Erratum

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There is an error in the ePress version of this article published on 19 October 2005.

On p. 5002, the sub-heading '*dsc-1* encodes a Q50 Paired-like (or Prd-like) homeodomain protein' should have been the first line of the paragraph that follows it, not a sub-heading.

This error has been corrected in both the published online and print versions.

We apologise to the authors and readers for this mistake.

Specification of muscle neurotransmitter sensitivity by a Paired-like homeodomain protein in *Caenorhabditis elegans*

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Summary

The effects of neurotransmitters depend on the receptors expressed on the target cells. In *Caenorhabditis elegans*, there are two types of GABA receptors that elicit opposite effects: excitatory receptors that open cation-selective channels, and inhibitory receptors that open anion-selective channels. The four non-striated enteric muscle cells required for the expulsion step of the defecation behavior are all sensitive to GABA: the sphincter muscle expresses a classical GABA-sensitive chloride channel (UNC-49) and probably relaxes in response to GABA, while the other three cells express a cation-selective channel (EXP-1) and contract. Here we show that the expression of the *exp-1* gene is under the control of *dsc-1*, which encodes a Paired-like homeodomain protein, a class of transcription factors previously associated with the terminal differentiation of neurons in *C. elegans*. *dsc-1* mutants have anatomically normal enteric muscles but are expulsion defective. We show that this defect is due to the lack of expression of *exp-1* in the three cells that contract in response to GABA. In addition, *dsc-1*, but not *exp-1*, affects the periodicity of the behavior, revealing an unanticipated role for the enteric muscles in regulating this ultradian rhythm.

Key words: Defecation, Homeobox, Paired-like, Enteric muscles, *dsc-1*, *clk-1*

Introduction

Homeodomain transcription factors are involved at numerous levels of animal development: while HOX genes specify the relative position of entire domains of gene expression in the anteroposterior axis, at the other extreme there are types of homeobox genes that appear to determine the expression of only a few genes in a single cell type. For example, in *Caenorhabditis elegans*, Paired-like homeodomain proteins have been found to be involved in the fine-tuning of neuronal differentiation, specifying such features as cell-type-specific synaptic inputs (Miller et al., 1992; White et al., 1992; Winnier et al., 1999), neurotransmitter synthesis (Eastman et al., 1999; Jin et al., 1999) and axonal outgrowth (Brockie et al., 2001; Pujol et al., 2000).

By relating behavioral outputs to patterns of gene expression and the expression of unique differentiated cellular features, it is possible to study the molecular, genetic and cellular bases of behavior in *C. elegans*. For example, neural circuits regulating behaviors such as pharyngeal pumping, egg-laying and locomotion have all been studied in this manner (reviewed by Rankin, 2002; Whittaker and Sternberg, 2004). The neuromuscular system that regulates the Defecation Motor Program (DMP) (Liu and Thomas, 1994) is also beginning to be studied at this level. The DMP consists of three distinct, stereotyped steps (Thomas, 1990): the posterior body muscle contraction (pBoc), the anterior body muscle contraction (aBoc) and the expulsion (Exp), which is the most complex step as it consists of the coordinated contractions of two specialized enteric muscles, the intestinal and anal depressor muscles, and probably the relaxation of a third enteric muscle, the sphincter. The defecation cycle period, which is the interval between two series of these three contractions, is very regular over time in single animals, and from animal to animal, but is modulated by sensory inputs such as touch (Liu and Thomas, 1994), food (Liu and Thomas, 1994) and temperature (Branicky et al., 2001).

Two GABAergic neurons, AVL and DVB, send processes to the enteric muscles (the intestinal, sphincter and anal depressor muscles) and are required for the proper execution of the DMP. Laser ablation studies have revealed that these two neurons are partially redundant for enteric muscle contractions. AVL, but not DVB, also appears to be required for the aBoc step (McIntire et al., 1993b). The pBoc step may be regulated by a non-neural pathway, as neuronal ablations have failed to identify any neuron required for the pBoc (E. Jorgensen, personal communication). Interestingly, the neurotransmitter GABA, normally an inhibitory neurotransmitter, is an excitatory neurotransmitter for enteric muscle contraction. Indeed, loss of the GABA biosynthetic enzyme glutamic acid decarboxylase (GAD), encoded by the *unc-25* gene, causes an expulsion defective (or Exp) phenotype, which can be rescued by exogenous GABA (Jin et al., 1999; McIntire et al., 1993a). In addition, mutations that disrupt GABA transport (*unc-47, gat-1*), and presynaptic GABA release (*unc-2*) also produce an Exp phenotype (Jiang et al., 2004; Mathews et al., 2003; McIntire et al., 1993a; McIntire et al., 1997). The excitatory effect of GABA on these muscles is mediated by *exp-1*, an unusual cation-selective GABA receptor, which is expressed in

the intestinal and anal depressor muscles (Beg and Jorgensen, 2003). There is little known about the development and specification of these muscles, except that they are non-striated muscles that require Twist, encoded by the *hlh-8* gene, for their formation. Loss of *hlh-8* also produces an Exp phenotype, because these muscles are missing or are severely reduced in the mutant (Corsi et al., 2000).

