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

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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,

1992; Thomas et al., 1993; Patterson et al., 1997; reviewed by Riddle and Albert, 1997). The upstream portion of this pathway is a typical TGFβ-like pathway consisting of a ligand encoded by *daf-7* (a TGFβ homolog in the bone morphogenetic protein subfamily) (Ren et al., 1996), type I and type II receptors encoded by *daf-1* and *daf-4* (Estevez et al., 1993; Georgi et al., 1990), and two Smads encoded by *daf-8* and *daf-14* (K. King and D. L. Riddle; T. Inoue and J. H. Thomas, personal communications). During non-dauer development, this upstream signal is active and functions to prevent dauer formation. Disruption of this part of the pathway by mutation of any of these genes results in a temperature-sensitive dauer constitutive (Daf-c) phenotype where dauers form under conditions that normally support reproductive growth (Riddle and Albert, 1997). We shall refer to this part of the dauer formation pathway as the Daf-c/TGFβ pathway.

During dauer development, the Daf-c/TGFβ pathway is inactive, allowing downstream genes such as *daf-3* and *daf-5* to promote dauer formation. Mutation of either *daf-3* or *daf-5* results in a dauer-defective (Daf-d) phenotype where dauers fail to form under conditions that normally promote dauer formation. Importantly, *daf-3* and *daf-5* mutations suppress the Daf-c phenotype of mutations of the Daf-c/TGFβ pathway (Riddle and Albert, 1997).

The sequence of *daf-3* suggests that it encodes a co-SMAD factor (Patterson et al., 1997). However, DAF-3 is unlike previously characterized co-SMADs in that it is inhibited rather than activated by upstream Daf-c/TGFβ signaling and it appears to be partially localized to the nucleus independent of the Daf-c/TGFβ signal. The expression pattern of a *daf-3::gfp* reporter suggests that DAF-3 is expressed in many tissues including the gut, nervous system and pharynx throughout development (Patterson et al., 1997).

Few DNA regulatory elements have been identified that are targeted by Smad binding. We have found that in *C. elegans* DAF-3 binds a unique organ-specific regulatory sequence called the *C* subelement from the pharyngeal muscle-specific myosin gene, *myo-2* (Okkema and Fire, 1994), and we have shown that *daf-3* negatively regulates enhancer activity of the *C* subelement in vivo. These results demonstrate that one function of *daf-3* is to modulate gene expression in the pharynx and they suggest that, in addition to activating downstream regulatory genes in the dauer formation pathway, *daf-3* directly affects structural genes such as *myo-2*. Our results also confirm that DNA binding is a general property of Smads and indicate that they can function as transcriptional repressors.

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 *Bam*HI-*Sal*I sites (B. Harfe, B. Kelly and A. Fire, personal communication). Each reporter plasmid was generated by inserting a *Xba*I-*Stu*I restriction fragment containing a concatenated oligonucleotide into *Spe*I and Klenow end-filled *Sac*II sites of pRS414/HIS.

To sequence the *daf-3* clone, pOK104.02 was constructed by cleaving *Bgl*II sites of the pACT1 polylinker and subcloning the cDNA insert into the *Bam*HI site of Bluescript KS+ (Stratagene). To produce DAF-3^N protein, pOK114.02 was constructed by inserting a *Bgl*II-*Sac*II fragment of *daf-3* cDNA into pGEX-3X. To produce DAF-3^C, pOK114.03 was constructed by inserting a *Bam*HI-*Sma*I 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. 4.9×10^6 pACT-RB1 transformants and 5.1×10^6 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 parental 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 reporters, 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^N protein, a GST fusion with the leader sequence, MH1 domain and linker region of DAF-3. pOK104.03 was used to produce DAF-3^C, a GST fusion with the linker region, MH2 domain and tail of DAF-3. *E. coli* 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 (Ausubel et al., 1990). DAF-3^N has a predicted M_r of 86.5×10^3; however the major induced protein migrated on SDS gels with an apparent M_r of 60×10^3, 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 DNase1 protection assays DAF-3^N was concentrated with Centron-30 columns to 19 µg/µl (Amicon).

