The DAF-3 Smad binds DNA and represses gene expression in the *Caenorhabditis elegans* pharynx

Jack D. Thatcher, Christina Haun and Peter G. Okkema*

Department of Biological Sciences (MC567), University of Illinois at Chicago, 900 S. Ashland Avenue, Chicago, IL 60607, USA

*Author for correspondence (e-mail: okkema@uic.edu)

Accepted 21 October; published on WWW 3 December 1998

SUMMARY

Gene expression in the pharyngeal muscles of *Caenorhabditis elegans* is controlled in part by organ-specific signals, which in the *myo-2* gene target a short DNA sequence termed the C subelement. To identify genes contributing to these signals, we performed a yeast one-hybrid screen for cDNAs encoding factors that bind the C subelement. One clone recovered was from *daf-3*, which encodes a Smad most closely related to vertebrate Smad4. We demonstrated that DAF-3 binds C subelement DNA directly and specifically using gel mobility shift and DNase1 protection assays. Mutation of any base in the sequence GTCTG interfered with binding in the gel mobility shift assay, demonstrating that this pentanucleotide is a core recognition sequence for DAF-3 binding. *daf-3* is known to promote formation of dauer larvae and this activity is negatively regulated by TGFβ-like signaling. To determine how *daf-3* affects C subelement enhancer activity in vivo, we examined expression a *gfp* reporter controlled by a concatenated C subelement oligonucleotide in *daf-3* mutants and other mutants affecting the TGFβ-like signaling pathway controlling dauer formation. Our results demonstrate that wild-type *daf-3* can repress C subelement enhancer activity during larval development and, like its dauer-promoting activity, *daf-3*’s repressor activity is negatively regulated by TGFβ-like signaling. We have examined expression of this *gfp* reporter in dauer larvae and have observed no *daf-3*-dependent repression of C activity. These results suggest *daf-3* directly regulates pharyngeal gene expression during non-dauer development.

Key words: DAF-3, TGFβ, Smad, DNA binding, *Caenorhabditis elegans*, Pharynx, Dauer

INTRODUCTION

Transforming growth factor beta (TGFβ)-like signaling pathways control critical developmental events in diverse animal groups (reviewed by Kingsley, 1994; Massagué et al., 1997; Heldin et al., 1997; Padgett et al., 1997). TGFβ family members, which include the TGFβs, activins and bone morphogenetic proteins, are secreted ligands that bind and activate heteromeric serine/threonine kinase receptors. The activated receptors transduce the signal to intracellular targets, thereby controlling gene expression within the nucleus.

A family of conserved proteins termed Smads (after *C. elegans* *sma-2, sma-3, sma-4* and *Drosophila mothers-against-dpp*) (Raftery et al., 1995; Sekelsky et al., 1995; Savage et al., 1996) plays a critical role in transducing TGFβ-like signals to the nucleus. Smad proteins share a highly conserved structure in which amino terminal and carboxy terminal domains, termed MH1 and MH2, are connected by proline-rich linker regions. Most Smads can be categorized into two functional subfamilies, receptor-regulated SMADs and co-SMADs. Recently, a model has emerged for how these two classes of Smads function in TGFβ-like signaling pathways (Heldin et al., 1997). Receptor-regulated SMADs are phosphorylated by specific receptors, resulting in formation of heteromeric complexes with co-SMADs. Co-SMADs associate with different receptor-regulated SMADs and so represent common factors used by different TGFβ-like signaling pathways. Upon association, the heteromeric Smad complexes are believed to translocate into the nucleus where they control expression of specific target genes.

Nuclear translocation of Smads suggested that these factors might function as transcription factors. Indeed, the MH2 region can serve as a transcriptional activation domain when fused to a heterologous DNA-binding domain (Liu et al., 1997; Kretzschmar et al., 1997; Wu et al., 1997). Likewise, a multimeric complex containing Smad2, Smad4 and a fork head family transcription factor FAST1, binds DNA in *Xenopus* (Chen et al., 1996, 1997; Liu et al., 1997). Recently, direct DNA binding has been demonstrated with *Drosophila* Mad and Medea (Kim et al., 1997; Xu et al., 1998), and mammalian Smad3 and Smad4 (Yingling et al., 1997; Zawel et al., 1998). With Mad, it has been shown that the DNA-binding activity resides in the MH1 domain. These results indicate that at least some Smads can function directly as DNA-binding transcription factors.

In *Caenorhabditis elegans*, a unique TGFβ-like signaling pathway (Fig. 1A) controls several phenotypes including formation of the dauer, an alternate larval stage specialized to survive harsh environmental conditions (Cassada and Russel, 1975; Riddle, 1977; Riddle et al., 1981; Vowels and Thomas,
produce DAF-3 \(^3\) \(^N\) protein, pOK114.02 was constructed by inserting a BglII-SacII fragment of daf-3 cDNA into pGEX-3X. To produce DAF-3 \(^3\), pOK114.03 was constructed by inserting a BamHI-Smal fragment of daf-3 cDNA into pGEX-1. The sequences of all plasmids described here are available from the authors.

The daf-3 cDNA in pOK104.02 was sequenced using the DNA Sequencing Core Laboratory at the University of Florida (Gainsville).

One-hybrid selection

One-hybrid selection was performed as described by Wang and Reed (1993). Two mixed staged C. elegans cDNA libraries (pACT-RB1 and pACT-RB2, the generous gift of R. Barstead) were screened. pACT-RB1 was generated with poly(dT) primers while pACT-RB2 was produced with random oligonucleotide primers. 4.9 x 10\(^6\) pACT-RB1 transformants and 5.1 x 10\(^6\) pACT-RB2 transformants were screened. S. cerevisiae YJL170 (the generous gift of I. Herskowitz) bearing the reporter plasmid pOK104.26 (marked with TRP-1) were transformed (Gietz et al., 1995) with library (marked with LEU-2) (Durfee et al., 1993) and grown for 9 days on synthetic dextrose plates lacking tryptophan, leucine and histidine (−TLH) to select for HIS3 activation. Faster growing colonies were replated both on −TLH, to confirm their His\(^+\) phenotype and to compare their relative growth rates, and on −TLH with 5 mM 3-aminotriazole, to compare their ability to resist inhibition of histidine biosynthesis. The clones were categorized based on these two criteria and selected cDNA plasmids from different categories were extracted from yeast and amplified in E. coli. To test whether the interaction with DAF-3 was reproducible and required C183, we retransformed these clones into YJL170 cells bearing either pOK104.26, the parent vector pRS414/HIS, or the plasmid pOK104.24 containing the unrelated oligonucleotide B207. Transformants were grown 4 days on −TLH plates to compare the ability of the cDNA clones to activate HIS3 expression with each of these reportors, and on −TL plates to monitor transformation efficiencies. Percentages of total transformants with His\(^+\) phenotypes were then computed and compared.

