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Hypodermal Expression of *Caenorhabditis elegans* TGF-β Type I Receptor SMA-6 Is Essential for the Growth and Maintenance of Body Length

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There are several transforming growth factor-β (TGF-β) pathways in the nematode *Caenorhabditis elegans*. One of these pathways regulates body length and 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. To further examine the molecular mechanisms of body-length regulation in the nematode by the TGF-β pathway, we examined the regional requirement for the type-I receptor SMA-6. Using a SMA-6::GFP (green fluorescent protein) reporter gene, *sma-6* was highly expressed in the hypodermis, unlike the type-II receptor DAF-4, which is reported to be ubiquitously expressed. We then examined the ability of SMA-6 expression in different regions of the *C. elegans* body to rescue the *sma-6* phenotype (small) and found that hypodermal expression of SMA-6 is necessary and sufficient for the growth and maintenance of body length. We also demonstrate that GATA sequences in the *sma-6* promoter contribute to the hypodermal expression of *sma-6*.

***Key Words:* *Caenorhabditis elegans*; TGF-β; Sma/Mab pathway; hypodermis; body length.**

INTRODUCTION

Intercellular signaling by growth factors coordinates many developmental decisions controlling the growth, differentiation, and morphogenesis of metazoans. The transforming growth factor-β (TGF-β) superfamily of secreted proteins has key roles in these signaling networks in many organisms. An 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 are 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

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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) pathway, the most well-characterised TGF-β-like pathway in this organism (Thomas *et al.*, 1993). Dauer larvae is the nonfeeding and developmentally arrested stage that is specialized for dispersal characteristics that promote survival in an unfavorable environment (Cassada and Russell, 1975). The dauer/nondauer developmental decision is influenced by 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, 1984a,b). The Daf pathway is a part of the pathway regulating 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* induce 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 defect). These phenotypes indicate that DAF-4 also functions in another well-characterized 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 *D*pp, *B*MP-*l*ike), and regulates body size, mail 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 signals. 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. Enhancement of the Daf-c phenotype, however, 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 that there is a low level of cross-talk between the Sma and Daf pathways at the level of ligand–receptor interaction. When green fluorescent protein (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. 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 (Morita *et al.*, 1999; Suzuki *et al.*, 1999). Thus, it seems likely that DBL-1 is secreted from neurons, then acts as a signal to other cell types in targeted tissues, which express downstream components. The cell types that respond to the DBL-1 signal to regulate body size have not yet been clarified.

We are interested in the mechanisms by which the ligand DBL-1 regulates the body length of *C. elegans*. Here, we examined the site of action of SMA-6 in the regulation of body length. We 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. We also examined the contribution of the GATA sequences that are present in the immediate upstream region of *sma-6* ATG to hypodermal expression.

MATERIALS AND METHODS

C. elegans Strains and General Methods

C. elegans strains were cultured and manipulated by using standard methods (Brenner, 1974). All strains were grown at 20°C 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* Genetics 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 (QIAGEN). All microinjections were performed 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.

