

# TGF $\beta$ -like Signaling and Spicule Development in *Caenorhabditis elegans*

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A TGF $\beta$ -like signal is required for spicule development in *Caenorhabditis elegans* males. This signal appears to originate in the male-specific musculature and is required for the migrations of cells within the proctodeum. The migrations of these cells form cellular molds, the spicule traces, in which the cuticle of the spicules is secreted. Mutations in *daf-4*, *sma-2*, *sma-3*, and *sma-4*, which disrupt TGF $\beta$ -like signaling, result in aberrant migrations and morphologically abnormal spicules. *daf-4*, and hence the TGF $\beta$ -like signal, is required prior to or during cell migrations. Therefore, the TGF $\beta$ -like signal may act to prime the migrating cells or as a guidance cue. Mutations in *lin-31* result in identical cell migration and spicule morphology defects. Thus, *lin-31*, which encodes a “winged helix” protein (Miller *et al.*, *Genes Dev.* 7, 933–947, 1993), may be a component of this TGF $\beta$ -like signaling pathway. © 1999 Academic Press

**Key Words:** TGF $\beta$ ; signal transduction; cell migration.

## INTRODUCTION

Members of the transforming growth factor  $\beta$  (TGF $\beta$ ) superfamily are secreted dipeptides that act as intercellular signals (Kingsley, 1994; Massagué, 1990). They are present in all metazoans and have diverse biological effects.

In the nematode *Caenorhabditis elegans*, there are at least two distinct TGF $\beta$ -like signaling pathways. One is required for the regulation of dauer development, egg-laying, social behavior, and intestinal pigmentation (Estevez *et al.*, 1993; Ren *et al.*, 1996; Thomas *et al.*, 1993). The other is required for the regulation of body size and for the development of the male-specific copulatory spicules and sensory rays (Savage *et al.*, 1996; Suzuki *et al.*, 1999; Krishna *et al.*, 1999).

Dauers are alternate third-stage larvae that form in response to adverse environmental conditions (Riddle, 1977, 1988; Riddle and Albert, 1997). Under favorable conditions, dauer formation is suppressed by a TGF $\beta$ -like signal produced in the ASI amphidial neurons (Ren *et al.*, 1996). This signal is the product of the *daf-7* gene (Ren *et al.*, 1996). Type II and type I receptors for the DAF-7 protein are coded for by *daf-4* and *daf-1*, respectively (Estevez *et al.*, 1993; Georgi *et al.*, 1990). Transducers of the DAF-7 signal are coded for by *daf-8* and *daf-14* (D. L. Riddle, personal

communication; J. H. Thomas, personal communication). Recessive mutations in any of these genes result in the inappropriate formation of dauers.

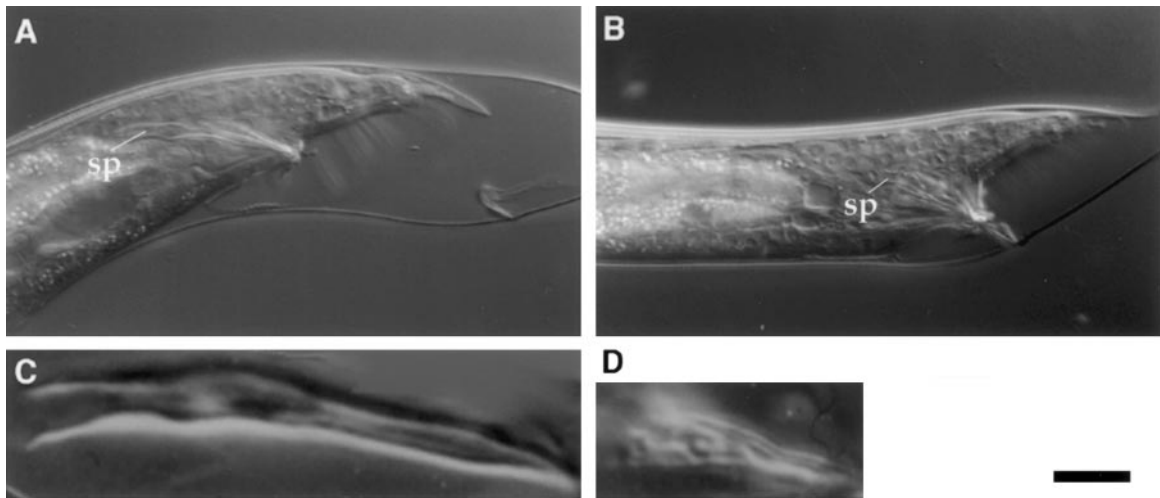
Mutations in *daf-4* also result in animals that are smaller than wild-type and in males that have short crumpled spicules and defects in sensory ray pattern (Savage *et al.*, 1996). These defects are not observed in *daf-7*, *daf-1*, *daf-8*, or *daf-14* mutant animals (Savage *et al.*, 1996), indicating the presence of a second TGF $\beta$ -like signaling pathway. The signal, type I receptor, and three transducers required for this pathway are coded for by the *dbl-1*, *sma-6*, *sma-2*, *sma-3*, and *sma-4*, genes respectively (Suzuki *et al.*, 1999; Krishna *et al.*, 1999; Savage *et al.*, 1996).