Expression and purification of DAF-3 proteins

pOK114.02 was used to produce DAF-3 \(^3\) \(^N\) protein, a GST fusion with the leader sequence, MH1 domain and linker region of DAF-3. pOK114.03 was used to produce DAF-3\(^{-}\)C, a GST fusion with the linker region, MH2 domain and tail of DAF-3. C. elegans BL21(DE3) transformed with either pOK114.02 or pOK114.03 were grown in M9 medium (Harwood and Cutting, 1990) at room temperature to A\(_{600}\) between 1.1 and 1.5 and protein expression was induced by adding 1 mM IPTG. Cells were grown an additional 4 hours and recombinant protein was purified by affinity chromatography using glutathione agarose (Ausbel et al., 1990). DAF-3\(^{-}\) has a predicted M\(_{r}\) of 86.5 x 10\(^3\); however the major induced protein migrated on SDS gels with an apparent M\(_{r}\) of 60 x 10\(^3\), although a faint band was observed with the correct M\(_{r}\). We believe the 60 kDa protein is a truncation product lacking the linker region. For DNAseI protection assays DAF-3\(^{-}\) was concentrated with Centron-30 columns to 19 \(\mu\)g/ml (Amicon).

DNA-binding assays

Gel mobility shift assays were performed essentially as described by Thatcher et al. (1995). 20 \(\mu\)l binding reactions contained 26.25 mM Hepes, pH 7.8, 3% glycerol, 60 mM KCl, 4 mM MgCl\(_2\), 4 mM spermidine, 0.5 mM DTT, 50 ng/\(\mu\)l poly(dIdC), 50 ng/\(\mu\)l poly(dAdT), 12.5 ng/\(\mu\)l protein and 1500 cts/minute/\(\mu\)l C183 probe. Gel mobility shift assays were performed using two sets of double-stranded oligonucleotides as probes and competitors. The first set had 5’ overhangs compatible with SfiI sites. The second set were blunt ended. Identical results were observed with both sets. C183 with 5’ overhangs was radiolabeled by Klenow end filling using \(\alpha\)\(^{32}\)P-ATP (6000 Ci/mmol). Blunt-ended C183 was radiolabeled with polynucleotide kinase using \(\alpha\)\(^{32}\)P-ATP (5000 Ci/mmol).

DNAse I protection assays were performed essentially as described by Thatcher et al. (1995). 20 \(\mu\)l binding reactions contained 5 mM

MATERIALS AND METHODS

Plasmid construction and sequencing

The parental vector used to produce each one-hybrid reporter plasmid was pRS414/HIS (the generous gift of B. Harfe, B. Kelly and A. Fire), which was derived from pRS314 (Sikorski and Hieter, 1989) by reversing the orientation of the polylinker between the T3 and T7 promoters (Christianson et al., 1992), and inserting a GAL1 minimal promoter and HIS3 reporter gene into the BamHI-SalI sites (B. Harfe, B. Kelly and A. Fire, personal communication). Each reporter plasmid was generated by inserting a XbaI-BamHI fragment containing GTF3 subelement into the pACT1 polylinker and subcloning the cDNA insert into the BamHI site of Bluescript KS+ (Stratagene). To

DNase I protection assays were performed essentially as described by Thatcher et al. (1995). 20 \(\mu\)l binding reactions contained 5 mM
Hepes, pH 7.8, 2.5 mM KCl, 87 mM NaCl, 1 mM MgCl₂, 1 mM DTT, 50 ng poly(ddC), 50 ng/μl poly(dAdT), 0.25-1.25 ng/μl DNaSe1, 0.05-1 μg/ml protein and 500 cts/minute/μl C subelement probe. Samples were preincubated at room temperature for 2 minutes prior to DNase I treatment at room temperature for 2 minutes. The probe was prepared by cleaving pPD20.97 (Okkema et al., 1993) with BssHII. Klenow end filling using a [32P]-dCTP (6000 Ci/mmol) and a [32P]-dGTP (3000 Ci/mmol), cleaving with HpaI, and gel isolating (5% acrylamide, 0.5× TBE) the 101 bp C subelement fragment.

**Strain construction**

All *C. elegans* strains were grown under standard conditions (Sulston and Hodgkin, 1988). Strains used in these experiments are:

- OK83: *cuIs5+ I; daf-1(m40) IV*
- OK66: *daf-4(m72) III; cuIs2+/+ III*
- OK70: *daf-4(m72) III*
- OK82: *daf-5(e1372) III; cuIs2+/+ III*
- OK84: *daf-8(e1393) I; cuIs2+/+ III*
- OK85: *daf-5+/+ I; daf-14(m77) IV*
- OK68: *daf-5(mg90) X*
- OK86: *daf-5(e1386) II; cuIs2+/+ III*
- OK67: *daf-4(m72) III; cuIs2+/+ III; daf-3(mg90) X*
- OK87: *daf-5(e1386) II; daf-4(m72) III; cuIs2+/+ III*

*cuIs2* and *cuIs5* are chromosomally integrated transgenes consisting of the *C183::gfp* reporter plasmid pOK100.03 and pRF4, a plasmid containing the dominant morphological marker rol-6 (su1006). These integrants were independently derived from a strain bearing the extrachromosomal array cuEx16 after 1500 rad gamma-irradiation and were backcrossed twice to N2 (Mello and Fire, 1995).

To construct Daf-c, *cuIs2* or *cuIs5* strains, males heterozygous for the transgene were crossed with Daf-c hermaphrodites. Rol cross progeny hermaphrodites were selected and allowed to self at 16°C. F₂ hermaphrodites were picked to individual plates and their progeny examined for Daf-c phenotype at 25°C.