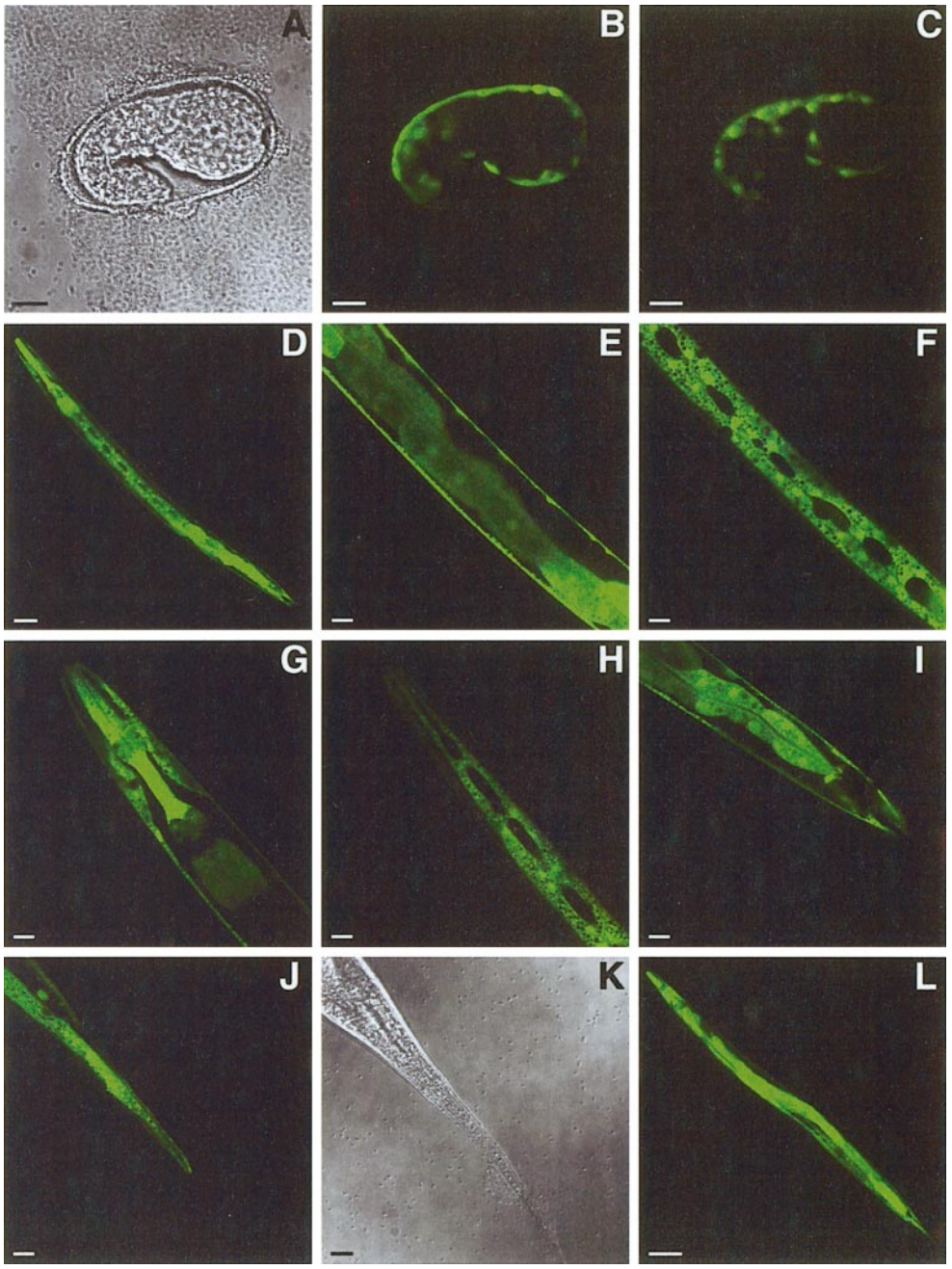
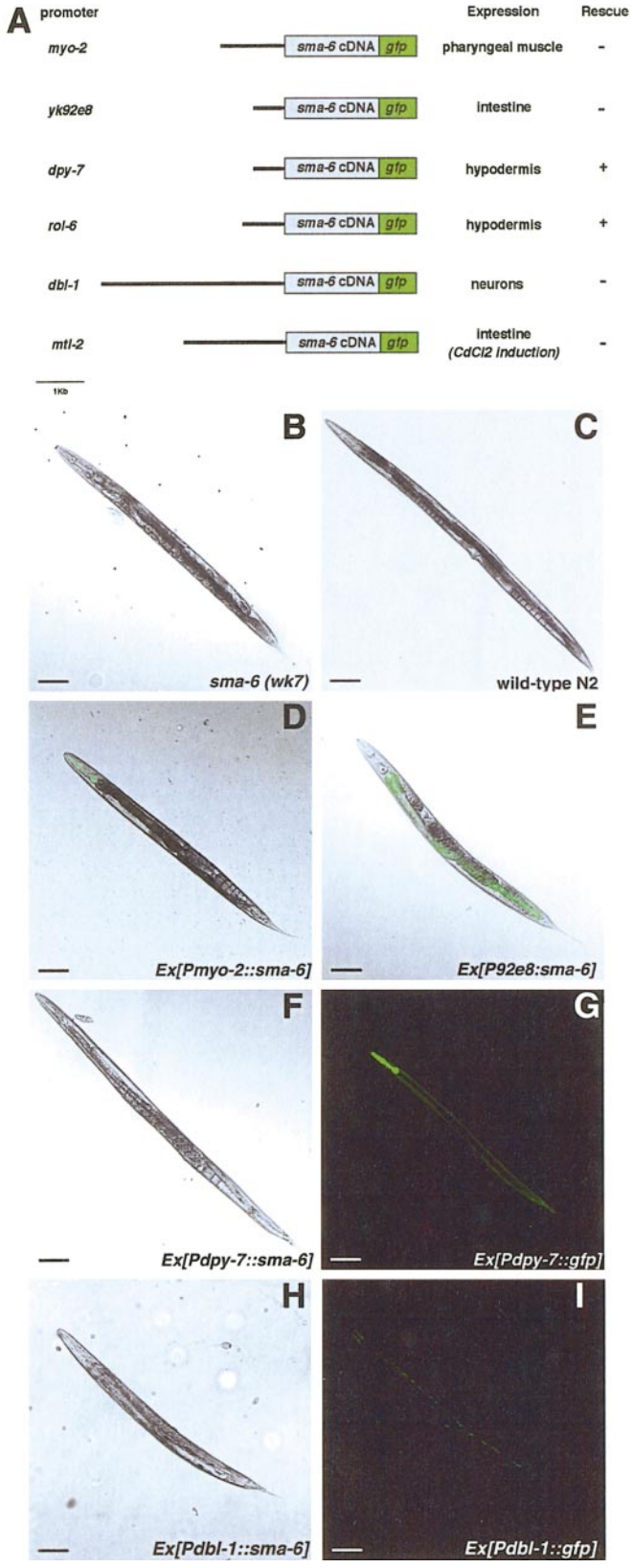


FIG. 1. *sma-6*::*gfp* is expressed in hypodermal cells. Confocal images of transgenic animals of an integrated line bearing $P_{sma\text{-}6(-3085)}$::*gfp*, a transcriptional *gfp* fusion construct with 3085 bp of the *sma-6* 5′ upstream region, are shown. (Details of the construct are given in Materials and Methods. See also Fig. 4A.) The construct includes the nuclear localization signal, but fluorescence can also be detected in the cytoplasm. Bar, 10 μm (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 observed in dorsal and ventral hypodermal cells. (D) L3 larva. Fluorescence is observed 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 observed 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 observed 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 observed 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 observed 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.



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 from the cosmid clone C32D5 with *Pst*I and *Eco*52I and encompassed the *sma-6* region was subcloned into pBluescript SK(−), which was digested with *Pst*I and *Not*I. A 6.0-kb *Bsm*I to *Sac*II region of pSQA13 was replaced with a 2-kb *Bsm*I to *Sac*II 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 corresponding regions of pSQA13. To make the *sma-6::gfp* fusion construct, the 3′ end of the *sma-6* coding region was amplified by using polymerase chain reaction (PCR). The primers used were 14282F ("F" suffix indicates forward direction primers), 5′-GTGTGTTGTATCGCTGATT TGGG-3′, and 15129R ("R" suffix indicates reverse direction primers), 5′-CGGGATCCAGATTGATTGGTGGCTGACTC-3′. The 15129R primer was designed to contain a *Bam*HI site (underlined), instead of a TAA stop codon. Amplified fragments from the *sma-6* cDNA clone or the cosmid clone C32D5 were digested with *Hpa*I and *Bam*HI, then cloned in-frame between the *Hin*cII and *Bam*HI sites of the GFP reporter vector pPD95.77 and named pQG5.1H4 and pQG1.0B1, respectively. A *Pst*I to *Hpa*I fragment, 4.5 kb from pSQF8 or 5.2 kb from pSQA13, containing upstream regulatory sequences and another part of the coding region of *sma-6,* was inserted between the *Pst*I and *Hin*cII sites of pQG5.1H4 or pQG1.0B1 and named pQG5.2I19 ($P_{sma-6(-3085)}$*::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 using PCR from the *sma-6* genomic clone pSQA13. The amplified products were digested with *Sal*I and *Bam*HI and the resulting 0.85-kb fragments were inserted into the GFP reporter vector pPD95.67 digested with *Sal*I and *Bam*HI. The primers used were: 11146F, 5′-CGCTTTTTGAAGCTTT CGGC-3′; and 12478R, 5′-CGGGATCCATTAAATC

