weighted back-projection was used to calculate the three-dimensional density map. The alignment was carried out with four projection views obtained by eigenvector-eigenvalue classification and averaging (see also Fig. 3B). Although these projections are not related by symmetry, further projections were incorporated by imposing 32-point group symmetry. A reconstruction imposing threefold symmetry only was virtually identical to the reconstruction using the full 32-point group symmetry, which indicates that the preparation does not suffer from significant preparation-induced distortions. For the isosurface representation, a threshold value was chosen that relates the volume of the model to a molecular weight of 730-kD if a density of 1.3 g cm^{-3} is assumed.
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18. We thank R. M. Glaeser (University of California, Berkeley), A. Lupas, and M. A. Kania for critically reading the manuscript; M. Boicu for DNA sequencing; and R. Mattes (University of Stuttgart) for providing the *dnaY* gene. T.T. acknowledges a Postdoctoral Fellowship for Research Abroad from the Japan Society for the Promotion of Science. This work was also supported by a grant from the Human Frontier Science Program to W.B.

14 August 1996; accepted 18 October 1996

Control of *C. elegans* Larval Development by Neuronal Expression of a TGF-β Homolog

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The *Caenorhabditis elegans* dauer larva is specialized for dispersal without growth and is formed under conditions of overcrowding and limited food. The *daf-7* gene, required for transducing environmental cues that support continuous development with plentiful food, encodes a transforming growth factor–β (TGF-β) superfamily member. A *daf-7* reporter construct is expressed in the ASI chemosensory neurons. Dauer-inducing pheromone inhibits *daf-7* expression and promotes dauer formation, whereas food reactivates *daf-7* expression and promotes recovery from the dauer state. When the food/pheromone ratio is high, the level of *daf-7* mRNA peaks during the L1 larval stage, when commitment to non-dauer development is made.

The soil nematode *C. elegans* develops to adulthood through four larval stages (L1 through L4) with abundant food, but as population density increases and bacterial food supply diminishes, the nonfeeding dauer larva may be formed at the second molt (*1*). Entry into, and exit from, the dauer stage are influenced by temperature, food supply, and a *Caenorhabditis*-specific dauer-inducing pheromone, the concentration of which reflects population density (*2*, *3*). Mutations in *daf-7* result in constitutive dauer larva formation even with abundant food (*4*). Killing amphid chemosensory neurons ASI, ADF, ASG, and ASJ with a laser microbeam results in a dauer-constitutive phenocopy at 20°C (*5*). Molecular evidence reported here suggests that DAF-7 acts as a negative regulator of dauer larva development by transducing chemosensory information from ASI neurons.

Using Tc1 transposon tagging (*6*) and DNA transformation rescue (*7*), we cloned the *daf-7* gene (*8*) (Fig. 1A). The predicted gene product contains the conserved characteristics of the TGF-β superfamily (*9*) (Fig. 1B). Within the ligand domain, *daf-7* contains the seven cysteines nearly invariant in the superfamily, and it has two additional cysteines previously found only in the vertebrate homologs TGF-β and activin. Comparisons of the ligand domain show that DAF-7 has 34% amino acid sequence identity with human bone morphogenetic protein–4 (BMP-4), 34% with *Drosophila decapentaplegic* (*dpp*) protein, and 28% with human TGF-β, but lacks several amino acids invariant in these subfamilies. Hence, it is a new subtype of the TGF-β superfamily.