In addition to mutants defective in the execution of the DMP steps, a number of mutants that affect the periodicity of the defecation cycle have been identified (e.g. Branicky et al., 2001; Iwasaki et al., 1995). In general, these mutants are distinct from those affecting the steps of the DMP, suggesting that the expression of the DMP is regulated by mechanisms that are distinct from those required for maintaining the periodicity of the DMP. The characterization of some of these genes has shown that calcium-dependent signals in the intestine play an important role in regulating the periodicity of the DMP. In particular, loss of *itr-1* (*dec-4/lef-1*), which encodes the worm inositol triphosphate receptor (IP_3 receptor) and regulates calcium release from the endoplasmic reticulum, slows down the defecation period (Dal Santo et al., 1999). Furthermore, the same authors showed that calcium levels peak in the intestine just before the first muscle contraction of the DMP, and that expression of the *itr-1* in the intestine is sufficient for normal rhythm generation. Moreover, mutations in *unc-43,* the *C. elegans* calcium-dependent calmodulin Kinase II (CaM Kinase II), which is widely expressed in neurons, muscles and the intestine, result in multiple behavioral defects, including defecation phenotypes (Reiner et al., 1999).

The characteristics of several other mutants also point to the intestine as the main modulator of the DMP periodicity. For example, *flr-1* and *flr-4* mutants, originally identified on the basis of their resistance to fluoride (Katsura et al., 1994), have very short defecation cycle lengths (Iwasaki et al., 1995). Intestinal expression of both *flr-1,* which encodes an ion channel of the degenerin/epithelial sodium channel superfamily, and *flr-4*, which encodes a serine/threonine protein kinase, is sufficient to rescue their respective defects (Take-Uchi et al., 1998; Take-Uchi et al., 2005). The *elo-2* gene, which encodes a fatty acid elongation enzyme that is expressed in the intestine, also appears to be involved in regulating the defecation cycle length as RNAi-mediated suppression of *elo-2* results in a shortened defecation cycle length (Kniazeva et al., 2003). Finally, loss of function of *dsc-4*, which encodes the worm microsomal triglyceride transfer protein (MTP), and is probably required in the intestine for lipid transport, also causes a shortened defecation cycle length (Branicky et al., 2001; Shibata et al., 2003).

We have studied the altered defecation cycle of the *clk-1* mutants, in which a number of behavioral, developmental and physiological features are abnormal (Branicky et al., 2001; Wong et al., 1995). *clk-1* encodes a highly conserved protein that is required for ubiquinone (UQ or CoQ) biosynthesis (Ewbank et al., 1997). We have analyzed the defecation behavior of *clk-1* mutants in some detail and found that in the mutants the cycle length is both increased and insensitive to changes in temperature. To investigate these defects further, we have carried out a screen to identify suppressor mutations (which we call *dsc*, for defecation suppressor of *clk-1*) and identified two classes of suppressor mutants. The class I *dsc* mutants (which include *dsc-4*) suppress both the lengthened defecation cycle and the temperature insensitivity; the class II mutants (which include *dsc-1*) suppress only the lengthened defecation cycle, but does not restore normal reaction to changes in temperature (Branicky et al., 2001).

Here we report our characterization of *dsc-1*. We find that the *dsc-1* mutants have a shortened defecation cycle length and are also expulsion defective. We cloned *dsc-1* and found that it encodes a Paired-like homeobox transcription factor that is expressed in some sensory neurons and in the enteric muscles. *dsc-1* is the first homeobox gene of its class that is found to be expressed in, and important for the differentiation of, non-neuronal cells in *C. elegans*. We show that expression of *dsc-1* in the intestinal and anal depressor muscles alone is sufficient to rescue both the defecation cycle length and expulsion defects. However, a variety of experimental manipulations allow for the uncoupling of the effects of *dsc-1* on these two phenotypes, suggesting a previously unanticipated role for the enteric muscles in regulating the defecation cycle length. We also show that *dsc-1(+)* activity is required for the expression of *exp-1* in the intestinal and anal depressor muscles, and that the expulsion defect of the *dsc-1* mutant is due to the loss of *exp-1* expression.

Materials and methods

General methods and strains

Animals were cultured at 20°C as described (Brenner, 1974), and were fed the *Escherichia coli* strain OP50. The *dsc-1(qm133)* allele was isolated in an EMS mutagenesis screen (Branicky et al., 2001) and was outcrossed five times; the *dsc-1(tm241)* allele was obtained from the National Bioresource Project for the nematode, and was outcrossed three times.

The mutations used to refine the genetic position of *dsc-1* include *lin-15(n765)*, *flr-4(ut7)* and *unc-7(e5)*. *dsc-1* was also mapped relative to the mnDF20 deficiency using the SP278 strain (mnDp1 (X;V)/+ V; mnDf20 X). Other mutations and strains used in this study include *exp-1(sa6)* I, EG1653 *oxIs22* [*Punc-49:UNC-49::GFP, lin-15(+)*] II (Bamber et al., 1999), *clk-1(qm30)* III, *unc-25(e156)* III and *hlh-8(nr2061)* X (Liu et al., 1999).