FIG. 2. 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–I) Transgenic animals bearing an extrachromosomal array expressing SMA-6 in pharyngeal muscle (D, P_{myo-2}*::sma-6*), intestine (E, P_{yk92e8}*::sma-6*), hypodermis (F and G, P_{dpy-7}*::sma-6*), and neurons (H and I, P_{dbl-1}*::sma-6*) in a *sma-6* (*wk7*) mutant background. GFP was fused to the C-terminal end of SMA-6, but fluorescence was extremely weak and sometimes was not detectable in these cases. The fluorescence observed in (D), (E), and (I) is due to a transcriptional *gfp* reporter construct with each promoter coinjected with the *sma-6* cDNA construct to reconfirm the promoter activity. (G) Fluorescence image of a L3 larvae of P_{dpy-7}*::sma-6* transgenic line. The fluorescence observed in the hypodermis is also due to a transcriptional *gfp* reporter P_{dpy-7}*::gfp* and the fluorescence in the pharynx is due to other *Pmyo-2::gfp.* Because of very weak expression of P_{dpy-7}*::gfp,* the reporter gene *Pmyo-2::gfp* was coinjected as another marker. (I) Fluorescence image of the same animal as in (H). The P_{dpy-7}*::sma-6* array rescued the Sma phenotype, but the P_{myo-2}*::sma-6*, P_{yk92e8}*::sma-6*, and P_{dbl-1}*::sma-6* arrays did not. Bar, 100 μm.

TGAAATTTGC-3′. The 12478R primer was designed to contain a *Bam*HI site (underlined) following the ATG start codon.

A 2.2-kb *Pst*I to *Sal*I fragment, further upstream from the *Sal*I site, was excised from pQG1.1C1 and inserted into pGFP4G31, which was digested with *Pst*I and *Sal*I. The resulting GFP reporter construct contained a 3-kb *sma-6* 5′ upstream region, and was designated pGFP4J1 ($P_{sma\text{-}6(-3085)}$*::gfp*). p414GFP ($P_{sma\text{-}6(-414)}$*::gfp*), a GFP reporter construct containing 414 bp of *sma-6* 5′ upstream sequence, was created by a deletion spanning from the *Pst*I site to the *Dra*III site of pGFP4J1. p414Q ($P_{sma\text{-}6(-414)}$*::sma-6*), which contained the same regulatory region as p414GFP and the *sma-6::gfp* fusion gene, was also made by deleting the *Pst*I to *Dra*III region from pQG5.2I19.

A series of further deletion constructs from −414 (the number indicates the bp relative to the ATG) were made by using the PCR method with 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′-AACTGCAGCCGCACTTCTACCTATTCTCCG-3′; −284F, 5′-AACTGCAGTCATACATATATCCATTTTGGC-3′; −277F, 5′-AACTGCAGTATATCCATTTTGGCGCAAC-3′; −265F, 5′-AACTGCAGGGCGCAACTTTTCCGTTCAG-3′; −248F, 5′-AACTGCAGCAGATCATTAGTATCATTAGG-3′; −234F, 5′-AACTGCAGCATTAGGCGGACAAGTTGTC-3′; −219F, 5′-AACTGCAGTTGTCAAAAAGAACATTAAGTG-3′; −209F, 5′-AACTGCAGGAACATTAAGTGAATGATGAG-3′; −157F, 5′-AACTGCAGAGGCCGTAGATTGAAATATTG-3′. These forward primers contained a *Pst*I site (underlined), while the reverse primer 12478R contained a *Bam*HI site, allowing the amplified fragments to be inserted into pPD95.67 digested with *Pst*I and *Bam*HI. 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 using the PCR method with p414GFP as the DNA template. The sequences of primers used were as follows: −157F, 5′-AACTGCAGAGGCCGTAGATTGAAATATTG-3′; −194R, 5′-GCTCTAGACATTCACTTAATGTTC-3′. Forward primer −157F contained a *Pst*I site, while reverse primer −194R contained a *Xba*I 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 and −161 bp was performed by using PCR with a mutant primer and p414GFP or p414Q as the DNA templates. The sequences of the primers used were as follows: Mutant GATA 1 F, Forward primer for mutant GATA 1, 5′-**CTCGCT**TTAAGAGAGATAGTGTACTGATAG-3′ annealing to positions −190 to −161; −191R, Reverse primer for mutant GATA 1, 5′-CATCATTCACTTAATGTTCTTTTTGAC-3′, complementary to positions −217 to −191; Mutant GATA 2 F, Forward primer for mutant GATA 2, 5′-**CTCGCT**TGTACTGATAGAAGAGGCCGTAG-3′, positions −177 to −149; −178R, Reverse primer for mutant GATA 2, 5′-CTCTT AACTATCTCATCATTCAC-3′, complementary to positions −200 to −178; −160F, Forward primer for mutant GATA 3, 5′-AAGAGGCCGTAGATTGAAATATTGTACG-3′, positions −160 to −133; Mutant GATA 3 R, Reverse primer for mutant GATA 3, 5′-**AGCGAC**GTACACTATCTCTCTTAACTATC-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 by using the *Kpn*I and *Sac*I site, then the *Hpa*I to *Spe*I 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, $P_{myo\text{-}2}$*::sma-6*. The *myo-2* promoter was obtained from the expression vector pPD30.69 using the *Nhe*I and *Spe*I sites. The 4-kb 5′ upstream sequence of *dbl-1* (Morita *et al.*, 1999) was used for a neuron-specific expression construct, $P_{dbl\text{-}1}$*::sma-6*. The 0.6-kb 5′ upstream sequences of Y105C5A.13 or *dpy-7* (Gilleard *et al.*, 1997; Johnstone and Barry, 1996) were used for intestine- or hypodermis-specific expression constructs, P_{yk92e8}*::sma-6* and $P_{dpy\text{-}7}$*::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. The promoter region of Y105C5A.13 had gut-specific activity. Each promoter region was amplified by using PCR from the genomic DNA and digested with *Pst*I and *Xba*I. The sequences of the primers used were as follows: 92e8-642F, 5′-AACTGCAGTGGCAAGTAGGAGGGAGG-3′; 92e8-3R, 5′-GCTCTAGATTCACGGAGTTGATAGGC-3′; *dpy-7*-603F, 5′-AACTGCAGTGAGTGTTCGAGCTCCAACC-3′; *dpy-7*-R, 5′-GCTCTAGATTTATCTGGAACAAAATGTAAG-3′. These forward primers (F) contained a *Pst*I site (underlined), while reverse primers (R) contained a *Bam*HI site. The amplified and digested fragments were inserted into the GFP reporter vector pPD95.69 or substituted for the heat shock promoter of pQGL11. The 0.8-kb 5′ upstream sequence of *rol-6* (Mello *et al.*, 1991) from pRF4 was also used for a hypodermis-specific expression construct, $P_{rol\text{-}6}$*::sma-6*, and the 2.2-kb of *mtl-2* promoter from the expression vector pPD54.01 was used for the other intestine-specific expression construct, $P_{mtl\text{-}2}$*::sma-6*.

