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A BMP homolog acts as a dose-dependent regulator of body size and male tail patterning in *Caenorhabditis elegans*

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SUMMARY

We cloned the *dbl-1* gene, a *C. elegans* homolog of *Drosophila decapentaplegic* and vertebrate *BMP* genes. Loss-of-function mutations in *dbl-1* cause markedly reduced body size and defective male copulatory structures. Conversely, *dbl-1* overexpression causes markedly increased body size and partly complementary male tail phenotypes, indicating that DBL-1 acts as a dose-dependent regulator of these processes. Evidence from genetic interactions indicates that these effects are mediated by a Smad signaling pathway, for which DBL-1 is a previously unidentified ligand. Our study of the *dbl-1* expression pattern suggests a role for neuronal cells in global size regulation as well as male tail patterning.

Key words: *Caenorhabditis elegans*, *decapentaplegic*, Bone morphogenetic protein, Body size determination, Male tail

INTRODUCTION

Regulation of groups of cells by secreted growth factors is a fundamental process in development. Bone morphogenetic proteins (BMPs) of the transforming growth factor-β (TGF-β) superfamily play major roles in this process (reviewed in Hogan, 1996). The diverse functions of BMP family members include specification of ventral fates in *Xenopus* embryos by BMP4, except where it is blocked by specific antagonists such as chordin that allow dorsal development (see Piccolo et al., 1996; Zimmerman et al., 1996), and specification of dorsal fates in *Drosophila* by Decapentaplegic (Dpp) in both the embryo (Irish and Gelbart, 1987) and leg imaginal discs (Held et al., 1994). The active forms of BMP molecules are dimers of polypeptides that are the processed C-terminal 'mature' domains of larger precursor proteins (reviewed in Hogan, 1996). BMP ligands promote the formation of a heteromeric complex of type II and type I serine/threonine protein kinase receptors, in which type I receptor is activated by phosphorylation by type II receptor. The activated type I receptor phosphorylates and activates Smad signal transducers that regulate transcription in the nucleus (reviewed in Heldin et al., 1997).

In *C. elegans*, mutations that result in small body size (Brenner, 1974; the Sma phenotype) and abnormal male tail structures (the Mab phenotype) have identified components of a putative TGF-β signal transduction pathway termed the Sma/Mab pathway (reviewed in Padgett et al., 1998). The *daf-4* and *sma-6* genes encode type II and type I receptors, respectively (Estevez et al., 1993; Krishna and Padgett, 1999). The *sma-2*, *sma-3* and *sma-4* genes encode Smad proteins (Savage et al., 1996). Since DAF-4 expressed in COS cells is capable of binding to human BMP2 and BMP4 (Estevez et al., 1993), it had been postulated that one or more *C. elegans* BMP-like ligands might function through this receptor to control body size and male tail patterning.

Two previously identified TGF-β family members in *C. elegans* did not appear to be candidates for this function. DAF-7, distantly related to the BMPs, inhibits entry into and promotes recovery from the dauer state, an alternative third larval (L3) diapause stage adopted by animals developing under conditions of starvation or overcrowding, or both (Ren et al., 1996). A second TGF-β family member, UNC-129, directs axon guidance (Colavita et al., 1998).

Here we show that a *C. elegans* BMP homolog encoded by the *dbl-1* gene (*dpp*, *BMP*-like) acts as a dose-dependent regulator of overall body size and male tail patterning, through the Sma/Mab pathway. The *dbl-1* expression pattern suggests that neurons play major roles in these processes.

MATERIALS AND METHODS

Cloning and molecular characterization of *dbl-1*

Degenerate PCR with primers V1 (Wharton et al., 1991) and WC [5′ GGA(CT)TCGG(CT)(ACT)GG(ACGT)(AG)C(AG)CA(AG)CA(AC-

GT)G 3′] corresponding to the conserved BMP-family amino acid sequence NH_2-CC(VA)P(TA)ES-COOH, amplified a 400 bp fragment from *C. elegans* genomic DNA. This amplification was performed in 50 μl reaction mixtures containing 1 μg of template DNA, 1.07 μg each of the primers, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 10 mM Tris-HCl, pH 8.3, 50 mM KCl and 0.5 U AmpliTaq DNA polymerase (Roche Molecular Systems) with 40 PCR cycles of 95°C (1 minute), 50°C (1 minute), 72°C (2 minutes). Hybridization of this fragment to an ordered grid of *C. elegans* YAC clones (Coulson et al., 1991) and subsequent PCR-based assay of individual overlapping cosmids (kindly provided by A. Coulson) in the region placed the sequence on the *C. elegans* physical map in the middle of cosmid ZC421 from a region near the center of linkage group (*LG*) *V*. Using the same PCR product as a probe, cDNA clones were identified from a *C. elegans* mixed-stage cDNA library (Barstead and Waterston, 1989). Sequencing of genomic and cDNA clones revealed eight exons (Fig. 1A) as well as a *C. elegans* splice acceptor consensus sequence, TTTTCAG (Blumenthal and Steward, 1997), upstream of the longest cDNA clone (GenBank accession number AF004395). Further PCR experiments showed that the SL1 splice leader is trans-spliced to this site and also identified a poly(A) sequence 868 bp 3′ to the termination codon (Fig. 1A).

BMP sequences were aligned with Pileup (GCG). Distance trees were prepared by PHYLIP (Felsenstein, 1986). Distance matrices were jumbled 100 times and the resulting trees were globally rearranged. Bootstraps (100) were performed using SEQBOOT and CONSENSE (Felsenstein, 1986).

C. elegans strains

All strains were cultured using standard methods (Brenner, 1974). The following mutations (all in the N2 wild-type background) were used: *unc-119(ed3)*, *fem-2(b245)*, *daf-4(m72)*, *sma-2(e502)*, *sma-3(e491)*, *sma-4(e729)*, *dpy-18(e364)*, *him-8(e1489)*, *unc-46(e177)*, *dpy-11(e224)*, *unc-42(e270)*, *him-5(e1490)*, *sDf20* and *eT1* (see Riddle et al., 1997) as well as *sma-6(wk7)* (Krishna and Padgett, 1999).