In this paper, we focus on the role of TGF $\beta$ -like signaling in spicule development. We describe the cellular basis of crumpled spicules, identify a potential source of the TGF $\beta$ -like signal, and identify a possible cofactor of the SMA-2, SMA-3, and/or SMA-4 signal transducers.

## MATERIALS AND METHODS

**Strains and strain maintenance.** *C. elegans* strains were grown on bacterial plates seeded with *Escherichia coli* OP50 (Brenner, 1974). Unless otherwise noted, *daf-4* homozygotes were grown at 15°C to prevent or limit dauer formation. All other strains were grown at 20 or 25°C. Strains used in this study were N2 = wild-type, STA5 = wild isolate, CB4088 = *him-5(e1490)*, CB1364 = *daf-4(e1364)*, DR63 = *daf-4(m63)*, PB55 = *daf-*

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**FIG. 1.** Spicule morphology in wild-type and TGF $\beta$ -like signal-defective mutant males. Left lateral views of wild-type (A) and *daf-4(m63)* mutant (B) adult male tails showing the morphology of the left spicule (sp). Isolated spicules (obtained by digestion of *C. elegans* males in 1 M acetic acid) from wild-type (C) and *daf-4(m63)* mutant (D) males. Scale bar, 10  $\mu$ m (A and B), 3.3  $\mu$ m (C and D).

*4(m592ts)*; *him-5(e1490)*; *daf-12(m20)*, CB502 = *sma-2(e502)*, CB491 = *sma-3(e491)*, DR1369 = *sma-4(e729)*, MT301 = *lin-31(n301)*, PB66 = *lin-31(n301)*; *him-5(e1490)*, and EM94 = *lin-31(bx31)*; *him-5(e1490)*. The *him-5* mutation increases the frequency of self-progeny males from approximately 0.5 to 33% by inducing high levels of X-chromosome nondisjunction during meiosis (Hodgkin *et al.*, 1979). The *daf-12* mutation suppresses dauer formation (Riddle and Albert, 1997). Neither *him-5(e1490)* nor *daf-12(m20)* has any effect on the development of male copulatory structures.

**Microscopic observations.** Individual animals or groups of animals were mounted in a small volume of M9 buffer on thin agar pads (Sulston and Horvitz, 1977) and observed by Nomarski differential interference contrast (DIC) microscopy (400 $\times$  or 1000 $\times$ ). Worms mounted for photomicroscopy or laser ablation were anesthetized by adding 0.1% sodium azide to the M9. All other observations were of nonanesthetized animals.

**Temperature shift experiments.** *daf-4(m592ts)III*, *him-5(e1490)V*, and *daf-12(m20)* mutant males were shifted between permissive (15 $^{\circ}$ C) and restrictive (25 $^{\circ}$ C) temperatures at various stages of development, allowed to grow until adulthood, and scored for spicule defects. Males were scored as mutant for spicules if either spicule was crumpled. Eight developmental stages, based primarily on the T cell lineage (Sulston and Horvitz, 1977; Sulston *et al.*, 1980), were identified: (i) late L2 (20–25 h posthatching), gonad straight, T.apap and T.appa present; (ii) early L3 (25–27.5 h posthatching), gonad reflexed, T.apap and T.appa present; (iii) mid L3 (27.5–30 h posthatching), R7 (T.apapp) R8 (T.appaa), and R9 (T.appap) present; (iv) late L3 (30–33 h posthatching), Rn.a and Rn.p cells present; (v) L3 lethargus (33–34 h posthatching), Rn.aa and Rn.ap cells present; (vi) early L4 (34–38 h posthatching), T cell lineage complete, no retraction of tail spike; (vii) early to mid L4 (38–40 h posthatching), tail spike retraction partial to complete, no ray papillae; (viii) mid L4 (40–41 h posthatching), tail spike retraction complete, ray papillae present.

**Laser ablations.** Laser ablations were accomplished using a MicroPoint laser system from Photonic Instruments, Inc. (Arling-

ton Heights IL). Cells were killed using multiple low-energy pulses from an attenuated beam to restrict collateral damage in surrounding cells (Bargman and Avery, 1995). The target cell, M, was identified from a published description of its morphology and location in newly hatched L1 larvae (Sulston and Horvitz, 1977).

**Nomenclature.** The Smad gene family nomenclature is followed in this paper (Derynck *et al.*, 1996). Specifically, Xmad2 (Graff *et al.*, 1996) is referred to as Smad2.

