Regulation of body length and male tail ray pattern formation of *Caenorhabditis elegans* by a member of TGF- β family

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SUMMARY

We have identified a new member of the TGF- β superfamily, CET-1, from *Caenorhabditis elegans*, which is expressed in the ventral nerve cord and other neurons. *cet-1* null mutants have shortened bodies and male tail abnormal phenotype resembling *sma* mutants, suggesting *cet-1*, *sma-2*, *sma-3* and *sma-4* share a common pathway. Overexpression experiments demonstrated that *cet-1* function requires wild-type *sma* genes. Interestingly, CET-1 appears to affect body length in a dose-dependent manner. Heterozygotes for *cet-1* displayed body lengths ranging between null mutant and wild type, and

overexpression of CET-1 in wild-type worms elongated body length close to *lon* mutants. In male sensory ray patterning, lack of *cet-1* function results in ray fusions. Epistasis analysis revealed that *mab-21* lies downstream and is negatively regulated by the *cet-1/sma* pathway in the male tail. Our results show that *cet-1* controls diverse biological processes during *C. elegans* development probably through different target genes.

Key words: TGF- β signaling, *Caenorhabditis elegans*, Body length, *cet-1*, *sma* pathway

INTRODUCTION

Members of the transforming growth factor- β (TGF- β) superfamily act as multifunctional ligands to control the proliferation and differentiation of a variety of cell types (Kingsley, 1994). Over the past decade, many have been shown to play essential roles during early embryogenesis in a broad range of animal species (Zhou et al., 1993; Weeks and Melton, 1987). A subfamily of these ligands, known as bone morphogenetic proteins (BMP) (Hogan, 1996), have been shown to be functionally conserved across distant species. In addition to the structural and functional conservation of the BMP ligands, the BMP receptor and signal transduction system are also highly conserved across species. Proper signaling requires complex formation of two distinct serine/threonine (ser/thr) kinase receptors, type I and type II. Upon ligand binding, a heteromeric complex of type I and type II receptor is formed. Type II receptor phosphorylates type I receptor at a specific sequence in the juxtatransmembrane region of the type I receptor, the (GS) box, resulting in signaling (Wrana et al., 1994). Activated type I receptor phosphorylates and activates intracellular component Smad protein that transduce its signal to the nucleus. (Massague, 1996; Heldin et al., 1997).

In the nematode *Caenorhabditis elegans*, at least two TGF- β -like signaling pathways exist: the dauer larva formation (*daf*) and small (*sma*) pathways. The *daf* pathway regulates dauer larva formation in response to exposure of larval animals to

starved or overcrowding conditions (Albert et al., 1981; Golden and Riddle, 1984). Dauer larva are stiff, motionless and appear to be developmentally arrested. Dauer formation-defective (*daf-d*) mutants fail to form dauer larvae under starved or overcrowding conditions. In contrast, constitutive dauer formation mutants (*daf-c*) are unable to resume a normal life cycle after the dauer-stimulating conditions are removed. Seven genes, *daf-1*, *daf-3*, *daf-4*, *daf-5*, *daf-7*, *daf-8* and *daf-14* have been proposed to act in a common signaling pathway in the regulation of dauer larva formation (Thomas et al., 1993). *daf-c* genes, *daf-1* (Georgi et al., 1990) and *daf-4* (Estevez et al., 1993) encode type I and type II ser/thr kinase receptors for the TGF- β superfamily, respectively. DAF-4 has been shown to bind to BMP-2 and BMP-4 in mammalian cells (Estevez et al., 1993). More recently, DAF-7, also a member of the TGF- β superfamily, has been reported to negatively regulate dauer larva formation. Loss-of-function *daf-7* mutants display a *Daf-c* phenotype (Ren et al., 1996). In the molecular analysis of *daf-d* genes, *daf-3*, which can suppress the phenotypes of *daf-1*, *daf-4*, *daf-7*, *daf-8* and *daf-14*, has been shown to encode an inhibitory SMAD protein (Patterson et al., 1997). In contrast, the *sma* pathway appears to control nematode body length as well as ray formation in the male tail. Mutants of *sma* such as *sma-2*, *sma-3* and *sma-4* have shortened body length (*Sma*) and male tail abnormal (*Mab*) phenotypes (Baird and Emmons, 1990). *sma-2*, *sma-3* and *sma-4* have been shown to encode proteins similar to the

SMADs identified in *Drosophila* and vertebrates (Savage et al., 1996). Interestingly, *daf-4*, which acts in the *daf* pathway also seems to be involved in the *sma* pathway. *daf-4* mutants display both *Sma* and *Mab* phenotypes. Mosaic analysis demonstrated that *sma-2* functions cell-autonomously and is downstream of *daf-4* (Savage et al., 1996). Although there has been considerable progress towards understanding the biological significance and signaling mechanisms of the TGF- β pathways in *C. elegans*, the ligand(s) that triggers the *sma* pathways has not yet been identified.

The male tail of *C. elegans* provides another model for studying TGF- β signaling. The male tail is a fan-shaped structure required for copulation, consisting of nine bilateral pairs of peripheral organs (sensory rays) formed in a precise pattern from the adult lateral epidermis (Sulston et al., 1980). The specification of ray identity has been shown to be a very complex process involving many genes. *mab-5* and *egl-5*, which encode homologues of *Drosophila antennapedia* and *abdominal-B*, respectively, were reported to be involved in cell fate determination of the sensory rays (Chow and Emmons, 1994; Cowing and Kenyon, 1996). Changes in the expression of MAB-5 protein, in particular its on-off switching, in the same cell lineage, is critical for proper region-specific patterning (Salser and Kenyon, 1996). The *mab-21* gene, which encodes a novel protein, has been shown to be involved in the regulation of ray 6 identity (Chow et al., 1995). *mab-21* mutants exhibit transformation of ray 6 to ray 4 identity, which results in subsequent ray fusion. In contrast, *sma-2*, *sma-3*, *sma-4* and *daf-4* mutants usually have rays 4-9 showing a fat morphology. Rays 4 and 5, rays 6 and 7 as well as rays 8 and 9 often fused to form fat rays (Baird et al., 1991; Savage et al., 1996). This implies that MAB-21 may be a component that can interact antagonistically with the *sma* pathway in determination of the ray morphology.