Worm Body-Length Measurement

Animals were grown at 20°C. Adult hermaphrodites were spread 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. For $P_{mtl\text{-}2}$*::sma-6* transgenic line, animals were grown on the plates supplemented with 10 μM $CdCl_2$ for induction. We confirmed the induction at the 10-μM concentration by using a transcriptional reporter $P_{mtl\text{-}2}$*::gfp* (data not shown). Six days after hatching, F_1 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 by using a dissecting microscope and micrometer.

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

TABLE 1

Hypodermis-Specific Expression of *sma-6* Can Elongate the Reduced Body Size of *sma-6* Mutant

Genotype	Transgene	Length (mm)[a]	Width (×10 mm)[a]	n[b]
Wild-type N2	None	1.33 ± 0.15	0.71 ± 0.04	43
dbl-1 (nk3)	None	0.98 ± 0.06	0.71 ± 0.03	58
sma-6 (wk7)	None	1.02 ± 0.06	0.69 ± 0.03	43
sma-6 (wk7)	*nkEx156[P_{myo-2}::sma-6]*	1.01 ± 0.10	0.72 ± 0.04	84
sma-6 (wk7)	*nkEx172[P_{yk92e8}::sma-6]*	0.99 ± 0.07	0.71 ± 0.04	86
sma-6 (wk7)	*nkEx188[P_{mtl-2}::sma-6]*	0.96 ± 0.05	0.70 ± 0.04	33
sma-6 (wk7)	*nkEx164[P_{dpy-7}::sma-6]*	1.36 ± 0.09	N.D.	54
sma-6 (wk7)	*nkEx176[P_{dpy-7}::sma-6]*	1.46 ± 0.09	0.62 ± 0.04	61
sma-6 (wk7)	*nkEx191[P_{rol-6}::sma-6]*	1.17 ± 0.08	0.62 ± 0.06	46
sma-6 (wk7)	*nkEx193[P_{rol-6}::sma-6]*	1.39 ± 0.29	0.68 ± 0.06	70

[a] Data are presented by mean ± SD. Body lengths were determined for each adult hermaphrodite at 6 days after hatched.
[b] Number of animals measured.

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, we first examined the expression pattern of *sma-6* during the course of *C. elegans* development. We fused 3 kb of the 5′ promoter region of *sma-6* (Krishna *et al.*, 1999) with GFP to create a transcriptional reporter gene (see Fig. 4A) and monitored *sma-6* expression in transgenic worms. The worms harboring the GFP reporter gene were fluorescent throughout the pharyngeal muscle, intestine, and hypodermis. The fluorescence was observed from around the 1.5-fold stage (Figs. 1A–1C) and continued to the adult stage (Figs. 1D and 1L). The expression in the hypodermis was detected in the dorsal (hyp-7) and ventral hypodermal cells (P cells) in the main body region (Figs. 1B–1J), but not in the lateral hypodermal H, V, and T cells (seam cells) (Figs. 1D, 1F, and 1H). The expression was also detected in a part of the hypodermal cells of the head (hyp-6) (Figs. 1G and 1H), but not in the hypodermal cells of the anterior (hyp-1, -2, -3) and posterior end (hyp-10) (Figs. 1G and 1J).

Our previous report using *in situ* hybridization of *sma-6* indicated expression only in the head region and intestine from L2 larva to the young adult stage (Mochii *et al.*, 1999). And, using a *lacZ* reporter, Krishna *et al.* (1999) reported that *sma-6* was also expressed during the embryonic stage as well as in the larval and adult stages and in the intestinal cells and pharyngeal muscle. In addition to this expression, our present study using the GFP reporter demonstrates that *sma-6* is also expressed in the hypodermis from the midembryonic stage to adult. The subtle discrepancy is probably due to a difference in the sensitivity of each method. This is especially true regarding hypodermal expression because the expression is weak compared to that in pharynx and intestine.

Unlike the expression of *dbl-1*, as revealed by the *dbl-1::gfp* reporter gene (Morita *et al.*, 1999; Suzuki *et al.*, 1999; see also Fig. 2I), we did not observe the expression of the GFP reporter with the *sma-6* promoter in the nervous system. This suggests that DBL-1 expressed in neurons is secreted and directed by an unknown mechanism to the SMA-6 expression site 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 by either 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 (see Figs. 4A–4C 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, we 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.