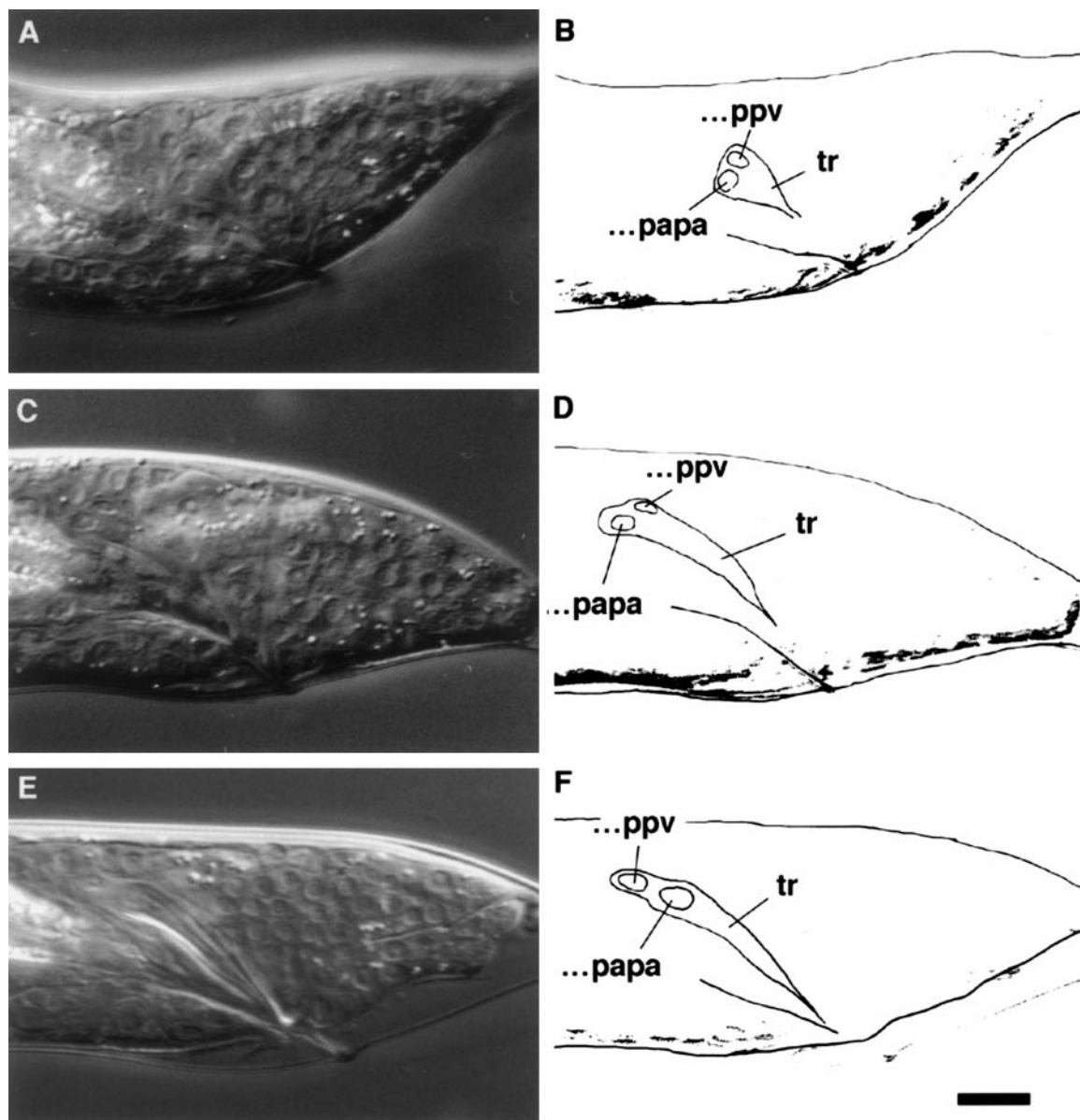
## RESULTS

### *Spicule Development in Wild-type Males*

The male-specific copulatory spicules are a pair of needle-like structures made of sclerotic cuticle (Figs. 1A and 1C). Each comprises two distinct sections, a posterior blade-like section and an anterior knob-like section. They are made by the cells of the proctodeum during the L4 larval stage (Sulston *et al.*, 1980).

The proctodeum, which forms the junction between the intestine, testis, and rectum, is composed primarily of descendants of the B cell. The B cell is a male-specific postembryonic blast cell (Sulston and Horvitz, 1977; Sulston *et al.*, 1980). During most of larval development, the shape of the proctodeum is determined by the pattern of B cell divisions. However, during the later half of the L4, there is a period of morphogenesis that determines the final shape of the adult male tail (Sulston and Horvitz, 1977; Sulston *et al.*, 1980; Baird *et al.*, 1991). During this period, in which there is a general anterior retraction of cells within the tail, the proctodeum elongates along its antero-posterior axis.