We sequenced *daf-7* mutant alleles (*10*) (Fig. 1B) and measured the severity of the temperature-sensitive (ts) dauer-constitutive (Daf-c) mutant phenotypes (*4*) (Table 1). The Tc1 transposon insertion strain (*m434::Tc1*) has residual *daf-7* activity, possibly due to removal of Tc1 during mRNA processing (*11*). Among six ethylmethane sulfonate–induced mutations, *m62* and *m88* are nonsense mutations suppressible by the *sup-7* amber suppressor (*12*), and *m83* generates a UGA codon in the pro-domain. Although these three mutant strains should produce truncated DAF-7 lacking most of the ligand domain and presumably are null mutants, they still exhibit a ts Daf-c phenotype

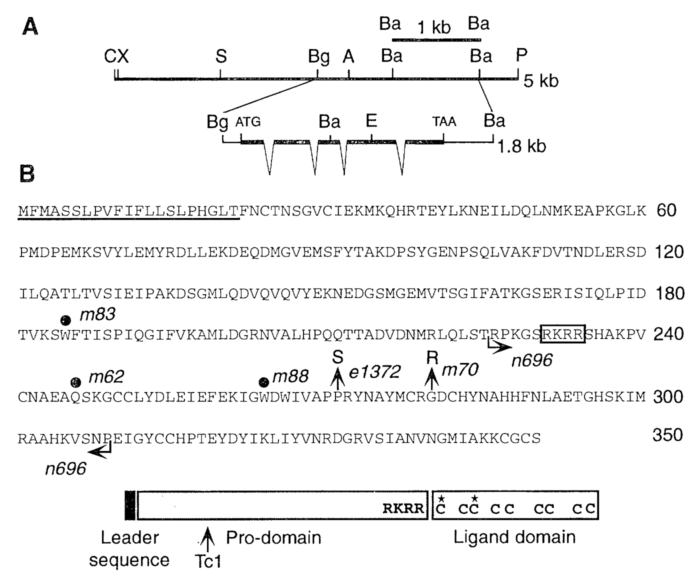

Fig. 1. *daf-7* encodes a TGF-β superfamily member. (**A**) Restriction map of a 5-kb genomic fragment that efficiently rescues *daf-7* mutants. Shown below is the intron-exon structure. C, Cla I; X, Xba I; S, Sal I; Bg, Bgl II; A, Acc I; Ba, Bam HI; E, Eco RI; P, Pst I. (**B**) Amino acid sequence of precursor protein beginning with the first predicted methionine, with leader sequence (underlined) and proteolytic cleavage site, RKRR (boxed). Mutation sites are indicated by arrows or dots. Shown below is a schematic of the *daf-7* protein indicating conserved TGF-β superfamily features and the point of Tc1 insertion. Two of the conserved cysteines (asterisks) in the ligand domain are found only in TGF-β and activin. GenBank accession numbers: U72883 for cDNA and U72884 for genomic DNA. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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reflecting the temperature sensitivity of wild-type dauer formation (*12*). The *n696* mutation results in an in-frame, 84–amino acid deletion that removes the proteolytic cleavage site and five of the nine cysteines. This deletion results in the most severe ts Daf-c phenotype, perhaps because the protein is toxic. Alternatively, if there is any translational read-through of nonsense codons, *n696* may be the only null allele. Crystallographic analyses of TGF-β2 and BMP-7 (*13*) revealed a conserved structure among TGF-β superfamily members, in which six conserved cysteines associate in a rigid "cysteine knot" that locks the base of β-sheet strands together. Mutation Gly^{280}→Arg (*m70*), which is in the cysteine knot and invariant in the superfamily, and mutation Pro^{271}→Ser (*e1372*), which is in the β sheet, could result in loss of function by causing conformational changes. On the basis of mutant phenotypes (Table 1), we conclude that DAF-7 function is required for non-dauer development at higher temperatures.

Expression of *daf-7* during development was analyzed by Northern (RNA) blot (Fig. 2). A rare 1.2-kb *daf-7* transcript was present in L1 larvae, present to a lesser extent in L2 larvae, and marginally detectable in L3 larvae and in pheromone-induced pre-dauer L2d larvae. Because commitment to non-dauer development is made during the L1 stage (*3*, *4*), when *daf-7* mRNA is most abundant, we postulate that *daf-7* functions as a signal to promote continuous development to adulthood.