We first generated a translational *sma-6::gfp* fusion construct and examined its ability to rescue the *sma-6* phenotype. When GFP was fused to the C-terminal end of SMA-6, the resulting product rescued the Sma phenotype (see Fig. 4), suggesting that the SMA-6::GFP was functional as a SMA-6 type-I receptor. The fluorescence of the GFP was extremely weak, however, and barely detectable in some

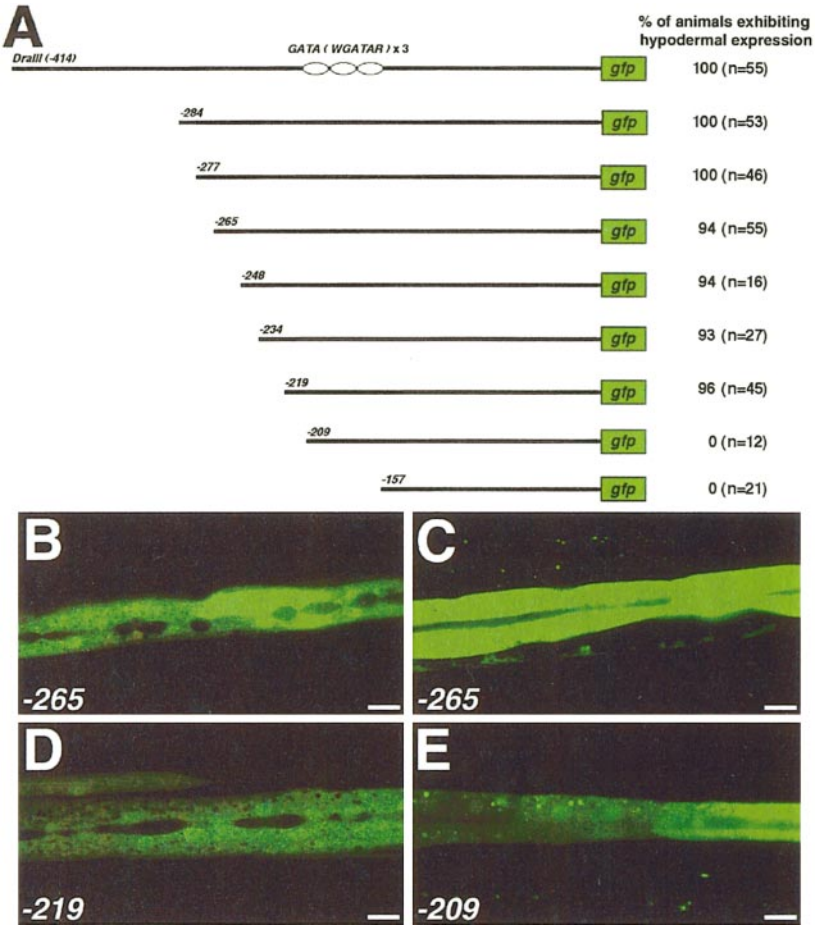


FIG. 3. Effect of deletions of the 5′ upstream region of *sma-6* on *gfp* expression in hypodermal cells. (A) Schematic diagram of the deletion constructs used and summary of the results. All fragments were subcloned into the *gfp* reporter vector pPD95.67 to create transcriptional fusions. Numbers refer 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 observed 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 (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 D, arrowhead) and some neurons (not shown).

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 tissue-specific activity of the promoter.

The *dpy-7* promoter (P_{dpy-7}) and *rol-6* promoter (P_{rol-6}) were used for the hypodermal-specific expression of *sma-6*. *dpy-7* and *rol-6* are the cuticle collagen genes that are expressed specifically in the hypodermis (Gilleard *et al.*, 1997; Kramer and Johnson, 1993; Fig. 2G). For pharyngeal muscle-specific expression, P_{myo-2}, the promoter region of *myo-2*, which codes for pharyngeal muscle-specific myosin (Okkema *et al.*, 1993; Fig. 2D), was used. 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 previously identified as a down-regulated gene in *sma-2* mutants in a cDNA microarray 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 (Fig. 2E), 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

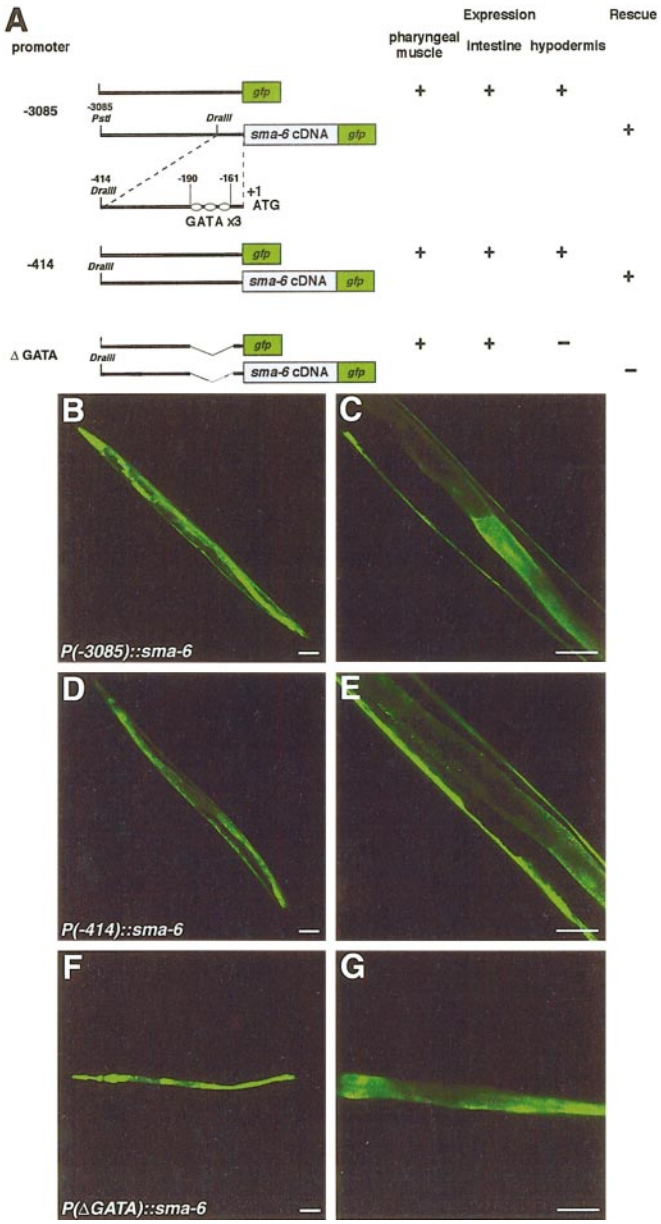


FIG. 4. Internal deletion of putative GATA recognition sequences from the *sma-6* promoter removes the hypodermal expression and ability to rescue the Sma phenotype. (A) Schematic diagram of the constructs used and summary of the results. The numbers indicate the 5′ end of the upstream region contained 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 (A). A *gfp* reporter construct with each promoter was coinjected for the same reason as in Fig. 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 did not affect 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 observed in the hypodermis, even at a higher magnification. There was no rescue of the reduced body length.