Isolation of *dbl-1* mutations

The *dbl-1(ev580)* mutation was identified as a PCR-amplified band from a pooled DNA sample of a Tc1 transposon insertion library (P. J. R. and J. Culotti, unpublished), using nested PCR with a pair of *dbl-1* 3′ UTR primers and a pair of Tc1 'right' primers (Plasterk, 1995). DNA samples used to make this pool were subsequently screened as above to find a DNA sample that contained DNA of *ev580*. The frozen worm stock corresponding to the positive DNA sample was thawed, and the worms were picked to individual plates and allowed to produce progeny. Subsequently, the parent worms were individually lysed and used for PCR analysis to identify an *ev580* line. Details of this procedure are available upon request. The *ev580* strain was extensively backcrossed prior to the phenotypic analysis described in Results.

The *dbl-1(wk70)* allele was found by complementation tests in which males heterozygous for unidentified *sma* mutations mapped to *LGV* (C. S.-D. and R. W. P., unpublished) were crossed with both *unc-46 dbl-1(ev580)* and *dbl-1(ev580)* homozygotes, and non-Unc Sma animals and Sma males, respectively, were sought among the progeny.

Genetic characterization of *dbl-1* alleles

24% of the non-Dpy cross progeny (n=495) from *ev580 unc-42*/+ + males and *unc-46 sDf20*/*dpy-11* (Dpy) hermaphrodites were Sma. A comparable experiment with *wk70* (n=924) also yielded 24% Sma cross progeny. The Sma phenotypes of *dbl-1* hemizygotes and homozygotes were indistinguishable, and no new phenotypes were apparent. The frequency of embryonic or larval lethality of the *dbl-1* hemizygotes was <0.5% for *ev580* (n=498) and <1.5% for *wk70* (n=938) in the population in which most animals were cross progeny. The lethality was 1% for *ev580* homozygotes (n=97) and 2% for *wk70* homozygotes (n=171).

ev580/+ and *wk70*/+ hermaphrodites produced Sma progeny at frequencies of 26% (n=1911) and 24% (n=1302), respectively. *ev580*/+, *wk70*/+ and *sDf20*/+ animals are slightly Sma, indicating that body size is sensitive to the dosage of *dbl-1*. Neither *ev580*/+ (n=32) nor *wk70*/+ (n=65) males had tail defects.

Plasmids and germ-line transformation

An 11 kb *Pst*I fragment containing *dbl-1* from cosmid ZC421 was cloned into the *Pst*I site of pSP72N2B (Powell-Coffman et al., 1994) to generate pYS1. pYS1 was digested with *Mlu*I, and the ends were blunted and ligated to generate pYS7, which was sequenced and confirmed to change the frame of the predicted translation product. In rescue experiments, ZC421, pYS1 or pYS7 was injected at 40 ng/μl as described (Mello et al., 1991) with pDP#MM016B, an *unc-119*-rescuing plasmid (Maduro and Pilgrim, 1995) or pTG96, a *sur-5::gfp* reporter plasmid (Gu et al., 1998). To create overexpression lines, these clones were injected at 100 or 200 ng/μl.

A plasmid containing a 5.3 kb *Xho*I fragment from ZC421 was cleaved with *Sac*I and *Alw*NI to generate a 4.5 kb fragment containing only the 5′ flanking sequence of the *dbl-1* gene; this fragment was blunted and subcloned into the *Msc*I site of the GFP vector TU#63 (Chalfie et al., 1994), which carries a nuclear localization signal, to generate pMY+nls. This signal was removed by *Kpn*I digestion to generate pMY-nls used in identification of cells by their morphologies.

Arrays were integrated into chromosomes using γ-irradiation, followed by at least two backcrosses with N2 or *unc-119; him-5*. Phenotypes or *dbl-1::gfp* expression patterns of corresponding non-integrated and integrated lines were indistinguishable. Arrays used were: *ctEx17* (ZC421, pTG96), *ctIs40 X* (ZC421, pTG96), *ctIs43 V* (pDP#MM016B, pMY+nls, pMY-nls).

Epistasis test

Sma self progeny from *daf/+;ctEx17* or *sma*/+; *ctEx17* animals (GFP-positive, Lon) were examined for presence of the GFP-expressing, *dbl-1*-overexpressing array *ctEx17*. This array is transmitted to about 60% of progeny at each generation; hence 60% of the Sma progeny are expected to carry the array if the *sma* mutation is epistatic to *dbl-1* overexpression, and 0% if it is not. To show that *ctEx17* in the GFP-positive Sma animals was functional, wild-type males were crossed to some of these animals, which produced Lon cross progeny. This array was subsequently integrated to produce *ctIs40*, which was shown to overexpress *dbl-1* (Fig. 2B). A *sma-6(lf)* mutation was also epistatic to *dbl-1* overexpression from *ctIs40* (data not shown).

Examination of the male tail phenotypes

For consistency in evaluating the male tail phenotypes to generate Table 1, all worms were observed from the ventral side. Adult male worms were washed in M9 solution (Brenner, 1974) on a depression slide and transferred, using a mouth pipette, to another well containing 10 mM sodium azide in M9. Each of the anesthetized worms was transferred to a 5% agar pad with roughly 1 μl of the sodium azide solution. Excess liquid was set aside with a pick, and the worm, normally resting on its side, was flipped with a pick to ventral side up and immediately covered with a 9×9 mm coverslip. The tail was observed under Nomarski optics at a magnification of 1000×.

Ray neuron openings were scored as dots at the ray tips (Sulston et al., 1980). Fusions of rays 5 to 4 and rays 9 to 8 were identified as rays near the normal positions of rays 4 and 8, respectively, with two openings on the ventral side of the fan. When two openings were not observed, the phenotype was scored as missing ray 5 or missing ray 9. The two openings of ray 9 fused to ray 8 were difficult to resolve, which may have resulted in a substantial portion of rays 9 to 8 fusions being scored as absence of ray 9. Fusions of rays 7 to 6 were identified as extra-thick rays near the normal position of ray 6. Fusions of rays 4 to 3 and rays 4 to 5 in *dbl-1* overexpression backgrounds were identified similarly.

Lineage analysis was performed on two *ev580* males as described by Sulston and Horvitz (1977). Worms were remounted when they flipped during molts, and only one side of the male tail was followed in a given worm, starting from the L2 stage until the non-epidermal ray group cells migrated internally during tail morphogenesis in L4. The analyzed sides of these *ev580* homozygotes produced fusions of rays 7 to 6 and rays 9 to 8 for worm 1, and transformation (non-fusion) of rays 7 to 6 and lack of ray 9 morphogenesis for worm 2. Absence of rays appeared to result from failure of morphogenesis of ray structural cells.