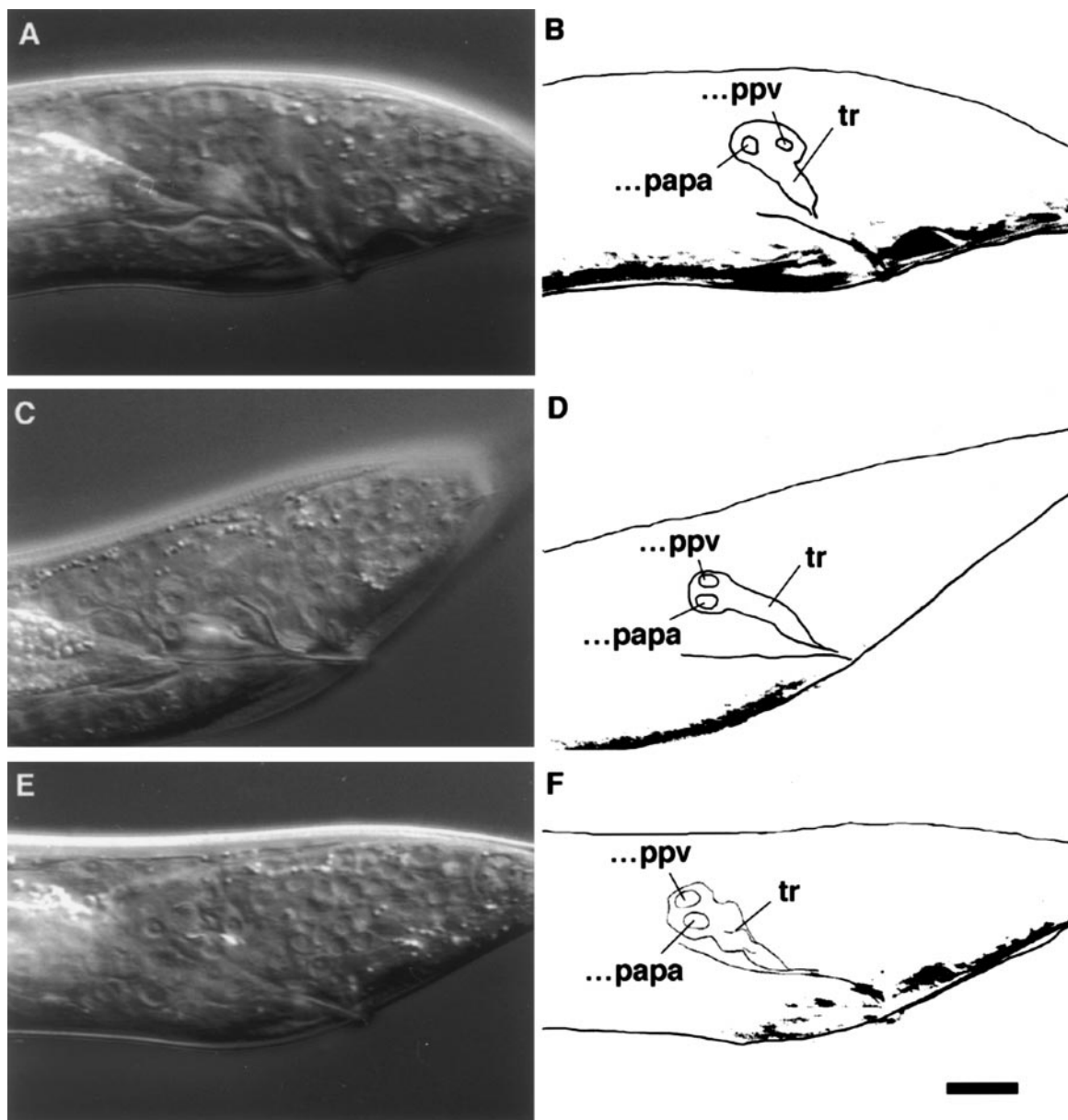
The proctodeal cells that secrete spicule cuticle also act as molds that determine spicule morphology (Sulston *et al.*,



**FIG. 2.** Development of the spicule trace in wild-type males. Left lateral views of wild-type L4 males at 37 (A and B), 40 (C and D), and 42 (E and F) h posthatching. The positions of B.alapapa (...papa), B.alappv (...ppv), and the left spicule trace are indicated in the line drawings (B, D, and F). Scale bar, 10  $\mu$ m.

