A study on the tissue specificity of DBL-1 signaling pathway that regulates body length in *Caenorhabditis elegans*

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2001

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ABBREVIATIONS

BMP	: bone morphogenetic protein
dbl-1	: dpp, BMP-like
C. elegans	: *Caenorhabditis elegans*
CAN	: cnal -associated neuron
daf	: dauer larva formation abnormal
daf-c	: constitutive dauer formation
daf-d	: defective dauer formation
DNA	: deoxyribonucleic acid
dpp	: decapentaplegic
dpy	: dumpy
dsRNAi	: double stranded RNA interference
egl	: egg laying defective
GFP	: green fluorescent protein
LG	: lateral ganglia
lon	: long
mab	: male tail abnormal
mRNA	: messenger RNA
PCR	: polymerase chain reaction
RGV	: retro-vesicular ganglion
RNA	: ribonucleic acid
sma	: small
TGF-β	: transforming growth factor-β
VNC	: ventral nerve cord
VG	: ventral ganglion

SUMMARY

Size control is one of the fundamental subjects of biology. In nematode *Caenorhabditis elegans*, a TGF-β-like signaling pathway, Sma pathway, which is composed of the ligand DBL-1, serine/threonine protein kinase receptors SMA-6 and DAF-4, and cytoplasmic signaling components SMA-2, SMA-3, and SMA-4, regulates body length of the worm. To further address the molecular mechanism of body length regulation in the nematode by the TGF-β-like signaling pathway, I examined the regional requirement for the type I receptor SMA-6. Using a SMA-6::GFP (green fluorescent protein) reporter gene, I found *sma-6* to be highly expressed in the hypodermis in addition to pharynx and intestine, while the type II receptor DAF-4 is reported to be more broadly expressed. I then examined the ability of SMA-6 expressed in different regions of the *C. elegans* body to rescue the *sma-6* phenotype (small) and found that hypodermal expression of SMA-6 is most important and sufficient for the growth and maintenance of body length. I also show that GATA sequences in the *sma-6* promoter contribute to the hypodermal expression of *sma-6*. Finally, I show that the DBL-1 signaling in middle larval stage is significant to control body length.

INTRODUCTION

Studying size control using C. elegans

How the size of an organism is determined is a fundamental aspect of biology. In spite of its importance, little attention had been paid for size control; it remains as mysterious as ever. The number and size of cells and the amount of extracellular matrix and fluid should have considerable impact on the size control of an animal. Although the changes in cell size and cell numbers during development may mainly contribute to the final size of an organism, it had been widely accepted that the size of an animal generally reflects the numbers of cells, but not size of cells.

Cell numbers depend on both cell proliferation and survival, which, as well as cell size, depend on both intercellular programs and extracellular signaling. These signaling molecules may regulate cell growth, cell division, and cell survival acting in endocrine, paracrine, or autocrine mode (reviewed in Conlon and Raff, 1999).

The transforming growth factor-β (TGF-β), for example, may be one such extrinsic factor. Members of TGF-β superfamily of secreted ligands play a central role in the signaling network regulating cell growth and proliferation (reviewed in Massague *et al.*, 2000). Indeed, several mutations in a TGF-β-like signaling pathway in *C. elegans*, Sma/Mab pathway, influence body length of the worm. Mutants of the pathway components, ligand, receptors, and cytoplasmic transducers, are smaller than normal (Estevez *et al.*, 1993; Krishna *et al.*, 1999; Morita *et al.*, 1999; Savage *et al.*, 1996; Savage-Dunn *et al.*, 2000; Suzuki *et al.*, 1999). As *C. elegans* have so many aspects of animal development in common, the investigation using *C. elegans* as a model organism, that is the

identification and studies using mutants of the genes, which involved in size control, is likely to provide the great leads.

Introduction to C. elegans as a model organism

C. elegans is a member of nematode. In the 1960s, Sydney Brenner took up the worm as a model organism to study the development, behavor and neurobiology genetically. The worm is a primitive organism, which shares many of the essential biological characteristics. Embryogenesis, morphogenesis, development, nerve function, behavor, aging and body size control, and how they are determined by genes. These are the fundamental mysteries of modern biology. *C. elegans* exhibits these phenomena, yet is only 1 mm in length. It produces sperm and eggs, mates and reproduces. A fertilized egg starts embryonic cleavage, then takes a form of worm through a complex process of morphogenesis and development, and growths to an adult through four larval molts (Figure I A). The generation time is about 3 days at 20°C and the life span is about 2-3 weeks. *C. elegans* is free-living nematode, lives and feed microbes in soil. In the laboratory, the worm can easily be handled such like a microbe, also be stored as freezing stock according to need, and be kept on petri plates seeded with bacteria, *Escherichia coli*.

It is still fresh in our minds that the genome is the first animal genome, which is essentially completely sequenced (The *C. elegans* Sequencing Consortium, 1998). The genome size is 97 Mb, while 12 Mb of budding yeast (saccaromyces cereviciae) and 3,000 Mb of human, and 19,099 of protein coding genes have been estimated. The worm is usually maintained as hermaphrodites, which are self-fertilizing and thus it makes easy to generate

and store homozygous mutant when it is not lethal. Male is also available and thus it enables genetic analysis. The hermaphrodite has five pairs of autosomal chromosomes (I to V) and one pair of sex chromosome, represented as XX, and the male has the autosomal chromosomes and single sex chromosome, represented as XO.

It is thought that the individual difference of total somatic cell number (more properly, nuclear number) is scarcely ever observed. By the time of hatch, during embryogenesis, 671 cells are generated and 113 of these disappear by programmed cell death. Thus, at the time of hatch from embryo, an L1 larva of hermaphrodite has 558 cells. 55 of these are somatic blast cells that produce additional somatic tissues during subsequent development to mature adult. Finally, an adult hermaphrodite has 959 somatic cells and male 1031 (Sulston and Horvitz, 1977; Sulston *et al.*, 1983).

The fundamental body frame is regarded as being comprised of three tubes. The outer tube, the hypodermis and the overlying exterior cuticle, and internal (hydrostatic) pressure maintain the cylindrical body shape. The outer tube bundles two smaller inner tubes, the pharynx and intestine, and the reproductive system (Figure I B, C). *C. elegans* molts four times after hatching. At each molt, a new cuticle of stage specific composition is secreted, and the old cuticle is cast off (reviewed Johnstone, 2000). The body size increases in size by about one-third during each four larval stage and again as an adult after the final molt (Byerly *et al.*, 1976). The body wall muscle is arranged in four longitudinal stripes, which are attached to the cuticle through a thin layer of hypodermis. Muscle cells make up 111 cells of the 959 cells. Worm moves by means of four longitudinal bands of muscle paired sub-dorsally and sub-ventrally. Alternative contraction and relaxation generates dorsal-ventral waved movement along the body, propelling the animal along. 338 are body

nervous system. Among the neural structures an anterior nerve ring with a ventral verve cord is running buck down the body. The cell bodies of most neurons are positioned around the pharynx, along the ventral mid line and in the tail. Most of their cell processes form a ring around the basement membrane that surrounds the pharynx, or they join the dorsal or ventral nerve cord. Most chemosensory and mechanosensory neurons extended afferent processes from the region of the nerve ring to sensory organs near the tip of the head. Other sensory nerons extend their processes along body or to the tail. The nerve ring receives and integrates sensory information and connects to motor neurons in the head or along the nerve cord.

Because of its transparent body, somatic cells are visible with a microscope and thus it makes easy to inject exogenous DNA or RNA into gonad. The DNA Injection is usually carried out with young adult or L4. Injected transgenes are usually maintained as an extra chromosomal array, which is transmissible and contains many copies of injected DNA and integration of the DNA into a chromosome may occur. The extrachromosomal arrays and integrated transgenes are represented by *Ex* and *Is*, respectively. DNA injection is used for rescue experiment with genomic and cDNA clone, analysis by overexpression of interested gene, and expression analysis using GFP and *lacZ* reporter gene. RNA interference (RNA-i) by injection of double stranded RNA (ds RNA) is also available for easy inhibition of function of the interested gene.

Thus, whereas in addition to common respects of essential biology, availability of genetic analysis, ease of handling, and facility of functional analysis, *C. elegans* achieves wide recognition as a good model organism for biological study.

TGF-β-like signaling pathway in C. elegans

As mentioned above, in *C. elegans*, several mutants, which reveal the phenotype of smaller than wild type had been identified (Figure II). Some genes for these small mutants (represented as Sma) has been cloned and it had become clear that some of them encode the components of a TGF-β -like signaling pathway. In many organisms, members of the TGF-β superfamily of secreted proteins play key roles in the intercellular signaling networks, which coordinates many developmental decisions controlling the growth, differentiation, and morphogenesis.

The accepted model for signaling by the TGF-β family has emerged recently (reviewed in Itoh *et al.*, 2000; Massague *et al.*, 2000; Massague and Chen, 2000; Massague and Wotton, 2000; Zimmerman and Padgett, 2000). The basic components of this model consist of two receptor serine/threonine protein kinases (receptor types I and II) and the Smad (for *C. elegans small* and *Drosophila mad*) proteins, a family of receptor substrates. According to this model, the binding of secreted ligands to the type II receptor promotes the formation of a heteromeric complex with a ligand-specific type I receptor. The type II receptor then phosphorylates a cytoplasmic domain, the GS domain (a glycine- and serine-rich stretch of residues), of the type I receptor, resulting in the activation of the type I receptor. The activated type I receptor of the heteromeric receptor complex then transduces the signal from the ligand to the downstream Smad proteins, which act as cytoplasmic mediators, by phosphorylation. Upon phosphorylation, the heteromeric complex containing the Smads is activated and translocates into the nucleus where the Smads control the transcription of specific target genes.

In the nematode *C. elegans*, four ligand proteins have been identified as members of the TGF-β superfamily, and two TGF-β-like pathways have been extensively characterized (reviewed in Patterson and Padgett, 2000). UNC-129, a TGF-β family member in *C. elegans* is required for the expression of the dorso-ventral polarity information that is essential for axon guidance and guided cell migration (Colavita *et al.*, 1998), although the mechanism of cellular migration guidance by UNC-129 and the downstream components involved in UNC-129 signaling remain to be elucidated. Another member of the TGF-β family in *C. elegans*, DAF-7 (Ren *et al.*, 1996), is involved in the Daf (for dauer larvae formation abnormal) pathway, the most well-characterised TGF-β-like pathway in this organism (Thomas *et al.*, 1993). Dauer larvae are the non-feeding and developmentally arrested stage that is specialised for dispersal characteristics that promote survival in an unfavorable environment (Cassada and Russell, 1975). The dauer/nondauer developmental decision is influenced by the temperature and the availability of food relative to the population density, as indicated by the concentration of a constitutively secreted dauer-inducing pheromone (Golden and Riddle, 1982; Golden and Riddle, 1984a; Golden and Riddle, 1984b). The Daf pathway is a part of the pathway regulating the entry into and recovery from the dauer larval stage in response to environmental cues (Ren *et al.*, 1996) and is also involved in egg-laying (Thomas *et al.*, 1993). The DAF-7 signaling is transduced through DAF-4 (Estevez *et al.*, 1993; Inoue and Thomas, 2000), the only type II receptor kinase identified in the *C. elegans* genome (*C. elegans* Sequencing Consortium, 1998). Mutations of *daf-4* cause small body size (Sma) and male tail abnormal (Mab) defects in addition to phenotypes indicating mutation of the Daf pathway, Daf-c (constitutive dauer formation) and Egl (egg-laying abnormal).

These phenotypes reflect that DAF-4 also functions in another well-characterised TGF-β-like pathway in *C. elegans*, the Sma/Mab pathway.