We report here the identification of a gene encoding a new TGF- β member, *cet-1*. By means of deletion mutations and genetic analysis, we demonstrate that *cet-1* is a ligand for the *sma* pathway and can regulate the *C. elegans* body length in a dose-dependent manner. We also show by epistatic analysis that the regulatory cascade of the *sma* pathway is highly conserved and negatively acts on *mab-21* during male tail patterning. We provide evidence that *cet-1* may also be involved in the *daf* pathway and will discuss the possibility of cross-talk between the *daf* and *sma* pathways at the level of ligand binding.

MATERIALS AND METHODS

General methods and strains

C. elegans strains were cultured as described by Brenner (1974) and grown at 20°C unless otherwise noted. Genetic cross were performed as described by Wood (1988). The following strains were used in this work: wild-type *C. elegans* variety Bristol strain (N2), *daf-4(m63)III*, *daf-7(m62)III*, *daf-7(e1372)III*, *sma-2(e502)III*, *sma-3(e491)III*, *sma-4(e729)III*, *lon-1(e185)III*, *mab-21(bx53)III*; *him-5(e1490)*, *him-5(e1467)V*, *dpy-11(e224)*; *unc-42(e270)V*, *unc-46(e177)*; *dpy-11(e224)V*, *him-5(e1490)*; *wxIs11*, *him-5(e1490)*; *wxIs22*, *sma-6(wk7)IV*; *him-5(e1492)V*.

Isolation of *cet-1* cDNA

A set of degenerate oligonucleotide primers were designed to target

conserved sequences present in the subfamily of TGF- β members including BMPs, Vg-1 and *dpp* (Basler et al., 1993). The forward polymerase chain reaction (PCR) primer was 5'-TGG(AG)A(AT)GA(CT)TGGAT(AT)(AG)T(AT)GC-3'; the reverse PCR primer was 5'-GT(CT)TG(GT)A(CT)(AG)AT(AGT)-GC(AG)TG(GT)TT-3'. These two primers were used in PCR for 40 cycles at 94°C for 1 minute, 50°C for 1 minute, 72°C for 1.5 minutes using *C. elegans* total cDNA as a template. The amplified PCR products were subcloned into pBluescript II KS- and sequenced by dideoxy chain-termination method with BcaBest (TAKARA). One of the 0.11 kb clones was used to screen a mixed stage *C. elegans* cDNA library to obtain a full-length clone.

Phylogenetic tree

Sequence alignment and construction of phylogenetic tree were performed by GENETIX-MAC software (SOFTWARE DEVELOPMENT CO., LTD). These results were confirmed by alignment with Pileup (GCG software), making distance tree by the method with Jukes-Cantor Distance and Kimura Two-Parameter Distance.

Germline transformation of *C. elegans*

Microinjection of DNAs into the gonadal syncytia of *C. elegans* hermaphrodites was carried out as described previously (Mello et al., 1991). Each transformation result was scored with multiple independent transgenic lines. All plasmids were injected at a concentration of 100 ng/ml. For GFP-expression, a translational *cet-1::GFP* fusion containing a nuclear localization signal was made by cloning the 4.1 kb *SphI-SalI* fragment derived from the cosmid T25F10 into the *SphI-SalI* site of pPD95.69 (a gift from A. Fire) and named pA05. For transformation, a 10:1 mixture of pA05 and pJM23 carrying *lin-15(+)* gene (Huang et al., 1994) was injected into *lin-15(n765)* hermaphrodites at a total concentration of 100 ng/ml. Non-Lin worms with normal vulvas grown at 25 were selected as transgenic animals. For rescue and overexpression experiments, a 9.3 kb *ApaI-PstI* fragment of T25F10 was subcloned into pBluescript SK- and named pA07. A high concentration (10:1) mixture of pA07 and pGFP-Myo3 and low concentration (100:1) mixture were injected into N2, *cet-1(kk3)* and *lon-1(e185)*. GFP expression was used to select for transgenic worms.

Isolation of *cet-1* null mutant

Transposable elements were targeted using a PCR and sib-selection scheme performed as described by Zwaal et al. (1993). Insertions were visualized by using nested PCR. The position of the Tc1 insertions were confirmed by multiple PCR reactions with flanking primers, followed by direct sequencing of the PCR products from both insertion junctions. Subsequently, Tc1 specific primers, Tc102 5'-AGCCAGCTACAATGGCTTTC-3', Tc103 5'-GATGCAAACGGA-TACGCGAC-3', Tc104 5'-CCAAACAAATCCAGTGCAAC-3', Tc105 5'-TGTCATTTCTTGCACCTC-3', and gene-specific primers MS7 5'-CATGGACAAACATCGGGGA-3', MS8 5'-AGGAACATTAGGAAGGAATCA-3', MS11 5'-CGTGTACAC-AAATCTGTTCG-3', MS12 5'-CGATTCTCGAGGTGGTCTCA-3', MS15 5'-GATATTCGATACGGAGACGG-3', MS16 5'-ACTCG-CAACTTCAAGAATGT-3' were used. To isolate the Tc1 insertion mutant, next primer sets were used, Tc102, Tc103, Tc104, Tc105, MS11 and MS12. To isolate the deletion allele, *kk3*, MS7, MS8, MS11 and MS12 were used. In this case, with no deletion, no band was visible but the visible band was amplified when deletion was introduced. To isolate the deletion allele, *kk4*, MS11, MS12, MS15 and MS16 were used. After isolation of the deletion mutants, they were backcrossed against N2 wild-type animals 10 times and then crossed with *dpy-11(e224)*; *unc-42(e270)V* and *unc-46(e177)*; *dpy-11(e224)V* respectively, to clean up chromosome V.

Body-length measurement of adult mutant worms

Animals were grown at 20°C. Newly laid embryos were collected over a 3–5 hour time period. 5 days later, F₁ adult animals, which were distinguishable from F₂ animals, were transferred to a new plate and anesthetized with 200 μ l of 10 mM sodium azide solution for 5 minutes. Body lengths of anesthetized animals were measured with a dissecting microscope and micrometer.

Dauer formation phenotype counts

The frequency of dauer formation was assessed under noninducing conditions essentially as described by Thomas et al. (1993). Noninducing conditions were defined as uncrowded animals on well-seeded 6 cm NG agar plates. Between 4 and 12 adult hermaphrodites were placed on a plate at the test temperature. After allowing egg laying for a limited time (less than 12 hours at 25°C), the parents were removed. As the progeny matured, the plates were checked frequently and L4 nondauers were counted and removed to prevent a new generation of eggs.