To construct a *cuIs2*/*daf-3* strain, *cuIs2+/+* males were mated to *daf-5(mg90) hermaphrodites* and the resulting *cuIs2+/+; daf-3(mg90)* 0 males were backcrossed with *daf-3(mg90) hermaphrodites* to produce *daf-3(mg90)* homozygous hermaphrodites carrying *cuIs2*. The same strategy was used to construct a *daf-4*/*cuIs2*/*daf-3* strain, using *daf-4(m72); daf-3(mg90)* hermaphrodites and selecting cross progeny with the small phenotype characteristic of *daf-4* mutants.

To construct *daf-5*, *cuIs2*, marker genes were used to follow the Daf-d alleles. *daf-5(e1386)* II hermaphrodites were crossed with *dpy-10(e1289)/+; unc-4(e120)/+ II; cuIs2+/IV* males. Rol cross progeny hermaphrodites segregating Dpy Unc progeny were identified and Rol non-Dpy non-Unc animals were picked to individual plates. Animals that segregated no Dpy Uncs were inferred to be homozygous Daf-d mutants.

To construct a *daf-5*, *daf-4*, *cuIs2+/+* strain, *daf-5(e1386)* hermaphrodites were mated with *daf-4(m72); cuIs2+/+* males. Rol cross progeny hermaphrodites were picked to 25°C, and Rol dauers were picked to recover *daf-4(m72)* homozygotes. These progeny were allowed to develop into adults at 16°C, and their eggs were transferred to 25°C to identify Rol Daf-d strains, homozygous for *daf-5(e1386)*.

**Analysis of *C183::gfp* expression**

To examine *C183::gfp* expression in *daf* mutant backgrounds, transgenic worms were grown at 16°C to allow non-dauer growth. Rol adults were picked, anesthetized in 0.5 mM levamisole, examined by fluorescence microscopy for GFP expression. Expression levels were judged by comparing fluorescence intensities to *daf-8*; *cuIs2/+* animals. Animals with greater fluorescence than the typical *daf-8*; *cuIs2/+* individual were scored as strong, while animals with equal or lower intensities were scored as weak.

*cuIs2* and *cuIs5* *daf-3(mg90) Dauer* dauers were produced on plates containing exogenous pheromone (the generous gift of G. I. Patterson and G. Ruvkun) as described by Golden and Riddle (1982), except that the animals were grown at 27°C instead of 25°C (M. Ailion and J. H. Thomas, personal communication).

**RESULTS**

**DAF-3 binds *C183* DNA in yeast and in vitro**

Expression of the pharyngeal muscle-specific myo-2 gene is controlled by a combination of cell-type- and organ-specific signals (Okkema and Fire, 1994). The organ-specific signals function through a segment of the myo-2 enhancer termed the *C* subelement, a unique transcriptional regulatory sequence that activates transcription in all pharyngeal cell types but is inactive outside the pharynx. The *C* subelement contains a binding site for PHA-4 (Kalb et al., 1998), a fork head family transcription factor essential for pharyngeal development (Mango et al., 1994; Kalb et al., 1998; Horner et al., 1998) and it thus serves as a target for signals specifying pharyngeal organ-identity.

To identify additional *C. elegans* genes controlling pharyngeal organ-specific expression, we performed a yeast one-hybrid screen for cDNAs encoding factors that bind the *C* subelement (Wilson et al., 1991; Wang and Reed, 1993). We had previously identified two overlapping 28 bp oligonucleotides from the *C* subelement, *C181* and *C183*, that retained organ-specific enhancer function (Okkema and Fire, 1994). A his3-200 yeast mutant strain bearing a reporter plasmid with four copies of the *C183* oligonucleotide upstream of a *HIS3* selectable marker (Fig. 1B) was transformed with

![Fig. 1. TGFβ-like pathway controlling dauer formation and one-hybrid reporter used to clone *daf-3*.](image)
plasmid library containing *C. elegans* cDNAs fused to the activation domain of yeast GAL4. Fusion proteins binding C183 should activate HIS3 expression, allowing cells to grow on plates lacking histidine. After screening approximately 10^7 yeast transformants, 12 plasmids were identified that reproducibly transformed yeast bearing the C183 reporter to a His* phenotype, but failed to transform cells bearing the parental reporter lacking C183 to His* (Table 1). Of these 12 plasmids eleven contained cDNAs from a single gene which will be described elsewhere (Table 1). Surprisingly, the remaining plasmid contained a cDNA from *daf-3*, a gene that promotes dauer larva formation and is most closely related to the vertebrate co-SMAD (Kim et al., 1997). To verify the interaction between DAF-3 and C183 is specific, we showed that gal4::daf-3 could not transform yeast with a reporter containing B207, an oligonucleotide unrelated to C183, to a His* phenotype.

To determine whether DAF-3 binds C183 directly, we performed an in vitro gel mobility shift assay with fusion proteins DAF-3 purified from *E. coli* as a glutathione-S-transferase (GST) fusion protein (Smith, 1993). Because the DNA-binding activity of *Drosophila* Mad resides in the MH1 domain (Kim et al., 1997), we expressed the amino-terminal region of DAF-3 containing the leader and MH1 domain fused to GST. DAF-3 N is a N-terminal fragment of DAF-3 containing the proline-rich linker and MH2 domain fused to GST. DAF-3 C is a C-terminal fragment of DAF-3 containing the proline-rich linker and MH2 domain fused to GST. Excess unlabeled C183 or the unrelated oligonucleotide B207 was added to the binding reaction as indicated to demonstrate specific binding. Fold Excess indicates the amount of competitor relative to the amount of labeled C183. The arrowhead indicates DAF-3$^N$/C183 complexes. The bracket indicates free probe. No shifted probe was observed with DAF-3$^N$ protein, despite the addition of 2.5x more DAF-3$^C$ than DAF-3$^N$; this pattern was indistinguishable from samples containing no added protein (data not shown).

regions including the MH2 domain have no detectable binding activity.

**DAF-3 binds a conserved DNA sequence distinct from the sequence recognized by *Drosophila* Mad**

To localize the DAF-3-binding site within C183, we performed a DNase1 protection assay using a restriction fragment encompassing the entire C subelement as a probe. DAF-3$^N$ protected a region of approximately 13 bases corresponding to the 3′ half of C183 (Fig. 3A,D). This region is not contained in the C subelement oligonucleotide C181 (Fig. 3B), and we found that DAF-3 fails to bind C181 both in yeast and in vitro (data not shown).