The cellular specificity of *daf-7* expression was analyzed in transgenic animals that express the green fluorescent protein reporter gene (*gfp*) under control of the *daf-7* promoter (*daf-7p::gfp*) (*14*). In the presence of ample food, both males and hermaphrodites expressed GFP in the ASI neuron pair, the sensory processes of which are exposed to the environment (*15*) (Fig. 3E). Expression was detected in larvae beginning 4 to 5 hours after hatching, through the four larval stages, and in adults (Fig. 3A). The absence of detectable *daf-7* mRNA in later stages (Fig. 2) could be due to very low expression levels or greater mRNA instability. Neither GFP expression nor *daf-7* mRNA was detected in embryos, suggesting that *daf-7* does not play a role in sensory neuron development.

In starvation-induced dauer larvae, GFP expression became undetectable. L1 larvae hatched in M9 buffer (*16*) did not develop without food, but they expressed GFP, al-

Table 1. *daf-7* mutations and ts Daf-c phenotypes. Mutants were maintained at 15°C on NG agar plates with *Escherichia coli* OP50 (*16*). Means for Daf-c phenotypes are each based on scoring 500 to 1000 animals. L4 larvae were transferred daily until egg laying ceased. Progeny were counted and scored as dauer or non-dauer (L4 to adult) 5 days (15°C), 4 days (20°C), or 3 days (25°C) after removal of the parent. Brood sizes were reduced relative to wild type at all three temperatures.

Mutant allele	Aa*	Codon†	Amino acid change	Percent constitutive dauer formation‡ (±SE)		
				15°C	20°C	25°C
m434::Tc1		Tc1 insertion in pro-domain		1 ± 1	9 ± 6	89 ± 3
m83	185	TG**G**→TG**A**	W→stop	7 ± 1	16 ± 4	100
m62	246	**C**AG→**T**AG	Q→stop	6 ± 2	18 ± 2	100
m88	264	T**G**G→T**A**G	W→stop	12 ± 2	12 ± 3	100
e1372	271	CC**A**→CC**G**	P→S	5 ± 12	5 ± 6	100
m70	280	**G**GG→**A**GG	G→R	15 ± 5	33 ± 6	100
n696	226–309	In-frame internal deletion		13 ± 4	62 ± 6	100

*Position of amino acid change as shown in Fig. 1B. †Nucleotides changed are given in bold. ‡*daf-7* mutants are also defective in dauer recovery. After *daf-7(n696)* dauer larvae (formed constitutively at 25°C) were transferred to fresh food, only 22 ± 1% recovered after 48 hours at 25°C, whereas 81 ± 10% and 85 ± 3% recovered at 20°C and 15°C, respectively.

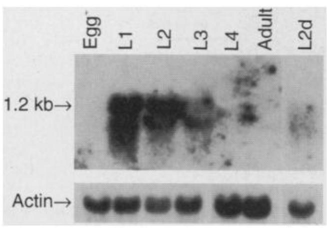

Fig. 2. Developmental Northern (RNA) analysis of *daf-7* gene expression. RNA was isolated from well-fed synchronous populations (*16*) of wild-type eggs, L1s, L2s, L3s, L4s, adults, and pheromone-induced L2ds at 20°C (*3*). Approximately 20 μg of $poly(A)^+$ RNA from each stage was fractioned on a 1% agarose–6% formaldehyde gel. A nitrocellulose blot was probed with the full-length *daf-7* cDNA under high-stringency conditions (6× saline sodium phosphate EDTA, 1% SDS, 10 μg of tRNA per milliliter at 65°C) and washed at high stringency [once with 2× saline sodium citrate (SSC), 0.5% SDS for 15 min at room temperature, then twice with 0.1× SSC, 0.5% SDS for 15 min at 65°C]. The blot was exposed with an intensifying screen at −80°C for 7 days. An autoradiogram of the blot probed with an actin cDNA under the same conditions was exposed for 3 hours to indicate loading in each lane.