RNA blot analyses

Well-fed animals were harvested to prepare mixed population RNA samples. To obtain semi-synchronized animals, well-fed animals were floated in 35% (w/v) sucrose, washed twice with M9, and filtered through a 52 μm Nitex screen to collect enriched gravid hermaphrodites trapped on the filter. These animals were treated with 0.8-1.2% NaOCl (Fisher), 0.5 M NaOH solution for roughly 7 minutes and washed three times with M9, to be able to collect a large number of semi-synchronized eggs. The eggs were hatched with food and harvested when they reached desired stages. RNA was prepared as described (Meyer and Casson, 1986). RNA blot analysis was performed as described (Schauer and Wood, 1990) except that Hybond-N filter was used (Amersham Life Science). The 1.7 kb insert of a *dbl-1* cDNA clone (pMYcDNA) was cut out with *Eco*RI and used as a *dbl-1* probe. The pCe7 plasmid linearized with *Hin*dIII was used as a ribosomal RNA probe (Files and Hirsh, 1981). The radioactive signals were analyzed using a Storm 860 phosphorimager and ImageQuant NT Ver. 2.0 software (Molecular Dynamics).

RESULTS

dbl-1 encodes a BMP homolog

We used degenerate PCR to clone a *C. elegans* member of the BMP family (see Materials and methods). Its predicted translation product (DBL-1) exhibits roughly 50% identity in the mature domain, including the seven conserved cysteines, to other BMP members (Fig. 1A,B). A UPGMA analysis of 45 mature-domain sequences from the TGF-β superfamily (data not shown), as well as a bootstrapped Fitch-Margoliash analysis (Fitch and Margoliash, 1987) on a smaller set of BMP mature domain sequences (Fig. 1C), indicates that DBL-1 belongs to the Dpp paralog class.

dbl-1 loss-of-function mutations result in reduced body size

For functional analysis, we obtained a mutation (*ev580*) in *dbl-1* by Tc1-transposon-mediated gene disruption (Plasterk, 1995; see Materials and methods), with a Tc1 insertion that introduces multiple termination codons near the beginning of the *dbl-1* mature domain (Fig. 1A,B). RNA blot analysis showed that Tc1 is present in the *dbl-1* mRNA from *ev580* mutant animals (Fig. 2A). Complementation tests with *ev580* against a set of newly isolated *sma* mutations (see below; C. S.-D. and R. W. P., unpublished) identified a second *dbl-1* allele, *wk70*, which changes Q^{314} in the mature domain to a termination codon (Fig. 1A,B). Therefore, both alleles are expected to act as severe loss-of-function (*lf*) mutations.

Both *dbl-1* mutations cause identical, fully-penetrant Small (Sma) phenotypes in hermaphrodites (Fig. 3A) as well as males (not shown). Since newly hatched (L1) wild-type and *dbl-1* mutant larvae are the same size (230±10 μm), this phenotype is probably due to abnormal post-embryonic growth. *dbl-1* is partially haploinsufficient for this function (see Materials and methods). Rates of embryonic and larval lethality in both *ev580* and *wk70* homozygous as well as hemizygous mutant strains were not significantly higher than the background rate seen in wild type (see Materials and methods).

dbl-1(lf) mutations cause defects in male copulatory structures

The *C. elegans* male tail includes a set of complex copulatory structures. Nine pairs of sensory rays are embedded in the cuticular fan (Fig. 3B). Each is derived from a precursor (Rn cell), which gives rise to one epidermal and four non-epidermal descendants including two neurons, one structural cell and one cell that undergoes programmed cell death (Sulston and Horvitz, 1977; Sulston et al., 1980; Zhang and Emmons, 1995). The wild-type rays are numbered 1-9 from the anterior. They are distinguishable by their thickness and the positions of their openings on the fan (with the exception of ray 6; see below). For example, ray 6 is thick, while all others are thin. Rays 1, 5 and 7 extend and open to the dorsal side; rays 2, 4 and 8 extend and open ventrally; and rays 3 and 9 extend medially and open to the margin of the fan (Table 4). Ray 6 extends medially and either does not open or has a narrower channel to the exterior (Chow et al., 1995). Also present are a pair of spicules, blade-like structures housed in the proctodeum, which are used in copulation (Sulston et al., 1980; Fig. 3B).

In males, both *ev580* and *wk70* mutations cause defects in rays and spicules (Fig. 3C). Using dorsal-ventral positions of extensions and openings of rays (and thickness in the case of ray 6) to assess their identities, the 5th, 7th and 9th rays in *dbl-1* mutants were found to resemble their neighboring rays 4, 6 and 8, respectively, with the frequencies indicated in Table 1. Some of these changes were accompanied by fusions of the neighbor ray pairs (frequencies shown in parentheses in Table 1), possibly because structural cells of these rays actually contacted and fused as described for ray fusions of a different type (Chow et al., 1995). These mutations also disrupted the morphology of spicules (Fig. 3C, Table 1). The tail defects, in contrast to the Sma phenotype, were fully recessive (see Materials and methods).

The cell lineages that generate rays in male tail development from the precursors V5.p, V6 and T.a were followed from late L2 to the L4 stage prior to ray morphogenesis in two *ev580* homozygous males. Despite the subsequent abnormalities described above, these lineages all produced the correct numbers of cells from the appropriate sublineages (see Materials and methods). Therefore, the *dbl-1* ray phenotypes are not caused by cell lineage defects, but rather appear to result from transformations of ray identities.

Sma and male tail phenotypes are consequences of severe loss of *dbl-1* activity

The Sma and male tail phenotypes of the *dbl-1* mutants were rescued by plasmid pYS1 (Fig. 3D), indicating that they result from the loss of *dbl-1* activity. The two alleles *ev580* and *wk70* cause equivalent phenotypes, and these phenotypes are no more severe in hemizygous animals of genotype *ev580/sDf20* or *wk70/sDf20* than in animals homozygous for *ev580* or *wk70*, indicating that these *dbl-1* mutations are both severe