1980). Thus, even before spicule cuticle is secreted, outlines of each spicule, or spicule traces, can be seen in the proctodeum (Fig. 2). The spicule traces first become apparent in early L4, between 37 and 38 h posthatching (Figs. 2A and 2B). They extend from the rectum to two bilateral pairs of proctodeal cells, B.a<sup>1/</sup>,apapa and B.a<sup>1/</sup>,appv, that define their anterior ends. At 38 h, the spicule traces are approximately one-quarter the length of mature spicules. Between 38 and 40 h posthatching, B.a<sup>1/</sup>,apapa and B.a<sup>1/</sup>,appv migrate anteriorly. As B.a<sup>1/</sup>,apapa and B.a<sup>1/</sup>,appv migrate, the

spicule traces elongate, tripling their original length (Figs. 2C and 2D). Beginning at 40 h cuticle is deposited along the traces forming the blade sections of the spicules. Between 41 and 42 h posthatching, B.a<sup>1/</sup>,apapa remain in place at the anterior end of the blade trace while B.a<sup>1/</sup>,appv resume their anterior migrations, forming the knob sections of the spicule traces (Figs. 2E and 2F). This secondary phase of cell migrations coincides with the onset of tail morphogenesis. The cuticle of the knob sections is secreted beginning at 42 h.

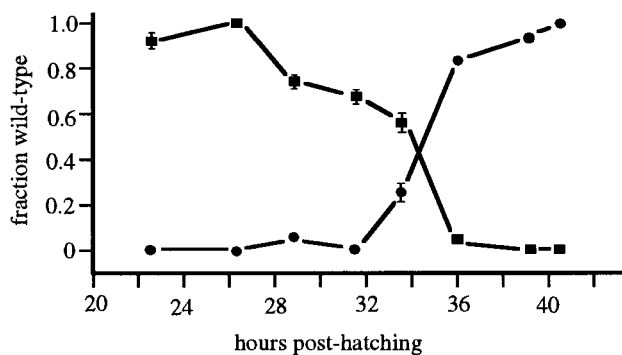


**FIG. 3.** Development of the spicule trace in *daf-4(m63)* males. Left lateral views of wild-type L4 males at 37 (A and B), 40 (C and D), and 42 (E and F) h posthatching. The positions of B.alapapa (... papa), B.alappv (... ppv), and the left spicule trace are indicated in the line drawings (B, D, and F). Scale bar, 10  $\mu$ m.

### TGF $\beta$ -like Signaling and Spicule Development

A TGF $\beta$ -like signal is required for the migrations of B.a<sup>1</sup>/<sub>r</sub>apapa and B.a<sup>1</sup>/<sub>r</sub>appv. This is apparent from the spicule defects observed in *daf-4* mutant males. The *daf-4* gene encodes a type II TGF $\beta$ -like receptor (Estevez *et al.*, 1993). In adult *daf-4* males, the spicules are short and crumpled (Savage *et al.*, 1996; Figs. 1B and 1D). This defect results from defects in the migrations of B.a<sup>1</sup>/<sub>r</sub>apapa and B.a<sup>1</sup>/<sub>r</sub>appv.

As in wild-type males, the spicule traces in *daf-4* males appear between 37 and 38 h posthatching (Figs. 3A and 3B). However, between 38 and 40 h posthatching, B.a<sup>1</sup>/<sub>r</sub>apapa and B.a<sup>1</sup>/<sub>r</sub>appv either fail to migrate or migrate only short distances when compared to wild-type (Figs. 3C and 3D). Consequently, the spicule traces do not elongate or elongate only slightly. Blade cuticle is deposited beginning at 40 h, but the deposition is patchy and commonly does not cover the entire blade trace. B.a<sup>1</sup>/<sub>r</sub>appv resume their migra-



**FIG. 4.** The *daf-4* temperature-sensitive period for spicule development. *daf-4(m592ts)* males were shifted between permissive (15°C) and restrictive (25°C) temperatures at various stages of development. The resulting adults were scored for defects in spicule morphology. Data for up-shifted (●) and down-shifted (■) males are plotted. (See Materials and Methods for criteria used to stage larval males.) Error bars represent  $\pm$  twice the SEM. Standard errors less than 0.015 are not indicated.

tions between 41 and 42 h posthatching but do not reach their wild-type locations (Figs. 3E and 3F).

A requirement for TGF $\beta$ -like signaling in spicule development is further supported by the spicule phenotypes observed in *sma-2*, *sma-3*, and *sma-4* mutant males. *sma-2*, *sma-3*, and *sma-4* are members of the Smad gene family (Savage *et al.*, 1996). Members of this gene family code for transducers of TGF $\beta$ -like signals (Sekelsky *et al.*, 1995; Savage *et al.*, 1996; Massagué, 1996; Wrana and Attisano, 1996). Mutations in *sma-2*, *sma-3*, and *sma-4* result in spicule morphology and B cell migration defects indistinguishable from those observed in *daf-4* mutant males (Hodgkin, 1983; Savage *et al.*, 1996; data not shown).