Defecation was scored as described in Branicky et al. (Branicky et al., 2001). Each animal was scored for five consecutive cycles at 20°C. To quantify the Exp phenotype, animals were also scored for five consecutive defecation cycles, which includes six consecutive posterior body contractions (pBocs), and the number of pBocs followed by an expulsion was determined. Student's *t*-test was used to determine statistical significance and was carried out using Graphpad Prism 4.0 software.

Positional cloning of *dsc-1*

dsc-1 had previously been mapped to the right arm of LG X, between *unc-3* and *lin-15* (Branicky et al., 2001). Using 2-point, 3-point and deficiency mapping strategies, we further refined the genetic position of *dsc-1* and determined that it is between the two cloned genes *pag-3* and *unc-7* (see www.wormbase.org). The six cosmids that correspond to this region were injected into the *dsc-1* mutants as a single pool, in pairs, and singly, and it was determined that the *dsc-1* mutants could be rescued by all injection mixes containing the C18B12 cosmid. The three predicted genes on the C18B12 cosmid were amplified from the cosmid by PCR and were tested for rescuing activity. A PCR product corresponding to the predicted gene C18B12.3 (from 30798 to 24611 on C18B12) was able to rescue the *dsc-1* mutants. All cosmids and PCR products were injected at a

concentration of 50 ng/μl with the co-injection marker *ttx-3::gfp* (Hobert et al., 1999) at a concentration of 150 ng/μl.

To determine the nature of the *qm133* mutation, genomic DNA was extracted from *dsc-1(qm133)* mutants and the predicted coding region was sequenced from both strands. We identified a G-to-A transition at position 695 of the coding region relative to the ATG (position 26250 of the C18B12 cosmid) resulting in an R-to-H substitution at residue 232 of the protein.

RT-PCR

First-strand cDNA libraries were generated by the reverse transcription of total RNA extracted from adult worms, using a polydT primer and reverse transcriptase. The *dsc-1* transcript was amplified from the library with primers corresponding to the predicted transcript and primers complementary to the polyA tail. The *dsc-1* transcript contains five exons and is 933 bp long. The 3′ UTR is 284 bp long, and probably uses one of two possible AAUAAA polyadenylation signals, located 14 or 18 bases upstream of the cleavage site. The *dsc-1* transcript does not seem to be trans-spliced, as the transcript could not be amplified using primers corresponding to either the SL1 or the SL2 splice-leader sequences, although there is a consensus trans-splice site located 14 bases upstream of the start codon.

Construction of plasmids and transgenic strains

For transcriptional and translational *dsc-1::gfp* reporter fusions, portions of the *dsc-1* promoter and genomic region were amplified from the C18B12 cosmid with primers containing synthetic *Pst*I and *Nhe*I sites, and were cloned into the *Pst*I and *Xba*I sites of the pPD95.77 vector, kindly provided by A. Fire. Unless otherwise indicated, constructs were injected into N2 and *dsc-1* at a concentration of 50 ng/μl along with the transformation marker pRF4[*rol-6(su1006)*] or *ttx-3::gfp* at a concentration of 150 ng/μl.

Pdsc-1::gfp

The insert contains bases 30284-28330 of C18B12, which includes ~2 kb upstream of the initiating ATG of *dsc-1* and the first 18 bp.

Pdsc-1::dsc-1::gfp

The insert contains bases 30284-25275 of C18B12, which includes ~2 kb upstream of the initiating ATG of *dsc-1* and the entire genomic sequence, excluding the stop codon.

For this construct, *gfp* expression could be observed in 4/4 lines, all of which were rescued. However, rescued lines also produced ~30% non-rescued transgenic worms.

Nde-box::dsc-1::gfp

The Nde box is a concatamerized element of the *ceh-24* promoter that was previously found (Harfe and Fire, 1998) and has been used to drive expression specifically in the enteric muscles (Bulow et al., 2002). It was amplified from the pBH10.21 clone and was cloned into the *Pst*I and *Xba*I sites of the pPD95.77 vector. (The *Pst*I site was introduced in the primer and the *Xba*I site was internal to the PCR product.) The *dsc-1* cDNA (–19 to +930) was amplified from a cDNA library with primers containing synthetic *Kpn*I sites, and was cloned into the *Kpn*I site of pPD95.77. This construct was injected into *dsc-1* at concentrations of 25 and 100 ng/μl along with the transformation marker *ttx-3::gfp* (at concentrations of 175 and 100 ng/μl). For this construct, *gfp* expression could only be observed in 3/5 transgenic lines; all three were rescued. However, rescued lines also produced ~30% non-rescued transgenic worms.

Pexp-1::gfp (pAB04)

This was kindly provided by E. Jorgensen (Beg and Jorgensen, 2003).

Nde-box::exp-1

This fusion was created using a PCR approach essentially as described previously (Hobert, 2002); the construct is not a GFP fusion, so as not to interfere with the function of EXP-1. The Nde-box portion was amplified from the pBH10.21 clone and the *exp-1* gene (from –60 to +4128, which includes the UTRs) from genomic DNA. The fusion PCR product was injected in *exp-1* and *dsc-1* mutants at a concentration of ~1 ng/μl together with the transformation marker *ttx-3::gfp* at a concentration of 100 ng/μl. For both backgrounds 4/4 lines were rescued. Rescued lines produced ~10% non-rescued transgenic worms.