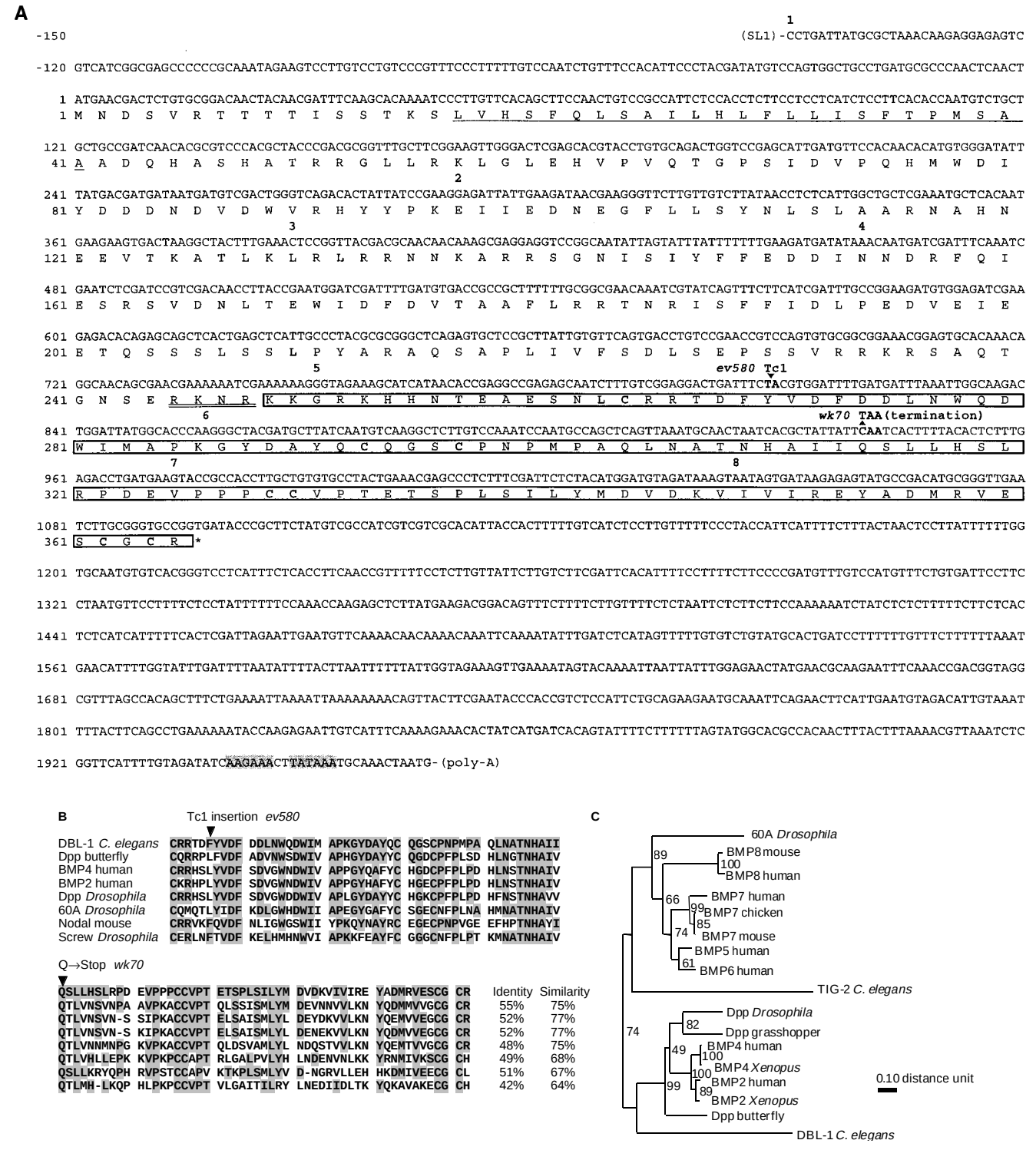

Fig. 1. Sequence of the *dbl-1* gene and comparisons with other BMP family members. (A) *dbl-1* cDNA sequence, including 5′ and 3′ untranslated regions, is shown with the amino acid sequence it is predicted to encode. The beginnings of exons (bold numbers), potential polyadenylation signals (Blumenthal and Steward, 1997; shaded), the predicted secretion signal (von Heijne, 1986; single-line underline), an RXXR cleavage site consensus (double line), the mature domain (boxed), and the *ev580* and *wk70* mutations (arrowheads) are indicated. (B) Comparison of predicted mature domain amino acid sequences beginning with the first conserved cysteine between DBL-1 and other BMP family members. Identical amino acids are highlighted; dashes indicate gaps. Residue 1 of the DBL-1 sequence shown corresponds to residue 264 of the sequence predicted from the *dbl-1* cDNA. The *ev580* mutation is a Tc1 insertion into a TA dinucleotide in the Y^{270} codon (TAC); *wk70* is a C→T substitution in the Q^{314} codon (CAA). (C) Bootstrapped distance tree based on Fitch-Margoliash analyses of 17 members of the BMP family from vertebrates, insects and *C. elegans*. A bootstrap value of >70 indicates significant relatedness (Hillis and Bull, 1993).

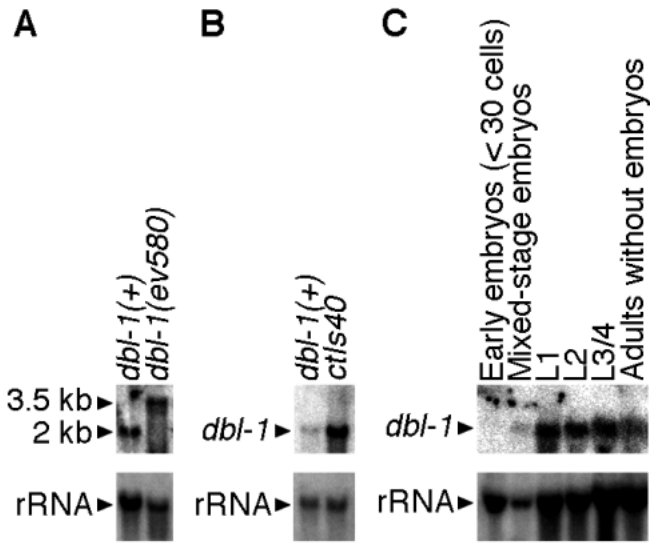

Fig. 2. RNA blot analyses. Filters were probed with a 1.7 kb *dbl-1* cDNA sequence. A control probe specific to ribosomal RNA was used to monitor RNA loading. (A) Blot of RNAs from mixed-stage populations of *him-8; dbl-1(+)* and *him-8; dbl-1(ev580)* animals. 3.5 kb and 2 kb bands are expected for *dbl-1* mRNA with and without the inserted Tc1 sequence, respectively. (B) Blot of RNAs from *him-8; dbl-1(+)* and *him-8; ctIs40* L1 larvae, showing *dbl-1* overexpression from the *ctIs40* array. Animals carrying the array contain 4.3-fold more *dbl-1* mRNA than the control animals, as measured by densitometry. (C) Developmental regulation of *dbl-1* message level. RNA samples of various stages were prepared from N2 except for the samples designated L1 (*him-8*) and adults without embryos (*fem-2*).

loss-of-function alleles (see Materials and methods). This conclusion is consistent with the molecular lesions in these alleles, both leading to premature termination in the mature domain of the protein, as described above. In further support of this view, a *dbl-1* deletion mutation that removes the entire *dbl-1* coding sequence results in phenotypes very similar to those we have reported (K. Morita and N. Ueno, personal communication).