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 $P_{sma\text{-}6(-3085)}$, the promoter region of *sma-6*, were also used for ubiquitous expression and for authentic *sma-6* expression, respectively.

The results are summarized in Table 1 and Fig. 2. Only hypodermal expression of SMA-6 with the *dpy-7* and *rol-6* promoters and ubiquitous expression with the *hsp16.41* promoter (data not shown) elongated the shortened body length of the *sma-6* mutant (Table 1 and Figs. 2A and 2F). Neither intestine nor pharyngeal muscle expression rescued the Sma phenotype (Table 1 and Figs. 2A, 2D, and 2E). Further, we also examined whether the expression of SMA-6 in neurons rescued the shortened body length by driving *sma-6* with a *dbl-1* promoter ($P_{dbl\text{-}1}$). Neuronal expression did not rescue the phenotype (Table 1 and Figs. 2A, 2H, and 2I). This results suggests that the Sma pathway is not active in neurons secreting DBL-1 and the ligand functions non-cell-autonomously to regulate body length. Finally, these results indicate that the hypodermal expression of SMA-6 is sufficient and thus the hypodermis is the key tissue for the regulation of body length. In addition to body length, we also tested the change of body width of each transgenic animal (Table 1). Although there was a slight decrease in the width of gravid adult animals of $P_{dpy\text{-}7}$*::sma-6* and $P_{rol\text{-}6}$*::sma-6* transgenic lines, hypodermal SMA-6 increased the body length more than the body width.

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 was removed and replaced by that of the vector (for details, see Materials and Methods). From the above observations, we 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 hypodermis-specific expression of SMA-6 must exist in the 5′ promoter region. Therefore, we 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.

Deletion to −414 bp (relative to the initiator ATG) did not affect hypodermal, intestinal, or pharyngeal expression (see Figs. 4A, 4D, and 4E). The expression level revealed by

TABLE 2
Deletion of GATA Recognition Sequences from Truncated *sma-6* Promoter Results in Loss of Ability to Rescue for the Sma Phenotype

Genotype	Transgene	Length (mm)[a]	Width (×10 mm)[a]	n[b]
Wild-type N2	None	1.33 ± 0.15	0.71 ± 0.04	43
dbl-1 (nk3)	None	0.98 ± 0.06	0.71 ± 0.03	58
sma-6 (wk7)	None	1.02 ± 0.06	0.69 ± 0.03	43
sma-6 (wk7)	*nkEx112*[$P_{sma\text{-}6(-3085)}$::*sma-6*]	1.33 ± 0.07	0.72 ± 0.04	26
sma-6 (wk7)	*nkEx127*[$P_{sma\text{-}6(-414)}$::*sma-6*]	1.24 ± 0.06	0.70 ± 0.04	38
sma-6 (wk7)	*nkEx180*[$P_{sma\text{-}6(\Delta GATA)}$::*sma-6*]	0.96 ± 0.06	0.70 ± 0.04	38

[a] Data are presented by mean ± SD. Body lengths were determined for each adult hermaphrodite at 6 days after hatched.
[b] Number of animals measured.

GFP in the hypodermis decreased gradually with further deletion, from −284 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 (Fig. 3). Deletion to −209 bp resulted in the disappearance of hypodermal expression but not of pharyngeal or intestinal expression (Fig. 3E). Thus, sequences between −219 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, we 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 Figs. 4A and 5A.

GATA Recognition Sequences Are Required for Hypodermal Expression of sma-6 and the Regulation of Body Length

We then deleted only a relatively short sequence of 30 bp including the three consecutive GATA elements from the truncated promoter (414 bp in length) to determine whether deletion of the GATA elements affected the hypodermal expression of *sma-6*. As we expected, the three GATA elements were necessary for hypodermal expression of the *gfp* reporter gene, but not for intestinal or pharyngeal expression (Figs. 4A, 4F, and 4G). 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. 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 ($P_{sma\text{-}6(\Delta GATA)}$::*sma-6*), failed to rescue the Sma phenotype (Fig. 4 and Table 2).

From these observations, we 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 (Figs. 3A and 3E) nor rescued the Sma phenotype (data not shown). This finding suggested that the GATA sequences were required but not sufficient to support hypodermal expression of *sma-6* and that the upstream flanking sequence was also required. To determine the contribution of each GATA element to hypodermal expression, we made a series of transcriptional reporter fusion genes with mutated promoter, in which all or each one of the three elements were mutated. Each mutation was designated as mutant GATA123 or mutant GATA1, GATA 2, and GATA 3 starting from the 5′ upstream and according to their positions, respectively (See Fig. 5A). We tested them for the ability to produce hypodermal expression. The introduction of the mutation in any one of the three GATA elements affected hypodermal, but not intestinal and pharyngeal, expression of gfp reporter gene (Fig. 5). When the mutation was in the most downstream GATA element ($P_{mutant\ GATA3}$::*gfp*), however, the effect was not as striking as those in the other two mutations, and the transgenic animals tested all expressed GFP in the hypodermis, even though the expression intensity was reduced to some degree (Figs. 5A, 5F and 5G). In contrast, when the function of the middle GATA element was ablated, the mutation ($P_{mutant\ GATA2}$::*gfp*) greatly reduced expression in the hypodermis, and in many cases hypodermal expression disappeared (Figs. 5A, 5D, and 5E). The mutation in the first GATA element ($P_{mutant\ GATA1}$::*gfp*), which is located most upstream of ATG, was also remarkably defective in its ability to drive hypodermal expression. Expression from $P_{mutant\ GATA1}$ did not disappear completely and some animals tested had hypodermal expression but it was barely detectable (Figs. 5A–5C). Mutant GATA2 often resulted in ectopic expression of GFP in the body-wall muscles and neurons (data not shown), but mutant GATA1 and mutant GATA3 did not. These results suggests that each of the GATA elements contributes to a different extent to drive the appropriate expression of *sma-6* in the hypodermis. The middle GATA element is the most important and the first one is also necessary for appropriate expression, but the contribution of the third element for hypodermal expression might be minimal. To confirm whether these reductions of promoter activities in the hypodermis correlate with body-length regulation,