RNAi

A portion of the *dsc-1* cDNA (+33 to + 933) was amplified from a first-strand cDNA library with primers containing synthetic *Nhe*I and *Pst*I restriction sites and was cloned into the corresponding sites in the pPD129.36 vector, kindly provided by A. Fire. The cDNA flanked by the T7 promoter sites was then amplified from the clone and was used as the template for in vitro transcription (Promega Ribomax). Double-stranded RNA was injected into wild-type animals at a concentration of ~1 μg/μl as described (Fire et al., 1998). The F1 progeny were scored for their defecation cycle length and number of pBocs associated with expulsions.

Results

dsc-1 mutants have a shortened defecation cycle and lack enteric muscle contractions

The *dsc-1(qm133)* mutant was identified in a screen for suppressors of the slow defecation phenotype of *clk-1* mutants (Branicky et al., 2001). In addition to decreasing the defecation cycle length of *clk-1* mutants, *dsc-1* also significantly shortens the defecation cycle length in a wild-type background (Fig. 1A). In addition to affecting the periodicity of the cycle, *dsc-1* is also required for the proper execution of the defecation motor program. *dsc-1* mutants have normal posterior and anterior body muscle contractions, but lack the enteric muscle contractions (Fig. 1B). As this step is necessary for the expulsion of the gut contents, *dsc-1* mutants also have a distended gut, as the gut lumen fills with bacterial debris (Fig. 2B). This phenotype, termed the ‘Constipated’ or Con phenotype, is common to most if not all expulsion defective mutants (Fig. 2C,D) (Hobert et al., 1999; McIntire et al., 1993b; Reiner and Thomas, 1995; Thomas, 1990).

dsc-1 encodes a Q50 Paired-like homeodomain protein

dsc-1 was previously mapped to LGX between *unc-3* and *lin-15* (Branicky et al., 2001). Using 2- and 3-point mapping strategies we determined that *dsc-1* is tightly linked to, and to the left of both *unc-7* and *flr-4*. Using the chromosomal deficiency *mnDf20*, which deletes *pag-3* but not *dsc-1*, we determined that *dsc-1* is right of *pag-3*. The six cosmids that cover the region between *pag-3* and *flr-4* were injected into *dsc-1* mutants as a single pool, then in pairs and then singly. All injection mixes containing the C18B12 cosmid were capable of rescuing all phenotypes of the *dsc-1* mutants. Of the three predicted genes on the C18B12 cosmid, a PCR product corresponding to only one, C18B12.3, was capable of rescuing *dsc-1* mutants (Fig. 1).

We sequenced the coding region of C18B12.3 and found a G-to-A point mutation that results in an R-to-H substitution at residue 232 of the protein (Fig. 3B). In addition, a strain harboring a deletion in the C18B12.3 gene, (*C18B12.3(tm241)*), phenocopied and failed to complement the *dsc-1(qm133)* mutant (Fig. 1 and data not shown). Finally, injection of double-stranded RNA corresponding to the

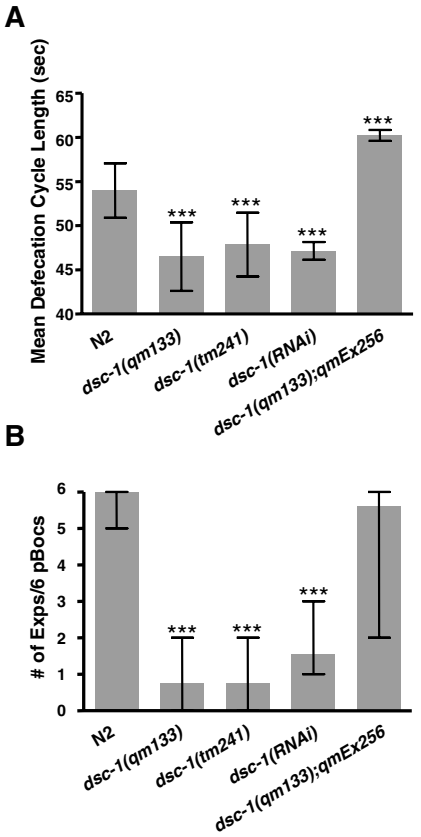


Fig. 1. The defecation phenotypes of *dsc-1* mutants. (A) *dsc-1* mutants have a shortened defecation cycle length. The bars represent the mean of the mean cycle lengths of *n* animals that had each been scored for five consecutive defecation cycles; the error bars represent the standard deviation of the animal means (*n*=20-30 animals). (B) *dsc-1* mutants have an expulsion defective (Exp) phenotype. The bars represent the mean number of expulsions per six pBocs; the error bars represent the range (of number of expulsions/six pBocs) observed (*n*=20 animals). For both A and B, the asterisks indicate that the data are significantly different from that of N2. All significant differences detected were at a level of $P<0.0001$.