DBL-1 may be a ligand for the Sma/Mab pathway

Sma and male tail phenotypes essentially identical to those described above also result from mutations in the *daf-4, sma-6, sma-2, sma-3* and *sma-4* genes, which encode receptors and Smad proteins of the Sma/Mab pathway thought to transduce TGF-β-like signals (reviewed in Padgett et al., 1998; Krishna and Padgett, 1999; Y. S. and W. B. W., unpublished), suggesting that *dbl-1* could encode its previously unidentified ligand. To test this possibility, we made doubly mutant animals carrying *dbl-1*(*ev580*) and each of the *daf-4* and *sma-6, -2, -3* and *-4* strong alleles. The Sma phenotypes of the double mutants were indistinguishable from those of single mutants (data not shown). The frequency of male tail defects in the double mutants was also not apparently higher than that in a *dbl-1* single mutant (Table 1). These results suggest that the role of the Sma pathway in body-size determination and male tail patterning is solely to transduce the DBL-1 signal.

Overexpression of *dbl-1* causes increased body size and male tail defects

To determine if *dbl-1* plays an instructive or permissive role, we generated transgenic animals carrying an integrated high-copy array of cosmid ZC421, which contains the *dbl-1* coding region and roughly 10 kb each of 5′ and 3′ flanking sequences (see Materials and methods). RNA blot analysis of one such line showed that the level of *dbl-1* message was 4.3-fold higher

Table 1. Male tail phenotypes resulting from *dbl-1* and Sma pathway mutations[a]

Strain	Ray 5[b,c] ventralized (ray 4-like)	Ray 7[b] ventralized (ray 6-like)	Ray 9[b] ventralized (ray 8-like)	Spicules crumpled	*n* (sides)
him-8	(0%)	0% (0%)	0% (0%)	8%	100
him-8;dbl-1(ev580)	(24%)	91% (51%)	92% (18%)	98%	100
him-8;dbl-1(wk70)	(29%)	94% (49%)	83% (15%)	100%	100
him-5[d]	(0%)	0% (0%)	0% (0%)	0%	50
dbl-1(ev580) him-5[d]	(32%)	82% (60%)	68% (18%)	100%	50
dbl-1(ev580)[e]	(38%)	100% (60%)	90% (34%)	100%	50
dbl-1(ev580)/sDf20[e]	(42%)	90% (80%)	64% (26%)	98%	50
dbl-1(wk70)/sDf20[e]	(34%)	84% (62%)	78% (34%)	100%	50
dbl-1(ev580)[f]	(17%)	100% (93%)	93% (27%)	97%	30
daf-4(m72);dbl-1(ev580)[f]	(10%)	97% (83%)	90% (23%)	100%	30
sma-6(wk7);dbl-1(ev580)[f]	(20%)	100% (97%)	77% (7%)	100%	30
sma-2(e502);dbl-1(ev580)[g]	(17%)	90% (80%)	67% (7%)	100%	30
sma-3(e491);dbl-1(ev580)[f]	(0%)	90% (80%)	83% (33%)	100%	30
sma-4(e729);dbl-1(ev580)[f]	(13%)	93% (83%)	90% (10%)	97%	30

[a]Percentages shown in parentheses are for ray transformations associated with ray fusions (see text).
[b]Occasionally, rays 5 (0-7%), 7 (0-3%), and 9 (7-36%) were absent in the mutants.
[c]Ray 5 was posteriorly displaced in the mutants to a position posterior to ray 6 at the frequency of 0-7%, which was accompanied by ventralization of its normal dorsal opening to a medial position.
[d]*him-5* strains were tested for comparison because in our *him-8* strain, rays 8 and 9 were frequently fused, although their openings retained normal positions.
[e]Strains were heterozygous for *unc-46*.
[f]Strains were homozygous for *unc-46* and *him-8*. Rates of embryonic or larval lethality were approximately constant for all genotypes shown (*dbl-1(ev580)*: 12%, *n*=194; double mutant genotypes: 8-14%, *n*>110 for each).
[g] Strain was *unc-46(+)* and homozygous for *him-8*.

than in control animals (Fig. 2B). The transgenic animals had a fully penetrant Long (Lon) phenotype (Brenner, 1974; see Fig. 4A,B).

The same Lon phenotype was observed in lines derived from animals injected with plasmid pYS1, which contained only the *dbl-1* coding region, 9 kb of 5′ and 0.5 kb of 3′ flanking sequences (see Fig. 3D). Comparable lines carrying pYS7, identical to pYS1 except for a frameshift in the first exon, were not Lon, indicating that this phenotype results from overproduction of DBL-1.

Moreover, this effect appears to be mediated by the Sma/Mab pathway. Epistasis tests showed that when a *dbl-1* overexpressing array was introduced into strains homozygous for mutations in any one of the above *sma* genes, all retained the same Sma phenotype (Table 2), indicating that these mutations block the effect of *dbl-1* overexpression.

dbl-1 overexpression also resulted in occasional dorsalization of ray 4, either without fusion (ventral to medial) or with fusion to the dorsal ray 5 (data not shown) or to the medial ray 3 (Fig. 4C). In general, these transformations were reciprocal to those resulting from *dbl-1(lf)* mutations (see Discussion), except that the latter did not appear to affect ray 3.

dbl-1 expression pattern in *dbl-1(+)* animals

RNA blot analysis showed that *dbl-1* transcript is either rare or absent in early embryos (<30 cells) and is present in moderate amounts in later embryos, larvae and adults (Fig. 2C), consistent with the role of DBL-1 in post-embryonic growth. No maternal contribution of *dbl-1* message from the hermaphrodite germ line was detected (data not shown).