RESULTS

Isolation and characterization of *cet-1* cDNA

The structure and function of the TGF- β family ligands are known to be highly conserved among animal species, so we designed degenerate oligonucleotide primers corresponding to conserved sequences within the C-terminal region of the vertebrate BMPs (Basler et al., 1993). Using these primers, we were able to amplify a 116 bp PCR fragment encoding a BMP-related sequence from cDNA derived from mixed stages of *C. elegans*. This fragment was used as a probe to screen a mixed stages cDNA library from which one positive clone was obtained from 6 $\times$ 10⁶ plaques. Sequence analysis revealed that the clone contains a typical SL-1 sequence (Huang and Hirsh, 1989) at the 5' end. Because northern blot analysis indicated a transcript of about 1.8 kb in length (data not shown), the isolated clone of approximately 1800 bp most likely represents a full-length transcript. This clone contains a single open reading frame of 1768 nucleotides predicting a polypeptide of 365 amino acids (Fig. 1A).

To date, two TGF- β -related ligands have been reported in *C. elegans*, DAF-7 and UNC-129 (Ren et al., 1996; Colavita et al., 1998). The predicated amino acid sequence identified this gene product as distinct from DAF-7 or UNC-129 and, thus, it represents a novel member of the TGF- β superfamily in the nematode. We named this new gene as *cet-1* (*C. elegans* TGF- β -1). It contains a RRKR sequence (underlined, position 233–236), a potential cleavage site for furin-like enzymes (Dubois et al., 1995) for the release of C terminus of the polypeptide. In addition, the predicted mature protein of 129 amino acids in the C terminus contains a conserved distribution of cysteine residues that is characteristic to TGF- β family members. Comparison of the amino acid sequence of CET-1 with other members of this family revealed that CET-1 is more closely related to members of the Vg1, DPP and BMP subfamily than to the TGF- β and activin subfamilies, and closest to Nodal in the region of the predicted mature peptide (Fig. 1B). When the phylogenetic tree was depicted by computer-assisted analysis based on the amino acid sequence of the C-terminal region (which are conserved within TGF- β superfamily), CET-1 was placed closest to Nodal, although it also shares high similarity with BMP-2/4 (Fig. 1C).

We examined the ability of CET-1 to function in the *Xenopus* embryo, taking advantage of the conserved function of TGF- β related factors across species. Overexpression of *cet-1* mRNA in the ventral blastomeres of *Xenopus laevis* caused duplication of the dorsal axis (data not shown). This result classifies CET-1 with activin, Vg1, Nodal and Nodal-related (NR) for the ability to induce a secondary body axis, when overexpressed ventrally (Thomsen et al., 1990). It differs from the other group of TGF- β -like molecules including the BMP-2/4, which ventralize embryos upon overexpression dorsally (Dale et al., 1992). This axis-inducing ability of CET-1, together with the structural information, indicates that CET-1 belongs to the Nodal subfamily rather than BMP-Dpp family gene. Alternatively, overexpressed CET-1, which may not be processed properly in vertebrate, might cause a dominant negative effect on endogenous BMPs. Thus, the possibility that CET-1 belongs to BMP-2/4 subfamily cannot be completely ruled out.

We next determined the genetic and physical map positions of the *cet-1* gene. Analysis of the genomic structure showed that the *cet-1* gene consists of eight exons. Search of the *C. elegans* database revealed that the genomic sequence of *cet-1* is contained within cosmid T25F10, which has been mapped on linkage group V (Fig. 1D). However, no known mutants have yet been localized in this region.

cet-1::GFP fusion reveals its expression pattern in wild-type animals

To examine the expression pattern of *cet-1* *in vivo* at the cellular level, we generated transgenic worms expressing a *cet-1::GFP* fusion gene (see Materials and Methods). Transgenic worms expressing this fusion protein under regulation of the *cet-1* promoter displayed green fluorescence in many neurons, including some amphid neurons and neurons in ventral nerve cord (VNC) (Fig. 2A). The cell bodies of VA, VB, DA and DB neurons were detected with fluorescence (Fig. 2B). The amphid neuron identified was AFD. Body wall muscles and neurons in the pharyngeal region were also detected (Fig. 2C). Signal was detected in cell bodies of another neuron, the canal associated neuron (CAN) (Fig. 2D), and DVA neuron at the anal region of the tail (Fig. 2E). Male-specific expression was observed in the neurons of the tail region (Fig. 2F). This expression pattern persisted through L1 to adult hermaphrodite. Interestingly, CET-1 is broadly expressed in neurons in contrast to DAF-7, which is thought to act as a neurotransmitter and whose expression is restricted to amphid neuron ASI (Ren et al., 1996; Schackwitz et al., 1996).

cet-1 null mutant shows small phenotype

To investigate the functional role of *cet-1*, we utilized the strategy of gene disruption by insertion of a Tc1 transposable element in the *cet-1* region. Subsequent excision and transposition of Tc1 would likely result in a deletion of the functional domains of the targeted gene (Zwaal et al., 1993). We screened pools of potential mutants for successful insertion of the Tc1 transposon in the region of *cet-1* gene by polymerase chain reaction (PCR) using specific oligonucleotide primers. Fig. 3A shows one case in which Tc1 was inserted into the 5' region 4081 bp upstream of the first methionine of the *cet-1* coding region. We then screened for worms in which transposition of Tc1 caused deletions that might affect the

To get further insight into the regulation of the *C. elegans* body length, we compared the body lengths of 5-day-old hatched hermaphrodites of N2 wild type and various mutants. In wild type and *daf-c* mutant, the body lengths ranged from 1.27 to 1.28 mm, respectively (Table 1). In the small phenotype mutants including *daf-4*, the body lengths of corresponding adult worms fell in the range of 0.71 to 0.81 mm. *sma-2(e502)* and *sma-3(e491)* have missense mutations in which an essential highly conserved glycine residue is substituted with