The Sma/Mab pathway is initiated by a recently identified third member of the TGF-β family in *C. elegans*, DBL-1 (for Dpp, BMP-like), and regulates body size, male tail patterning, and the patterning of dopaminergic neurons (Lints and Emmons, 1999; Morita *et al.*, 1999; Suzuki *et al.*, 1999). DBL-1 signaling is also received and transduced through DAF-4. The type I receptor kinase, SMA-6 (Krishna *et al.*, 1999), and three Smad proteins, SMA-2, SMA-3, and SMA-4 (Savage *et al.*, 1996; Savage-Dunn *et al.*, 2000) relay the signaling (Figure III). Mutations of these components all result in the Sma and Mab phenotypes. Among the components of the two TGF-β-like pathways, DAF-4 is the only one that, when mutated, produces both the Daf and Sma phenotypes, and is required for both pathways. However, enhancement of the Daf-c phenotype is observed in the double mutant strains *daf-1; sma-6* (Krishna *et al.*, 1999) and *daf-7; cet-1(dbl-1)* (Morita *et al.*, 1999). These observations suggest there might be a low level of cross-talk between the Sma and Daf pathways at the level of ligand-receptor interaction. When GFP is expressed under the control of the *daf-4* promoter (Gunther *et al.*, 2000) or as a *daf-4::gfp* translational fusion gene (Patterson *et al.*, 1997), GFP fluorescence is found in a subset of neurons that overlaps with GFP expression driven by the *daf-1* (type I receptor; Georgi *et al.*, 1990; Gunther *et al.*, 2000) promoter. In contrast, in the pharynx, intestine, hypodermis, and body wall muscles, the *daf-4* promoter is active, but the *daf-1* promoter is not (Gunther *et al.*, 2000). This wide expression of DAF-4 is consistent with the hypothesis that it is required for pathways in addition to the Daf pathway. Interestingly, assays using *lacZ* reporter fusion constructs suggested that the type I receptor SMA-6 and one of the SMAD proteins, SMA-3, which are components of the Sma/

Mab pathway, are also expressed in the intestine, pharynx, and other tissues (Krishna *et al.*, 1999; Savage-Dunn *et al.*, 2000). In contrast, the expression patterns of the *dbl-1::gfp* reporter fusion product showed *dbl-1* expression to be restricted primarily to neurons, including the ventral nerve cord (Figure IV ; Morita *et al.*, 1999; Suzuki *et al.*, 1999). Thus, it seems likely that DBL-1 is to be secreted from neurons then acts as a signal may be led to other cell types in targeted tissues, which is expressing downstream components (Table I). However, the cell types that respond to the DBL-1 signal to regulate body size have not yet been clarified.

I have been interested in the mechanisms by which the ligand DBL-1 regulates the body length of *C. elegans*. Here I have examined the site of action of SMA-6 in the regulation of body length. I show that *sma-6* is highly expressed in the hypodermis and that this hypodermal expression is necessary and sufficient for the regulation of body length. I have also examined the contribution of the GATA sequences, which are present in the immediate upstream region of *sma-6* ATG to the hypodermal expression.

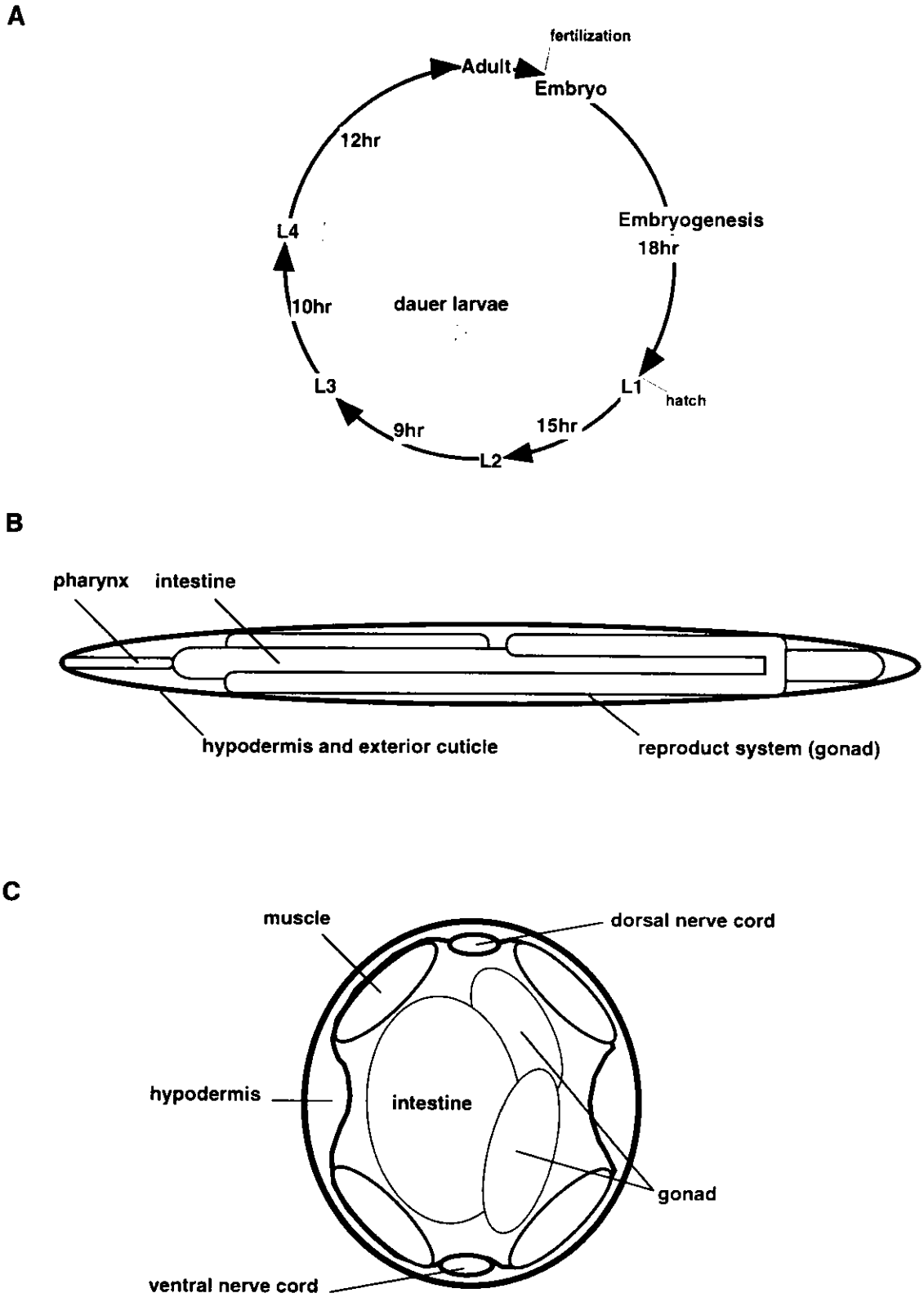

Figure I Schematic diagrams for introduction to *C. elegans*

(A) Life cycle of *C. elegans*. A fertilized egg grows to adult through four larval molts in about 3 days at 20 ℃. In unfavorable environment, the worm forms dauer larva, which is developmentally arrested stage, to survival. (B) Schematic diagram of the worm at lateral view and (C) cross section diagram. The fundamental body frame is regarded as being comprised of three tubes. The outer tube, the hypodermis and the overlying exterior cuticle, bundles two smaller inner tubes, the pharynx and intestine, and the reproductive system. Muscles, neurons and other tissues are left out in (B).

Figure II Small mutants.
Transmitted light images of worms of adult hermaphrodites 5-6 days old after hatching. The mutants, *sma-6 (wk7)* (middle) and *dbl-1 (bx5)* (bottom), are represent the phenotype of smaller than wild type N2 (top). Anterior is to the left and dorsal is up. Bar, 100μm.

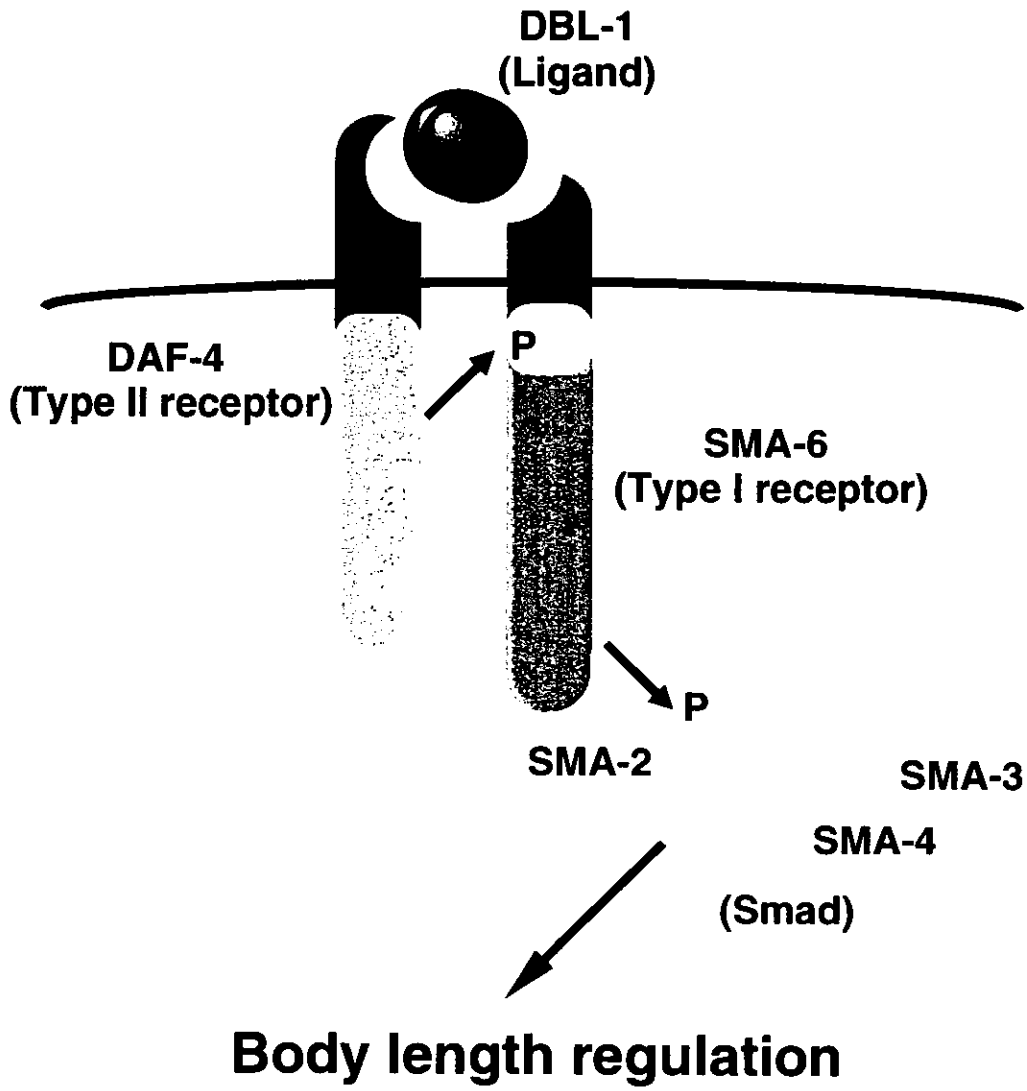

Figure III Schematic diagram of DBL-1 signaling pathway.
One of the TGF-β-like signaling pathways in C. elegans, which involves the regulation of body length of worm.

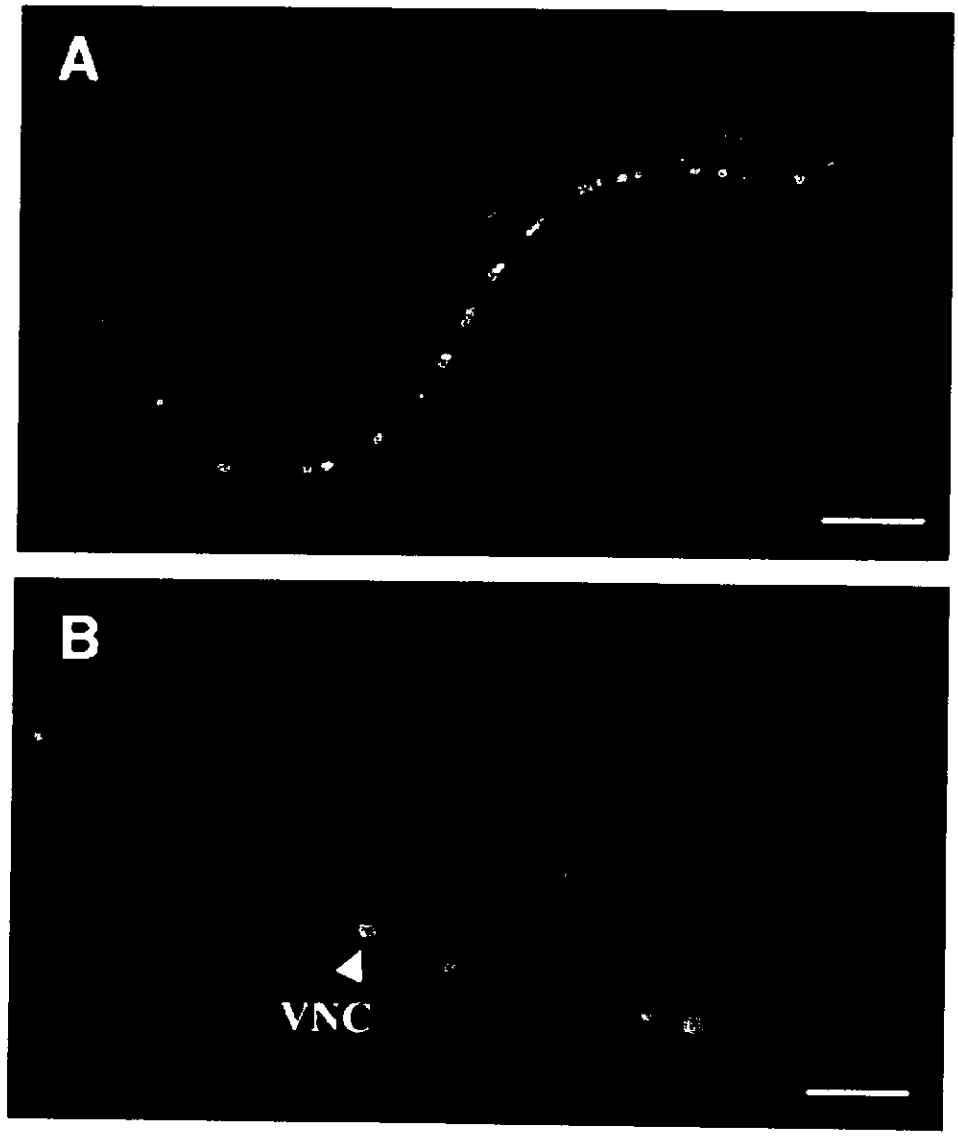

Figure IV DBL-1::GFP expresses in neurons.
(A) Fluorescence image of L2 larva. (B) Image at higher magnification. Strong fluorescence is seen in ventral nerve cord (VNC, white arrowhead). Bra, 50 μm, 20 μm. (Adopted from Morita *et al.*, 1999).