### **A TGF $\beta$ -like Signal Is Required Prior to or during B Cell Migrations**

The temporal requirement for TGF $\beta$ -like signaling in spicule development was determined using a temperature-sensitive allele of *daf-4*, *m592*. At a permissive temperature (15°C) the spicules of *daf-4(m592)* males are wild-type, whereas at a nonpermissive temperature (25°C) their spicules are short and crumpled. To determine when *daf-4*, and hence a TGF $\beta$ -like signal, is required for spicule development, *daf-4(m592)* males were shifted between permissive and restrictive temperatures at various stages of development. The temperature-shifted males were allowed to mature and then scored, as adults, for spicule morphology (Fig. 4).

From these data, a temperature-sensitive period from 33 to 34 h posthatching was defined for the role of *daf-4* in spicule development. This period follows the completion of the B cell lineage but precedes the onset of the B.a<sup>1</sup>/<sub>apapa</sub> and B.a<sup>1</sup>/<sub>appv</sub> cell migrations. In downshifted animals, the

*daf-4* tsp extended to the period between 28 and 33 h posthatching. During this period, the last three rounds of division within the B cell lineage are completed. B.a<sup>1</sup>/<sub>apapa</sub> and B.a<sup>1</sup>/<sub>appv</sub> are derived from these divisions and are born at 32 and 31 h posthatching, respectively (Sultson and Horvitz, 1977; Sulston *et al.*, 1980). These temperature-sensitive periods most likely correspond to either the periods in which DAF-4 is synthesized or the periods in which DAF-4 function is required.

### **M Cell Derivatives Are a Possible Source of a TGF $\beta$ -like Signal**

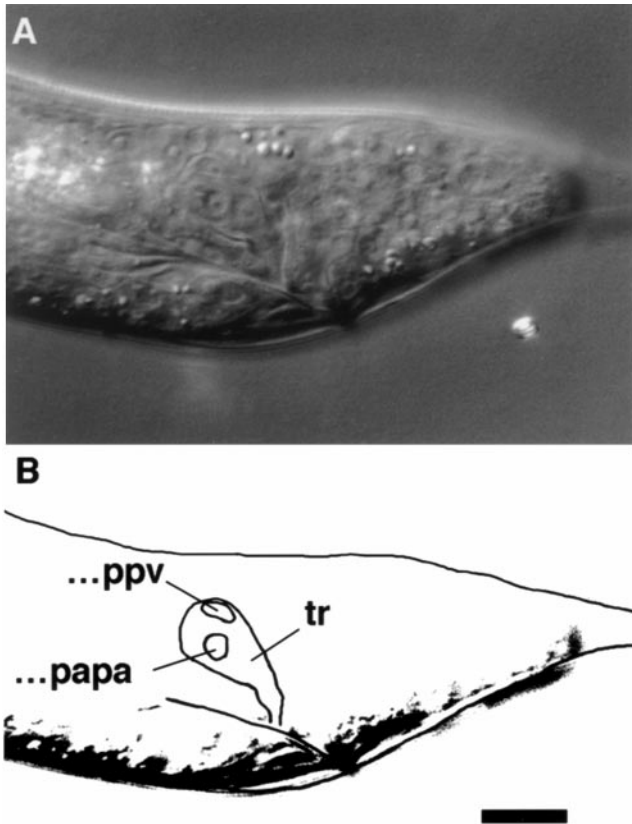
Sulston *et al.* (1980) reported that laser ablation of the M cell or of the spicule retractors, which are descendants of M, resulted in crumpled spicules. This defect was attributed to the failure of the proctodeum to elongate during tail retraction in M-ablated animals. It is also possible that derivatives of the M cell are the source of the TGF $\beta$ -like signal that is required for B.a<sup>1</sup>/<sub>apapa</sub> and B.a<sup>1</sup>/<sub>appv</sub> cell migrations. If this is the case, ablation of M should phenocopy these defects.

To investigate this possibility, M was ablated in eight L1 males. These males were allowed to develop to the early L4 larval stage and then scored for defects in B.a<sup>1</sup>/<sub>apapa</sub> and B.a<sup>1</sup>/<sub>appv</sub> cell migrations. In each case, ablation of M resulted in B.a<sup>1</sup>/<sub>apapa</sub> and B.a<sup>1</sup>/<sub>appv</sub> cell migration defects identical to those observed in mutant males with defective TGF $\beta$ -like signal transduction pathways (Fig. 5).

### **LIN-31 Is Required for Spicule Development**

In *Xenopus* and in mammals, *fork head*/HNF-3 $\beta$  homologs are utilized as transcriptional cofactors of Smads proteins (Chen *et al.*, 1996, 1997; Zhou *et al.*, 1998; Liu *et al.*, 1999). In *C. elegans*, there are multiple genes that code for *fork head* homologs (Miller *et al.*, 1993; Hope, 1994; Azzaria *et al.*, 1996; Ogg *et al.*, 1997). One of these is *lin-31*. *lin-31* is best characterized for its role in vulval development (Ferguson and Horvitz, 1985; Kim and Horvitz, 1990; Miller *et al.*, 1993). However, we have observed that *lin-31* also is required for spicule development; *lin-31(n301)* mutant males have short, crumpled spicules (Fig. 6A). Defects in spicule development are not specific to the *n301* allele of *lin-31* as an identical spicule phenotype is observed in *lin-31(bx31)* mutant males (data not shown).