CAC	TGC	CGCGCCGCTG	CGACCAAG	TTT	GAGCCTG	ATTATG	CGCTAA	ACAAGAGG	AGAGATG	CTCATCGG	CGAGCCCC	CGCAAATAG	AAGT	90																		
CCT	TGT	CTCTGT	CCC	GT	TTCC	CTTTT	TGTCCA	ATCTGT	TTCCAC	ATTCCT	CACGATAT	GTCCAG	TGGCTGC	CTGATG	CGCCCA	ACTCA	ACT	180														
ATGA	ACG	ACTCTGT	GCGG	ACA	ACTACA	ACGATTT	CAAGCA	AAAA	TCC	TTGTT	CACAG	CTTCCA	ACTGT	C	CGCC	ATTCT	CCAC	CTT	270													
M	N	D	S	V	R	T	T	T	T	I	S	S	T	K	S	L	V	H	S	F	Q	L	S	A	I	L	H	L	F	30		
CTC	TCAT	CTC	CTT	CAC	ACCA	ATGT	CTG	CTG	CGC	GATCA	ACACG	CGTCC	CAC	GCTAC	CCG	GACG	CGG	TTG	CTT	CGG	AAG	T	TGG	GACTC	360							
L	L	I	S	F	T	P	M	S	A	A	A	D	Q	H	A	S	H	A	T	R	R	G	L	L	R	K	L	G	L	60		
GAG	CAG	TAC	CTGT	G	CAG	ACTGG	TCC	GAG	CATTG	ATG	T	TCC	ACA	ACAC	ATG	TGG	GATAT	T	TAT	G	AC	GATG	ATA	TG	ATG	T	G	ACT	GGG	T	450	
E	H	V	P	V	Q	T	G	P	S	I	D	V	P	Q	H	M	W	D	I	Y	D	D	D	N	D	V	D	W	V	90		
AG	ACT	ATTAT	CCGA	AGG	AGATT	TATTG	AAG	ATA	ACGA	AGGG	TCTT	GTG	TCTT	A	ACCT	CTC	ATT	GGCT	GCT	CG	AA	TGCT	CACA	AT	540							
R	H	Y	Y	P	K	E	I	I	E	D	N	E	G	F	L	L	S	Y	N	L	S	L	A	A	R	N	A	H	N	120		
GA	AAG	TGACTA	AGG	CTACT	TTG	AA	ACTCC	G	TTAC	G	ACG	CA	ACA	CA	AA	AG	C	GAG	GAG	G	TCC	G	CA	ATAT	TAG	TAT	T	T	T	T	630	
E	E	V	T	K	A	T	L	K	L	R	L	R	R	N	N	K	A	R	R	S	G	N	I	S	I	Y	F	F	E	150		
GAT	GAT	ATAA	ACA	ATGAT	CGATT	T	CAA	ATC	GAT	CCG	T	CG	ACA	AC	CTTAC	CGA	AT	GGAT	CGATT	T	T	GAT	GTG	AC	CG	CG	CT	T	T	720		
D	D	I	N	N	D	R	F	Q	I	E	S	R	S	V	D	N	L	T	E	W	I	D	F	D	V	T	A	A	F	180		
TTG	CGG	CGA	ACA	AA	TCGT	ATCAG	T	T	TC	GATCG	ATT	TG	CGG	AAG	ATGT	GG	AG	ACG	GAAG	AGAC	ACAG	AG	CAG	CTC	ACT	G	AG	CT	CATT	G	810	
L	R	R	T	N	R	I	S	F	C	I	D	L	P	E	D	V	E	T	E	E	T	Q	S	S	S	L	S	S	L	210		
CCT	ACG	CGCG	GGTCT	CAG	AGT	GCT	CCG	CTTAT	TGT	GTT	CAG	TG	AC	TGT	CCG	A	CCG	TCC	AGT	GTG	CGG	CGG	AA	CG	GAG	TCG	ACA	AA	CA	900		
P	T	R	G	S	Q	S	A	P	L	I	V	F	S	D	L	S	E	P	S	S	V	<u>R</u>	<u>R</u>	<u>K</u>	<u>R</u>	S	R	Q	T	240		
GG	CA	AC	AG	CGA	ACG	AAAA	ATCG	AAAA	AGGG	TAG	AA	AG	CA	T	CATA	AC	ACC	GAG	GCCG	AG	AG	CA	ATC	T	T	TG	T	CG	GAG	GACT	GATT	990
G	N	S	E	R	K	N	R	K	K	G	R	K	H	H	N	T	E	A	E	S	N	L	C	<u>R</u>	<u>R</u>	<u>T</u>	<u>D</u>	<u>F</u>	<u>Y</u>	270		
GTG	GAT	TTT	TGAT	GATT	TAA	ATTGG	CA	AG	ACT	TG	GATTAT	TG	CA	CCCA	AGGG	CTAC	GAT	GCTT	AT	CA	ATG	T	CA	AGG	CTCT	T	TG	CCAA	AT	CC	1080	
V	D	F	D	D	L	N	W	<u>Q</u>	<u>D</u>	<u>W</u>	<u>I</u>	<u>M</u>	<u>A</u>	<u>P</u>	<u>K</u>	<u>G</u>	<u>Y</u>	<u>D</u>	<u>A</u>	<u>Y</u>	<u>Q</u>	C	<u>Q</u>	<u>G</u>	<u>S</u>	C	<u>P</u>	<u>N</u>	<u>P</u>	300		
ATG	CCAG	CTCAG	TAA	ATG	CA																											

Fig. 1. Structural analysis of *cet-1*. (A) Nucleotide and predicted amino acid sequences of *cet-1* cDNA. Nucleotide and amino acid number are indicated at the right. The seven cysteine residues conserved among the TGF- β superfamily members are boxed. The degenerate oligonucleotide primers that were used for cloning of *cet-1* are underlined. The potential N-linked glycosylation site is indicated by double underline. GenBank accession number for *cet-1* is AF074395. (B) Alignment of the C-terminal amino acid sequences of CET-1 and representative members of the TGF- β superfamily. Residues that are conserved in at least 3 of 5 proteins are shaded and the seven conserved cysteine residues are marked with asterisks. Gaps introduced to optimize the alignment are represented by dashes. (C) A phylogenetic tree was constructed based on the amino acid sequence similarities of the C-terminal region after the first cysteine residue in the C termini. (D) Physical and genetic maps of the *cet-1* region. The top line is the gene structure with exons represented by boxes, separated by introns. The second line depicts the restriction map of the genomic cosmid clone T25F10. Restriction sites are shown for *Eco*RI (E), *Eco*RV (V), *Hind*III (III), *Kpn*I (K), *Sac*I (S), *Xba*I (Xb) and *Xho*I (Xh). The third line is the physical map indicating the location of cosmid clones. The fourth line is the genetic map of *cet-1* on linkage group V.

other amino acids, resulting in strong loss-of-function alleles (Hoodless et al., 1996). Therefore, it is possible that the shortened body lengths of *sma-2*, *sma-3* and *sma-4* mutants are due to the decrease in Smad signals to almost background level, similar to our *cet-1(kk3)* null mutant. *daf-c* background does not seem to affect the body length, because the body lengths of *daf-7(m62)* mutant are nearly wild type, and those of *daf-7(m62); cet-1(kk3)* double mutant are indistinguishable from *cet-1(kk3)* alone.