TABLE 3

The Reduction of Hypodermal Expression and Loss of the Ability to Rescue by the Mutations in GATA Element Are Correlated

Genotype	Transgene	Length (mm)[a]	Width (×10 mm)[a]	n[b]
Wild-type N2	None	1.33 ± 0.15	0.71 ± 0.04	43
dbl-1 (nk3)	None	0.98 ± 0.06	0.71 ± 0.03	58
sma-6 (wk7)	None	1.02 ± 0.06	0.69 ± 0.03	43
sma-6 (wk7)	*nkEx201[$P_{mutantGATA1}$::sma-6]*	1.03 ± 0.06	0.71 ± 0.04	46
sma-6 (wk7)	*nkEx205[$P_{mutantGATA2}$::sma-6]*	1.05 ± 0.06	0.72 ± 0.04	40
sma-6 (wk7)	*nkEx212[$P_{mutantGATA3}$::sma-6]*	1.27 ± 0.10	0.71 ± 0.06	81
sma-6 (wk7)	*nkEx218[$P_{mutantGATA123}$::sma-6]*	1.03 ± 0.06	0.70 ± 0.04	52

[a] Data are presented by mean ± SD. Body lengths were determined for each adult hermaphrodite at 6 days after hatched.
[b] Number of animals measured.

we also generated some transgenic lines carrying *sma-6* expression constructs with these mutated promoters. Consistent with the above failures of hypodermal expression, the mutations in the GATA elements 1 and 2 resulted in loss of the ability to rescue the Sma phenotype in spite of the presence of intestinal and pharyngeal expression. The third mutated promoter construct retained the ability reflecting the intactness of the activity in the hypodermis (Table 3). As in the case of deletion of all the GATA elements (Figs. 4A, 4F, and 4G), triple mutation of all three GATA elements (mutant GATA123) resulted in both loss of the ability to rescue (Table 3) and hypodermal expression, but expression in the other tissues was maintained (not shown). Together with the result of experiments using tissue specific promoters, these results again suggest that the Sma pathway alone in the pharynx and intestine cannot regulate body size and the hypodermis is the primary target tissue for the regulation.

DISCUSSION

How an organism's body size or length is determined is a fundamental question in biology. In mammals, it is widely accepted that body size is determined by the number of cells but not by the cell size. In nematodes, however, there are a number of cases in which the cell numbers do not correlate with body length (Flemming *et al.*, 2000). We examined 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*.

We demonstrated that the type-I receptor gene *sma-6* is expressed in the hypodermis at a significant level (Fig. 1), and confirmed its previously reported expression in the intestine and pharynx (Krishna *et al.*, 1999; Mochii *et al.*, 1999). We 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. These GATA elements are required for the proper expression pattern of *sma-6* in the hypodermis and the expression correlates with the ability to regulate body length (Fig. 4, Table 2, Fig. 5, and Table 3). 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 required for 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 are suggested to be required for hypodermal expression of this gene. Furthermore, 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 (*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, 1999; Zhu *et al.*, 1997, 1998). Others, *elt-1* and *elt-3*, are 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, might also be required for the development of the hypodermis (Gilleard *et al.*, 1999). Therefore, ELT-1 and ELT-3 might participate in the hypodermis-specific regulation of *sma-6* expression. Although no obvious phenotype was reported from the suppression of *elt-3* by RNA-i (Gilleard *et al.*, 1999) and, also, the RNA-i for *elt-3* did not cause any defect on the hypodermal expression of *sma-6* (data not shown), other GATA factors might compensate for the function of *elt-3*. We did 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