Table I. Summary of the expression patterns of DBL-1 signaling pathway components

	DBL-1 [a]	DAF-4 [b]	SMA-6 [c]	SMA-3 [d]
	Ligand	Receptor TypeII	Receptor TypeI	Smad
Neurons				
Head gangliae [e]	+	+	-	-
Phasmid neurons	-	+ [f]	-	-
VNC	+	+ [g]	-	-
Pharyngeal neurons	+	-	-	-
DVA	+	-	-	-
CAN	+	-	-	-
Dorso-rectal ganglion	+	-	-	-
Pre-anal ganglion	+	-	-	-
Dorsal nerve cord	+	-	-	-
Body wall muscle	+ [h]	+ [i]	-	-
Pharynx	+	+	+	+
Intestine	-	+	+	+
Hypodermis	-	+	-	-
Other tissues	-	-	-	+ [j]

[a] Suzuki *et* al., 1999; Morita *et al.*, 1999

[b] Patterson *et al.*, 1997; Gunter *et al.*, 2000

[c] Krishna *et al.*, 1999; Mochii *et al.*, 2000

[d] Suvage-Dunn *et al.*, 2000

[e] LG, VG, and RVG

[f] PLN and PLM

[g] PVT

[h] Head region

[i] Adult body

[j] Not described in detail

RESULTS

sma-6 *is expressed in hypodermal cells*

In contrast to DAF-4, whose mutation affects dauer larvae formation (Daf), body length (Sma), and male-specific structures (Mab) (Baird and Ellazar, 1999; Flemming *et al.*, 2000; Riddle *et al.*, 1981; Suzuki *et al.*, 1999), *sma-6* mutants display only the Sma and Mab phenotypes (Jiang and Sternberg, 1999; Krishna *et al.*, 1999). Therefore, SMA-6 is considered to be involved exclusively in the regulation of body length and establishes the specificity of this TGF-β pathway (Krishna *et al.*, 1999). To reveal how the ligand *dbl-1*, which is mainly expressed in neuronal cells such as the ventral nerve cord and AFD neurons (Morita *et al.*, 1999; Suzuki *et al.*, 1999), acts on the type I receptor SMA-6 to regulate body length, I first examined the expression pattern of *sma-6* during the course of *C. elegans* development. I fused 3 kb of the 5′ promoter region of *sma-6* (Krishna *et al.*, 1999) with GFP to create a transcriptional reporter gene (Figure 5A) and monitored the *sma-6* expression in transgenic worms. The worms harboring the GFP reporter gene showed fluorescence broadly in the pharyngeal muscle, intestine, and hypodermis. The fluorescence was seen from around the 1.5-fold stage (Figure 1A-C) and continued to the adult stage (Figure 1D, L). The expression in the hypodermis was detected in the dorsal (hyp-7) and ventral hypodermal cells (P cells) in the main body region (Figure 1B- J), but not in the lateral hypodermal H, V, and T cells (seam cells) (Figure 1D, F, H). The expression was also detected in a part of the hypodermal cells of the head (hyp-6) (Figure 1G, H), but not in the hypodermal cells of the anterior (hyp-1, -2, -3) and posterior end (hyp-10) (Figure 1G, J). Although, Krishna *et al.* (1999) reported that *sma-6* was

expressed in the intestinal cells and pharyngeal muscle using a *lacZ* reporter, the present study demonstrates that *sma-6* is also expressed in the hypodermis.

Unlike the expression of *dbl-1*, as revealed by the *dbl-1::gfp* reporter gene (Morita *et al.*, 1999; Suzuki *et al.*, 1999), I did not observe the expression of the GFP reporter with the *sma-6* promoter in the nervous system (Figure 2. This suggests that DBL-1 expressed in neurons is secreted and directed by an as yet unknown mechanism to the sites of SMA-6 expression to activate SMA-6/DAF-4 signaling. This apparent long-range action, which was predicted by the expression profiles of *dbl-1* and *sma-6*, prompted us to determine which site of SMA-6 expression was required for body length regulation.

Hypodermal expression is sufficient for restoring body length

The Sma phenotype of *sma-6* was rescued either by the transgenesis of cosmid C32D5 containing the entire *sma-6* coding region (data not shown; see Krishna *et al.*, 1999) or a *sma-6* cDNA with the promoter region described above (Figure 5A-C and Table 2). To examine the site of SMA-6 expression that was required for body length regulation, i.e., the target tissue for DBL-1, I attempted to rescue the Sma phenotype of a null mutant of *sma-6* (*wk7*) by generating transgenic worms carrying the *sma-6* cDNA driven by different tissue-specific promoters.

I first generated a translational *sma-6::gfp* fusion construct examined its ability to rescue for the *sma-6* phenotype. When GFP was fused to the C-terminal end of SMA-6, the resulting product rescued the Sma phenotype (Figure 5), suggesting that the SMA-6::GFP was functional as SMA-6 type I

receptor. However, the fluorescence of the GFP was extremely weak and barely detectable in some cases. Thus, to trace the region of expression, a GFP reporter gene fused only with each tissue-specific promoter was always coinjected with the rescuing construct (*sma-6::gfp*) ,which was fused with the same promoter. This enabled detectable fluorescence to be monitored to ensure the tissue-specific activity of the promoter.

I chose the *dpy-7* promoter (P_{dpy-7}) and the *myo-2* promoter (P_{myo-2}) for the hypodermal- and pharyngeal-muscle-specific expression of *sma-6*, respectively. *dpy-7* is a cuticle collagen gene that is expressed specifically in the hypodermis (Gilleard *et al.*, 1997; Figure 3G) and *myo-2* codes for the pharyngeal-muscle-specific myosin (Okkema *et al.*, 1993; Figure 3D). P_{yk92e8}, the promoter region for a predicted gene, Y105C5A.13, which encodes *yk92e8*, was used for gut-specific expression. *yk92e8* is one of the cDNA clones that were previously identified as a down-regulated gene in *sma-2* mutants in a cDNA macroarray experiment (Mochii *et al.*, 1999). The expression pattern of the GFP reporter gene with P_{yk92e8} suggested that the activity of the promoter is intestine-specific (Figure 3E), and the expression was detected through all the developmental stages from the late embryonic stage (data not shown). The promoter of *mtl-2*, a metallothionein gene expressed exclusively in intestinal cells upon cadmium induction, was also used as an intestine-specific promoter (Freedman *et al.*, 1993; Moilanen *et al.*, 1999). $P_{hsp16.41}$, the promoter for a heat shock gene *hsp16.41*, and $P_{sma-6\ (-3088)}$, the promoter region of *sma-6* were also used for ubiquitous expression and for the authentic *sma-6* expression, respectively.

The results are summarized in Table 1 and Figure 3. Interestingly, only hypodermal expression of SMA-6 with the *dpy-7* promoter and

ubiquitous expression with the *hsp16.41* promoter (data not shown) elongated the shortened body length of the *sma-6* mutant (Table 1 and Figure 3A, F), and neither gut- nor pharyngeal-muscle expression rescued the Sma phenotype (Table 1 and Figure 3A, D, E). These results indicate that hypodermal expression of SMA-6 is sufficient for the regulation of body length.

Minimum sequence required for hypodermal expression of sma-6

Because the backbone of the constructs using *sma-6* (cDNA)*::gfp* for the rescue experiments was the GFP vector (obtained from A Fire), the original 3'UTR of the *sma-6* gene had been removed and replaced by that of the vector (for details, see Materials and Methods). From the above observations, I assumed that the sequences in either the introns or the 3' UTR of the *sma-6* gene are dispensable and that the cis element that drives the hypodermamis-specific expression of SMA-6 must exist in the 5'promoter region. Therefore, I prepared stepwise deletions of the 5' upstream region of the *sma-6* promoter to identify the minimum element that was sufficient for its hypodermis-specific expression.

I found that deletion to -414 bp (relative to the initiator ATG) did not affect the hypodermal, intestinal, or pharyngeal expression (Figure 5A, D, E). The expression level revealed by GFP in the hypodermis decreased gradually with further deletion, from –284 bp to -219 bp, but the promoter was still active in the tissue to a certain extent. The expression of GFP in the pharynx and intestine was also retained (Figure 4). The deletion to -209 bp resulted in the disappearance of hypodermal expression but not of pharyngeal or intestinal expression (Figure 3 E). Thus, sequences between -219 bp and -209 bp appeared to be necessary for the hypodermal cell expression, but no

conserved *cis* elements could be identified in this region. Instead, immediately downstream of this region, I identified three typical GATA recognition sequences (hereafter referred to as GATA elements), WGATAR (Ko and Engel, 1993; Merika and Orkin, 1993; Omichinski *et al.*, 1993), in tandem within a short region, as shown in Figures 5A and 6A.

GATA recognition sequences are required for hypodermal expression of sma-6 and the regulation of body length

I next deleted only a relatively short sequence of 30 bp including the three consecutive GATA elements from the truncated promoter (414 bp in length) to see if the deletion of the GATA elements affected the hypodermal expression of *sma-6*. As I expected, the three GATA elements were necessary for the hypodermal expression of the *gfp* reporter gene, but not for intestinal and pharyngeal expression (Figure 5A, F, G). To test its ability to drive SMA-6 to rescue the small phenotype of the *sma-6* mutant, these truncated 5′ regulatory regions were used to express the *sma-6* cDNA. Interestingly, expression with the 414 bp 5′ upstream sequence ($P_{sma\text{-}6\ (-414)}$::*sma-6*) rescued the reduced body length of the *sma-6* mutants, whereas *sma-6* cDNA with the promoter that lacked the three GATA sequences ($P_{sma\text{-}6\ (\Delta GATAx3)}$::*sma-6*), failed to rescue the Sma phenotype (Figure 5 and Table 2).

From these observations, I expected that the three GATA sequences were necessary and sufficient for hypodermal expression of *sma-6*. However, -209 bp of the *sma-6* 5′ upstream sequence (relative to ATG), including the GATA elements and about 20 bp of flanking sequence, neither supported hypodermal expression of the *gfp* reporter gene (Figure 4A, E) nor rescued the

Sma phenotype (data not shown). This finding suggested that the GATA sequences were required but not sufficient to support the hypodermal expression of *sma-6* and that the upstream flanking sequence was also required. To determine the contribution of each GATA element to the hypodermal expression, I next made a series of transcriptional reporter fusion genes in which the mutations were introduced in each of the three elements (designated as mutant GATA1, 2, 3 from upstream), and tested them for the ability to produce hypodermal expression (Figure 6). I found that the introduction of the mutation in any one of the three GATA elements reduced the hypodermal expression (Figure5); however, when the mutation was in the most downstream GATA element ($P_{mutant\ GATA3}$::*gfp*), the effect was not as striking (Figure 6 A, F and G). All animals tested carrying the $P_{mutant\ GATA3}$::*gfp* construct expressed GFP in hypodermis but the intensity of fluorescence were weakened than that of wild type construct. In contrast, when the function of the middle GATA element was ablated, the mutation ($P_{mutant\ GATA2}$::*gfp*) caused massive reduction of the expression in the hypodermis, and in many cases the hypodermal expression was diminished (Figure 6 A, D and E). The mutation in the first GATA element ($P_{mutant\ GATA1}$::*gfp*), which is located the most upstream of ATG, was also remarkably defective in its ability to drive hypodermal expression, but the expression did not disappear completely (Figure 6 A-C). Mutant GATA2 often resulted in the ectopic expressions of GFP in the body-wall muscle and neurons (data not shown), but mutant GATA1 and mutant GATA3 did not. These results suggest that each of the GATA elements contribute to a different extent to drive the appropriate expression of *sma-6* in the hypodermis; the middle GATA element is most important and the first GATA element is also

necessary for appropriate expression but the contribution of the third GATA element for hypodermal expression is minimal.