To further localize the sequence required for DAF-3 binding, we examined a set of mutated derivatives of C183 for the ability to bind DAF-3 in yeast and in vitro. We began with four C183 derivatives with 5 bp alterations that had been previously examined for organ-specific enhancer activity in transgenic *C. elegans* (Fig. 3B; Okkema and Fire, 1994). In the one-hybrid assay, his3-200 mutant yeast bearing a reporter with concatenates of either *Cmut1*, *Cmut2* or *Cmut3* were efficiently converted to a His* phenotype by transformation with gal4::daf-3. In contrast, yeast bearing a
DAF-3 binds DNA to repress transcription

reporter containing concatenated Cmut4 were not converted to His⁺ (data not shown). Similarly, Cmut1, Cmut2 and Cmut3 competed effectively for DAF-3³ binding using the in vitro gel mobility shift assay, whereas Cmut4 competed poorly (data not shown). Thus, in yeast and in vitro DAF-3 binding requires bases altered in Cmut4.

To determine specific base pairs important for DAF-3 binding, we used the gel mobility shift assay to examine...
binding to mutated derivatives of \(C183\) with single base pair alterations affecting the DAF-3 protected region (Table 2). There was no reduction in the ability of seven of the these mutated oligonucleotides to compete with wild-type \(C183\) for DAF-3\(^{3N}\) binding (Fig. 3C). In contrast, five of the oligonucleotides competed less effectively, indicating the base pairs altered in these mutated derivatives are important to DAF-3 binding (Fig. 3C,D). The mutated derivatives were also radiolabeled and used directly as probes for gel mobility shifts and, consistent with the competition experiments, there was no reduction in the ability to form complexes with the oligonucleotides that competed efficiently with \(C183\), while reduced complex formation was observe with the oligonucleotides that competed poorly (data not shown). These results demonstrate that the sequence GTCTG is a core recognition site for DAF-3 binding (Fig. 3C,D).

The DAF-3 core recognition site is similar to sequences in \(C183\) implicated in Smad binding in vertebrates (Fig. 3D). These include the activin response element of the \(Xenopus\) Mix.2 gene (Chen et al., 1996, 1997; Liu et al., 1997), the mammalian TGF\(\beta\) responsive p3TP-Lux promoter (Yingling et al., 1997) and a palindromic sequence derived for optimal binding of mammalian Smad4 and Smad3 (Zawel et al., 1998). This sequence similarity suggests \(C183\) contains a conserved Smad-binding site recognized by DAF-3. Adjacent to the DAF-3-binding site in \(C183\) is a consensus binding site for \(Drosophila\) Mad (GCCGnCG) (Fig. 3D; Kim et al., 1997). DAF-3 does not appear to recognize this site because mutations that disrupt this sequence in the oligonucleotides \(Cmut2\), \(Cmut3\), \(C17A\) and \(G18T\) do not to interfere with DAF-3\(^{3N}\) binding in yeast or in vitro (Fig. 3B,C).

daf-3 negatively regulates \(C183\) enhancer activity in \(C.\) elegans

To facilitate examining \(C183\) enhancer activity in various genetic backgrounds, we constructed a reporter with an enhancer consisting of 8 copies of \(C183\) fused to \(gfp\) reporter gene with a basal myo-2 promoter (Okkema and Fire, 1994) (\(C183::gfp\), Fig. 4A). This reporter was transformed into wild-type \(C.\) elegans and independently integrated at two different chromosomal locations, which are termed \(cuIs2\) and \(cuIs5\). Animals homozygous or heterozygous for either \(cuIs2\) or \(cuIs5\) exhibited strong GFP expression in the pharynx from mid-embryogenesis through adulthood (Fig. 4B,C; Table 3; data not shown). Although the cytoplasmic localization of GFP made it difficult to verify expression in every cell, fluorescence was observed throughout the pharynx consistent with the function of \(C183\) as an organ-specific enhancer (Okkema and Fire, 1994).

\(daf-3\) appears to be expressed the \(C.\) elegans pharynx, as well as many other tissues (Patterson et al., 1997), suggesting it could be a direct regulator of \(C183\) enhancer activity in vivo. To determine how \(daf-3\) affects \(C183\) enhancer activity, we examined expression of the \(C183::gfp\) reporter in \(daf-3(mg90)\) null mutants (Patterson et al., 1997). The mutation in \(Cmut4\) eliminates enhancer function in vivo, suggesting this site binds an essential activator (Okkema and Fire, 1994; Fig. 3B). Because \(Cmut4\) also fails to bind DAF-3 in vitro (Fig. 3B), we expected that DAF-3 might be this activator and that \(C183\) enhancer activity would be reduced in \(daf-3(mg90)\). However, we were surprised to find that the level, pattern and timing of GFP expression in \(cuIs2\); \(daf-3(mg90)\) was indistinguishable from that in +/+; \(cuIs2\) throughout development (Fig. 4D; data not shown). As adults, 100% of the \(daf-3(mg90)\) mutants bearing \(cuIs2\) exhibited strong GFP fluorescence similar to wild-type \(cuIs2\) animals (Table 3). Thus, \(daf-3\) is not required for \(C183\) enhancer activity, and we believe an as yet unidentified activator may also bind near the DAF-3 site in \(C183\).

In the dauer pathway, \(daf-3\)’s dauer-promoting activity is negatively regulated by \(daf-4\), such that \(daf-4(m72)\) mutants have a temperature-sensitive Daf-c phenotype (Riddle and Albert, 1997). To determine if deregulation of \(daf-3\) might also affect \(C183\) enhancer activity, we examined expression of the...
sequences are I overhangs used for cloning and end filling.

5(e1386) suppressed the reduction in position in the dauer formation pathway as dauer formation and epistasis analyses place it at the same necessary for proper daf-3(mg90) (Riddle and Albert, 1997). As in daf-3, GFP expression in daf-4(m72) increased expression observed in daf-8(e1393) adults (Table 3). These genes constitute a complete signal transduction pathway including ligand, receptors and a Smad (Riddle and Albert, 1997). The fact that disruption of this signal at any point reduces C183 activity strongly suggests that this TGFβ-like pathway inhibits C183 repression by daf-3 and daf-5.

The only gene in the Daf-c/TGFβ pathway not required for maintaining strong C183 enhancer activity is daf-14. No reduction in GFP fluorescence was observed in cuIs3/+; daf-14(m77) adults (Table 3). While we cannot rule out a minor role for daf-14, this result suggests daf-14 is not necessary to regulate the repression of C183 activity by daf-3, whereas it is necessary to regulate the dauer-promoting activity of daf-3.