We also examined whether a plasmid construct encoding the genomic DNA that covers the region deleted in *cet-1(kk3)* could rescue the null mutant upon DNA injection. The shortened body length distinctive of the *Sma* phenotype was rescued to normal length by overexpression of exogenous *cet-1* gene in a dose-dependent manner (Table 1). When injection of a high concentration of the *cet-1* plasmid was performed, rescued animals, *cet-1(kk3); kkEx8[*cet-1*(+) 10:1]*, had an average rescued body length of 1.15 mm. However, when injection was performed at a lower dose, rescued animals, *cet-1(kk3); kkEx9[*cet-1*(+) 100:1]*, were average of 1.04 mm in

length. In addition, overexpression of *cet-1* in wild-type animals resulted in a *lon* (Long) phenotype (average body length of 1.43 mm) resembling the elongated body seen in *lon-1* and *lon-2* mutants. Furthermore, overexpression of *cet-1* in *lon-1* mutants resulted in even longer worms, although with quite low viability. Heterozygous *cet-1* mutant worms (*cet-1/+*), in which the dosage of *cet-1* is half the level of wild type, are shorter in length (1.12 mm) than wild type, but longer than the *cet-1* homozygous (*cet-1/cet-1*) mutant. Taken together, these results suggest that CET-1 can control the body length of *C. elegans* in a dose-dependent manner.

To determine the qualitative changes in body length, we measured the individual lengths of each body region. Comparison of *cet-1(kk3)* mutants with N2;*EX10[*cet-1*(+)]* worms shows an overall change in body length of almost twofold (0.75 to 1.43 mm). Interestingly, the length of the pharynx and gonad was not drastically affected; the relative lengths of the pharynx to total length were 9 and 13% in *cet-1(kk3)* and N2;*EX10[*cet-1*(+)]*, respectively. The measurement is comparable to that of wild-type N2 (11%). In contrast, the

B

C

D

Table 1. Body length of mutants

Mutant	Body length (mm)*	n‡
N2	1.28±0.03	85
<i>kk4</i>	1.27±0.04	67
<i>daf-7(m62)</i>	1.28±0.04	68
<i>cet-1(kk3)</i>	0.75±0.03	103
<i>sma-2(e502)</i>	0.73±0.03	57
<i>sma-3(e491)</i>	0.71±0.03	134
<i>sma-4(e729)</i>	0.79±0.02	86
<i>daf-4(m63)</i>	0.82±0.05	75
<i>daf-7(m62); cet-1(kk3)</i>	0.76±0.04	88
<i>cet-1/+</i>	1.12±0.04	48
§ <i>cet-1(kk3); kkEx8[cet-1(+)] 10:1</i>	1.15±0.05	87
¶ <i>cet-1(kk3); kkEx9[cet-1(+)] 10:1</i>	1.04±0.10	100
N2; <i>kkEx10[cet-1(+)]</i>	1.43±0.04	58
<i>lon-1(e185)</i>	1.45±0.06	87

Body length were measured of various mutants of 5-day-old hatched adult hermaphrodite, with objective microscope and micrometer (see Materials and methods).
*Data are means±s.d.
‡Numbers of measured animals.
§High concentration of plasmids of *cet-1* rescuable fragment were injected.
¶Low concentration of plasmids of *cet-1* rescuable fragment were injected (see Materials and methods).

length of pharynx to gonad, and gonad to anus were remarkably expanded 5- to 10-fold between *cet-1(kk3)* and N2;*EX10[cet-1(+)]* (2 to 22% and 3 to 19 %, respectively) (data not shown). It is noteworthy that there was no change in number of nuclei as revealed by DAPI staining but, rather,

nuclei appeared to be dispersed in the region (data not shown). This observation suggests the hypodermal cells that form the synticium changed their volume but not their number (see also Discussion).

***cet-1* function requires wild-type *sma* genes**

Because the null mutant of *cet-1* shows an indistinguishable phenotype from those of *sma-2*, *sma-3* and *sma-4*, we carried out an epistatic analysis by injecting the genomic fragment capable of rescuing *cet-1* into *sma-2*, *sma-3* and *sma-4* mutants. The *cet-1* genomic fragment failed to rescue the *sma-2*, *sma-3* and *sma-4* mutant phenotypes (data not shown). These results indicate that CET-1 likely acts upstream of SMA-2, SMA-3 and SMA-4. *cet-1* function, either in wild-type background or when it is overexpressed, requires the SMAD proteins to transduce the signal.

***cet-1* null mutant shows male tail abnormality**

In addition to the shortened body length, *cet-1(kk3)* mutants exhibited abnormalities in the male tail. Crumple spicules were found in almost all the males (38/40, data not shown). More profound phenotype was also observed in the patterning of the sensory rays (Fig. 3C,D). The majority of tails had fusions of rays 4 and 5, rays 6 and 7 as well as rays 8 and 9 resulting in fat fused rays (Fig. 3D). This ray fusion was often observed on both sides of the body (Table 2). In rare cases, multiple fusions at 4 and 5, 6 and 7, and 8 and 9 occurred laterally. These phenotypes are also reminiscent of those in *sma-2*, *sma-3*, *sma-4* and *daf-4* mutants. However, the higher frequency and severity of ray fusions seen in the *cet-1(kk3)* mutant, as