DBL-1 signaling at middle larval stage is critical for body length regulation

From the analysis of expression pattern of *sma-6* using the *gfp* reporter gene, *sma-6* promoter is active in hypodermis even in adult stage (Figure 1L). Whereas, *dpy-7*, of which promoter region was used for hypodermis specific expression of *sma-6*, has been reported to be expressed in late embryonic stages and in an oscillating pattern during larval development, but not expressed in adult (Johnestone and Barry, 1996; Johnstone, 2000). Indeed, the fluorescence derived from $P_{dpy\text{-}7}$ :: *gfp* is extremely weak in adult. Although only slight fluorescence is seen in hypodermis of adult worm carrying the $P_{dpy\text{-}7}$ :: *gfp* transgene. Thus the *dpy-7* promoter used seems to be hardly active in the adult hypodermis. Nonetheless, *sma-6* expressed under the control of the *dpy-7*promoter can rescue the small phenotype of *sma-6* mutant. This observation promoted me to ask whether, in addition to the tissue specificity of the signaling, at least a part of DBL-1 signaling involved in body length regulation is influenced temporally during worm development or not. To investigate the temporal effect, I examined the ability of transient expression of ligand, DBL-1, using heat shock promoter to rescue the Sma phenotype of *dbl-1* mutant. Single heat shock was performed at each developmental stage for the *dbl-1* mutant carrying an expression construct *P hsp16.42* :: *dbl-1* (*ctIs40*, obtained from Suzuki), which was growing at 16 C.

In result, the effect of elongation of body length varied and depended on stages when heat shock was given (Table 3). The induction of DBL-1 by heat shock at L2 and L3 stages fully restored the reduced body length to wild

type level. Heat shocks at the preceding L1 stage and following L4 stage had certain degree of, but not enough, effect. To my surprise, the induction at embryonic stage and adult stage did not cause any changes in body length. These results suggest that the brought information by the DBL-1/SMA-6 signaling during the middle larval development is definitive to control the final body length in spite of considerable growth after final molt from L4 to adult.

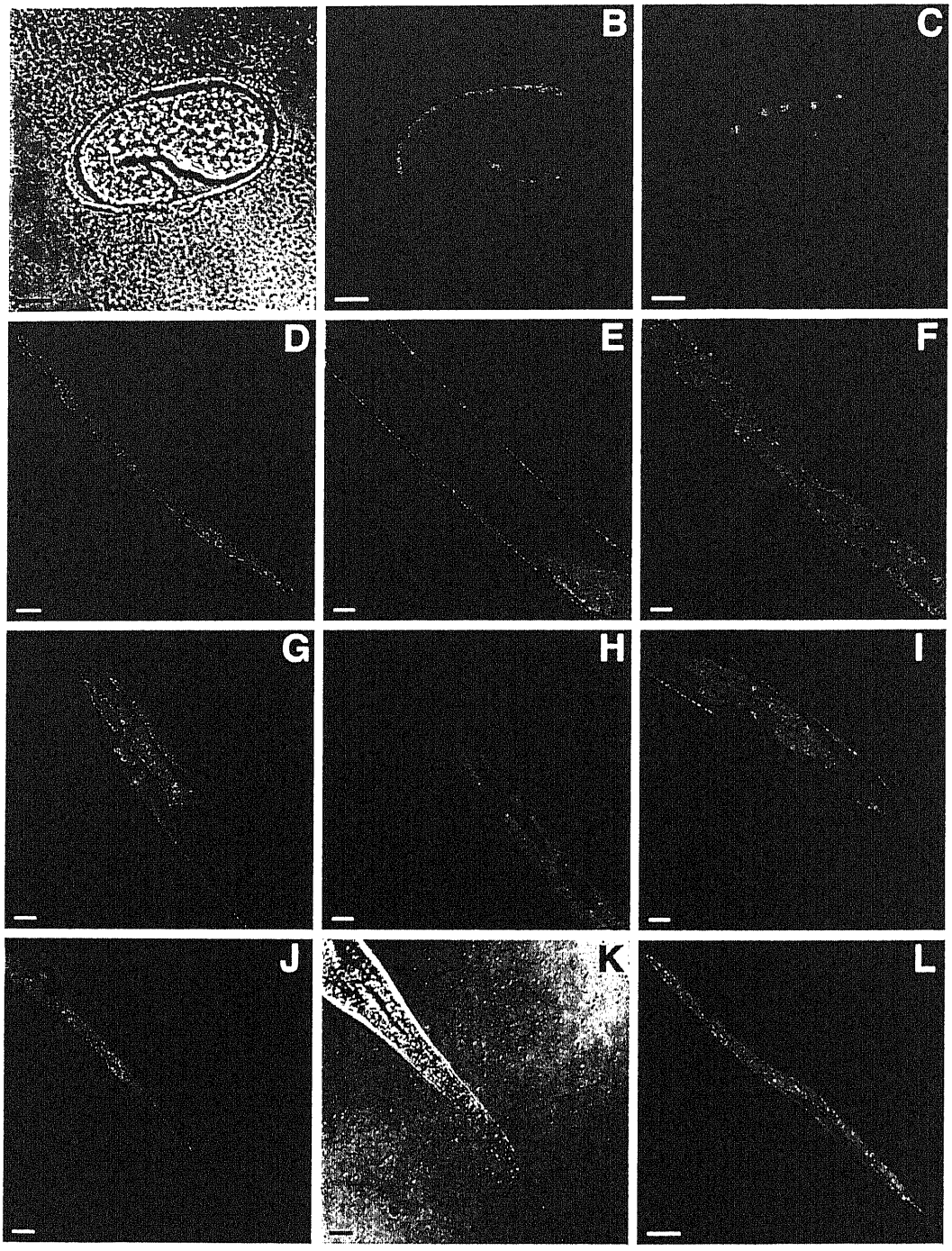

Figure 1. *sma-6::gfp* is expressed in hypodermal cells.

Figure 1. ***sma-6::gfp*** **is expressed in hypodermal cells.**

Confocal images of transgenic animals of an integrated line bearing $P_{sma\text{-}6}$ *(-3065)::gfp*, a transcriptional *gfp* fusion construct with 3065 bp of the *sma-6* 5′ upstream region, are shown. (Details of the construct are given in Materials and Methods. See also Fig. 4A.) The construct includes the nuclear localization signal, but fluorescence can also be detected in the cytoplasm. Bar, 10 _m (A-C, E-K), 50 μm (D), 100 μm (L). (A) Transmitted light image of a 1.5-fold stage embryo. (B, C) Fluorescence images of the same embryo as in A, midline plane and lateral plane of focus, respectively. Fluorescence is seen in dorsal and ventral hypodermal cells. (D) L3 larva. Fluorescence is seen in the pharynx, intestine, and hypodermis. (E-I) Images of each part of the same larva as in D, at higher magnification. (E, F) Middle trunk region, midline plane, and left lateral plane of focus, respectively. Fluorescence is seen in the intestine (E) and main body hypodermal syncytium surrounding the seam cells (V cells), which appear as dark ovals (F). (G, H) Anterior of the same larva as in D, midline plane and left lateral plane of focus, respectively. Fluorescence is seen in the pharynx (E) and hypodermal syncytium in the head but not in H cells (seam cells in the head) (H) or V cells as in F. (I) Fluorescence is seen in the posterior gut and immediately posterior to the gut. (J, K) The most posterior end of a young adult worm, fluorescence and transmitted light images, respectively. Fluorescence is not seen in the most posterior part of the hypodermis. (L) The expression patterns of GFP in the pharynx, intestine, and hypodermis are maintained in the adult worm.

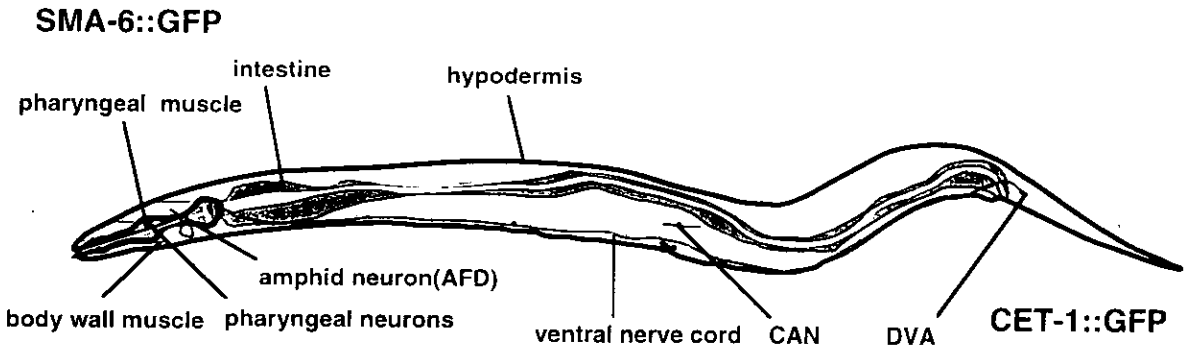

Figure 2 Schematic diagram of the expression patterns of ligand DBL-1 and type I receptor SMA-6 in *C. elegans*. The expression pattern of DBL-1 :: GFP and SMA-6 :: GFP are represented in red and green color, respectively. While DBL-1 :: GFP is detected mainly in some neurons, SMA-6 :: GFP is seen in pharynx, intestine, and hypodermis, not in neurons.

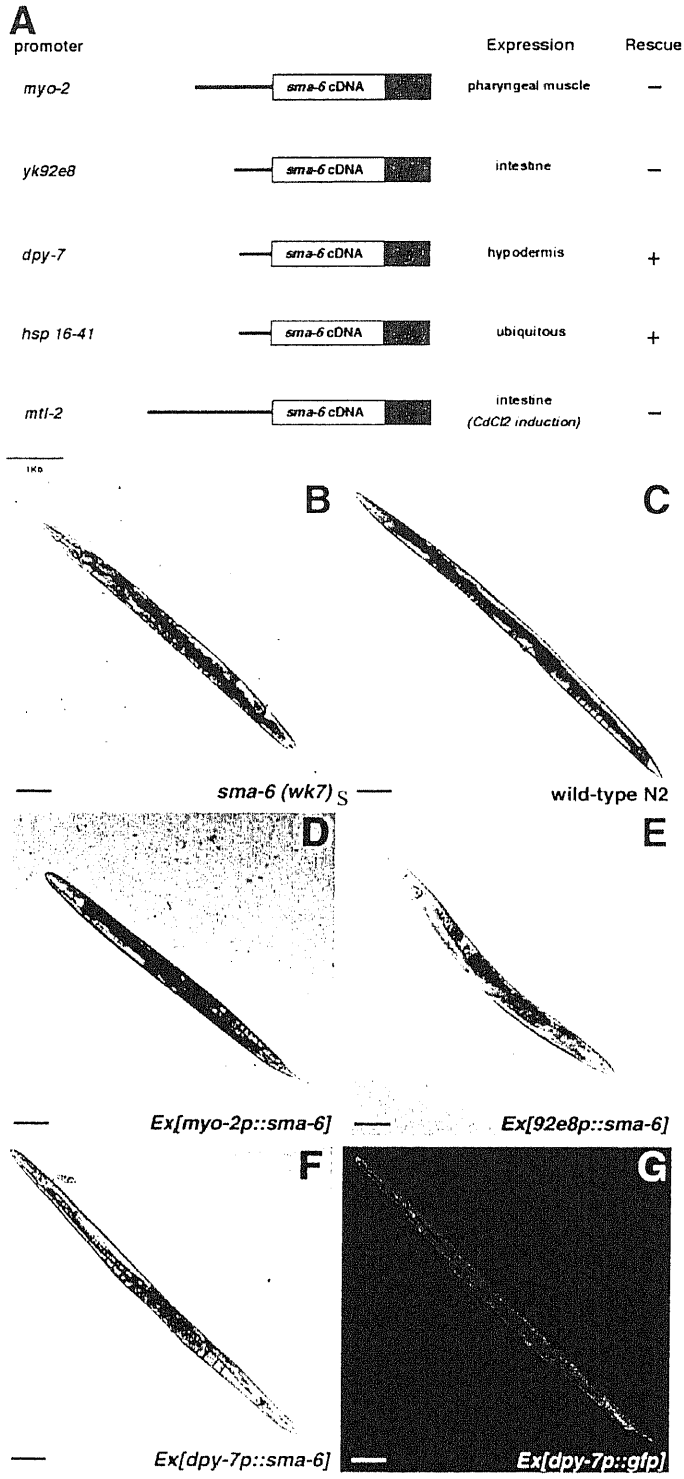

Figure 3. Hypodermal SMA-6 can elongate the reduced body length of the *sma-6* mutant.