No daf-3-dependent decrease of C183::gfp expression in dauers daf-3 promotes dauer formation and could repress C183 enhancer activity to downregulate pharyngeal gene expression during the dauer stage. Indeed, GFP expression in wild-type or Daf-c mutant dauers bearing the C183::gfp reporter is weakly reduced compared to non-dauer L3 larvae (data not shown). To determine if daf-3 is necessary for this slight reduction, we compared expression of the C183::gfp reporter in wild-type and daf-3(mg90) dauers induced by growth at elevated temperature in the presence of exogenous dauer pheromone

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Percent transformants with strong gfp expression*</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>cuIs2</td>
<td>100</td>
<td>124</td>
</tr>
<tr>
<td>cuIs2/+</td>
<td>94</td>
<td>118</td>
</tr>
<tr>
<td>cuIs5/+</td>
<td>97</td>
<td>118</td>
</tr>
<tr>
<td>daf-7(e1372); cuIs2/+</td>
<td>2</td>
<td>128</td>
</tr>
<tr>
<td>cuIs5; daf-1(m40);</td>
<td>8</td>
<td>114</td>
</tr>
<tr>
<td>daf-4(m72); cuIs2/+;</td>
<td>36</td>
<td>110</td>
</tr>
<tr>
<td>daf-8(e1393); cuIs2/+</td>
<td>3</td>
<td>134</td>
</tr>
<tr>
<td>cuIs5; daf-14(m77)</td>
<td>98</td>
<td>115</td>
</tr>
<tr>
<td>cuIs2; daf-3(mg90)</td>
<td>100</td>
<td>107</td>
</tr>
<tr>
<td>daf-4(e1386); cuIs2/+</td>
<td>97</td>
<td>107</td>
</tr>
<tr>
<td>daf-4; cuIs2/+; daf-3</td>
<td>84</td>
<td>136</td>
</tr>
<tr>
<td>daf-5; daf-4; cuIs2/+; daf-3</td>
<td>99</td>
<td>168</td>
</tr>
</tbody>
</table>

*Strong expression was defined as fluorescence intensities greater than the typical daf-8(e1393); cuIs3/+ level, which was greater than the typical daf-7(e1372); cuIs2/++; cuIs5/+; daf-1(m40); or daf-4(m40); cuIs2/+ levels, but still markedly less than wild-type cuIs2 or cuIs5. Counts for each strain were conducted on 5 different days and the sums were pooled. Shown are the percentage of animals with strong expression and the total number scored for each strain (n). Reduced fluorescence was most often observed throughout the pharynx of the daf-7, daf-1, daf-4 and daf-8 strains, although stronger GFP expression was occasionally observed in the region of the posterior bulb occupied by the gland cells bodies.

Additional genes in the dauer formation pathway affect C183 enhancer activity

We next asked if additional genes affecting dauer formation are necessary for proper C183 enhancer activity. daf-5 promotes dauer formation and epistasis analyses place it at the same position in the dauer formation pathway as daf-3 (Fig. 1A; Riddle and Albert, 1997). As in daf-3, GFP expression in daf-5(e1386); cuIs2/+ was indistinguishable from wild type (Table 3). Also like daf-3, daf-5(e1386) largely suppressed the reduction in C183::gfp expression observed in the daf-4(m72) single mutant. 99% of daf-4(m72); daf-5(e1386) adults bearing cuIs2 exhibited strong GFP expression (Table 3). Therefore, both daf-5 and daf-3 function to repress C183 enhancer activity.

daf-4 is a component of the Daf-c/TGFβ signaling pathway that negatively regulates the dauer-promoting activities of daf-3 and daf-5 (Fig. 1A). If this signaling pathway also inhibits the transcriptional repressor activity of daf-3, then mutation of other genes in the Daf-c/TGFβ pathway should decrease C183 activity. As in daf-4(m72), decreased expression of the C183::gfp reporter was observed in daf-7(e1372), daf-1(m40) and daf-8(e1393) adults (Table 3). These genes constitute a complete signal transduction pathway including ligand, receptors and a Smad (Riddle and Albert, 1997). The fact that disruption of this signal at any point reduces C183 activity strongly suggests that this TGFβ-like pathway inhibits C183 repression by daf-3 and daf-5.

Additional genes in the dauer formation pathway affect C183 enhancer activity

We next asked if additional genes affecting dauer formation are necessary for proper C183 enhancer activity. daf-5 promotes dauer formation and epistasis analyses place it at the same position in the dauer formation pathway as daf-3 (Fig. 1A; Riddle and Albert, 1997). As in daf-3, GFP expression in daf-5(e1386); cuIs2/+ was indistinguishable from wild type (Table 3). Also like daf-3, daf-5(e1386) largely suppressed the reduction in C183::gfp expression observed in the daf-4(m72) single mutant. 99% of daf-4(m72); daf-5(e1386) adults bearing cuIs2 exhibited strong GFP expression (Table 3). Therefore, both daf-5 and daf-3 function to repress C183 enhancer activity.

daf-4 is a component of the Daf-c/TGFβ signaling pathway that negatively regulates the dauer-promoting activities of daf-3 and daf-5 (Fig. 1A). If this signaling pathway also inhibits the transcriptional repressor activity of daf-3, then mutation of other genes in the Daf-c/TGFβ pathway should decrease C183 activity. As in daf-4(m72), decreased expression of the C183::gfp reporter was observed in daf-7(e1372), daf-1(m40) and daf-8(e1393) adults (Table 3). These genes constitute a complete signal transduction pathway including ligand, receptors and a Smad (Riddle and Albert, 1997). The fact that disruption of this signal at any point reduces C183 activity strongly suggests that this TGFβ-like pathway inhibits C183 repression by daf-3 and daf-5.

The only gene in the Daf-c/TGFβ pathway not required for maintaining strong C183 enhancer activity is daf-14. No reduction in GFP fluorescence was observed in cuIs3/+; daf-14(m77) adults (Table 3). While we cannot rule out a minor role for daf-14, this result suggests daf-14 is not necessary to regulate the repression of C183 activity by daf-3, whereas it is necessary to regulate the dauer-promoting activity of daf-3.