DNA-binding assays

Gel mobility shift assays were performed essentially as described by Thatcher et al. (1995). 20 µl 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/µl poly(dIdC), 50 ng/µl poly(dAdT), 12.5 ng/µl protein and 1500 cts/minute/µl *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 *Sty*I sites. The second set were blunt ended. Identical results were observed with both sets. *C183* with 3′ overhangs was radiolabeled by Klenow end filling using α[^{32}P]-dCTP (6000 Ci/mmol). Blunt-ended *C183* was radiolabeled with polynucleotide kinase using γ[^{32}P]-ATP (5000 Ci/mmol).

DNase1 protection assays were performed essentially as described by Thatcher et al. (1995). 20 µl binding reactions contained 5 mM

Hepes, pH 7.8, 2.5 mM KCl, 87 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT, 50 ng poly(dIdC), 50 ng/µl poly(dAdT), 0.25-1.25 ng/µl DNase1, 0.05-1 µg/µl protein and 500 cts/minute/µl *C* subelement probe. Samples were preincubated at room temperature for 2 minutes prior to DNase1 treatment at room temperature for 2 minutes. The probe was prepared by cleaving pPD20.97 (Okkema et al., 1993) with *Bss*HI, Klenow end filling using $\alpha[^{32}P]$-dCTP (6000 Ci/mmol) and $\alpha[^{32}P]$-dGTP (3000 Ci/mmol), cleaving with *Hpa*I, 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	*cuIs5 /+ I; daf-4(m72) III*
OK82	*daf-7(e1372) III; cuIs2/+ III*
OK84	*daf-8(e1393) I; cuIs2/+ III*
OK85	*cuIs5 /+ I; daf-14(m77) IV*
OK68	*cuIs2 III; daf-3(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. F_2 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-3(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 bearing *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(e128)/+ 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 segregate 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 *cuIs2; daf-3(mg90)* 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

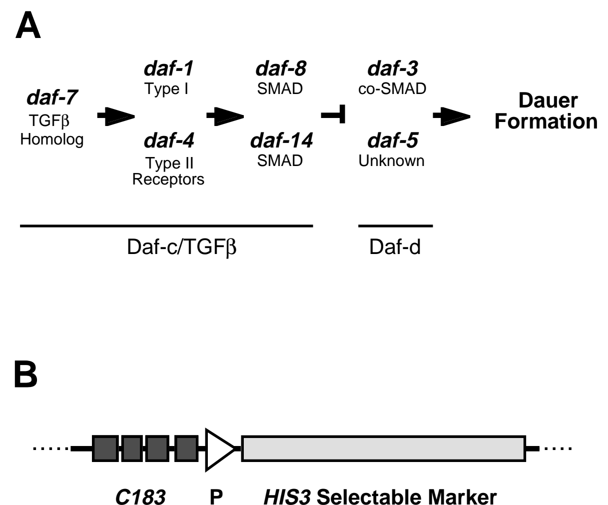

Fig. 1. TGFβ-like pathway controlling dauer formation and one-hybrid reporter used to clone *daf-3*. (A) The TGFβ-like signaling pathway controlling dauer formation. During non-dauer development, the upstream part of this pathway, which we refer to as the Daf-c/TGFβ pathway, is active and antagonizes *daf-3* and *daf-5* dauer-promoting activity (reviewed by Riddle and Albert, 1997). During dauer development, the Daf-c/TGFβ pathway is inactive, allowing *daf-3* and *daf-5* to promote dauer formation. (B) The one-hybrid reporter used to clone *daf-3*. This reporter contains four copies of the *C183* oligonucleotide (Okkema and Fire, 1994) upstream of a *gal-1* minimal promoter (P), fused to the *HIS3* selectable marker (Sikorski and Hieter, 1989). One-hybrid reporters containing other oligonucleotide concatenates have a similar structure.

Table 1. Results of one-hybrid selection

Total transformants*	1×10^7
Colonies analyzed‡	300
cDNA clones reintroduced§	62
Positives¶	12
Clone a	11
daf-3@	1

**S. cerevisiae* YJL170 cells were transformed with one of two mixed stage *C. elegans* cDNA libraries, pACT-RB1 – poly(dT) primed, and pACT-RB2 – random oligonucleotide primed (the generous gift of R. Barstead). Approximately an equal number of clones were screened from each library. Shown is the sum of all transformants.

‡Most rapidly growing colonies were replated and their growth rates were compared to each other, as well as their ability to resist inhibition of histidine biosynthesis by 3 aminotriazole (5 mM). The clones were categorized based on these two criteria.