Figure 3. Hypodermal SMA-6 can elongate the reduced body length of the *sma-6* mutant.

(A) Schematic diagram of the constructs used and summary of the results.

(B) *sma-6(wk7)*. (C) wild-type N2.

(D-G) Transgenic animals bearing an extrachromosomal array expressing SMA-6 in pharyngeal muscle (D, $P_{myo\text{-}2}$*::sma-6*), intestine (E, P_{yk92e8}*::sma-6*) and hypodermis (F and G, $P_{dpy\text{-}7}$*::sma-6*) in a *sma-6 (wk7)* mutant background. GFP was fused to the C-terminal end of SMA-6 but fluorescence was exceedingly weak and sometimes was not detectable in these cases. The fluorescence seen in panels D and E due to the transcriptional *gfp* reporter construct with each promoter co-injected with the *sma-6* cDNA construct to reconfirm the promoter activity. (G) Fluorescence image of the same animal as in F. The fluorescence seen in hypodermis due to the $P_{dpy\text{-}7}$*::gfp*, transcriptional *gfp* reporter construct co-injected. The intestinal autofluorescence is also seen due to increased setting for amplification of gain for that that the expression of GFP from $P_{dpy\text{-}7}$*::gfp* was very week and scarcely ever detectable in adult. And GFP in pharynx due to the $P_{myo\text{-}2}$*::gfp*, which was co-injected as an injection marker for the same reason.

The $P_{dpy\text{-}7}$*::sma-6* array rescued the Sma phenotype, but the $P_{myo\text{-}2}$*::sma-6* and P_{yk92e8}*::sma-6* arrays did not. Bar, 100 μm

Table 1. Hypodermis-specific expression of *sma-6* can elongate the reduced body size of *sma-6* mutant.

Genotype	Transgene	Length (mm)[a]	n[b]
Wild-type N2	None	1.28 ± 0.09	67
dbl-1 (nk3)	None	0.91 ± 0.07	108
sma-6 (wk7)	None	0.91 ± 0.06	122
sma-6 (wk7)	$P_{myo\text{-}2}$ :: *sma-6*	0.90 ± 0.10	113
sma-6 (wk7)	P_{yk92e8} :: *sma-6*	0.87 ± 0.07	121
sma-6 (wk7)	$P_{dpy\text{-}7}$ :: *sma-6*	1.36 ± 0.09	54
sma-6 (wk7)	$P_{dpy\text{-}7}$ :: *sma-6*	1.36 ± 0.09	55

[a] Data are presented by mean ± SD. Body length were determined for each adult hermaphrodite at six days after hatched.

[b] Number of animals measured.

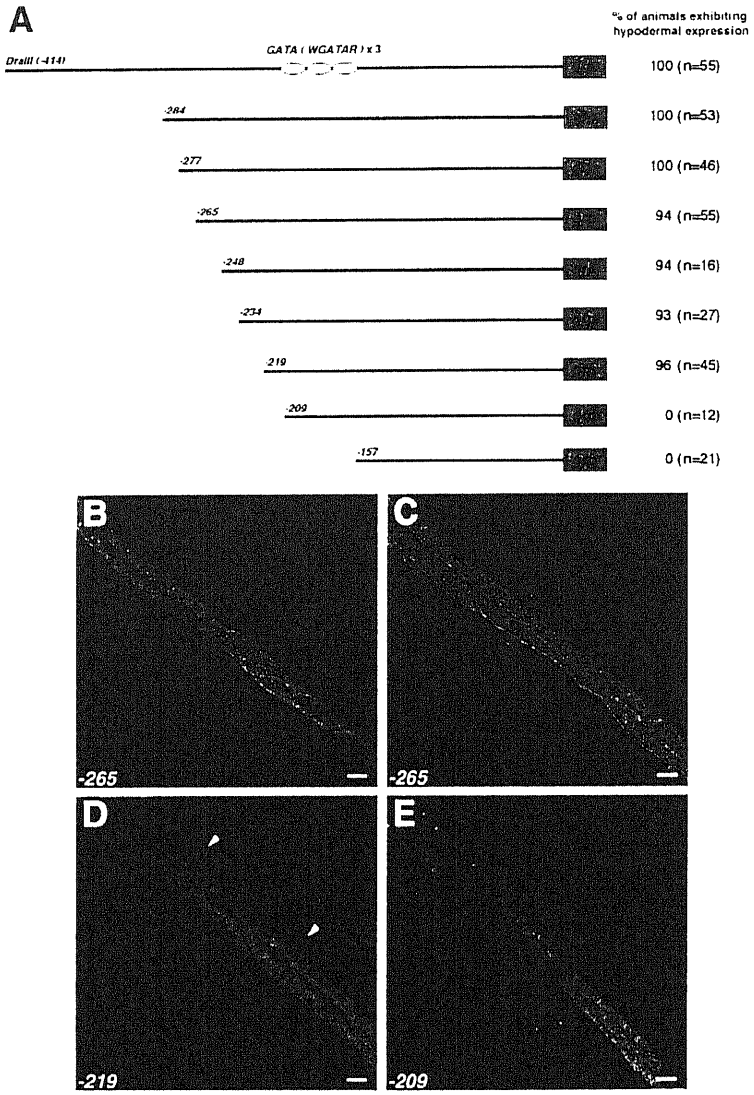

Figure 4. Effect of deletions of the 5′ upstream region of *sma-6* on gfp expression in hypodermal cells.

Figure 4. Effect of deletions of the 5′ upstream region of *sma-6* on gfp expression in hypodermal cells.

(A) Schematic diagram of the deletion constructs used and summary of the results. All fragments were subcloned into the *gfp* reporter vector pPD95.67 to create transcriptional fusions. Numbers refer to the 5′ end position of the fragments relative to the A of ATG, as +1. Clear ovals indicate the consensus recognition sequence of GATA-type transcription factors. The vector includes a nuclear localization signal but fluorescence was also seen in the cytoplasm. (B- E) Confocal images of the middle trunk region of transgenic animals (L2-L3) bearing an extrachromosomal array of a deletion construct. The numbers correspond to the 5′ end as indicated in panel A. Bar, 10 μm. (B, C) midline plane and left lateral plane of focus of an animal, respectively. The intensity of fluorescence in the hypodermis is weakened according to deletions (B, D). GFP expression in the intestine remains in spite of the deletion to -209 bp (C, E), but hypodermal expression has disappeared (E). Deletions from -277 bp sometimes led to ectopic GFP expression in the body wall muscle (see panel D, arrowhead) and some neurons (not shown).

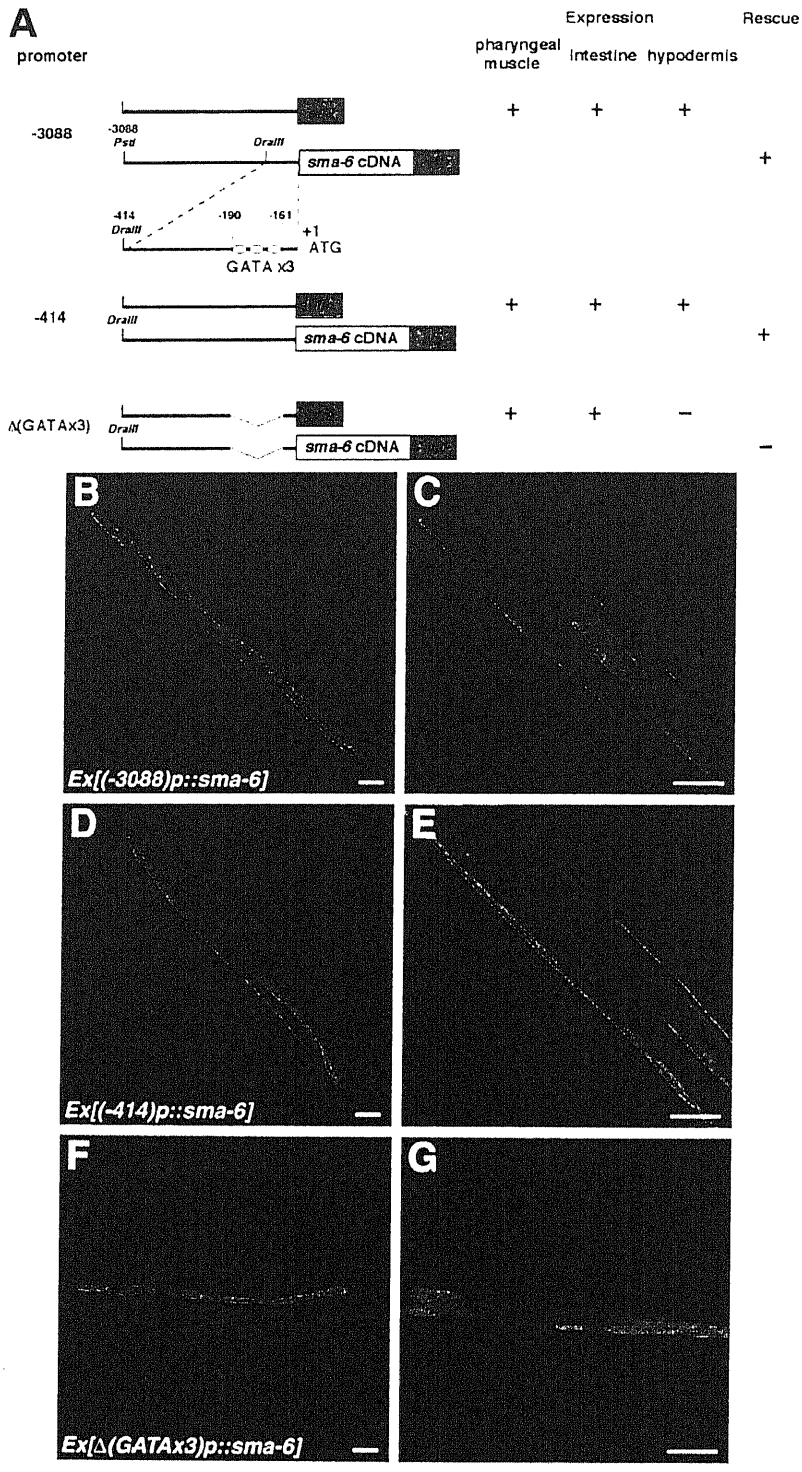

Figure 5. Internal deletion of putative GATA recognition sequences from the *sma-6* promoter removes the hypodermal expression and ability to rescue the Sma phenotype.

Figure 5. Internal deletion of putative GATA recognition sequences from the *sma-6* promoter removes the hypodermal expression and ability to rescue the Sma phenotype.

(A) Schematic diagram of the constructs used and summary of the results. The numbers indicate the 5′ end of the upstream region contained in each construct, relative to ATG. The internal deletion of the three GATA recognition sequences is referred to as Δ(GATAx3). (B-G) Transgenic *sma-6 (wk7)* animals bearing an extrachromosomal array expressing *sma-6* cDNA from each 5′ upstream sequence shown in panel A. A *gfp* reporter construct with each promoter was co-injected for the same reason as in Figure 2. The left panels (C, E, G) show confocal images of the middle trunk region of the same 6-day-old adult worms as in the right panels (B, D, F), at a higher magnification and with a midline plane focus. Bar, 100 μm (B, D, F), 50 μm (C, E, G). (B, C) $P_{sma\text{-}6(-3085)}$::*sma-6* was used as a control.

(D, E) $P_{sma\text{-}6(-414)}$::*sma-6*. Deletion to -414 bp showed no effect on GFP expression or ability to rescue the Sma phenotype. (F, G) $P_{sma\text{-}6(\Delta GATAx3)}$::*sma -6*. *gfp* reporter expression persists in the pharynx and intestine but is not seen in the hypodermis, even at a higher magnification. No ability to rescue the reduced body length is observed.

Table 2. Deletion of GATA recognition sequence from truncated *sma-6* promoter results in loss of ability to rescue for the Sma phenotype.

Genotype	Transgene	Length (mm)[a]	n[b]
Wild-type N2	None	1.28 ± 0.09	67
sma-6 (wk7)	None	0.91 ± 0.06	122
sma-6 (wk7)	$P_{sma\text{-}6(-3085)}$::*sma-6*	1.19 ± 0.10	83
sma-6 (wk7)	$P_{sma\text{-}6(-414)}$::*sma-6*	1.18 ± 0.06	106
sma-6 (wk7)	$P_{sma\text{-}6(\Delta GATA)}$::*sma-6*	0.89 ± 0.06	118

[a] Data are presented by mean ± SD. Body length were determined for each adult hermaphrodite at six days after hatched.

[b] Number of animals measured.