To further investigate the expression of *dbl-1*, we constructed two GFP reporter constructs, both containing 4.5 kb of upstream sequence but differing in the presence or absence of a nuclear localization signal (see Materials and methods). Analysis of live animals that carry a mixture of these two constructs allowed us to identify cells by cell-body and axonal morphology as well as by nuclear position. Consistent with the RNA blot result, the reporter expression was first detected in embryos just prior to hatching and remained constant in most cells throughout the larval and adult stages.

Cells expressing the reporters are predominantly neurons (Fig. 5; Table 3). Expressing cells in the ventral nerve cord appear to be of the DA, DB, VA and VB classes (Fig. 5A,B,E,F), throughout the entire length of the cord. Although several pharyngeal neurons express the reporters (Fig. 5C,D), there is so far no phenotypic indication of a role for *dbl-1* in the pharynx. In the male tail, the reporters were expressed, as in the hermaphrodite, in the DVA neuron and also in the glial cells (Bα.l/rd and Bγ.pl/r) that form the sockets for the spicules starting from the L4 stage (Fig. 5G,H).

DISCUSSION

We have shown that *dbl-1* encodes a member of the Dpp/BMP class of signaling molecules, and that *dbl-1(lf)* mutations result in reduced body size and male-tail-defective phenotypes. Opposite effects on post-embryonic growth and, to some extent, on male tail patterning result from *dbl-1* overexpression, indicating that DBL-1 functions as a dose-dependent regulator of these processes.

The other *C. elegans* TGF-β-like genes in which mutations are available, *daf-7* (Ren et al., 1996) and *unc-129* (Colavita et al., 1998), exhibit only Daf-c (dauer-formation constitutive) and Unc (axon guidance) defective phenotypes, respectively. Conversely, *dbl-1(lf)* mutations do not result in Daf-c or Unc phenotypes. These observations suggest that there is little if any overlap in functions of TGF-β family members in *C.*

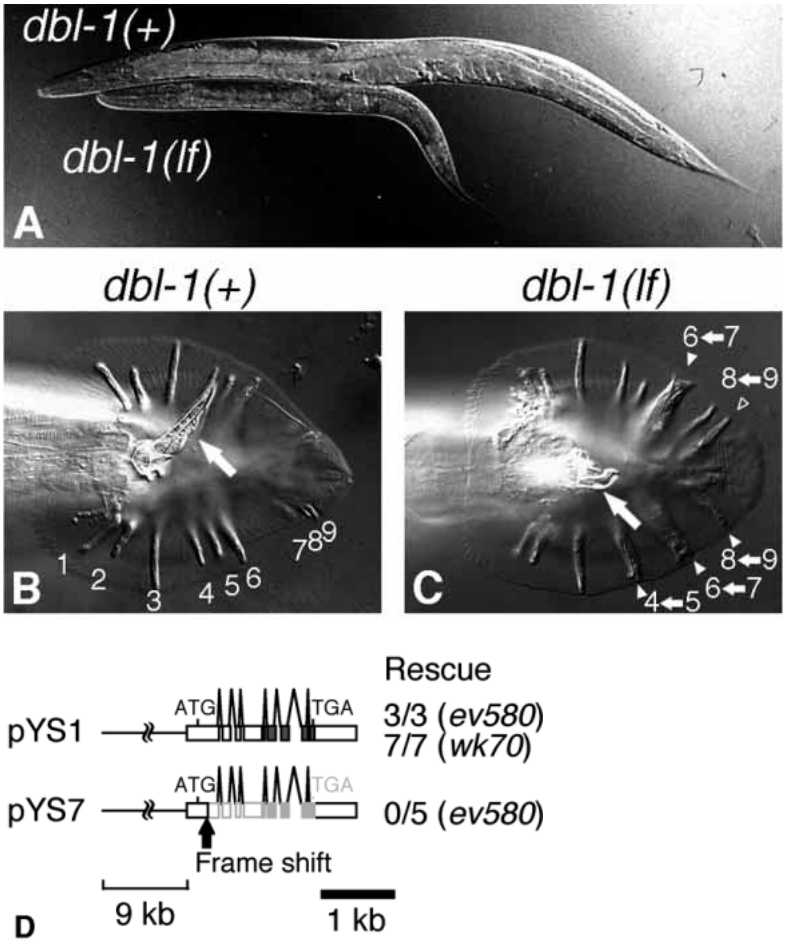

Fig. 3. *dbl-1* loss-of-function phenotypes. (A) The Sma phenotype. The photograph shows adult wild-type (*dbl-1(+)*) and *dbl-1(ev580)* (*dbl-1(lf)*) hermaphrodites immediately after start of egg laying. (B) Ventral view of a phenotypically wild-type male tail. Rays are numbered. Note the thicker appearance of ray 6. One spicule is indicated by the arrow. (C) A *dbl-1(ev580)* mutant male tail. Ray transformations accompanied by ray fusions are indicated with filled arrowheads along with types of ray transformations. Ray transformation not accompanied by ray fusion is indicated by the open arrowhead; ray 9 has a ray-8-like ventral opening instead of its normal marginal opening. One of the pair of crumpled spicules is indicated by the arrow. (D) Diagrams of clones used in rescue experiments. Mature domains are shaded. The coding region rendered non-functional by the frameshift is shown in gray.

Table 2. Epistatic relationships between *dbl-1* overexpression and *sma* mutations

Mutation	Phenotype of mutant homozygotes carrying *dbl-1* array	Percentage of Sma animals carrying *dbl-1* array (*n*)*
dbl-1(ev580)	Lon	0% (301)
daf-4(m72)	Sma	49% (261)
sma-6(wk7)	Sma	51% (302)
sma-2(e502)	Sma	49% (293)
sma-3(e491)	Sma	59% (147)
sma-4(e729)	Sma	53% (137)

*See Materials and methods for explanation. *daf-4(m72), sma-6(wk7), sma-2(e502), sma-3(e491)* and *sma-4(e729)* are strong *lf* alleles (Estevez et al., 1993; Savage et al., 1996; Hoodless et al., 1996; Shi et al., 1997; Krishna and Padgett, 1999; S. K, C. S.-D. and R. W. P., unpublished).