No daf-3-dependent decrease of C183::gfp expression in dauers daf-3 promotes dauer formation and could repress C183 enhancer activity to downregulate pharyngeal gene expression during the dauer stage. Indeed, GFP expression in wild-type or Daf-c mutant dauers bearing the C183::gfp reporter is weakly reduced compared to non-dauer L3 larvae (data not shown). To determine if daf-3 is necessary for this slight reduction, we compared expression of the C183::gfp reporter in wild-type and daf-3(mg90) dauers induced by growth at elevated temperature in the presence of exogenous dauer pheromone

Table 2. C183 derivatives with single base pair mutations

<table>
<thead>
<tr>
<th>C183</th>
<th>caagTCTGGTTGTTGCCGAGATCCTGCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>C17A</td>
<td>caagTCTGGTTGTTGCCGAGATCCTGCC</td>
</tr>
<tr>
<td>G18T</td>
<td>caagTCTGGTTGTTGCCGAGATCCTGCC</td>
</tr>
<tr>
<td>G19T</td>
<td>caagTCTGGTTGTTGCCGAGATCCTGCC</td>
</tr>
<tr>
<td>A20C</td>
<td>caagTCTGGTTGTTGCCGAGATCCTGCC</td>
</tr>
<tr>
<td>T21G</td>
<td>caagTCTGGTTGTTGCCGAGATCCTGCC</td>
</tr>
<tr>
<td>G22T</td>
<td>caagTCTGGTTGTTGCCGAGATCCTGCC</td>
</tr>
<tr>
<td>T23G</td>
<td>caagTCTGGTTGTTGCCGAGATCCTGCC</td>
</tr>
<tr>
<td>C24A</td>
<td>caagTCTGGTTGTTGCCGAGATCCTGCC</td>
</tr>
<tr>
<td>T25G</td>
<td>caagTCTGGTTGTTGCCGAGATCCTGCC</td>
</tr>
<tr>
<td>G26T</td>
<td>caagTCTGGTTGTTGCCGAGATCCTGCC</td>
</tr>
<tr>
<td>C27A</td>
<td>caagTCTGGTTGTTGCCGAGATCCTGCC</td>
</tr>
<tr>
<td>C28A</td>
<td>caagTCTGGTTGTTGCCGAGATCCTGCC</td>
</tr>
</tbody>
</table>

Altered bases are in bold face and lower case; the lower case caag sequences are StyI overhangs used for cloning and end filling.
complexes with DAF-3.
Smads bind the Mad consensus of SMAD. It will be interesting to determine if other of the DAF-3 co-SMAD and an unidentified receptor-regulated contains a bipartite binding site for hetero-oligomers consisting the DAF-3-binding site suggests that Drosophila (GCCGnCG) derived for Mad, a receptor-regulated SMAD in DAF-3 core recognition site is a consensus binding sequence binding sites for a number of other factors. Adjacent to the sequence to the DAF-3-binding site suggests that DAF-3 binds DNA and we have demonstrated this activity to control transcription of various target genes (Heldin et al., 1997).
Isolation of a daf-3 cDNA in a one-hybrid screen suggested that DAF-3 binds DNA and we have demonstrated this activity in vitro. These results are consistent with recent reports that Drosophila Mad and Medea, and vertebrate Smad3 and Smad4 bind DNA directly (Kim et al., 1997; Yingling et al., 1997; Zawel et al., 1998; Xu et al., 1998). Our in vitro DNA-binding assays used a N-terminal fragment of DAF-3 containing the conserved MH1 domain. Thus, like Drosophila Mad (Kim et al., 1997), a DNA-binding domain appears to be localized to the MH1 region of DAF-3. We have not yet examined DNA binding of full-length DAF-3 in vitro; however the daf-3 cDNA isolated in the one-hybrid screen contains both the MH1 and MH2 domains suggesting that full-length DAF-3 can also bind DNA.
The pentanucleotide sequence GTCTG near the 3' end of C183 was identified as the core recognition site for DAF-3 binding. This sequence is also contained in a larger sequence repeated in the activin response element of the Xenopus Mix.2 gene that is essential for binding activin response factor, a complex containing Smad4, Smad2 and the fork head domain factor FAST-1 (Fig. 3D; Chen et al., 1996, 1997; Liu et al., 1997). Likewise, the DAF-3 recognition sequence is contained in a sequence in the TGFβ-responsive p3TP-Lux promoter that is required for binding of the mammalian co-SMAD Smad4 (Fig. 3D; Yingling et al., 1997), and it contains a match with half of a palindromic sequence derived for optimal mammalian Smad4 and Smad3 binding, which is sufficient for TGFβ responsiveness (Fig. 3D; Zawel et al., 1998). The similarity between these sites suggests the core recognition site for DAF-3 binding may be a general Smad recognition sequence.
In addition to the DAF-3-binding site, C183 contains binding sites for a number of other factors. Adjacent to the DAF-3 core recognition site is a consensus binding sequence (GCCGnCG) derived for Mad, a receptor-regulated SMAD in Drosophila (Fig. 3D; Kim et al., 1997). The proximity of this sequence to the DAF-3-binding site suggests that C183 contains a bipartite binding site for hetero-oligomers consisting of the DAF-3 co-SMAD and an unidentified receptor-regulated SMAD. It will be interesting to determine if other C. elegans Smads bind the Mad consensus of C183 and if they form complexes with DAF-3.

Slightly distal to the DAF-3-binding site is a sequence bound by PHA-4, a C. elegans fork head domain transcription factor (Fig. 3D; Kalb et al., 1998; Horner et al., 1998). pha-4 is known to activate the C183 enhancer, and it is essential for pharyngeal organogenesis (Kalb et al., 1998; Mango et al., 1994). The proximity of the PHA-4-binding site with the Mad consensus sequence and the DAF-3-binding site is provocative, because Fork Head and Smad proteins have been shown to interact. In Xenopus, the fork head factor FAST-1 forms an activin-inducible complex with Smad2 and Smad4, which binds the activin response element in the Mix.2 gene (Chen et al., 1996, 1997; Liu et al., 1997). None of the known pha-4 cDNAs encode a sequence similar to the Smad interaction domain of FAST-1 (Chen et al., 1997; Azzaria et al., 1996), suggesting that if PHA-4 does interact with Smads, it employs a novel domain. An interaction between PHA-4 and Smad complexes could be a mechanism coupling TGFβ-like signaling and pharyngeal development.