§Selected cDNA plasmids from different categories were extracted from yeast, amplified in bacteria and transformed into the original *C183* reporter strain used to perform the screen, as well as a strain with the parental reporter plasmid lacking *C183* sequences.

¶Clones that promoted growth of yeast cells with the *C183* reporter, but failed to promote growth with the parental vector.

@The *daf-3* cDNA that we isolated is identical to an alternatively spliced form reported by Patterson et al. (1997) (accession number AF005205).

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 *Smad4* (Fig. 1A; Patterson et al., 1997; Riddle and Albert, 1997; Hahn et al., 1996). 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 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 including its MH1 domain. This protein (DAF-3^N) produced a slowly migrating complex when incubated with radioactively labeled *C183* oligonucleotide (Fig. 2). Competition with 250-fold excess of unlabeled *C183* markedly reduced complex formation (Fig. 2), while competition with even a 2000-fold excess of *B207* oligonucleotide failed to eliminate binding (Fig. 2). In contrast, DAF-3^C, a GST fusion containing the linker and carboxy terminal regions including the MH2 domain, failed to bind *C183* (Fig. 2).

Taken together, our results examining DAF-3 in yeast and in vitro demonstrate that DAF-3 binds *C183* specifically and directly. Furthermore, like *Drosophila* Mad (Kim et al., 1997), a DAF-3 DNA-binding domain is localized to the amino-region including the MH1 domain, while the linker and carboxy-regions including the MH2 domain have no detectable binding activity.

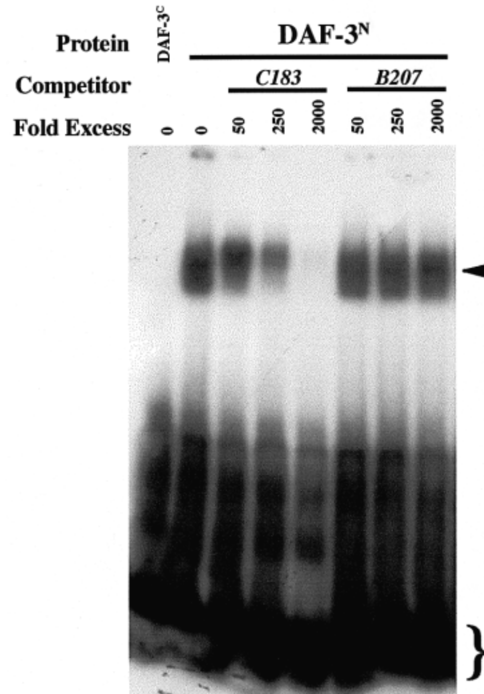

Fig. 2. DAF-3 specifically binds *C183* in gel mobility shift assays. DAF-3^C is a C-terminal fragment of DAF-3 containing the proline-rich linker and MH2 domain fused to GST. DAF-3^N is a N-terminal fragment of DAF-3 containing the leader and MH1 domain fused to GST. Excess unlabeled *C183* or the unrelated 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^C protein, despite the addition of 2.5× more DAF-3^C than DAF-3^N; this pattern was indistinguishable from samples containing no added protein (data not shown).