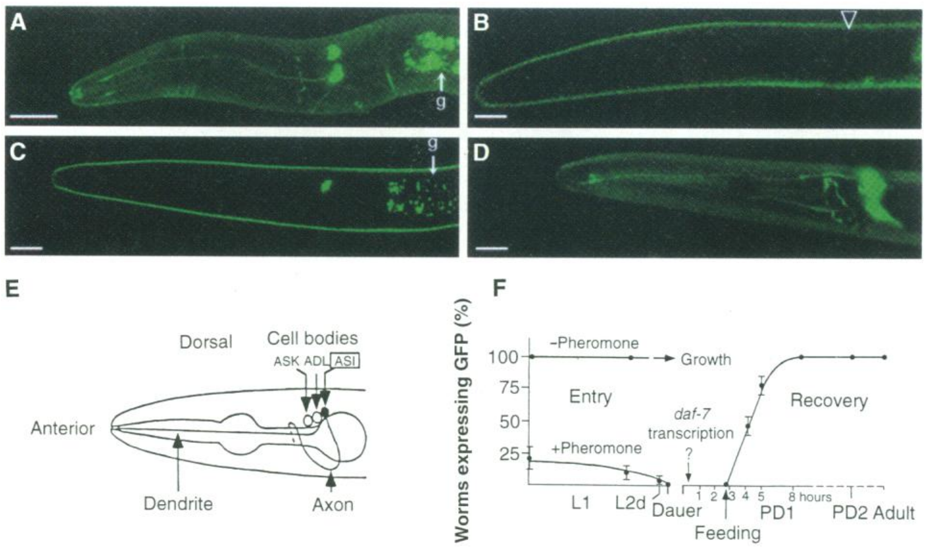

Fig. 3. *daf-7p::gfp* expression in ASI neurons. (**A** to **C**) Wild-type background. (A) L1 larva; (B) dauer larva induced by pheromone; (C) recovering dauer larva about 8 hours after transfer to food at 25°C. (**D**) *daf-7(n696)* mutant background. Daf-c dauer larva, 20°C. g indicates gut autofluorescence. Arrowhead in (B) indicates the expected position of ASI cell bodies. Bars, 10 μm. (A) and (D) are three-dimensional confocal images. Faint fluorescence is apparent in the ASI axons and dendrites. (**E**) Diagrammed positions of the pharynx and cell bodies of ASI and neighboring neurons used for reference (one side shown). (**F**) GFP expression during dauer entry and recovery at 25°C. Preparation of crude pheromone extract and induction of dauer formation were as described (*3*). An average of 70% dauer larvae were induced with 100 μl of pheromone extract. A culture without pheromone was used as a control. Pheromone-induced dauer larvae were transferred to fresh food for recovery. PD1 and PD2 are the post-dauer equivalent of L3 and L4, respectively. Data are means ± SD for three replicates. About 20 animals were examined for each replicate.

beit at a lower level than with abundant food. Animals hatched in M9 buffer with pheromone (*17*) did not express GFP. These data suggest that pheromone regulates *daf-7* expression during dauer entry. During pheromone-induced dauer formation at 25°C (Fig. 3, B and F), GFP expression was suppressed in L1 larvae, both in the percentage of animals expressing GFP and in the intensity of fluorescence. Faint GFP fluorescence became undetectable as animals entered the dauer stage. In the absence of pheromone, animals expressed GFP from the L1 stage through adulthood.

To test whether *daf-7p::gfp* expression resumes during exit from the dauer stage, we transferred pheromone-induced dauer larvae to fresh food without pheromone at 25°C. Wild-type dauer larvae become committed to recovery within 1 hour of transfer, and resume feeding within 3 hours (*3*). By 4 hours, weak GFP expression was detected in about 50% of the recovering animals; after 8 hours, all animals expressed GFP at higher levels (Fig. 3, C and F). Because formation of the GFP fluorophore requires about 4 hours at 22°C (*18*), we infer that *daf-7* transcription must be initiated very early in dauer recovery. When transferred to M9 buffer (lacking both pheromone and food) at 25°C, dauer larvae did not recover or express GFP even after 2 days, indicating that food is required for resumption of *daf-7* expression and for resumption of development under these conditions.