**DISCUSSION**

DAF-3 binds C183 directly
In a yeast one-hybrid screen to identify genes controlling the pharyngeal organ-specific enhancer activity of C183, we recovered the Smad gene daf-3. daf-3 belongs to the subfamily of Smads referred to as co-SMADs (Patterson et al., 1997), which include vertebrate Smad4 (Hahn et al., 1996; Lagna et al., 1996), Drosophila Medea (Raftery et al., 1995; Wisotzkey et al., 1998) and C. elegans sma-4 (Savage et al., 1996). Upon ligand stimulation, co-SMADs are thought to form heteromeric complexes with receptor-regulated SMADs allowing them to control transcription of various target genes (Heldin et al., 1997).
Isolation of a daf-3 cDNA in a one-hybrid screen suggested that DAF-3 binds DNA and we have demonstrated this activity in vitro. These results are consistent with recent reports that Drosophila Mad and Medea, and vertebrate Smad3 and Smad4 bind DNA directly (Kim et al., 1997; Yingling et al., 1997; Zawel et al., 1998; Xu et al., 1998). Our in vitro DNA-binding assays used a N-terminal fragment of DAF-3 containing the conserved MH1 domain. Thus, like Drosophila Mad (Kim et al., 1997), a DNA-binding domain appears to be localized to the MH1 region of DAF-3. We have not yet examined DNA binding of full-length DAF-3 in vitro; however the daf-3 cDNA isolated in the one-hybrid screen contains both the MH1 and MH2 domains suggesting that full-length DAF-3 can also bind DNA.

The pentanucleotide sequence GTCTG near the 3' end of C183 was identified as the core recognition site for DAF-3 binding. This sequence is also contained in a larger sequence repeated in the activin response element of the Xenopus Mix.2 gene that is essential for binding activin response factor, a complex containing Smad4, Smad2 and the fork head domain factor FAST-1 (Fig. 3D; Chen et al., 1996, 1997; Liu et al., 1997). Likewise, the DAF-3 recognition sequence is contained in a sequence in the TGFβ-responsive p3TP-Lux promoter that is required for binding of the mammalian co-SMAD Smad4 (Fig. 3D; Yingling et al., 1997), and it contains a match with half of a palindromic sequence derived for optimal mammalian Smad4 and Smad3 binding, which is sufficient for TGFβ responsiveness (Fig. 3D; Zawel et al., 1998). The similarity between these sites suggests the core recognition site for DAF-3 binding may be a general Smad recognition sequence.
In addition to the DAF-3-binding site, C183 contains binding sites for a number of other factors. Adjacent to the DAF-3 core recognition site is a consensus binding sequence (GCCGnCG) derived for Mad, a receptor-regulated SMAD in Drosophila (Fig. 3D; Kim et al., 1997). The proximity of this sequence to the DAF-3-binding site suggests that C183 contains a bipartite binding site for hetero-oligomers consisting of the DAF-3 co-SMAD and an unidentified receptor-regulated SMAD. It will be interesting to determine if other C. elegans Smads bind the Mad consensus of C183 and if they form complexes with DAF-3.

Slightly distal to the DAF-3-binding site is a sequence bound by PHA-4, a C. elegans fork head domain transcription factor (Fig. 3D; Kalb et al., 1998; Horner et al., 1998). pha-4 is known to activate the C183 enhancer, and it is essential for pharyngeal organogenesis (Kalb et al., 1998; Mango et al., 1994). The proximity of the PHA-4-binding site with the Mad consensus sequence and the DAF-3-binding site is provocative, because Fork Head and Smad proteins have been shown to interact. In Xenopus, the fork head factor FAST-1 forms an activin-inducible complex with Smad2 and Smad4, which binds the activin response element in the Mix.2 gene (Chen et al., 1996, 1997; Liu et al., 1997). None of the known pha-4 cDNAs encode a sequence similar to the Smad interaction domain of FAST-1 (Chen et al., 1997; Azzaria et al., 1996), suggesting that if PHA-4 does interact with Smads, it employs a novel domain. An interaction between PHA-4 and Smad complexes could be a mechanism coupling TGFβ-like signaling and pharyngeal development.

**daf-3 and daf-5 negatively regulate C183 enhancer activity**
Wild-type daf-3 promotes dauer formation and daf-3 mutants have a dauer-defective (Daf-d) phenotype (Riddle and Albert, 1997). In reproductively growing C. elegans daf-3 and daf-5 negatively regulate C183 enhancer activity is negatively regulated by genes in the Daf-c/TGFβ signaling pathway (daf-7, daf-1, daf-4, daf-8 and daf-14). When this...
upstream signaling pathway is inactivated by mutation, daf-3 dauer-promoting activity is deregulated resulting in a temperature-sensitive dauer constitutive (Daf-c) phenotype. The Daf-c phenotypes of these mutants are dependent on wild-type daf-3 and they are efficiently suppressed by mutation of daf-3 (Riddle and Albert, 1997).

Our results indicate that wild-type daf-3 negatively regulates C183 enhancer activity and this repressor activity is similarly negatively regulated by TGF-like signaling (Fig. 6). Interestingly, daf-5 also negatively regulates C183 enhancer activity (Fig. 6). daf-5 and daf-3 are positioned at the same point in the dauer formation pathway and mutations in either of these genes result in identical Daf-d phenotypes (Riddle and Albert, 1997), suggesting daf-3 and daf-5 function in a common process that is negatively regulated by the Daf-c/TGF-like signaling pathway. Previous genetic analyses could not distinguish the relative order of daf-3 and daf-5 gene function in the dauer formation pathway (Riddle and Albert, 1997). For repression of the C183 enhancer, daf-5 cannot function downstream of daf-3, because DAF-3 binds directly to C183. Therefore, daf-5 likely functions upstream or in parallel to daf-3 to repress C183, and perhaps similarly to promote dauer formation. While daf-5 has not yet been molecularly identified, we suggest that daf-5 may act in a DNA-binding complex with daf-3. daf-5 may encode a receptor-regulated SMAD or another class of transcription factor that interacts with daf-3.

daf-14 is the only gene in the Daf-c/TGF-like signaling pathway that is not necessary to regulate daf-3's repression of C183 enhancer activity (Fig. 6). This situation is distinct from dauer formation, where daf-14 is necessary to repress daf-3. However, we cannot exclude the possibility that daf-14 makes a minor contribution to C183 enhancer activity; daf-14 and daf-8 both encode Smads and they could be partially redundant (K. King and D. L. Riddle; T. Inoue and J. H. Thomas, personal communication). Indeed, we did observe a slightly weaker reduction in C183 activity in daf-8 mutants than in other Daf-c strains.