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

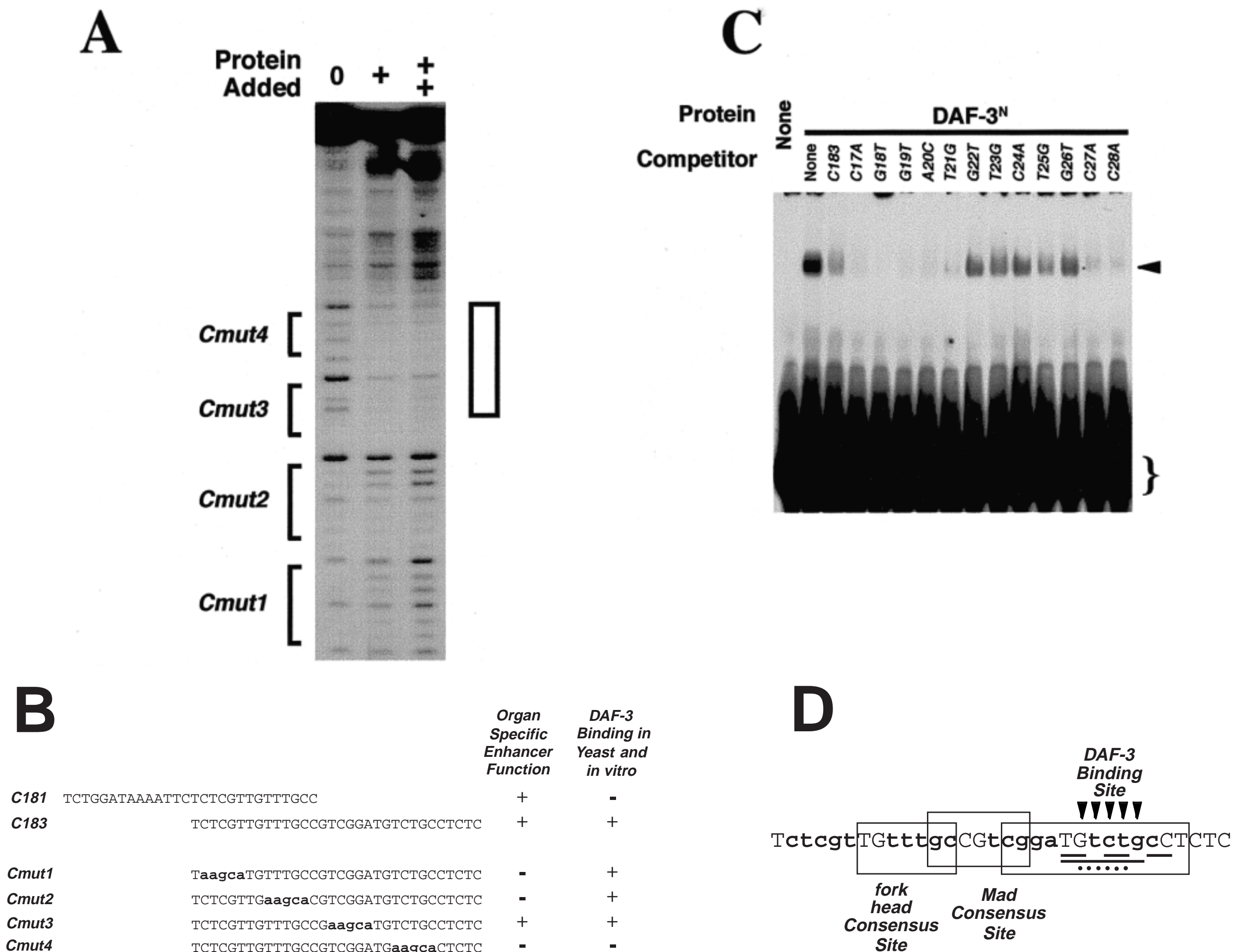

Fig. 3. DAF-3 binds a 5 bp sequence near the 3′ end of *C183*. (A) DNase1 protection assay mapping the DAF-3N-binding site within a restriction fragment corresponding to the entire wild-type *C* subelement. The box to the right indicates the region protected by DAF-3N. For reference, the regions altered in *Cmut1* through *Cmut4* are indicated to the left. The '0' indicates that no DAF-3N was added, while a '+' or double '+' indicates 1 µg or 5 µg, respectively, of partially purified DAF-3N was added. (B) Sequences of wild-type and various mutated *C* subelement oligonucleotides. *C181* and *C183* are overlapping *C* subelement oligonucleotides that retain organ-specific enhancer activity. *Cmut1* through *Cmut4* are mutated derivatives of *C183*; the altered bases are shown in bold face and lower case. Organ-specific enhancer function indicates the ability to enhance pharyngeal gene expression in transgenic *C. elegans* (Okkema and Fire, 1994). DAF-3 binding in yeast and in vitro indicates the ability of DAF-3 to bind the various oligonucleotides using the one-hybrid and gel mobility shift systems. In one-hybrid assays, *gal-4::daf-3* was used to transform yeast carrying reporter vectors with concatenates of the different oligonucleotides. A '+' indicates that at least 24% of the transformants bearing *gal-4::daf-3* and the reporter grew on plates lacking histidine. A '–' indicates that no transformants bearing both plasmids grew on plates lacking histidine. (C) Gel mobility shift competition with oligonucleotides containing single base pair mutations of *C183*. 250-fold excess of the indicated oligonucleotides were used for competitions. The oligonucleotide sequences are in Table 2. This figure is labeled similarly to Fig. 2. (D) Sequence of the *C183* region of the *C* subelement indicating the DAF-3-binding site and sites implicated in Smad binding in other systems. The box on the right shows the bases protected against DNase1 digestion by DAF-3N. The arrowheads denote bases required for strong DAF-3 binding in the gel mobility shift assay. The dashed underline marks bases matching a sequence in the activin response element of the *Xenopus Mix.2* gene, which is essential for binding of a complex containing Smad4, Smad2 and the fork head domain factor FAST-1 (Chen et al., 1996, 1997; Liu et al., 1997). The solid underline marks bases matching a sequence in the TGFβ-responsive p3TP-Lux promoter that is essential for Smad4 binding (Yingling et al., 1997). The dotted underline marks bases matching half of a palindromic sequence derived for optimal binding of mammalian Smad3 and Smad4 (Zawel et al., 1998). Also boxed is a sequence matching the consensus binding site for *Drosophila* Mad (GCCGnCG) (Kim et al., 1997) and a sequence matching the consensus binding site for mammalian fork head domain transcription factors (TGTTTGC) (Costa, 1994; Pierrou et al., 1994; Kaufmann et al., 1995). PHA-4, a *C. elegans* fork head family member required for pharyngeal organogenesis, binds this region and activates to *C183* enhancer activity (Mango et al., 1994; Kalb et al., 1998; Horner et al., 1998).