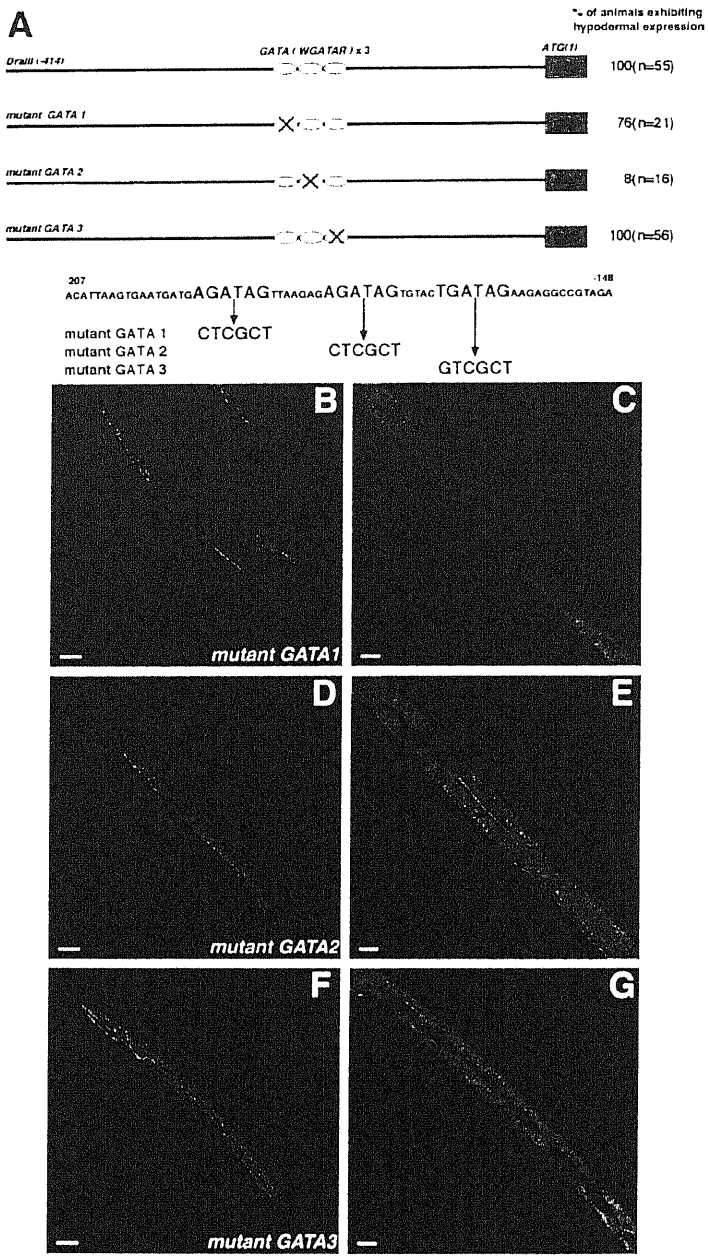

Figure 6. Analysis of effect of mutations in the putative GATA recognition sites on hypodermal expression.

Figure 6. Analysis of effect of mutations in the putative GATA recognition sites on hypodermal expression.

Each of three GATA sites within 414 bp of the 5′ upstream region of *sma-6* was mutated individually. (A) Schematic diagram of the constructs used and summary of the results. Clear ovals indicate the putative GATA recognition sites, and the mutated sites are represented by an X. the numbers on the sequence are locations relative to the A of ATG. The vector includes a nuclear localization signal but fluorescence was seen also in the cytoplasm. Mutated promoters are referred to as *Pmutant GATA1, 2,* and *3,* according to the position of the GATA site from the 5′ to 3′ side, respectively. (B-G) Transgenic L2-L3 animals bearing an extrachromosomal array of *gfp* reporter fusion with the mutated promoter. The left panels show confocal images of the middle trunk region of the same animals as in the right panels (B, D, F), at a higher magnification and focus on the left lateral (C, G) or midline (E) plane. Bar, 50 μm (B, D, F), 10 μm (C, E, G). (B, C) $P_{mutant\ GATA1}$::*gfp*. Extremely weak fluorescence can be seen in the main body hypodermal syncytium only at a higher magnification. (D, E) $P_{mutant\ GATA2}$::*gfp*. No fluorescence can be seen in the hypodermis even at a higher magnification. (F, G) $P_{mutant\ GATA3}$::*gfp*. The clear expression pattern, dark seam cell areas surrounded by intensely fluorescent hypodermal syncytium, is seen even at lower magnification. In either case, expression of the *gfp* reporter gene in the pharynx and intestine is maintained.

Table 3. Transient expression of *dbl-1* under the control of heat shock promoter at larval stage rescues the Sma phenotype of *dbl-1* mutant.

Genotype	Transgene	heat shock[a]	Length (mm)[b]	n[c]
Wild-type N2	None	None	1.20 ± 0.05	21
dbl-1 (bx5)	None	None	0.93 ± 0.04	25
dbl-1 (bx5)	$P_{hsp16.41}$ :: *dbl-1*	None	0.99 ± 0.07	35
dbl-1 (bx5)	$P_{hsp16.41}$ :: *dbl-1*	embryo	0.97 ± 0.12	57
dbl-1 (bx5)	$P_{hsp16.41}$ :: *dbl-1*	L1	1.05 ± 0.06	14
dbl-1 (bx5)	$P_{hsp16.41}$ :: *dbl-1*	L2	1.20 ± 0.06	23
dbl-1 (bx5)	$P_{hsp16.41}$ :: *dbl-1*	L3	1.20 ± 0.08	32
dbl-1 (bx5)	$P_{hsp16.41}$ :: *dbl-1*	L4	1.13 ± 0.09	24
dbl-1 (bx5)	$P_{hsp16.41}$ :: *dbl-1*	young adult	0.97 ± 0.09	23

[a] Heat shock was performed at30 ℃ for 1 hr at each stage.

[b] Data are presented by mean ± SD. Body length were determined for each adult hermaphrodite at six days after hatched.

[c] Number of animals measured.

DISCUSSION

How an organism's body size or length is determined is a fundamental problem in biology. In mammals, it is widely accepted that body size is determined by the number of cells but not by the cell size. However, in nematodes, there are a number of cases in which the cell numbers do not correlate with body length (Flemming *et al.*, 2000). Here, I address how nematode growth is regulated by focusing on a polypeptide growth factor belonging to the TGF-β superfamily and its signaling pathway in *C. elegans*.

I have shown that the type I receptor gene *sma-6* is expressed in the hypodermis at a significant level, and confirmed its previously reported expression in the intestine and pharynx (Krishna *et al.*, 1999). I also identified three putative GATA transcription factor binding sites, WGATAR (Ko and Engel, 1993; Merika and Orkin, 1993; Omichinski *et al.*, 1993), present in the region immediately upstream of the *sma-6* translation start site. These elements are situated in tandem in the forward orientation. I showed these GATA elements to be required for the proper expression of *sma-6* in the hypodermis. Multiple GATA elements are found in the upstream region of intestine-specific genes, *vit-2*, *ges-1*, *cpr-1*, *mtl-2*, and *elt-2*, and some of them are be required for the intestine-specific expression of these genes. (Britton *et al.*, 1998; Egan *et al.*, 1995; Fukushige *et al.*, 1999; MacMorris *et al.*, 1992; Moilanen *et al.*, 1999). GATA elements also exist in the upstream sequence of *dpy-7*, one of the cuticle collagen genes expressed in the hypodermis, and have been suggested to be required for the hypodermal expression of this gene. As in the case of *sma-6*, deletion of the 5′ upstream region of *dpy-7* containing the GATA consensus sequence results in the loss of *lacZ* reporter expression in the hypodermis (Gilleard *et al.*, 1997).

In the *C. elegans* genome, seven GATA factors have been identified and four others have been predicted (Table 4; The *C. elegans* Sequencing Consortium, 1998). Two of the identified GATA factors, END-1 (Zhu *et al.*, 1997) and ELT-2 (Hawkins and McGhee, 1995) are expressed exclusively in the endoderm. A requirement of END-1 and ELT-2 for endoderm specification has been demonstrated (Fukushige *et al.*, 1998; Fukushige *et al.*, 1999; Zhu *et al.*, 1998; Zhu *et al.*, 1997). MED-1 and MED-2 seem likely to be involved in specification of MS and E identity (Maduro MF *et.* al, 2001). The only two, ELT-1 and ELT-3, in the identified and perdicted GATA factors in *C. elegans* genome has been reported to be required for development of the hypodermis. *elt-1* is expressed during the early embryonic stages and is required for epidermal cell fates in the embryo (Page *et al.*, 1997; Spieth *et al.*, 1991) and *elt-3*, which is expressed from the late embryo to adult stages, may also be required for the development of the hypodermis (Gilleard *et al.*, 1999). Therefore, ELT-1 and ELT-3 may participate in the hypodermis-specific regulation of *sma-6* expression. I could not examine the possible genetic interaction between mutants of these GATA factors and *sma-6* mutants, because the *elt-1* mutation in particular is embryonically lethal (Page *et al.*, 1997) and mutant for *elt-3* is not available. Thus, I examined whether the inhibition of *elt-3* by dsRNA-i causes any effect on the hypodermal expression of *sma-6*. dsRNA prepared from *elt-3* cDNA were injected to the transgenic animal carrying integrated array of $P_{sma\text{-}6}$::*gfp* construct, but no noticeable effect was detected on the expression (data not shown). Also, no obvious phenotype was reported from the suppression of *elt-3* by ds RNA-i (Gilleard *et al.*, 1999). However, other GATA factors might compensate for the function of *elt-3* and it is very likely that the expression of *sma-6* is regulated by such

GATA factors expressed in the hypodermis or other transcription factors, which recognize GATA elements and bind DNA.

Our results suggested that sequences adjacent to the GATA elements are also essential for hypodermal expression. Deletion of a sequence immediately upstream of the GATA elements dramatically reduced the transcriptional activity of the *sma-6* promoter in the hypodermis. Interestingly, deletion of the 5′ flanking sequence from –265 bp (relative to ATG) as well as $P_{mutant\ GATA2}$::*gfp* sometimes resulted in the ectopic expression of the GFP reporter in part of the body wall muscle (Figure 4 D) and in some neurons (not shown). These results suggest that the flanking sequence is important for the hypodermal specific expression. Requirement of the sequence upstream of the three GATA elements for the hypodermal expression may suggest that other transcription factors act with the GATA factor at the site, although no known transcription factor binding sequence has been identified there. Several studies in vertebrates and *Drosophila* have shown that other transcription factors and co-factors interact with GATA factors (reviewed in Charron and Nemer, 1999; Molkentin, 2000). Some of these interactions are required for full activation of transcription, and tissue- or developmentally restricted factors may fulfill the role of giving specificity to GATA-type transcription factors (Durocher *et al.*, 1997; Gong and Dean, 1993; Kawana *et al.*, 1995; Merika and Orkin, 1995).

While the DBL-1 ligand is expressed mainly in some neurons (Morita *et al.*, 1999; Suzuki *et al.*, 1999), the Type II receptor DAF-4 is expressed rather broadly in the hypodermis, intestine, and pharynx, in addition to the nervous system (Gunther *et al.*, 2000; Patterson *et al.*, 1997). It is also known that the expression pattern of *sma*-3, which codes for one of the three SMAD proteins that function downstream of SMA-6 in the Sma pathway, overlaps with that

of *sma-6* (Krishna *et al.*, 1999; Savage-Dunn *et al.*, 2000). Thus, the hypodermis, pharynx, and gut, where both type I and type II receptors for Sma signaling are expressed, are expected to be good candidates for target tissues of DBL-1 (Table 5). If this is the case, the ligand may be secreted and act nonautonomously on these target cells, which express a set of downstream molecular components for body length regulation.

In this study, I have shown that the hypodermis is the most important target tissue for the regulation of body length by region-specific expression of a functional SMA-6 fusion protein and by the rescue of the Sma phenotype of the *sma-6* mutant. Whereas, by rescue of *daf-4* Sma phenotype using some promoter fusion constructs with *daf-4* gene, Inoue and Thomas showed that the DBL-1 signaling pathway regulate body size in multiple tissues. The expression of *daf-4* in body wall muscle, pharynx, and hypodermis partially rescues the Sma phenotype of *daf-4* mutant differently (Inoue and Thomas, 2000). The variance from my results may be attributable to the difference of the conditions of measurement. Alternatively, as SMA-6 is not expressed in body wall muscle, DAF-4 might have its unique function independent of type I receptor SMA-6 (Gunther *et al.*, 2000). Also, the incomplete rescue of size of *daf-4* mutant with hypodermal expression may due to the activity of *rol-6* promoter they used. Recently, our concurrent efforts to investigate the molecular mechanism of body length regulation identified a target gene that is normally suppressed by DBL-1 signaling and is also expressed in the hypodermis. Overexpression of this gene in the hypodermis but not in other tissues including intestine, pharynx, and body wall muscle, was found to shorten the body length (Morita, K. *et al.*, submitted for publication). Taken together, these results indicate that the hypodermis is a key tissue involved in determining body length.