Fig. 2. Expression pattern of CET-1 as studied with a *cet-1::GFP* fusion gene. (A) Fluorescence image of L2 larva. Lateral view. Strong signals are seen in the ventral nerve cord (VNC) and canal-associated neurons (CAN). (B) *cet-1::GFP* expression in the VNC and (C) in the head of adult animals. Body wall muscles (arrowhead), pharyngeal neurons and amphid neuron, AFD show GFP fluorescence. (D) Cell body of a CAN (arrow). (E) Tail region of adult hermaphrodite. VNC (arrowhead) and DVA neuron (arrow) are positive. (F) Tail region of L4 male. Two pairs of spicule associated neurons (arrow) and DVA neuron (arrowhead) are detected. All images are taken using fluorescence microscopy. Anterior is to the left and dorsal is up except F), which are ventral view. Scale bars (A) 50 µm, (B-F) 20 µm.

compared to those of known *sma* pathway mutants, suggests that *cet-1* has more severe effect than single mutations of *Smad* genes.

mab-21* is epistatic to genes in the *sma* pathway and is negatively regulated by *cet-1

In *sma* pathway mutants, rays 4, 5 and 7, which are normally thin rays, become fat rays like ray 6. Fusion between these rays are observed frequently. Mutation of *mab-21* results in specific transformation of ray 6 into a more anterior ray 4. The displacement of the transformed thin ray 6 leads to the formation of a very thick ray in which both thin rays 6 and 4 are fused (Baird et al., 1991; Chow et al., 1995). This transformation appears opposite to the phenotype of mutants in the *sma* pathway. To analyze the relationship between *sma* and *mab-21*, we constructed double mutants of *sma* pathway mutant and *mab-21*. Table 3 shows that *mab-21* is epistatic to all of the *sma* pathway mutants for ray pattern formation, indicating that *mab-21* is downstream of the *sma* pathway and is negatively regulated by *cet-1*. In the absence of *mab-21* product, the generation of thick ray in the small mutants could not be accomplished. However, it is interesting to note that, while *mab-21* mutant phenotype suppressed the Mab phenotypes of *sma* pathway genes, the body length of the double mutants remained short. These results suggest that sensory ray patterning and body length regulation are acting through the *sma* pathway, but bifurcate at the control of their target genes.

Because *mab-21* is necessary for determination of the identity of rays (Chow et al., 1995), we analyzed the expression pattern of *mab-21::GFP* in wild-type and *cet-1* null mutant backgrounds to see if expression level or pattern is altered in the mutant. *mab-21* reporter gene is expressed in the structural and hypodermal cells of rays 2 to 7. In the *cet-1* null mutant background, the expression pattern was not altered as compared with wild type (data not shown). While no significant increase of gene activity was detected with the reporter and no additional cell expressing the reporter gene was noticed, the results suggest that the mechanism by which the *cet-1* signal impinges on *mab-21* activity may not be acting at the transcriptional level.

CET-1 interacts with the *daf-7* TGF- β pathway

As mentioned above, there are two distinct TGF- β pathways in *C. elegans*. To examine whether the *cet-1/sma* pathway cross-talks with the *daf* pathway at the level of ligand-receptor interaction, we generated double mutants of *cet-1* and *daf-7*. Dauer formation of the *daf-c* mutant is stimulated at non-permissive temperature even under healthy growth and feeding conditions. *daf-*

Fig. 3. *cet-1* null mutant. Inactivation of *cet-1*. To isolate the *cet-1* null mutant, we used targeted gene inactivation by screening for insertion and deletion of Tc1 transposable elements in the *cet-1* region using PCR. (A) The position of the Tc1 insertion in *cet-1* is indicated above the genomic structure (a). (Tc1 was inserted 4081 bp upstream of the first methionine of *cet-1*.) *kk4* was identified to be a 1846 bp deletion of *cet-1* 5' upstream region (b). *kk3* was identified to be a 5728 bp deletion that extended from the Tc1 insertion site to encompass the entire *cet-1* coding region (c). (B) Photomicrographs of adult hermaphrodite 5 days after hatching. N2; *kkEx10[cet-1(+)]* (top), N2 wild type (middle) and *cet-1(kk3)* mutant (below). Lateral view. Anterior is to left and dorsal is up. Scale bars, 100 μ m.; bright-field illumination. (C) Male tail phenotype of adult *him-5(e1467)* animal. (D) Male tail phenotype of *cet-1(kk3); him-5(e1467)* double mutant. Rays 4 and 5, ray 6 and 7 as well as ray 8 and 9 are fused with each other, respectively. (C,D) Photographs of male tails are from Nomarski differential interference contrast microscopy. Scale bars, 10 μ m.

Table 2. Frequency of male tail ray fusions in mutants (%)

Mutant	Ray 1	Ray 2	Ray 3	Ray 4	Ray 5	Ray 6	Ray 7	Ray 8	Ray 9	n*
<i>cet-1(kk3)</i>	0	0	0	46	47	85	85	18	18	153
<i>sma-2(e502)</i>	0	0	0	22	22	70	70	12	12	86
<i>sma-3(4491)</i>	0	0	0	18	19	54	53	11	11	74
<i>sma-4(e729)</i>	0	0	0	30	30	68	68	9	9	53
<i>daf-4(m63)</i>	0	0	0	24	30	63	63	15	13	46

The frequency in percentage at which each ray is fused with another ray.
*Numbers of sides scored.

7 mutants show Daf-c phenotype and constitutively form dauer larvae at 20° and 25°C (Table 4). At 20°C, 21% of both *daf-7(e1372)* and *daf-7(m62)* mutants formed dauer larvae and this dauer formation was enhanced in the double mutants of *daf-7(e1372); cet-1(kk3)* and *daf-7(m62); cet-1(kk3)* at a frequency of 77 and 56%, respectively, suggesting that *cet-1* may also be participating as a suppressor of dauer formation in combination with *daf-7*. To examine whether this interaction occurs at the ligand-receptor level or the intracellular signaling level, downstream of the receptor, we constructed *daf-7; sma-2* double mutants. Since *sma-2* is known to act as an intracellular signaling component of the *sma* pathway, we hypothesize that, if the interaction between *cet-1* and *daf-7* acts through the intracellular signals, *daf-7; sma-2* double mutants should also result in an increased penetrance of the Daf phenotype at 20°C. Table 4 shows that the interaction between *daf-7* and *sma-2*, if it is present, is a rather weak one. It argues that *cet-1* is interacting with the *daf* pathway extracellularly at the ligand receptor level. This in turn suggests that CET-1 may interact with a complex of receptors comprising of DAF-1, DAF-4 or SMA-6 and participate in the regulation of *daf* pathway. However, we cannot rule out the potential presence of cross-talk occurring within the cell at the level of SMAD proteins.