reporter containing concatenated *Cmut4* were not converted to His^+ (data not shown). Similarly *Cmut1*, *Cmut2* and *Cmut3* competed effectively for DAF-3N 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^{N} 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 TGFβ 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^{N} 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 a *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,E; 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

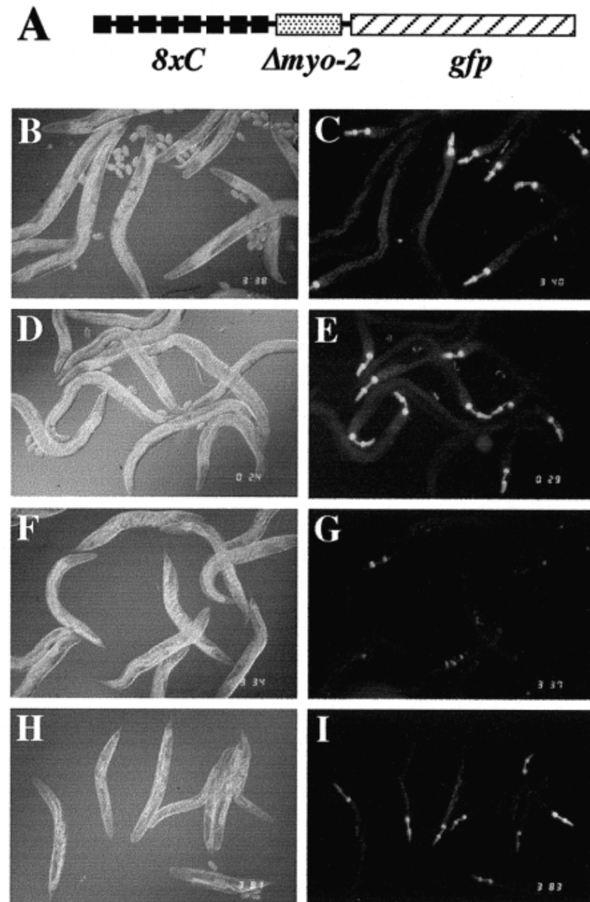

Fig. 4. *daf-3* negatively regulates *C183* enhancer activity. (A) The *C183::gfp* reporter used to examine *C183* enhancer function in wild-type and mutant backgrounds. This reporter contains 8 copies of *C183* upstream of the basal *myo-2* promoter (Okkema and Fire, 1994) fused to *gfp*. It was chromosomally integrated and crossed into different mutants. (B,D,F,H) Differential interference contrast photomicrographs of representative fields of adult *C. elegans* bearing *C183::gfp*. (C,E,G,I) Fluorescence photomicrographs of GFP fluorescence. (B,C) Wild type, *cuIs2*, (D,E) *cuIs2; daf-3(mg90)*, (F,G) *daf-4(m72); cuIs2/+*, (H,I) *daf-4(m72); cuIs2/+; daf-3(mg90)*. Most *daf-4(m72); cuIs2/+* adults exhibited weak fluorescence throughout the pharynx. Shown is a field with one adult pharynx with moderately strong fluorescence. *daf-4(m72)* mutants have an egg-laying defect and strong GFP expression can be observed in the embryos accumulating in the *daf-4(m72); cuIs2/+* mutants.