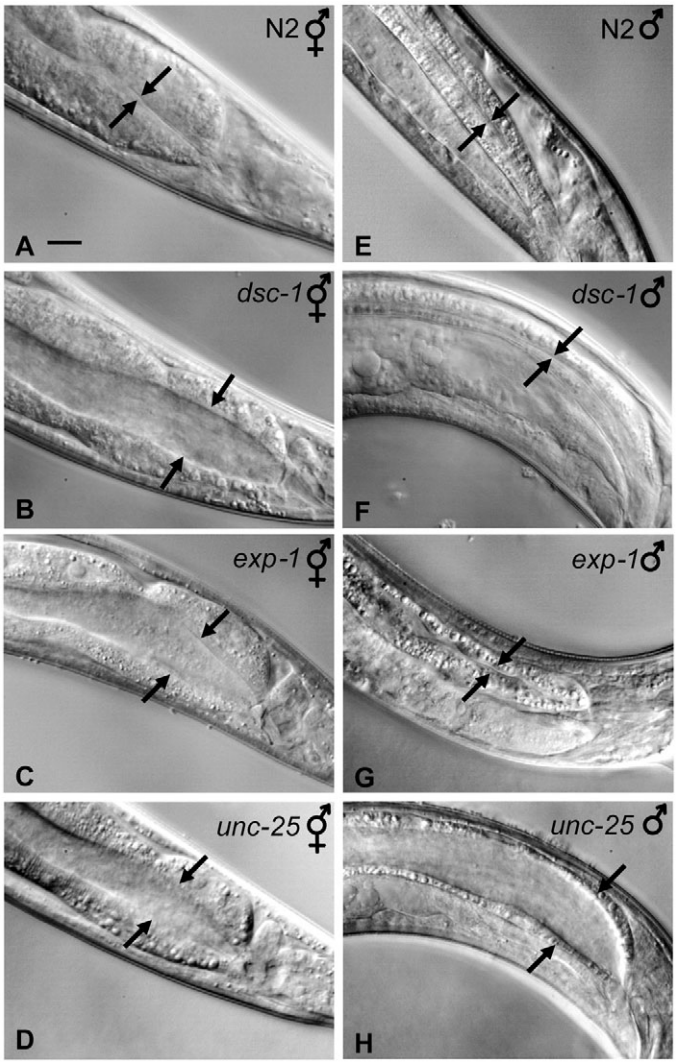


Fig. 2. The Con phenotype of Exp mutants. Nomarski images of the tail region of wild-type and Exp mutant hermaphrodites (A-D) and their male counterparts (E-H). Arrows indicate the width of the intestinal lumen. N2 hermaphrodites and males are neither expulsion defective (Exp) nor Constipated (Con). *dsc-1(qm133)* (B) *exp-1(sa6)* (C) and *unc-25(e156)* (D) hermaphrodites are Exp and, as a result, exhibit the Con phenotype, as the gut lumen fills with bacterial debris and becomes distended. Neither *dsc-1* (F) nor *exp-1* (G) males are Con, indicating that they are not expulsion defective. By contrast, *unc-25(e156)* males are Exp, and as a result are severely Con (H). Scale bar: 10 μm.

C18B12.3 coding region into wild-type animals also phenocopied the *dsc-1(qm133)* mutation (Fig. 1). Together these observations indicate that *dsc-1* corresponds to the predicted gene C18B12.3 and suggest that the *dsc-1(qm133)* mutation may be a genetic null.

dsc-1 encodes a Q50 Paired-like (or Prd-like) homeodomain protein. Paired-class proteins are defined by the presence of a homeodomain (HD) that resembles that encoded by the *Drosophila prd* gene and are characterized by six diagnostic residues in the HD. Three sub-classes can be defined according to the residue at position 50 of the HD. The Pax or Prd-type genes are characterized by a serine at position 50 and also contain a second DNA-binding domain, the so-called Paired domain (PD). The other two sub-classes lack the PD (and are therefore Prd-like) and have either a lysine (K_{50}) or a glutamine (Q_{50}) at position 50, the latter being considered to be the most ancestral of the three sub-families (reviewed by Galliot et al., 1999). Comparison between the homeodomain of DSC-1 with the consensus Q_{50} Prd-like homeodomain (Fig. 3C) reveals that the DSC-1 HD contains only four of the six diagnostic residues for the Prd class, indicating that it is somewhat diverged. In addition, *dsc-1* does not appear to be orthologous to any of the more than 12 Q_{50} Prd-like sub-families already described, suggesting that *dsc-1* encodes a novel Q_{50} Prd-like protein. In addition, *dsc-1* does not appear to have orthologs in any species other than *C. briggsae*, suggesting that it might be a recently evolved, nematode-specific, or even *Caenorhabditis*-specific, gene.