Under growth-favoring conditions, expression of the *daf-7p::gfp* transgene in *daf-7(n696)* (*19*) is the same as in wild-type animals, suggesting that *daf-7* transcription is not dependent on its functional product. At 20°C, GFP expression became undetectable in starvation-induced dauer larvae, but GFP was expressed in constitutive dauer larvae formed with abundant food (Fig. 3D). Hence, *daf-7* expression is not down-regulated by dauer formation; mutant animals expressed GFP even though they formed dauer larvae in the absence of *daf-7(+)* activity. Thus, *daf-7* expression is regulated by pheromone and food stimuli, and DAF-7 is a signaling molecule required for transducing environmental cues that inhibit dauer formation or promote growth (or both). The pheromone is, in fact, required for dauer formation; a mutant that does not produce pheromone does not form dauer larvae (*20*). GFP expression results suggest that ASI neurons express *daf-7* soon after hatching to signal non-dauer development in the presence of low amounts of pheromone, then again use *daf-7* to signal recovery from the dauer state in response to fresh food at higher growth temperatures.

At 20°C, laser microsurgery indicated that neurons ASI, ADF, and ASG were apparently redundant (*5*). The temperature itself may account for this difference, or the surgery may not have killed ASI cells soon enough to prevent *daf-7* expression, so that additional neurons (perhaps DAF-7 target cells) had to be disrupted to cause dauer formation. Alternatively, unknown transcriptional or translational regulatory elements for expression of GFP in other cells may be absent from the reporter construct.

Other mutations that result in a ts Daf-c phenotype are positioned together with *daf-7* in the genetic pathway for dauer formation (*21*). Of these, *daf-1* and *daf-4* encode receptor serine-threonine kinases of the TGF-β superfamily, and *daf-8* encodes a Mad (Mothers against *dpp*) homolog that may function as a downstream target of these receptors (*22*, *23*). When expressed in COS cells, the *daf-4* receptor binds human BMP-2 and BMP-4 (*23*). Therefore, the *daf-7* protein is a candidate ligand for *daf-4* or *daf-1* receptors, or both. In favorable growth conditions, the *daf-7* ligand may be secreted from ASI neurons to bind *daf-1* or *daf-4* receptors to activate DAF-8, resulting in production of an endogenous growth-promoting or dauer-inhibiting signal. As precedent for this type of neural function, activin has been implicated as a neurotransmitter or neuromodulator in central neural pathways in rats for the release of oxytocin in response to suckling (*24*).

Many parasitic nematodes form infective larvae, analogous to the dauer stage, that are adapted to seek a new host (*25*). If *daf-7*–related proteins play a role in processing sensory cues for entry into or exit from these states of diapause, their analysis would be potentially valuable to design agents for specific control of parasitic nematode dispersal.

Note added in proof: Expression of *daf-7::gfp* in ASI neurons and its regulation by pheromone also have been observed by Schackwitz *et al.* (*26*).

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27. We thank C. Bargmann and J. White (neuron identification and discussion), M. Chalfie (*gfp* vector), J. Kramer (pRF4 plasmid), R. Barstead (cDNA library), H. R. Horvitz [*daf-7(n696)*], P. Hoppe and M. Estevez (help with microinjection), M. Estevez (preparing the Fig. 2 blot), M. Stanley and E. Norton (processing Fig. 3, A to D), V. Spate (γ source), D. Gems and C. Gunther (helpful discussions), and L. Hatley (manuscript processing). Supported by U.S. Department of Health and Human Services grant HD11239 to D.L.R.

23 August 1996; accepted 3 October 1996