The hypodermis is a group of cells underlying the cuticle, most of which are multinucleated syncytia (Hedgecock and White, 1985; Sulston and Horvitz, 1977). The hypodermis and cuticle are thought to be important for maintenance of worm morphology. Indeed, it is widely known that some mutations of cuticle collagen genes show morphological changes and phenotypes of Dpy, Lon, and Rol, but not Sma. Hypodermal cells also define the change of shape from an oval to a worm shape during embryogenesis (reviewed in Simske and Hardin, 2001). Again, hypodermis may be the most critical tissue to control body length. In particular, hyp-7 is the largest hypodermal cell and covers a large part of the body. I observed a high level of *sma-6* expression in hyp-7, which implies that hyp-7 contributes largely to the regulation of body length. During larval development, hyp-7 grows and increases the number of nuclei as a consequence of cell fusions with the daughter cells of lateral and ventral seam cells (hypodermal blast cells) (Figure 7A; Sulston and Horvits, 1977). These seam cells synthesize DNA just before each molt and divide. The proliferation occur only during larval growth and the daughter cells derived from the blast cells endoreplicate soon after the cells are born and then these daughter cells fuse with hyp-7 (Figure 7B; Hedgcook 1982 and 1985). Thus, in *C. elegans*, lateral seam cells, where *sma-6* is not expressed (Figure 7C, see also Figure 1F and H), are diploid and not syncytical but the main body hypodermis is syncytial polyploid tissue as a consequence of endoreplication and cell fusion during larval development. In the meanwhile, by heat shock experiment, I also showed that the DBL-1 signaling at middle larval stage is critical to regulate body length in addition to the importance of hypodermis (Table 3). Therefore, DBL-1 signaling maybe involved in the body-length determination probably through the regulation of

hypodermal cell proliferation and/or endoreplication during larva development.

A recent investigation suggests that endoreduplication in hypodermal nucleus is involved in the body length determination of *C. elegans* (Flemming *et al.*, 2000). Endoreplication, which consists of repetitive nuclear DNA synthesis cycles without cell division, leads to polyploidy and the endoreplication cycles are seen in many animals and plants (Conlon and Raff, 1999; Traas et al., 1998; Vidwans and Su, 2001). An adult worm continues to grow without cell divisions after the last molt and Flemming *et al.* also showed that some hypodermal nuclei undergo postlarval rounds of endoreplication. The continuous growth and progression of polyploidization in adulthood are not inconsistent with the expression of SMA-6 in adult. They concluded that, when focused on volume (mm^3) of the animal, around 60% of the volume depend on the acellular growth in adult. Even though, the result of heat shock experiment suggests that information of the signal input during larval development may be important to determine the final body size.

In case that the DBL-1 signaling regulates the endoreplication, how is it achieved? In *Drosoplila*, endoreplication is likely to be required for massive gene amplification and large-scale transcription (reviewed in Vidwans and Su, 2001). A component necessary for initiation of endreplication in *Drosophila*, Fizzy-related (FZR) has been identified (Sigrist and Lehner, 1997). FZR is normally promotes the degradation and inactivation of mitotic cyclins to ensure the absence of them during G1 phase. Thus, the inhibitory factor for endoreplication, E cyclin, is eliminated during the cycles. Homolog of FZR seems to be conserved over the spices. It is found in *C.elegans* genome (The C. elegans genome sequencing consorcium) as well as in yeast, although the study on the function in *C. elegans* has not been reported. Thus, the possibility

that TGF-beta like signalling in hypodermis of *C. elegans* may be involved in endoreplication cycles via cell cycle regulators like is raised.

On the one hand, in *Drosophila*, components of the insulin signaling pathway have been reported to be involved in the regulation of cellular growth and/or cell size (Bohni *et al.*, 1999; Gao *et al.*, 2000; Goberdhan *et al.*, 1999; and reviewed in Day and Lawrence, 2000; Leevers, 1999). However, this may not be the case in *C. elegans*, because mutants of the insulin receptor pathway in this organism reveal Daf phenotype rather than Sma phenotype and it has been shown that the pathway is involved in aging in *C. elegans*. And any genetic interactions between the insulin receptor pathway and Sma pathway have also not been shown yet.

Though, significance of the DBL-1 signaling pathway in hypodermis to control body length of worm is clearly evident, *sma-6* expresses in the pharynx and intestine as well as in the hypodermis. What is the exact role of the signaling pathway in the pharynx and intestine? However the role of the pathway in these tissue is not clear, the signaling may actually play a part, at least in the intestine. Because, by the analysis using differential hybridization with arrayed cDNA, we have identified several genes, which are expressed in intestine and regulated by DBL-1 signalimg. In addition, the *sma-6* expression in the intestine is also regulated by the DBL-1 signaling itself, thereby constituting an autoregulatory circuit (Mochii *et al.*, 1999). Since the intestine is also a poliploid tissue (Hedgecock and White, 1985; Sulston and Horvitz, 1977), DBL-1 signaling might be involved in the endorepliction cycle also in the tissue. DBL-1 signaling in pharynx and intestine might contribute to reflect the availability of nutrition to growth. Environmental factors, in particular, such as nutrition play an important part to control animal size. It has been known that the information of nutrient availability affects the

decision of the dauer larva formation through DAF-7 signaling (Ren *et al.* , 1996), other TGF-b-like signaling pathway in *C. elegans*. At the same time, or after the commitment time period to form dauer larva during the L1 and L2 larval stages, the information of food intake might have an effect on growth through DBL-1 signaling.

Although clarification of the precise mechanism of body length determination awaits further studies, the present study furthers our understanding of the basis of intercellular interaction in this morphologically fundamental and dynamic event.

Table 4. Zn-finger GATA factors in *C.elegans*

Gene	Domains	Site of expression
ELT-1	GATA x2	Epidermis
ELT-3	GATA x1	Hypodermis
END-1	GATA x1	Endoderm
END-3	GATA x1	Endoderm [a]
ELT-2	GATA x1	Endoderm
MED-1	GATA x1	Endoderm and mesoderm
MED-2	GATA x1	Endoderm andmesoderm
F52C12.5 (ELT-5 [b])	GATA x1 anterior and	Seam cells, sheath and socket, and ventral cells in embryogenesis [b]
F55A8.1 (ELT-6 [b])	GATA x1 anterior and	Seam cells, sheath and socket, and ventral cells in embryogenesis [b]
C18G1.2 (ELT-7 [c])	GATA x1	Endoderm [c]

[a] Maduro *et al.*, unpublished data

[b] Koh and Rothman, unpublished data

[c] Strohmaier *et al.*, unpublished data

Table 5. Summary of the expression patterns of DBL-1 signaling pathway components

	DBL-1 [a]	DAF-4 [b]	SMA-6 [c]	SMA-3 [d]
	Ligand	Receptor TypeII	Receptor TypeI	Smad
Neurons				
Head gangliae [e]	+	+	-	-
Phasmid neurons	-	+ [f]	-	-
VNC	+	+ [g]	-	-
Pharyngeal neurons	+	-	-	-
DVA	+	-	-	-
CAN	+	-	-	-
Dorso-rectal ganglion	+	-	-	-
Pre-anal ganglion	+	-	-	-
Dorsal nerve cord	+	-	-	-
Body wall muscle	+ [h]	+ [i]	-	-
Pharynx	+	+	+	+
Intestine	-	+	+	+
Hypodermis	-	+	+ [k]	-
Other tissues	-	-	-	+ [j]

[a] Suzuki *et* al., 1999; Morita *et al.*, 1999
[b] Patterson *et al.*, 1997; Gunter *et al.*, 2000
[c] Krishna *et al.*, 1999; Mochii *et al.*, 2000
[d] Suvage-Dunn *et al.*, 2000
[e] LG, VG, and RVG
[f] PLN and PLM
[g] PVT
[h] Head region
[i] Adult body
[j] Not described in detail
[k] In this study

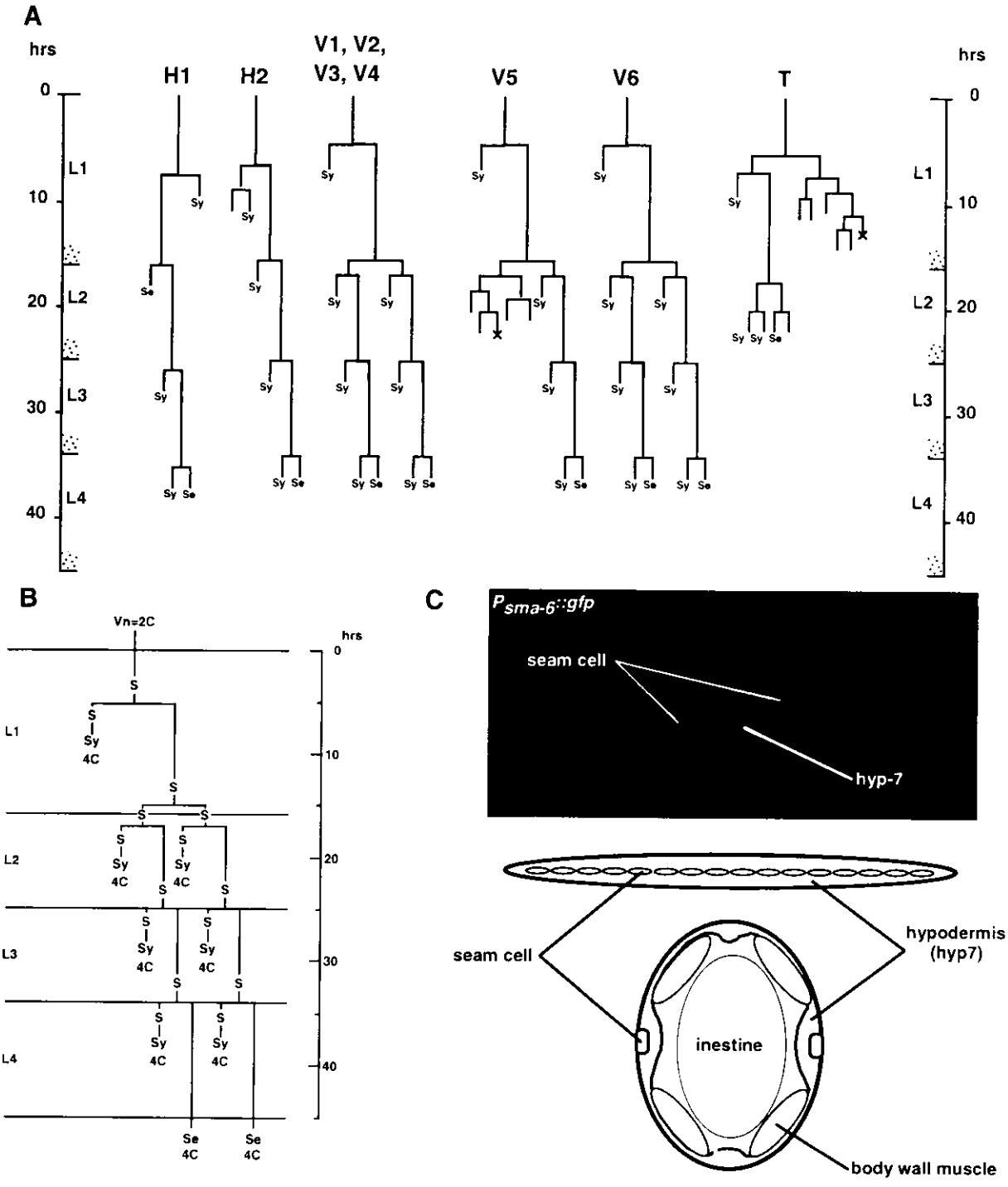

Figure 7. Schematic diagrams of hypodermal cell development and the hypodermal expression of *sma-6*.

(A) Post embryonic H, V, and T lineages: development of hypodermal cells. Molts are indicated by solid lines on the time axis and the adjacent stippling indicating the period of lethargus (Adopted from *C. elegans* II, 1997). Only seam cell (Se) and syncytial hypodermal cell (Sy) lineage are given the accounts.

(B) Endoreplications in the lateral hypodermal cells (V lineages). DNA synthesis is indicated by an S. Se and Sy is represents lateral seam cell and syncytial nuclei, respectively. C is the haploid genome content. (Adopted from Hedgecock and White, 1985)

(C) Schematic diagrams of the *Psma-6 :: gfp* expression in the hypodermis at L3 stage. Fluorescence is seen in the syncytial hypodermal cell (hyp7), but not in seam cells.

MATERIALS AND METHODS

C. elegans strains and general methods

C. elegans strains were cultured and manipulated using standard methods (Brenner, 1974). All strains were grown at 20 ℃ unless otherwise noted. Strains used in this study were the wild-type *C. elegans* variety Bristol N2 strain and the *sma-6* mutant strain LT186 (*sma-6* (*wk7*) II) (Krishna *et al.*, 1999). All strains were provided by the *Caenorhabditis* Genetic Center.