Table 3. Cells expressing *dbl-1::gfp

Sex-non-specific expression	
Lateral ganglia	One unidentified neuron in each
Ventral ganglion	AVKL/R
Retro-vesicular ganglion	DB1, DB2, VB1, VB2
Ventral cord	VA2-11,VB3-11, DA3-7, DB3-7
Pre-anal ganglion	VA12, PDB
Dorso-rectal ganglion	DVA
Canal-associated neurons	CANL/R
Pharyngeal neurons	I5, M1, M2L/R, M4, M5
Body wall muscle	Two most anterior cells in each quadrant
Male-specific expression	
Proctodeum	Spicule socket cells 1/2 (L/R)

*Tissues in which expressing neurons are listed are based on locations of the cell bodies. Expression is also seen in the dorsal cord, from cytoplasmic reporter expression in the processes of (presumably) DA3-7, DB1-7, PDB.

elegans. One other BMP-like molecule, *tig-2*, has been identified by sequence similarity (R. W. P., unpublished), but its function is not yet known.

Role of DBL-1 signaling in body size determination

Size regulation of body components is a poorly understood aspect of animal growth and development. In comparisons of wild-type and mutant L4 larvae at the same stage, the *ev580* mutation caused no decrease in the number of seam cell nuclei and an approximately 35% decrease in seam-cell internuclear distances as observed by Nomarski microscopy, as well as no obvious deficit in total somatic nuclear number as roughly estimated in DAPI-stained animals (Y. S. and W. B. W., unpublished). These observations suggest that decreases in cell size rather than cell number are primarily responsible for the smaller body size of *dbl-1* mutant animals. In *C. elegans*, cell number does not necessarily correlate with the overall body size. For example, mutations in a negative regulator of the cell cycle encoded by the *cul-1* gene result in hyperplasia (Kipreos et al., 1996), but do not cause overall increases in body size similar to that caused by *dbl-1* overexpression.

Another class of mutations that cause changes in body size are those in the *dpy* and *sqt* genes, many of which encode cuticle collagens (e.g. von Mende et al., 1988; Kramer et al., 1988). Mutations in these genes cause shorter body length, and a few alleles and allelic combinations result in Lon animals (Kusch and Edgar, 1986). It is possible that these changes are related to *dbl-1*-mediated effects; however, Dpy phenotypes of *dpy* and *sqt* mutant animals appear short but are generally no thinner than wild type, in contrast to Sma phenotypes, which are thinner than wild type (see Fig. 3A).

Our evidence from reporter constructs indicates that *dbl-1* is expressed primarily in neurons. Their cell bodies are distributed along the length of the animal and so are candidates for sources of DBL-1 signal that could globally regulate body size.

DBL-1 may be a dorsalizing factor in male ray patterning

Rays are pluripotent entities that acquire characteristic morphologies during development (Zhang and Emmons, 1995). These differences are presumably generated by intrinsic factors and local interactions rather than by long-range positional signals, because displaced ray precursor cells retain their normal fates in ray development (Chow and Emmons, 1994). Our results, together with those of Savage et al. (1996), provide the first evidence for a signaling mechanism that specifies ray identities.

In *dbl-1(lf)* mutants, the dorsal rays 5, 7 and the medial ray 9 are transformed to resemble their neighbors, rays 4, 6 and 8, respectively. These morphological changes are correlated with the loss of expression of two ray-specific marker genes (M. Fleischmann and F. Mueller, personal communication; R. Lints and S. Emmons, personal communication). In transgenic strains overexpressing *dbl-1*, the ventral ray 4 occasionally appears dorsalized to resemble ray 3 or ray 5.

Rays 5 and 7 are dorsal rays, and their transformation products (rays 4 and 6) in *dbl-1(lf)* mutants are ventral and medial, respectively. Ray 9 is medial, and its transformation product (ray 8) is ventral (Table 4). Thus all the observed defects involve apparent dorsal-ventral transformations (in a dorsal-to-ventral direction), whereas not all involve anterior-posterior transformations. For example, when ray 5 is posteriorly displaced in *dbl-1(lf)* mutants, it opens to a medial position instead of its normal dorsal position (Table 1). Moreover, overexpression of *dbl-1* results in dorsalization of

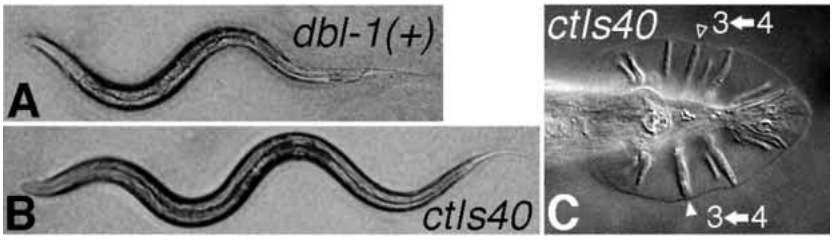

Fig. 4. *dbl-1* overexpression phenotypes. (A,B) *him-8* hermaphrodites of the same age without (A) and with (B) *ctIs40*, respectively (see Materials and methods). (C) Overexpression phenotypes in the tail of a male from the transgenic strain in B. Ray 4 (normally ventral) is dorsalized and fused with ray 3 on one side (filled arrowhead); on the other, ray 4 has a marginal opening like ray 3 but is not fused (open arrowhead). In other animals (not shown), ray 4 was also found dorsalized and fused to ray 5.

Table 4. Summary of male tail ray positions in wild-type, *dbl-1(lf)* and *dbl-1* overexpression backgrounds*

	Position		
Ray	Wild type	*dbl-1 (lf)* (ventralized)	*dbl-1* overexpression (dorsalized)
1	Dorsal	–	–
2	Ventral	–	–
3	Medial	–	–
4	Ventral	–	Medial or dorsal
5	Dorsal	Ventral or medial	–
6	Medial (thick)	–	–
7	Dorsal	Medial (thick)	–
8	Ventral	--	–
9	Medial	Ventral	–

*See text for further description of ray positions and morphologies. Dash indicates that no change from the wild type was observed.

ray 4, regardless of the direction of displacement along the anterior-posterior axis. These observations suggest that DBL-1 may normally act as a dorsalizing factor in tail patterning.

The rays are differentially sensitive to *dbl-1(lf)* mutations, with rays 9 and 7 being affected most frequently, followed by ray 5. The more anterior medial ray 3 and dorsal ray 1 are not affected (Tables 1, 4). The ventral ray 4 is also responsive, as shown by its dorsalization when *dbl-1* is overexpressed. This result supports the view that DBL-1 could act as a general dorsalizing factor (required only for morphogenesis of the more posterior rays). However, it seems unlikely that DBL-1 acts as a morphogen to specify the R5, R7 and R9 cells (or their descendants), but rather that these cells are pre-programmed by other signals or intrinsic factors to become responsive to DBL-1. Consistent with this view is not only the evidence cited above that misplaced Rn cells develop normally, but also our failure to find cell bodies of DBL-1-expressing cells in the immediate vicinity of the ray precursor cells. In the male tail, the *dbl-1* reporters were expressed only in the spicule socket cells and the DVA neuron. The receiving end of the Sma/Mab pathway is thought to function in ray cells as demonstrated for ray 7 (Savage et al., 1996). Although cell bodies of cells expressing *dbl-1* reporters in the male tail are positioned dorsally, their distance makes it unlikely that they produce a gradient of DBL-1 that could be differentially detected by the posterior three dorsal-most Rn cells and those just beneath them. Such a gradient might, on the other hand, be produced by extracellular proteins that regulate DBL-1 activity.