The severity of phenotype of the *qm133* mutant, which harbors an R-to-H substitution at position 53 of the

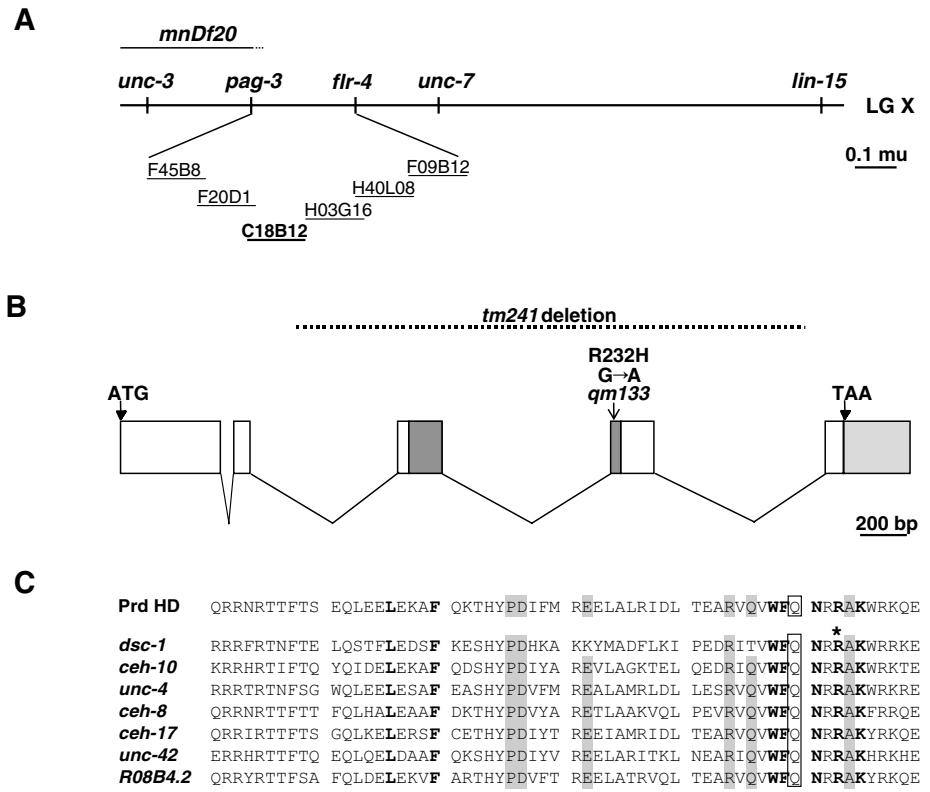


Fig. 3. Positional cloning of *dsc-1*. (A) Mutations, deficiencies and cosmids used for the positional cloning of *dsc-1*. (B) Genomic structure of *dsc-1* and the lesions of the two *dsc-1* alleles. The dark gray box indicates the homeobox, and the light gray box indicates the 3′ UTR. (C) Comparison between the homeodomain of DSC-1 with the consensus Q_{50} Paired-like homeodomain (Prd HD) (see Galliot et al., 1999), and the homeodomains of the six other Q50 Paired-like homeodomain proteins in *C. elegans*. The residues shaded in gray indicate the six residues that are diagnostic of the Paired class; the residues in bold indicate invariant or highly conserved residues that are not specific to the Paired class; the box indicates the Q_{50}. The *qm133* mutation results in an R-to-H substitution in an absolutely conserved residue at position 53 of the homeodomain (indicated by an asterisk).

homeodomain is the same as that of the *tm241* deletion mutant, which lacks the HD entirely. This suggests that the *qm133* mutation may entirely abolish the binding of the *dsc-1* HD to its target DNA. Indeed, the Arg53 residue is one of the most highly conserved residues of the HD from a broad range of species and has been shown to contact the DNA phosphate backbone in the paired- and engrailed-DNA crystal structures (Kissinger et al., 1990; Wilson et al., 1995). In addition, it has been shown that mutations at position R53 of human Q_{50} Prd-like genes CHX10 and HESX1, which result in microphthalmia and septo-optic dysplasia, respectively, entirely abolish binding to target DNA in vitro (Dattani et al., 1998; Ferda Percin et al., 2000).

dsc-1 is expressed in enteric muscles

In *C. elegans*, there are 13 genes encoding Prd-like homeobox proteins (six are K_{50} Prd-like and seven are Q_{50} Prd-like genes), eight of which (*unc-4*, *unc-30*, *unc-42*, *ceh-10*, *ceh-17*, *ceh-36*, *ceh-37* and *ttx-1*) have been studied genetically, and all have been shown to be required for particular features of a subset of neurons (Altun-Gultekin et al., 2001; Baran et al., 1999; Eastman et al., 1999; Jin et al., 1994; Lanjuin et al., 2003; Miller et al., 1992; Pujol et al., 2000; Satterlee et al., 2001; Svendsen and McGhee, 1995; White et al., 1992). Based on some of these analyses, it has been suggested that this class of homeoproteins may have essential roles in defining neuron subtype identity (Baran et al., 1999). In order to determine the focus of action of *dsc-1*, we examined the expression pattern of transcriptional and translational *gfp* reporter fusions.

We found that both the transcriptional and translational fusions are expressed in a subset of bilateral sensory neurons. Based on the positions of the cell bodies and processes we believe we have identified these neurons as AWA, AWB, AWC, ASE, FLP and PVD (Fig. 4A,B,D,E). GFP expression could also occasionally be observed in a sixth pair of unidentified neurons, located to the anterior of AWC (not shown). As expected, we observed expression of the transcriptional fusion in both the cell bodies and axons, and in the cytoplasm as well as in the nucleus, whereas expression of the translational fusion was mostly confined to the nucleus.