DISCUSSION

CET-1 is a ligand in the *sma* pathway

In the past several years, genetic studies in *C. elegans* have identified at least 5 components, *sma-2*, *sma-3*, *sma-4*, *sma-6* and *daf-4*, involved in the *sma* pathway (Savage et al., 1996; R. W. Padgett, personal communication). However, the ligand(s) that triggers the signaling pathway was unknown. In this study, we show that a newly identified gene, *cet-1*, encodes

a ligand for the *sma* pathway. We were able to demonstrate that its null mutation resulted in *Sma* and *Mab* phenotypes reminiscent to mutants of *sma-2*, *sma-3*, *sma-4*, *sma-6* and *daf-4*. The same phenotype was also observed independently in two other *cet-1* alleles (Y. Suzuki and W. B. Wood, personal communication). In addition to the *sma* pathway, recent studies have identified components that mediate the *daf* pathway: DAF-7 as a ligand, DAF-1 as a type I receptor, DAF-4 as a type II receptor, and DAF-3 as SMADs. It appears that DAF-4 functions as a common type II receptor for both *sma* and *daf* pathways. By analogy with the dual role of activin type II receptors IIA (ActRIIA) and IIB (ActRIIB) being utilized in both activin and BMP pathways in vertebrates (Schulte-Merker et al., 1994; Oh and Li, 1997), DAF-4 has been suggested to function similarly in mediating signals from multiple ligands. DAF-7 has 9 conserved cysteine residues in the predicted mature peptide, which place it in the TGF-β or activin subfamily (Ren et al., 1996). In contrast, CET-1 has only 7 cysteine residues, like the BMP *dpp* subfamily members. In this regard, it is interesting to note that the dual specificity of the common type II receptor in recognizing two types of ligand, is also conserved between *C. elegans* DAF-4 and vertebrate ActRIIs.

Taken together, we propose that, in *C. elegans*, there are at least two TGF-β-like pathways which are triggered by distinct ligands, DAF-7 and CET-1. Each of these pathways uses a pathway-specific type I receptor, a common type II receptor, DAF-4, and receptor-specific SMAD-like components (Fig. 4). In this study, we have demonstrated a genetic interaction between *daf-7* and *cet-1*, suggesting that the CET-1 ligand is not exclusively utilized for the *sma* pathway but can also participate in the *daf* pathway, if it is minimal (arrow with broken line, Fig. 4). Such a functional redundancy of the TGF-β family ligands has previously been reported for activin and

Table 3. Epistasis analysis of *mab-21* with *sma-2*, *sma-3*, *sma-4*, *sma-6*, *daf-4* and *cet-1*

	Male tail phenotype	
	Thin ray 6, ray 4, 6 fusion (Mab-21)	Thick ray 4,5,6,7 (cet-1/Sma/Daf-4)
<i>mab-21(bx53); him-5(e1490)</i>	+(>500*)	—
<i>mab-21(bx53); sma-2(e502); him-5(e1490)</i>	+(350)	—
<i>mab-21(bx53); sma-3(e491); him-5(e1490)</i>	+(300)	—
<i>mab-21(bx53); sma-4(e491); him-5(e1490)</i>	+(300)	—
<i>mab-21(bx53); daf-4(e491); him-5(e1490)</i>	+(250)	—
<i>mab-6(wk7); mab-21(bx53); him-5(e1490)</i>	+(150)	—
<i>mab-21(sy155); cet-1(kk3); him-5(e1467)</i>	+(100)	—

*Numbers of animals scored given in parentheses.

Table 4. Dauer formation of *daf-7; cet-1(kk3)* double mutants

Genotype	Percentage of dauer formation	
	20°C	25°C
<i>daf-7(e1372)</i>	21 (777)*	100 (769)
<i>daf-7(m62)</i>	21 (624)	100 (720)
<i>daf-7(e1372); cet-1(kk3)</i>	77 (673)	100 (606)
<i>daf-7(m62); cet-1(kk3)</i>	56 (595)	100 (279)
<i>daf-4(m63)</i>	63 (364)	100 (432)
<i>cet-1(kk3)</i>	0 (480)	0 (226)
<i>daf-7(e1372); sma-2(e502)</i>	40 (206)	100 (160)
<i>daf-7(m62); sma-2 (505)</i>	29 (231)	100 (202)

*The percentage of animals forming dauers is given, with the total number of animals counted given in parentheses.

Fig. 4. Two distinct TGF- β signal pathways in the nematode *C. elegans*. Two distinct signaling cascades, the *daf* pathway (regulation of dauer formation) and the *cet-1/sma* pathway (regulation of body length and male tail ray pattern formation) have been identified. A weak functional redundancy was observed between the *daf* and *cet-1/sma* pathways (dashed arrow). *mab-21* appears to be negatively regulated by *cet-1/sma* pathway in the pattern formation of male tail rays.

OP-1 (BMP-7), both of which are capable of inducing dorsal mesoderm in animal caps of early *Xenopus* embryo through a common type I receptor ALK2 (ActRI) and ActRIIA (Yamashita et al., 1995).

How body length is determined by CET-1

It is intriguing to find that body length in *C. elegans* can be controlled by CET-1 in a dose-dependent manner. Namely, the extent to which the shortened body length of *cet-1* mutants could be rescued was dependent upon the amount of exogenous *cet-1* introduced. In addition, *lon* mutants as well as N2 wild-type worms could be elongated by overexpression of CET-1. Absence or reduction of the CET-1 level to half of that in the wild-type animal also resulted in shortening of body length proportionally. The exact molecular mechanism by which body length is determined remains to be solved. However, it is clear that a diffusible signaling molecule can act as modifier of the developmental regulation. The total amount of CET-1 ligand present in an individual worm might guide the determination of the body length.