In both *lin-31(n301)* and *lin-31(bx31)* males, the adult spicule defects result from aberrant B.a<sup>1</sup>/<sub>apapa</sub> and B.a<sup>1</sup>/<sub>appv</sub> cell migrations (Figs. 6B and 6C; data not shown). These B cell migration defects are identical to those observed in *daf-4*, *sma-2*, *sma-3*, *sma-4*, and M-ablated males. This requirement for *lin-31* in spicule development indicates a possible involvement in TGF $\beta$ -like signaling.



**FIG. 5.** Spicule trace defects in an M-ablated male. Left lateral views of an L4 male (40 h posthatching) in which M had been ablated with a laser microbeam during L1. The positions of B.alapapa (...papa), B.alappv (...ppv), and the left spicule trace are indicated in the line drawing (B). Scale bar, 10  $\mu$ m.

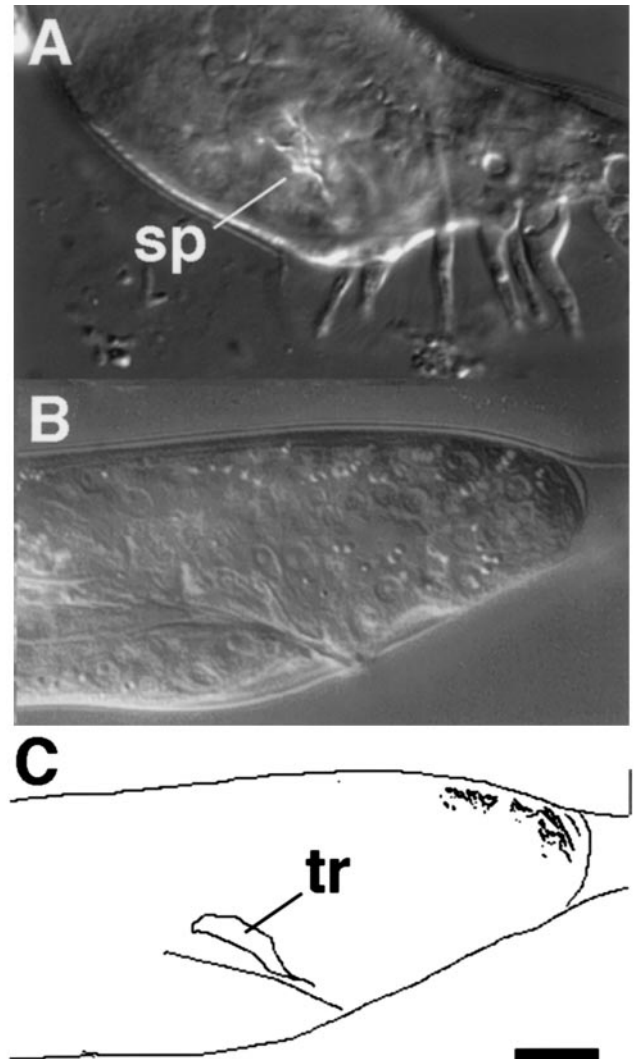
## DISCUSSION

We have demonstrated that a TGF $\beta$ -like signal is required for spicule development in *C. elegans* males. This is evident from the spicule defects observed in *daf-4*, *sma-2*, *sma-3*, and *sma-4* males. In these animals, which are defective in TGF $\beta$ -like signal transduction, the spicules are short and crumpled due to B.a<sup>1</sup>’,apapa and B.a<sup>1</sup>’,appv migration defects during the L4 larval stage. The primary tsp for *daf-4* is after the completion of the B cell lineage but prior to the onset of B cell migrations. This tsp probably correlates with the synthesis of DAF-4 and/or with the requirement for DAF-4 function. Thus, this TGF $\beta$ -like signal may prime B.a<sup>1</sup>’,apapa and B.a<sup>1</sup>’,appv for their migrations or may act as a guidance cue.

The TGF $\beta$ -like signal required for spicule development most likely is encoded by the *dbl-1* gene. Mutations in *dbl-1* result in adult spicule, ray pattern, and body size defects identical to those resulting from mutations in *daf-4*, *sma-2*, *sma-3*, and *sma-4* (Savage *et al.*, 1996; Suzuki *et al.*,

1999). Spicule development in *dbl-1* mutant males has not been analyzed.