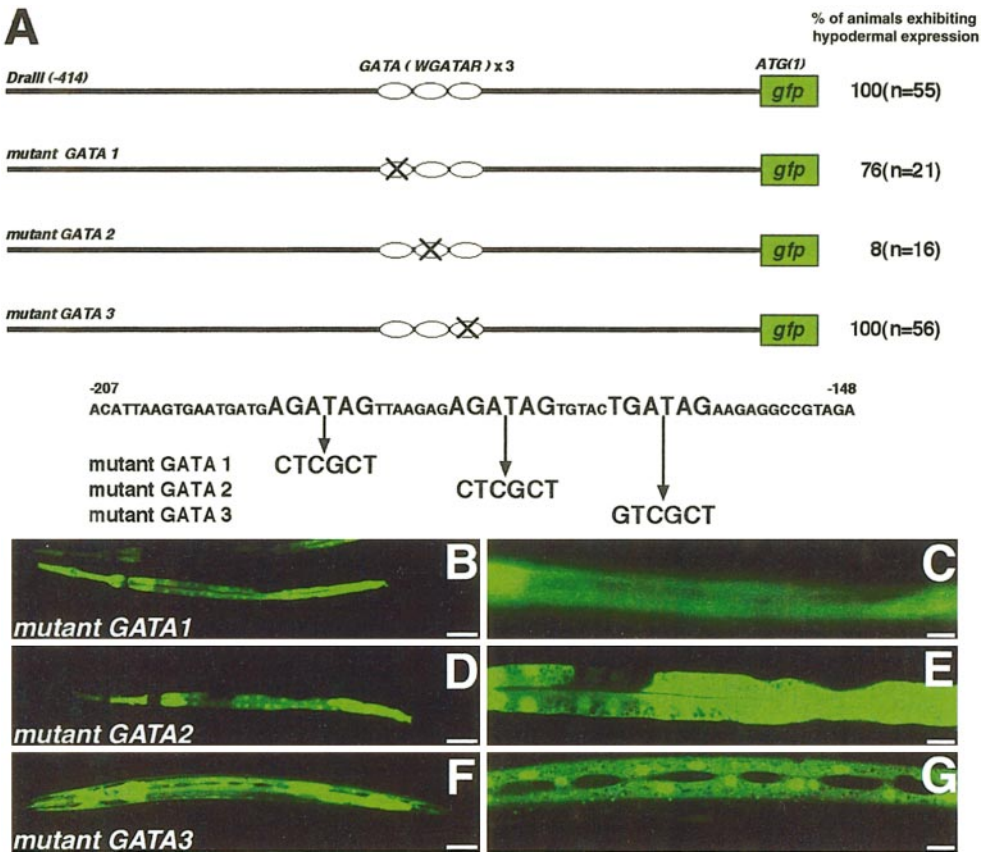


FIG. 5. Analysis of effect of mutations in the putative GATA recognition sites on hypodermal expression. Each of three GATA sites within 414 bp of the 5′ upstream region of *sma-6* was mutated individually. (A) Schematic diagram of the constructs used and summary of the results. Clear ovals indicate 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 also observed 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 observed in the main body hypodermal syncytium only at a higher magnification. (D, E) $P_{mutant\ GATA2}$*::gfp*. No fluorescence is observed 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 evident even at lower magnification. In either case, expression of the *gfp* reporter gene in the pharynx and intestine is maintained.

(Page *et al.*, 1997); however, it is very likely that the expression of *sma-6* is regulated by such GATA factors expressed in the hypodermis.

Our results suggest 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. Deletion of the 5′ flanking sequence from −265 bp (relative to ATG) as well as $P_{mutant\ GATA2}$*::gfp* sometimes resulted in ectopic expression of the GFP reporter in part of the body wall muscle and in some neurons (Fig. 3D). 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 hypodermal expression suggests 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* demonstrated that other transcription factors and cofactors 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 might fulfill the role of conferring 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). 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. If this is the case, the ligand might be secreted and act nonautonomously on these target cells, which express a set of downstream molecular components for body-length regulation. It is noteworthy that DBL-1 signaling itself regulates *sma-6* expression after the initiation of *sma-6* gene expression, thereby constituting an autoregulatory circuit (Mochii *et al.*, 1999).

In the present study, expression of *sma-6* in neurons secreting DBL-1 does not contribute to regulate body length. The hypodermis is the most important target tissue for 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. The hypodermis is a group of cells underlying the cuticle, most of which are multinucleated syncytia (Hedgecock and White, 1985; Sulston and Horvitz, 1977). In particular, hyp-7 is the largest hypodermal cell and covers a large part of the body. We observed a high level of *sma-6* expression in hyp-7, which suggests that hyp-7 contributes largely to the regulation of body length. Hypodermal cells also define the change of shape from an oval to a worm shape during embryogenesis (reviewed in Simske and Hardin, 2001). Recently, in our efforts to investigate the molecular mechanisms of body-length regulation, we 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 the intestine shortens body length (K.M. *et al.*, unpublished data). Taken together, the results indicate that the hypodermis is a key tissue involved in determining body length.

Although, in contrast to our results, Inoue and Thomas (2000) reported that hypodermal expression of *daf-4* using the *rol-6* promoter could only partially rescue the small body size of *daf-4* mutants, the difference might be due to a difference in the expression level in different transgenic lines. Indeed, our transgenic lines carrying *Prol-6::sma-6* seem to differ somewhat in the extent of body-length elongation depending on the line (Table 1). Alternatively, the difference between these results might be due to a difference in the conditions of growth and measurement of size. They maintained animals at 15°C to prevent dauer larva formation and measured them at the young adult stage, while we maintained them at 25°C and measured them when they were adults (6 days after hatching).

Additionally, they also reported that expression of *daf-4* in other tissues was involved in body-size regulation. Especially, they demonstrated that pharyngeal expression of *daf-4* participates in the regulation of worm body width and length. Therefore, we also measured the body width of all transgenic lines. The width of the worm, which was extended by hypodermis-specific expression of *sma-6* using the *dpy-7* or *rol-6* promoters, was slightly less than that of the wild-type and *sma-6* mutants (Table 1). The result suggests that the worm body extended lengthwise. Neuronal expression of *sma-6* did not affect either length or width, as previously shown with *aex-3*::*daf-4* (Table 1). The pharyngeal and/or intestinal expression of *sma-6* by each tissue-specific promoter (Table 1) or mutated *sma-6* promoters (Tables 2 and 3) might increase width. These differences are very small, however, or undetectable. As Inoue and Thomas (2000) suggested, different tissues might contribute to the different aspects of body-size regulation, but we were not able to establish this in our experiments.

Components of the insulin signaling pathway in *Drosophila* are 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). This might not be the case in *C. elegans*, however, because mutants of the insulin receptor pathway in this organism do not have the Sma phenotype and have not been shown to have any genetic interaction with mutants of the Sma pathway.

A recent investigation suggests that endoreduplication is involved in the body-length determination of *C. elegans* (Flemming *et al.*, 2000). Endoreduplication, which consists of repetitive nuclear DNA synthesis cycles without cell division, leads to polyploidy. In *C. elegans*, the main body hypodermis is syncytial polyploid tissue, but lateral seam cells, where *sma-6* is not expressed, are not (Figs. 1F and 1H). Therefore, regulation of polyploidy might be involved in body-length determination. The role of DBL-1 signaling in the pharynx and intestine, where downstream components including SMA-6 are expressed, is unclear. Because endoreduplication is also observed in intestinal nuclei (Hedgecock and White, 1985; Sulston and Horvitz, 1977), the intestine might also be involved in the regulation of body length. The intestine is not the primary tissue defining body length, however, because intestinally expressed SMA-6 could not rescue the Sma phenotype (Figs. 2A and 2E and Table 1).

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

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