Table 2. *C183* derivatives with single base pair mutations

C183	caagTCTGGTTGTTTGCCGTCGGATGTCTGCC
C17A	caagTCTGGTTGTTTGCCGT**a**GGATGTCTGCC
G18T	caagTCTGGTTGTTTGCCGTC**t**GATGTCTGCC
G19T	caagTCTGGTTGTTTGCCGTCG**t**ATGTCTGCC
A20C	caagTCTGGTTGTTTGCCGTCGG**c**TGTCTGCC
T21G	caagTCTGGTTGTTTGCCGTCGGA**g**GTCTGCC
G22T	caagTCTGGTTGTTTGCCGTCGGAT**t**TCTGCC
T23G	caagTCTGGTTGTTTGCCGTCGGATG**g**CTGCC
C24A	caagTCTGGTTGTTTGCCGTCGGATGT**a**TGCC
T25G	caagTCTGGTTGTTTGCCGTCGGATGTC**g**GCC
G26T	caagTCTGGTTGTTTGCCGTCGGATGTCT**t**CC
C27A	caagTCTGGTTGTTTGCCGTCGGATGTCTG**a**C
C28A	caagTCTGGTTGTTTGCCGTCGGATGTCTGC**a**

Altered bases are in bold face and lower case; the lower case caag sequences are *Sty*I overhangs used for cloning and end filling.

C183::gfp reporter in *daf-4(m72)* mutants. We found that, while GFP expression was normal in *daf-4(m72)* embryos and early larvae bearing *C183::gfp*, it was markedly reduced in *daf-4(m72)* adults bearing the reporter, even though these animals were maintained at the permissive temperature and did not form dauers (Fig. 4F,G). Only 2% of the adult *daf-4(m72)* mutants bearing *cuIs2* exhibited strong GFP throughout the pharynx (Table 3). These results demonstrate that wild-type *daf-4* is required for maintaining strong *C183* enhancer activity during larval development, perhaps by negatively regulating a transcriptional repressor activity of *daf-3*.

To test this hypothesis, we examined expression of the *C183::gfp* reporter in a *daf-4(m72); daf-3(mg90)* double mutant. The strong reduction in GFP expression observed in *daf-4(m72)* single mutants was largely suppressed in the double mutant (Fig. 4H,I). As adults, 84% of *daf-4(m72); daf-3(mg90)* animals bearing *cuIs2* exhibited much stronger GFP expression than *daf-4* single mutants (Table 3). Thus the reduction of *C183* enhancer activity observed in *daf-4(m72)* requires wild-type *daf-3*. Although this suppression was clearly evident, it appeared incomplete because GFP expression in a typical *daf-4(m72); daf-3(mg90)* double mutant was slightly weaker than in either wild-type or *daf-3(mg90)* single mutants (compare Fig. 4C,E,I) suggesting additional genes may also contribute to repression of *C183*.

Taken together our results indicate that *daf-3* is a repressor of *C183* enhancer activity and, as with its dauer-promoting activity, *daf-3*'s transcriptional repressor activity is negatively regulated by *daf-4*. Reduced *C183* enhancer activity is observed in *daf-4* mutants because the repressor activity of *daf-3* is deregulated and possibly activated in inappropriate stages.

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(mg90)*, GFP expression in *daf-5(e1386); cuIs2/+* was indistinguishable from wild type (Table 3). Also like *daf-3(mg90)*, *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 *cuIs5/+; 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 3. *C183* enhancer activity in *daf* mutants

Genotype	Percent transformants with strong *gfp* expression*	*n*
cuIs2	100	124
cuIs2/+	94	118
cuIs5/+	97	118
daf-7(e1372); cuIs2/+‡	2	128
cuIs5/+; daf-1(m40)‡	8	114
daf-4(m72); cuIs2/+‡§	2	158
daf-8(e1393); cuIs2/+‡	3	134
cuIs5/+; daf-14(m77)	98	115
cuIs2; daf-3(mg90)	100	107
daf-5(e1386); cuIs2/+	97	107
daf-4; cuIs2/+; daf-3	84	136
daf-5; daf-4; cuIs2/+	99	168

*Strong expression was defined as fluorescence intensities greater than the typical *daf-8(e1393); cuIs2/+* 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.