Short, crumpled spicules also result from ablation of the F and U cells (Chamberlin and Sternberg, 1993). This ablation causes a premature termination of the B cell lineage; hence B.a<sup>1</sup>’,apapa and B.a<sup>1</sup>’,appv never are born. This effect cannot explain the spicule defects observed in TGF $\beta$ -like signal defective animals. The last divisions in the B cell lineage occur at 33 h posthatching (Sulston and Horvitz, 1977; Sulston *et al.*, 1980). However, 70% of the *daf-4(m592ts)* males shifted from 15 to 25°C between 33 and 34 h posthatching, after the completion of the B lineage,



**FIG. 6.** Spicule defects in a *lin-31* male. (A) Ventrolateral view of an adult *lin-31(n301)* male in which short, crumpled spicules (sp) are apparent. (B) Left lateral view of an *lin-31(n301)* L4 male (41 h posthatching). The position of the left spicule trace is indicated in the line drawing. The B.alapapa and B.alappv nuclei are not visible in this focal plane. Scale bar, 10  $\mu$ m.

exhibit spicule defects. Rather, F cell ablation results indicate that B.a<sup>1</sup>/<sub>apapa</sub> and B.a<sup>1</sup>/<sub>appv</sub> are partially replaced by their parents (spicule cuticle is deposited) but these cells do not migrate in response to a TGF $\beta$ -like signal.

A possible source of the TGF $\beta$ -like signal required for spicule development is the male-specific musculature. These muscles are derived from a single postembryonic myoblast, M (Sultson and Horvitz, 1977). When M is ablated in L1 males, or when the spicule retractors are ablated in early L4 males, adults with crumpled spicules result (Sulston *et al.*, 1980). This defect can be partially attributed to the failure of the proctodeum to elongate during tail morphogenesis in the later half of L4 (Sulston *et al.*, 1980). However, we have shown that ablation of M in L1 males phenocopies the B.a<sup>1</sup>/<sub>apapa</sub> and B.a<sup>1</sup>/<sub>appv</sub> migration defects of *daf-4*, *sma-2*, *sma-3*, and *sma-4*. These migration defects occur during early L4, prior to tail morphogenesis. Hence, the spicule defects observed in M-ablated males may be explained by an M descendent being the source of a TGF $\beta$ -like signal. The recent identification of *dbl-1* as the likely TGF $\beta$ -like signal required for spicule development will allow this issue to be addressed further (Suzuki *et al.*, 1999).

Expression of the TGF $\beta$ -like signal in M cell descendants may require the *egl-5* gene product. *egl-5* is an abdominal B homolog that provides positional information in the posterior region of the body (Wang *et al.*, 1993; Chisholm, 1991; Chow and Emmons, 1994). *egl-5* mosaic males that are mutant in the M cell lineage, but wild-type in most other cells including B cell descendants, have short, crumpled spicules (Chisholm, 1991). This nonautonomous requirement is consistent with *egl-5* being required to regulate the expression of a TGF $\beta$ -like signal in M cell descendants.

Finally, *lin-31* has been identified as a possible component of the TGF $\beta$ -like signaling pathway required for spicule development. *lin-31* mutant males have short crumpled spicules. These spicule defects result from B.a<sup>1</sup>/<sub>apapa</sub> and B.a<sup>1</sup>/<sub>appv</sub> migration defects identical to those observed in *daf-4*, *sma-2*, *sma-3*, *sma-4*, and M-ablated males.

*lin-31* codes for a "winged helix" transcription factor that is homologous to *fork head* and HNF-3 $\beta$  (Miller *et al.*, 1993). *fork head*/HNF-3 $\beta$  homologs have been implicated in activin signal transduction (Chen *et al.*, 1996, 1997; Zhou *et al.*, 1998; Liu *et al.*, 1999). In activin-stimulated cells, these winged helix proteins interact with Smad proteins to form a transcriptional activation complex. SMA-2, SMA-3, and SMA-4 all are members of the Smad family of TGF $\beta$ -like signal transducers (Savage *et al.*, 1996; Graff *et al.*, 1996). Thus, LIN-31 may act as a transcriptional cofactor of SMA-2, SMA-3, and/or SMA-4. Alternatively, LIN-31 may act not in TGF $\beta$ -like signaling, but rather in a parallel signaling pathway. This is true of *daf-16*, which also encodes *fork head*/HNF3b homolog (Ogg *et al.*, 1997). DAF-16, like the DAF-7 TGF $\beta$ -like signaling pathway, is required for regulation of dauer development (Riddle and Albert, 1997). However, DAF-16 is not a component of the DAF-7

pathway. Rather, DAF-16 acts in an insulin-like signaling pathway that functions parallel to DAF-7 signaling (Gottlieb and Ruvkun, 1994; Morris *et al.*, 1996; Ogg *et al.*, 1997; Kimura *et al.*, 1997; Tissenbaum and Ruvkun, 1998; Gems *et al.*, 1998). At this point, we cannot discriminate between these possible roles for LIN-31 in spicule development.

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