this clone (position −2147 to +2443 relative to the *fgf* gene) was inserted in the *Sma*I site of the pPD95.75 vector which was obtained from A Fire (Carnegie Institute, Baltimore, MD, USA). This clone is referred to as pJP6. It encodes a modified FGF in which the sequence encoding the last six residues NYQRYP has been replaced by the predicted 15 residue peptide GIGQRTQRRTLGGYR, read from the vector sequence.

The constructs were coinjected at respectively 10 and 30 ng/μl with pRF4 containing the rol-6 (*su*1006) dominant allele (at 110–150 ng/μl) in wild type animals for the complete gene and the *s2613* containing strain BC4253 for the truncated form. Four different transgenic strains (referred to as *fEx 50* to *53*) were obtained with pKS-RV-7 and eight strains with pJP6 (*fEx 54* to *63*). Some of these strains were tested for the presence of the transgene by genomic Southern blot and by amplification using internal primer J14 and a T3 primer contained in the vector.

Isolation and sequencing of fgf *mutant animals*

The three alleles of *let-756* were generated on a *dpy-17* (*e164*) *unc-32* (*e-189*) chromosome after a 18 mM ethyl methane sulfonate (EMS) mutagenesis for alleles *s2613* and *s2809* (Brenner, 1974); or ultraviolet irradiation (UV), 110 J/m^2 from a 30-watt Germicidal lamp (254 nm), for allele *s2887* (Stewart *et al*., 1991). Dpy Unc homozygous animals (i.e. free of *sDp3*) were isolated from strains BC4253, BC4839 and BC5147, and PCR was done on three single larvae according to Williams *et al*. (1992), using four primer pairs covering the entire *fgf* genomic region, J6-J7 (position −107 to +573 on genomic), J8-J9 (+684 to +1175 on genomic), J10-J11 (+1354 to +1881) and J12 (+2039) -J2 (see Figure 3). For alleles *s2613* and *s2809*, and the wild type, DNA was amplified from all four regions and sequenced. For the *s2887* allele, the 5′ region could not be amplified. Genomic DNA containing this allele was extracted from a mixed population of the BC5147 strain (some containing *sDp3*), digested with *Cla*I, transferred and probed with the region which could not be amplified (defined by primer pair J1-J7). Two fragments not present in a wild type digest were detected. To clone one of the rearranged fragments, the *s2887* genomic DNA digested with *Cla*I was ligated to the adaptor of the Marathon cDNA Amplification Kit (Clontech). Amplification was done using a primer in the adaptor (AP1) and a primer specific for *fgf* (J7). The second rearranged fragment was amplified using a specific *fgf* primer (J1) along with a primer located position 43616 of the R13A5 cosmid. Both products were sequenced.

Abbreviations

EMS, ethylmethanesulfonate; UV, ultraviolet. For FGF (fibroblast growth factor), we follow the Genetic Nomenclature Guide published in the 1998 special issue of *Trends in Genetics* where FGF is the human, mouse or *C. elegans* protein, and the italic *FGF* and *fgf* are the human and *C. elegans* genes, respectively.

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