The use of the *cet-1::GFP* fusion construct allowed observation that CET-1 expression in hermaphrodites is mainly neuronal, in the amphid neurons and ventral nerve cord, and that this expression remains consistent throughout the four

larval stages and in adults. The DAF-4 type II receptor, an essential component of the *sma* pathway, is broadly expressed in many anterior pharyngeal and tail neurons, ventral nerve cord, intestine and pharynx as revealed by *daf-4::GFP* (Patterson et al., 1997). This observation is consistent with the idea that this receptor is used not only in the *sma* but also in the *daf* pathway. It is therefore unlikely that DAF-4 expression would determine the regional specificity of CET-1 activity. In contrast to DAF-4, *sma-6::GFP* is expressed mainly in anterior pharyngeal and tail hypodermis (R. W. Padgett, personal communication). Given that the signaling specificity of the TGF- β pathway is determined by type I receptors (Wrana et al., 1992), regional activation of the *sma* pathway may be determined by restricted expression of the SMA-6 type I receptor. However, hypodermal cells are known to form syncytium. Therefore, signals from the receptor might also be regionally restricted by localized effectors downstream of SMA-6. We thus hypothesize that the target cells lie in the hypodermis of anterior pharyngeal and tail regions where SMA-6 is expressed.

The next question to address is why the body length is controlled by the amount of CET-1. Morphological changes, including alteration of body length, often attribute to defects in cuticle formation. For example, mutations in *dpy-2*, *dpy-7* and *dpy-10*, all of which encode collagen (Levy et al., 1993; Johnstone et al., 1992), a major component of cuticle, result in a short body length and dumpy phenotype. These results suggest that cuticle formation might be altered depending on the dosage of CET-1 ligand. CET-1 belongs to the dpp/Vg1 subfamily, members of which are poorly diffusible when examined in cultured cells. They have the tendency to be retained on the surface of expressing cells due to attachment to extracellular matrix (Panganiban et al., 1990). In *Drosophila* wing discs, however, DPP can act as a morphogen and influence the behavior of cells positioned over 20 cells away. This response is thought to be due to the direct action of the secreted DPP protein (Nellen et al., 1996), although it might also involve cell proliferation and cell communication. In our case, while the target tissue is a hypodermal syncytium, the CET-1 can act through extracellular diffusion to reach the specific receptor. Diffusion of the intracellular signaling components, such as the SMAD proteins, can also facilitate further propagation of developmental signal within regional domains of the hypodermal sheath. The proportional alteration of the body length in specific region in mutant animals would certainly argue for this notion.

Male tail ray pattern formation

mab-21 acts as a component that regulates pattern formation of male tail ray by directing seam cells to become specific ray during the late L3 and early L4 larval stages (Chow et al., 1995). We demonstrate that *mab-21* acts downstream of the *sma* signaling pathway and is negatively regulated by *cet-1*. In the male tail of double mutants of *mab-21* and *sma* genes, only *mab-21* mutant ray pattern was displayed. This fat-to-thin ray transformation phenotype in the absence of *sma* pathway genes implies that the fat ray morphology requires functional *mab-21* gene product. Our results on the *mab-21* reporter gene expression argues that the negative regulation of *mab-21* by *cet-1* may be at the post-transcriptional level. In wild-type animal, *mab-21* expression is present in the V6 rays and ray 7.

Among these, only ray 6 can bypass the repression by the *cet-1* signal and take up a fat morphology. In the other rays, however, *mab-21* function is suppressed by the *cet-1* signal. Thus, they all take up a thin morphology. Upon mutations of the *sma* pathway components, the repression is relieved, and upregulation of the *mab-21* biological activity leads to a thin-to-fat morphology transformation. However, in the absence of *mab-21* gene function, the repressing signal becomes irrelevant. All the rays become thin. What makes ray 6 different from the rest? Although it remains speculative, *mab-5*, *egl-5* and *mab-18* (Chow and Emmons, 1994), which encode *antennapedia*, *abdominal B* type homeobox-containing proteins (Wang et al., 1993) and a *Pax-6* homologue (Zhang and Emmons, 1995), respectively, would be strong candidates that help define the uniqueness of ray 6 in response to the repressing signal.

mab-21 gene is highly conserved in various animal species from vertebrates to invertebrates (Margolis et al., 1996; K. L. C., unpublished data). In vertebrates, its expression is most prominent in the brain and, in *C. elegans*, *mab-21* is expressed mainly in neuron, hypodermis and ray cells. These observations suggest that *mab-21*, which is acting downstream of TGF- β -type signal, may be involved in the regulation of neuronal/hypodermal development. In addition, *mab-21* is thought to be the functional modulator of the homeobox genes *mab-5*, *egl-5* and *mab-18*. Since Hom-C/Hox genes are the key players in cell fate decision and body patterning, our results suggest that male tail may provide a good model system to examine how upstream regulatory signals like the *cet-1* ligand can pattern the sensory rays through these homeodomain-containing transcription factors and their modulators.

Bifurcation and convergence of signals

From the epistatic analysis, *mab-21* mutant phenotype suppresses the ray patterning defects of the *sma* pathway mutants. However, suppression does not occur with regard to the body length abnormality. Our results argue that body morphology regulation and the ray patterning are two distinct processes in normal nematode development. The specificity of these biological responses is not modulated by the signaling molecules. These processes act through a common extracellular ligand secreted from neurons, common receptors and intracellular SMAD molecules in the target cells. It is through different target genes present in these cells at a specific time frame that alternative but specific responses are generated.

Variation on expressivity of phenotype of mutant signaling molecule can be found in the *daf* pathway. Null mutant *daf-7* strain exhibits a temperature-sensitive Daf-c phenotype. It suggests more than only the temperature sensitivity of wild-type dauer formation process (Golden and Riddle, 1984) but also the potential presence of an alternative dauer inductive signal. Although *cet-1* mutation alone gives no indication of its role in dauer formation, our double mutant analyses clearly illustrate that *cet-1* can be that second signal (see Table 5). In fact, a similar phenomenon is also found in ray patterning. While the degree of ray fusions in *cet-1* null mutants are significantly higher than those of *sma-2*, *sma-3*, *sma-4* and *daf-4* mutants, the severity still varies among individuals. It implies that an alternative ray patterning signal may be present. The recent identification of a novel TGF- β like ligand UNC-129 (Colavita et al., 1998) eludes to the presence of a related

molecule that may act in concert with *cet-1* in certain developmental processes, although their cooperative functionality remains to be tested.

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