**Possible relevance for control of C183 enhancer by daf-3**

Our results indicate that DAF-3 modulates C183 enhancer activity; however it is unlikely that DAF-3 mediates the organ-specificity of C183. The daf-3(mg90) mutation does not affect the pharyngeal-specific expression pattern of the C183::gfp reporter, nor does this mutation result in any apparent pharyngeal defects. What then is the function of daf-3 repression of C183 enhancer activity? One possibility is that, during dauer formation, daf-3 reduces myo-2 expression via C183 to conserve energy or to contribute to pharyngeal remodeling (Vowels and Thomas, 1992). However, we found no evidence for a daf-3-dependent reduction in C183 enhancer activity in dauers. Furthermore, Daf-c mutant dauers produced at the non-permissive temperature exhibited little reduction in enhancer activity compared to wild-type larvae, even though C183 activity was reduced in the adults of these mutants grown at permissive temperature (data not shown). Therefore, we suggest that daf-3 repression of C183 enhancer activity may be distinct from its role in dauer formation. The C. elegans genome likely contains additional DAF-3 responsive elements involved in dauer formation and these may be identified based on the DAF-3 core recognition sequence.

Previous work has identified several other phenotypes exhibited by daf TGF-like signaling mutants that are also under distinct control from dauer formation, including defective egg-laying, clumping behavior, dark intestines and inappropriate expression of L1-specific surface antigens (Vowels and Thomas, 1992; Thomas et al., 1993; Grenache et al., 1996). Like regulation of C183 enhancer activity, these phenotypes are controlled by TGF-like signaling via a branch point in the dauer formation pathway at the position of daf-3 and daf-5 (Thomas et al., 1993; Grenache et al., 1996). We suggest that, like the C183 enhancer, genes affecting these phenotypes might be under direct transcriptional control of daf-3. Perhaps, in addition to its role in dauer formation, daf-3 functions as a regulator of many genes in response to TGF-like signals that maintain homeostasis during larval and adult stages.

Pharyngeal development in C. elegans shares a number of similarities with heart development in other species (Haun et al., 1998). In particular CEH-22, an NK-2 class homeodomain factor that acts in combination with factors binding the C subelement to promote myo-2 transcription in pharyngeal muscle cells, is structurally and functionally related to the Drosophila Tinman and vertebrate Nkx2.5 factors that promote heart development in other systems (Okkema and Fire, 1994; Haun et al., 1998). Repression of C subelement activity by DAF-3 suggests another similarity between pharyngeal and heart development. Like CEH-22 and DAF-3 targeting the myo-2 enhancer, Drosophila Tinman acts in combination with Smads to regulate the activity of the DPP-responsive enhancer of the tinman gene (Xu et al., 1998). Perhaps combinatorial control by Smads and NK-2 factors may be a conserved feature of pharyngeal and cardiac genes.

**Possible mechanisms for antagonism with Daf-c/TGF-like signaling and repression of C183 enhancer activity by DAF-3**

DAF-3 functions antagonistically to the Daf-c/TGF-like signaling
pathway (Patterson et al., 1997), and this antagonistic activity is reminiscent of the activity of the anti-SMADs, which include Smad6 and Smad7 in mammals (Imamura et al., 1997; Nakao et al., 1997; Hata et al., 1998), and Dad in Drosophila (Tsuneizumi et al., 1997). Could DAF-3 function like these anti-SMADs? Anti-SMADs have been reported to block TGFβ-like signaling both by binding to receptors (Imamura et al., 1997; Nakao et al., 1997) and by competing for activated receptor-regulated SMADs (Hata et al., 1998). Our results demonstrating DAF-3 binds DNA suggests DAF-3 functions differently than the reported anti-SMADs. Moreover, previous genetic evidence indicates dad-3 functions downstream of both the receptors and the DAF-8/DAF-14 Smads during dauer formation. Thus it seems unlikely that DAF-3 antagonizes TGFβ-like signaling by the same mechanisms proposed for the anti-SMADs.

Patterson et al. (1997) have proposed a model predicting that, during dauer development, when the Daf-c/TGFβ signal is inactive, DAF-3 homo-oligomers repress genes for growth and metabolism. During non-dauer development, signaling causes DAF-8 or DAF-14 to form heteromeric complexes with DAF-3 that activate expression of these genes. Our results examining C183 enhancer activity are consistent with the first part of this model. When TGFβ-like signaling is disrupted by mutation, we observed a dad-3-dependent repression of C183 enhancer activity. However, we observed no reduction in C183 activity in a dad-3 single mutant, demonstrating that wild-type dad-3 is not required for C183 activity. Thus, it seems unlikely that Daf-c/TGFβ signaling antagonizes DAF-3 activity by converting it into an activator of C183, although it is possible that DAF-8 could associate with DAF-3 to block repression.

How does DAF-3 repress the C183 enhancer? Any of three mechanisms proposed for eukaryotic transcriptional repressors could be employed by DAF-3 (Johnson, 1995). First, DAF-3 may compete with a transcriptional activator for binding to C183. Indeed, the mutation in Cmut4 that interferes with DAF-3 binding also interferes with C183 enhancer activity in transgenic C. elegans, suggesting an unidentified activator targets the same region of C183 as DAF-3 (Okkema and Fire, 1994). Second, DAF-3 may inhibit an activator bound to another site in C183 by direct interaction. PHA-4 and the second gene identified in our one-hybrid screen are both candidate activators that may interact with DAF-3. Third, DAF-3 may directly interact with the transcriptional machinery preventing it from responding to a bound activator. Whatever the mechanism, Daf-c/TGFβ signaling could antagonize the DAF-3 repressor by phosphorylating a factor that competes or interacts with DAF-3, or by phosphorylating an unidentified site of DAF-3 itself.

We would like to thank David Stone, Susan Lieberman, Bill Kelly, Brian Harfe, Andy Fire and Robert Barstead for providing us with advice and materials to perform one-hybrid selection, as well as Garth Patterson and Gary Ruvkun for providing us with advice and materials for examining dauer formation mutants. We are grateful to Elizabeth Goodwin, Henry Epstein, Thomas Bürglin and two anonymous reviewers for their critical reading of this paper. This work was supported by the NIH (R01-GMS3996). Some nematode strains used in this work were provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR).

REFERENCES