In addition to expression in neurons, we also observed expression in the enteric muscles. Expression of the transcriptional fusion could be observed only in the sphincter muscle (Fig. 4C), whereas expression of the translational fusion was observed in the nuclei of the two intestinal muscles (IM) and the anal depressor muscle (AD), but not in the sphincter. This suggests that sequences upstream and internal to the *dsc-1* gene might participate in a combinatorial code of expression that identifies as a group all the muscles that are involved in defecation, and that there are sequences in the coding region that prevent expression of *dsc-1* in the sphincter and promote expression of *dsc-1* in the IM and AD (see Discussion).

Expression of *dsc-1(+)* in two enteric muscles is sufficient to rescue the defecation defects of *dsc-1* mutants

In order to determine whether it was the expression of *dsc-1* in the neurons, the enteric muscles or both that was necessary for the defecation phenotypes of *dsc-1* mutants, we used a synthetic promoter element (the so-called NdE-box), derived from the concatamerization of an element in the *ceh-24* promoter (Harfe and Fire, 1998) to specifically drive expression of the *dsc-1* cDNA in the intestinal and anal

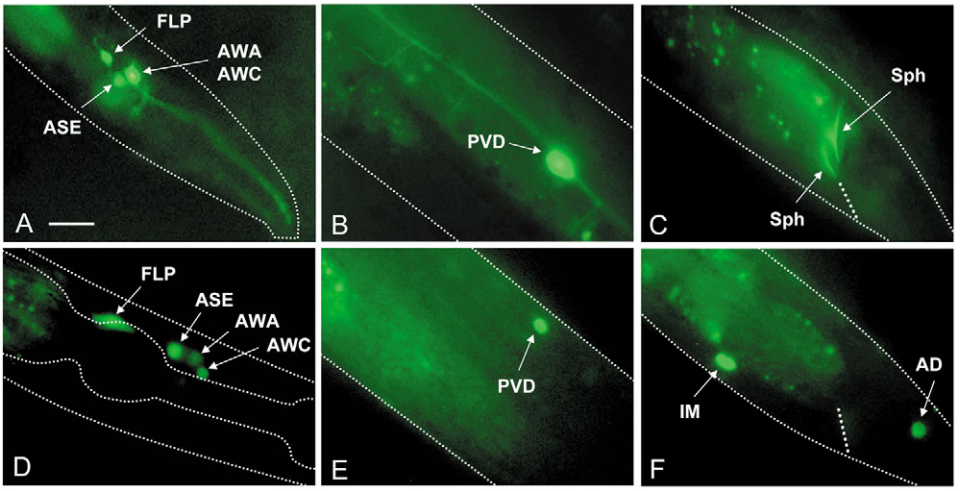
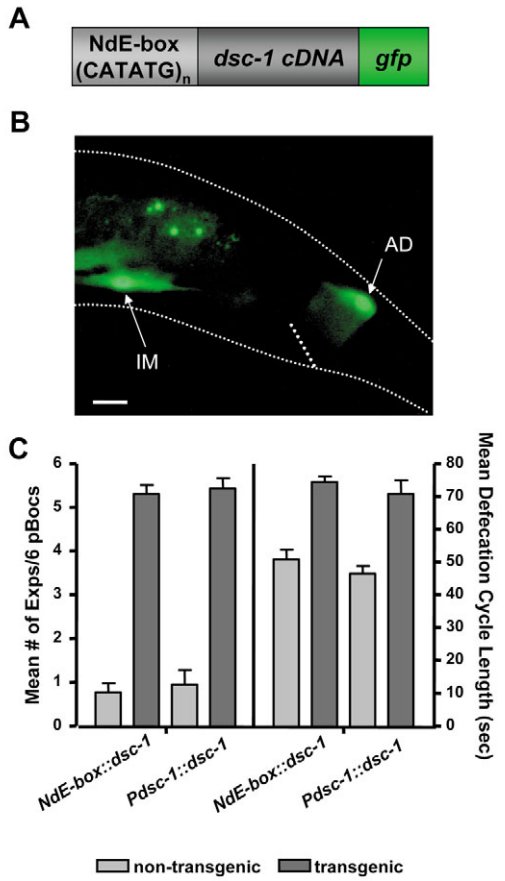


Fig. 4. The expression patterns observed for *dsc-1* transcriptional and translational reporter *gfp* fusions. (A-C) The pattern of expression observed with a transcriptional *gfp* reporter. *dsc-1* is expressed in four pairs of sensory neurons in the head (A), the PVD neuron (B) and the sphincter muscle (C). (D-F) The pattern of expression observed with a fully functional translational *gfp* reporter. Expression of a translational fusion of *dsc-1* can be detected in the nuclei of the same sensory neurons that express the transcription fusion (D,E); however, by contrast to the transcriptional fusion, the translational fusion is expressed in the intestinal and anal depressor muscles, but not in the sphincter (F). All animals were adults, albeit of different ages and sizes. Scale bar: 10 μm.

depressor muscles (Fig. 5A,B). Indeed, we found that expression of *dsc-1(+)* in only these two muscle types was sufficient to rescue both the defecation cycle length and expulsion defects of *dsc-1* mutants. Moreover, we found that the degree of rescue obtained with this construct was the same as that obtained by a translational fusion that uses the *dsc-1* promoter and the entire genomic region containing *dsc-1* to drive *dsc-1* expression in both the enteric muscles and neurons.