Role of DBL-1 in male spicule development

In contrast to our knowledge of signal transduction pathways that determine lineage patterns to produce the correct number of spicule-producing cells, the mechanisms of spicule morphogenesis are largely unknown (reviewed in Emmons and Sternberg, 1997). Our results show that DBL-1 may play an important role in this process. The expression of *dbl-1* reporter genes suggests a role for spicule socket cells in spicule morphogenesis. Consistent with this suggestion, laser ablation of the ancestors (Bα and Bγ) of these cells results in a crumpled spicule phenotype (Y. S. and W. B. W., unpublished) which, however, could simply indicate a structural role for the socket cells.

Previous experiments showed that laser ablation of either the M cell or the spicule retractor muscles derived from M results in a crumpled spicule phenotype. The spicule retractors are connected to cells (B.al/rappv) at the leading edge of spicules undergoing morphogenesis (Sulston et al., 1980). It is possible that the DBL-1 signal could induce the retractor muscles or the leading cells to connect with each other.

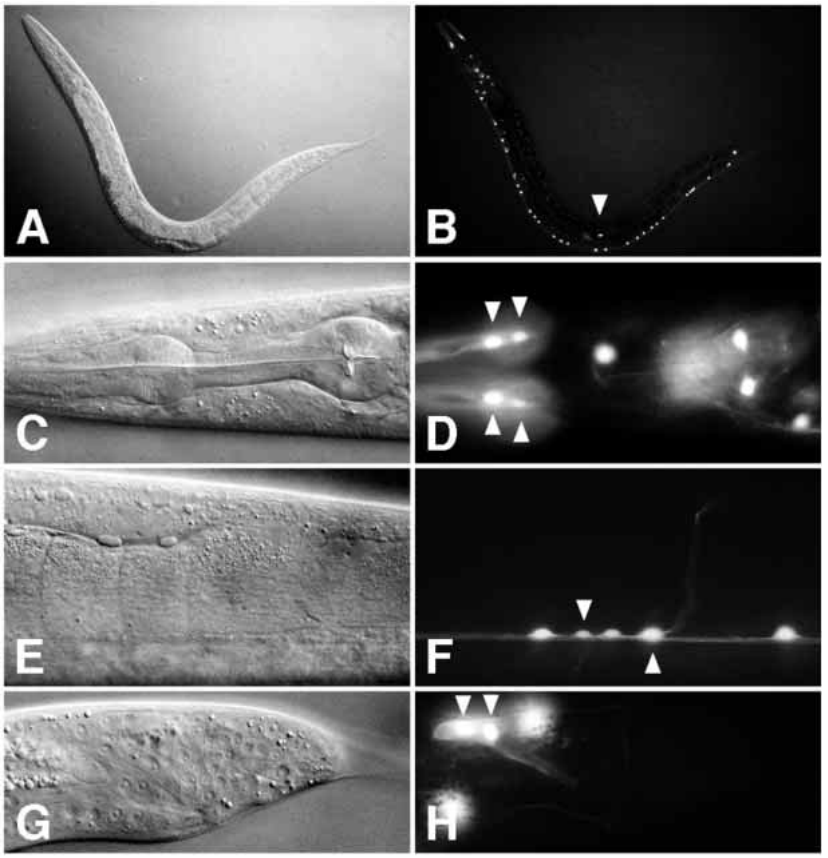

Fig. 5. Expression pattern of *dbl-1::gfp* reporter transgenes. All photographs are oriented anterior-left and ventral-down except (E) and (F), which are ventral views. Left panels, Nomarski and right panels, fluorescence images. (A,B) An L4 hermaphrodite. Note the line of *dbl-1*-expressing neurons in the ventral nerve cord. Arrowhead indicates CANL. (C,D) Head region of an L4 hermaphrodite. Cells tentatively identified as anterior muscle cells (arrowheads) and pharyngeal neurons express *dbl-1::gfp*. (E,F) Ventral view of posterior trunk (P9.p-P10.p region). Cell bodies of five neurons are seen to express the reporter. The axon bundle of the ventral cord is also visible due to the accumulation of cytoplasmic GFP from the reporter lacking a nuclear localization signal. Arrowheads indicate the cell bodies of the DB7 and DA7 neurons, which send processes circumferentially to the dorsal nerve cord. (G,H) An L4 male tail undergoing morphogenesis. Arrowheads indicate expression of *dbl-1::gfp* in the spicule socket cells along developing spicules. Another dorsally located expressing cell, seen out of focus, is the DVA neuron.

Comparison of DBL-1 and Dpp functions

In summary, *dbl-1* appears to be expressed primarily in neurons arrayed along the body length. The presumably processed and secreted DBL-1 protein acts as a ligand for the Sma/Mab pathway to control male tail morphogenesis as well as body size. In the male tail, although DBL-1 seems unlikely to act as a morphogen as argued above, its apparent activity as a general dorsalizing factor would be consistent with the role of its homolog Dpp in *Drosophila* embryogenesis and leg development (Irish and Gelbart, 1987; Held et al., 1994). Regarding body size, we have shown that DBL-1 acts in a dose-dependent manner to control the size of the animal as a whole, probably by influencing cell size. Its mechanism may relate to the role of Dpp in *Drosophila* wing size control. The wing phenotypes resulting from changes in *dpp* expression are associated with changes in cell number; however, no direct link has been demonstrated between Dpp and the cell cycle (reviewed in Edgar and Lehner, 1996). Possibly, Dpp may regulate cell growth, which in turn triggers cell division at a critical cell size. In *C. elegans*, where the number of cell divisions appears to be strictly controlled (Sulston and Horvitz, 1977), the result of inhibiting or stimulating cell growth may be to produce smaller or larger cells, respectively, with concomitant effects on body size. Further exploration of DBL-1 functions could provide new insights into cell-size and body-size regulation.

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