Germ line transformation of C. elegans

Transformation of *C. elegans* was performed by microinjection of plasmid DNA into the distal arms of the hermaphrodite gonad as described previously (Mello *et al.*, 1991). Each plasmid was prepared for microinjection using the QIAGEN plasmid purification kit. All microinjections were carried out at a final DNA concentration of 100 ng/μl.

Stable transgenic lines were generated through the selection of transformed progeny exhibiting GFP expression. For the rescue experiment, rescue of the body length defect was assessed. Each transformation result was examined with multiple independent transgenic lines.

Transformation constructs

All GFP reporter vectors and expression vectors were obtained from A. Fire (A. Fire, personal communication).

To generate the rescue construct pSQA13, a 9.2 kb fragment that was cut out from cosmid clone C32D5 with *PstI* and *Eco52I* and encompassed the *sma-6* region was subcloned into pBluescript SK(-) that was digested with *PstI* and *NotI*. A 6.0 kb *BsmI* to *SacII* region of pSQA13 was replaced with a 2 kb *BsmI* to *SacII* fragment from the *sma-6* cDNA clone (obtained from R. Padgett). In the resulting construct, named pSQF8, the coding region and 3' UTR of the *sma-6* cDNA clone was substituted for the correspond regions of pSQA13. To make the *sma-6::gfp* fusion construct, the 3' end of the *sma-6* coding region was amplified by PCR. The primers used were 14282F ("F" postfix indicates forward direction primers), 5'-GTGTGTTGT ATCGCTGATT TGGG-3', and 15129R ("R" postfix indicates reverse direction primers), 5'-CGGGATCCAGA TTGATTGGTGGCTGACTC-3'. The 15129R primer was designed to contain a *BamHI* site (underlined), instead of a TAA stop codon. Amplified fragments from the *sma-6* cDNA clone or the cosmid clone C32D5 were digested with *HpaI* and *BamHI*, then cloned in-frame between the *HincII* and *BamHI* sites of the GFP reporter vector pPD95.77 and named pQG5.1H4 and pQG1.0B1, respectively. A *PstI* to *HpaI* fragment, 4.5 kb from pSQF8 or 5.2 kb from pSQA13, containing upstream regulatory sequences and another part of coding region of the *sma-6*, was inserted between the *PstI* and *HincII* sites of pQG5.1H4 or pQG1.0B1 and named pQG5.2I19 ($P_{sma\text{-}6(-3088)}$*::sma-6*) and pQG1.1C1, respectively. To generate pGFP4G31, a transcriptional GFP reporter construct, 1.3 kb fragments containing the 5' upstream sequence and the initiator methionine codon of SMA-6 were amplified by PCR from the

sma-6 genomic clone pSQA13. The amplified products were digested with *SalI* and *BamHI* and the resulting 0.85 kb fragments were inserted into the GFP reporter vector pPD95.67 digested with *SalI* and *BamHI*. The primers used were; 11146F, 5'-CGCTTTTTGAAGCTTT CGGC-3'; and 12478R, 5'-CGGGAT CCATTAAATCT GAAATTTGC-3'. The 12478R primer was designed to contain a *BamHI* site (underlined) following the ATG start codon.

A 2.2 kb *PstI* to *SalI* fragment, further upstream from the *SalI* site, was excised from pQG1.1C1 and inserted into pGFP4G31 that had been digested with *PstI* and *SalI*. The resulting GFP reporter construct contained a 3 kb *sma-6* 5' upstream region, and was designated pGFP4J1 ($P_{sma-6(-3088)}$::*gfp*). p414GFP ($P_{sma-6(-414)}$::*gfp*), a GFP reporter construct containing 414 bp of *sma-6* 5' upstream sequence, was created by a deletion spanning from the *PstI* site to the *DraIII* site of pGFP4J1. p414Q ($P_{sma-6(-414)}$::*sma-6*), which contained the same regulatory region as p414GFP and the *sma-6*::*gfp* fusion gene, was also made by deleting the *PstI* to *DraIII* region from pQG5.2I19.

A series of further deletion constructs from –414 (the number indicates the bp relative to ATG) were made by the PCR method using forward primers together with the reverse primer, 12478R. The sequences of the forward primers used were as follows, with the number of each referring to its position relative to ATG: -308F, 5'-AACTGCAGCCGCACTTCTACCTATT CTCCG-3'; -284F, 5'-AACTG CAGTCATACATATATCCATTTTGGC-3'; -277F, 5'-AACTGCAG TATATCCATTTTGGCGCAAC-3'; -265F, 5'-AACTGCAGGGCGCAACTTTT CCGTTCAG-3'; -248F, 5'-AACTGCAGCAGATCATTAGTAT CATTAGG-3'; -234F, 5'-AACT GCAGCATTAGGCGGACAAGTTGTC-3'; -219F, 5'-AACTGC AGTTGTCAAA AAGAACATTAAGTG-3'; -209F, 5'-AACT GCAGGAACATT AAGTGAATGA TGAG-3'; -157F, 5'-AACTGCAGAGGCCGTAGATTGAAAT

ATTG-3′. These forward primers (F) contained a *PstI* site (underlined), while the reverse primer 12478R contained a *BamHI* site, allowing the amplified fragments to be inserted into pPD95.67 digested with *PstI* and *BamHI*. The resulting GFP reporter constructs were designated according to the position of their 5′ end relative to the ATG.

pΔGATA-GFP ($P_{sma\text{-}6(\Delta GATA\ )}$ *::gfp*) was generated by the PCR method using p414GFP as the DNA template. The sequences of primers used were as follows: -157F, 5′-AACTGCAGAGGCCGTAGATTGAA ATATTG-3′; -194R, 5′-GCTCTAGACATTCACTTAATGTTC-3′. Forward primer –157F contained a *PstI* site, while reverse primer -194R contained a *XbaI* site (underlined). The resulting construct contained 414 bp with an internal deletion of three GATA elements of the *sma-6* 5′ upstream sequence. The same primers and p414Q as the template DNA were used to create pΔGATAQ ($P_{sma\text{-}6(\Delta GATA\ )}$*::sma-6*) , which had the same upstream sequence as pΔGATA-GFP and the *sma-6::gfp* fusion gene.

Mutation of each of three GATA elements located between –190 bp and –161 bp was carried out by PCR using a mutant primer and p414GFP as the DNA template. The sequences of the primers used were as follows: Mutant GATA 1 F, Forward primer for mutant GATA 1, 5′-**CTCGCTTTAAG** AGAG ATAGT GTACTGATAG -3′ annealing to positions –190 to –161; -191R, Reverse primer for mutant GATA 1, 5′-CATCATTCACTTAATGTTCTTTTTG AC-3′, complementary to positions –217 to –191; Mutant GATA 2 F, Forward primer for mutant GATA 2, 5′-**CTCGCT**TGTACTGATAGAAGAGGCCGTA G-3′, positions –177 to –149; -178R, Reverse primer for mutant GATA 2, 5′-CT CTT AACTATCTCATCATTCAC -3′, complementary to positions –200 to -178; -160F, Forward primer for mutant GATA 3, 5′- AAGAGGCCGTAGATTG AAATATTGTACG -3′, positions –160 to –133; Mutant GATA 3 R, Reverse

primer for mutant GATA 3, 5'-**AGCGAC**G TACACTATCTCTCTTAACTATC -3', complementary to positions –189 to –161. Substituted nucleotides in the GATA consensus sequences are indicated in bold. All mutations were verified by DNA sequencing.

Transformation constructs for tissue-specific expression

The *sma-6* cDNA clone was subcloned into the heat shock promoter vector pPD49.83 using the *KpnI* and *SacI* site, then the *HpaI* to *SpeI* fragment of pQG5.2I19 was subcloned into it. The resulting construct, designated pQGL11, contained a *sma-6::gfp* fusion gene driven by the *hsp16.41* promoter ($P_{hsp16.41}$*::sma-6*) . Replacing the heat shock promoter with a *myo-2* promoter made the pharyngeal-muscle-specific expression construct, P_{myo-2}*::sma-6*. The *myo-2* promoter was obtained from the expression vector pPD30.69 using the *NheI* and *SpeI* sites. The 0.6 kb 5'upstream sequences of Y105C5A.13 and *dpy-7* (Gilleard *et al.*, 1997; Johnstone and Barry, 1996) were used for gut- and hypodermis-specific expression constructs, P_{yk92e8}*::sma-6* and P_{dpy-7}*::sma-6*, respectively. The cDNA clone *yk92e8* was originally obtained as a gene that was down-regulated by DBL-1 signaling (Mochii *et al.*, 1999) and was encoded by Y105C5A.13 . I found the promoter region of Y105C5A.13 had gut-specific activity. Each promoter region was amplified by PCR from the genomic DNA and digested with *PstI* and *XbaI*. The sequences of the primers used were as follows: 92e8-642F, 5'-AACTGCAGTGGCAAGTAGGAGGGAG G -3'; 92e8-3R, 5'-GCTCTAGATTC ACGGAGTTGATAGGC-3'; *dpy-7*-603F, 5'-AACTGC AGTGAGTGTTCGAGC TCCAACC-3'; *dpy-7*-R, 5'-GCTCTAGATT TATCTGGAACAAAATGTAAG-3'. These forward primers (F) contained a

PstI site (underlined), while reverse primers (R) contained a *BamHI* site. The amplified and digested fragments were inserted into the GFP reporter vector pPD95.69 or substituted for the heat shock promoter of pQGL11.

Worm body length measurement

Animals were grown at 20 ℃. Adult hermaphrodites were strewed on seeded plates and allowed to lay eggs for several hours. The adults were then removed and the embryos were allowed to hatch and develop. Six days after hatching, F1 adults were transferred to a fresh plate and anesthetized with 200 µl of 10 mM sodium azide solution. Anesthetized animals were linearized and their body lengths were measured with a dissecting microscope and micrometer.

CONCLDING REMARKS

The signaling by the members of TGF-β superfamily plays many roles in development and growth. In nematode *Caenorhabditis elegans*, DBL-1 signaling pathway, which is one of the TGF-β-like signaling pathways, regulates the body length of the worm.

In this study, I first made it appear that type I receptor for DBL-1 signaling, SMA-6, is highly expressed in the main body hypodermis, but not lateral hypodermal blast cells, in addition to the expression in pharynx and intestine. Then I represented the sufficiency of the hypodermal expression of SMA-6 to restore the reduced body length of *sma-6* mutant using transgenic lines of each tissue specific promoter fusion with *sma-6* gene. I also showed the importance of the hypodermal expression to regulate body length using *sma-6* own promoter. I found that The triple consensus sequence recognized by GATA type transcription factor get lined up in immediate upstream of *sma-6* coding region. The deletion or mutation of these putative GATA biding sequence resulted in loss of the expression in hypodermis and the ability to restore the reduced body length. At the same time, it became clear that the signaling in pharynx and intestine are not critical for the control of body length however the actual role of the signaling in these tissues. Finally, I showed the signal input at L2-L3 stage is most necessary to define the final body length. Together with the previous works by now, these results give raise the possibility that BDL-1 signaling in the hypodermis is involved in the regulation of endoreplication. To elucidate the mechanism by which the DBL-1 signaling control body length, further investigation including identification and analysis of targets of the signaling, which directly regulate body length, will be desired. I wish that the study of body size control using *C. elegans*

would throw new light upon the fundamental aspects of biology.

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ACKNOWLEDGMENT

I wish to make an address of thanks to all who supported and encouraged me to accomplish this study.

First, I express my unbounded gratitude to my research advisor, Professor Naoto Ueno for his exact guidance and continuous encouragement throughout this study. I also express my deep appreciation to Drs. Kenichiro Morohashi, Masaharu Noda, and Ikue Mori, for their review and critical reading of my manuscript.

Furthermore, I really appreciate Dr. Kiyokazu Morita for his advises and helpful discussions.

And I would like to thank A. Fire, S. Xu, J. Ahnn, and G. Seydoux for providing various vectors, A. Coulson for cosmids, and R. Padgett for *sma-6* cDNA. I also thank the *Caenorhabditis* Genetics Center, which is funded by the National Institutes of Health National Center for Research Resources (NCRR) for some *C. elegans* strains used in this work, *the C. elegans* Genome Sequencing Center for invaluable genome sequences, and the *C. elegans* community for numerous personal communications.

I express my special thanks to Mrs. Mariko Ichikawa for her technical support. And I am also grateful to all the members of the Division of Morphogenesis, Department of Developmental Biology, National Institute for Basic Biology (NIBB) for their helpful discussion and generous supports.

Finally, I cannot thank my mother and father enough for their support and encouragement.

The study described in this thesis was all carried out at the Department of Developmental Biology, NIBB.