§*daf-4(m72)* was also crossed into a *cuIs5/+* background. The *gfp* fluorescence was indistinguishable from the *daf-4(m72); cuIs2/+* strain.

(Golden and Riddle, 1982; M. Ailion and J. H. Thomas, personal communication). We found that GFP expression was indistinguishable in wild-type and *daf-3(mg90)* dauers (Fig. 5). Therefore, *daf-3* does not appear to repress *C183* activity during the dauer stage.

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.

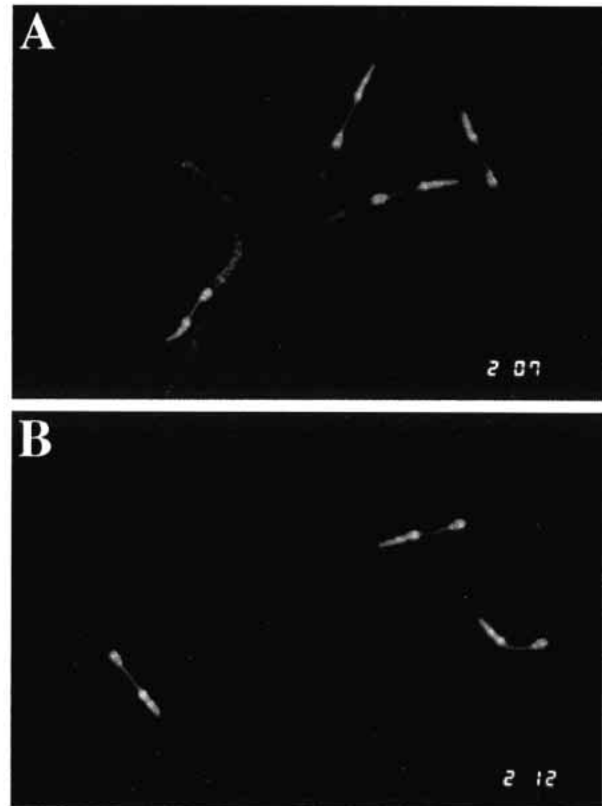

Fig. 5. Wild-type and *daf-3* dauers express *C183::gfp* similarly. (A) Fluorescence photomicrographs of *cuIs2* dauers. (B) *cuIs2; daf-3(mg90)* dauers. *cuIs2* and *cuIs2; daf-3(mg90)* animals were induced to produce dauers by hatching on plates with dauer pheromone (Golden and Riddle, 1982), and incubating at 27°C for 4 days (M. Ailion and J. H. Thomas, personal communication).

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* 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 TGFβ-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/TGFβ 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/TGFβ 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 regulate *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.

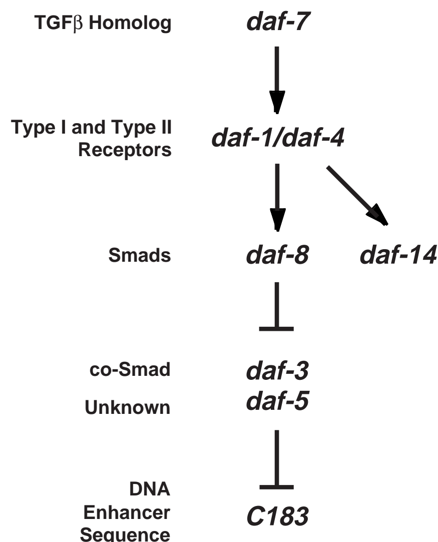

Fig. 6. Model for control of pharyngeal-specific *C183* enhancer activity by TGFβ-like signaling. *daf-3* and *daf-5* repress *C183* activity. This inhibitory activity is antagonized by the TGFβ-like pathway controlling dauer formation. The TGFβ-like signal acts through *daf-8*, but *daf-14* has little or no affect on *C183*.

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* TGFβ-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 TGFβ-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 TGFβ-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/TGFβ signaling and repression of *C183* enhancer activity by DAF-3

DAF-3 functions antagonistically to the Daf-c/TGFβ 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 *daf-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 *daf-3*-dependent repression of *C183* enhancer activity. However, we observed no reduction in *C183* activity in a *daf-3* single mutant, demonstrating that wild-type *daf-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.

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