This suggests that, for the defecation phenotypes that we have been studying in the *dsc-1* mutants, *dsc-1(+)* is required only in the IM and AD. Consistent with this, we observed that *dsc-1(RNAi)* only knocks down the expression of *Pdsc-1*::*dsc-1::gfp* in these two enteric muscles and not in the *dsc-1* expressing neurons. However, *dsc-1(RNAi)* can completely phenocopy the *dsc-1(qm133, tm241)* mutations (Fig. 1), indicating that loss of *dsc-1* in only these two enteric muscles is responsible for the defecation defects in the mutants.

dsc-1(+) is required for the expression of *exp-1*

After having established that the expression of *dsc-1(+)* in the AD and IM is necessary for the regulation of the defecation cycle length and the expulsion step, we sought to determine the nature of the defect in the mutant muscles. Using a combination of polarized light microscopy, rhodamine-phalloidin staining and expression of a non-rescuing *dsc-1(qm133)::gfp* reporter, we could observe that the IM, AD and sphincter were all present in the mutants, and had an overall wild-type appearance (data not shown). Given the role of homeoproteins of the Prd-like

Fig. 5. Expression of *dsc-1(+)* in two enteric muscles in sufficient to rescue the defecation defects of *dsc-1* mutants. (A) Schematic of construct used to express *dsc-1* specifically in the AD and IM. (B) Expression of the construct depicted in (A) in *dsc-1(qm133)* animals. The dotted line represents the anus. (C) Comparison of the degree of rescue of *dsc-1(qm133)* mutants obtained by expression of the *dsc-1* cDNA specifically in the IM and AD under the NdE-box promoter in comparison to the expression of the *dsc-1* genomic region under its own promoter, which expresses *dsc-1* in sensory neurons as well as in the two enteric muscles (shown in Fig. 3). Dark gray bars represent transgenic worms, and light gray bars represent the non-transgenic siblings from the transgenic lines. For the left panel, the bars represent the average number of expulsions per six pBocs observed for three independent transgenic lines, and the error bars represent the standard deviations of the means obtained for each transgenic line. For the right panel, the bars represent that average defecation cycle length observed for three independent transgenic lines, and the error bars represent the standard deviations of the means obtained for each transgenic line. Twelve transgenic and 12 non-transgenic animals were scored per line. Scale bar: 10 μm.

class in defining neuron sub-type identity, we wondered whether *dsc-1* could have a similar role in these muscles. A number of genes affecting the expulsion step at the neuronal level, as well as some genes required for the development of the enteric muscles have been identified (Corsi et al., 2000; Hobert et al., 1999; Jin et al., 1999; Mathews et al., 2003). However, only one gene, *exp-1*, has been shown to be required in these muscles for their function in the execution of the expulsion step. *exp-1* encodes an unusual cation-selective GABA receptor, which is expressed in the intestinal and anal depressor muscles as well as in a few other cells (Beg and Jorgensen, 2003).

We found that expression of an *exp-1::gfp* transcriptional reporter could not be detected in the IM and AD in either *dsc-1(qm133)* or *dsc-1(tm241)* backgrounds, while *gfp* expression could be observed when the same transgene was transferred into a *dsc-1(+)* background (Fig. 6A,B and data not shown). *exp-1::gfp* expression could also be restored when a *dsc-1*-rescuing construct was introduced into the *dsc-1* mutant lines (data not shown). In addition, we observed that *dsc-1(+)* was not required for the expression of *exp-1* in the PDA neuron, a neuron that is known to express *exp-1* but is not known to have a role in defecation. Taken together, these results indicate that *dsc-1(+)* is required for the expression of *exp-1* in an enteric-muscle-specific manner.

As a control, we also examined the expression of *unc-49* in *dsc-1* mutants (Fig. 6C,D). *unc-49* encodes a typical anion-gated GABA channel (Bamber et al., 1999), which is expressed in the sphincter, and should promote relaxation of this muscle in response to GABA. However, the exact role of the sphincter in hermaphrodite defecation is less clear than for the other two muscles. Given that *unc-49* mutants are not expulsion defective (McIntire et al., 1993a), the sphincter probably plays only a minor role in the expulsion step in hermaphrodites. However, in males there is a remodeling of the system in adulthood such that the expulsion step is achieved by relaxation of the sphincter, rather than by contraction of the IM and AD (Reiner and Thomas, 1995). The expression of *unc-49* does not appear to be in any way altered in *dsc-1* mutants, which is consistent with the expression pattern of *dsc-1*. Moreover, we found that *dsc-1* mutant males do not have the Con phenotype that is associated with the Exp phenotype (Fig. 2F). This further supports a requirement for *dsc-1* only in the IM and AD muscles, and only for the expression of genes such as *exp-1*, that act in the IM and AD. As has previously been reported (Reiner and Thomas, 1995), we found that *exp-1* males are also not Con, whereas *unc-25* males are severely Con. This is consistent with a requirement for GABA for the expulsion in males, not by acting on EXP-1 to promote contraction of the IM and AD, but rather by acting on UNC-49 to promote relaxation of the sphincter.

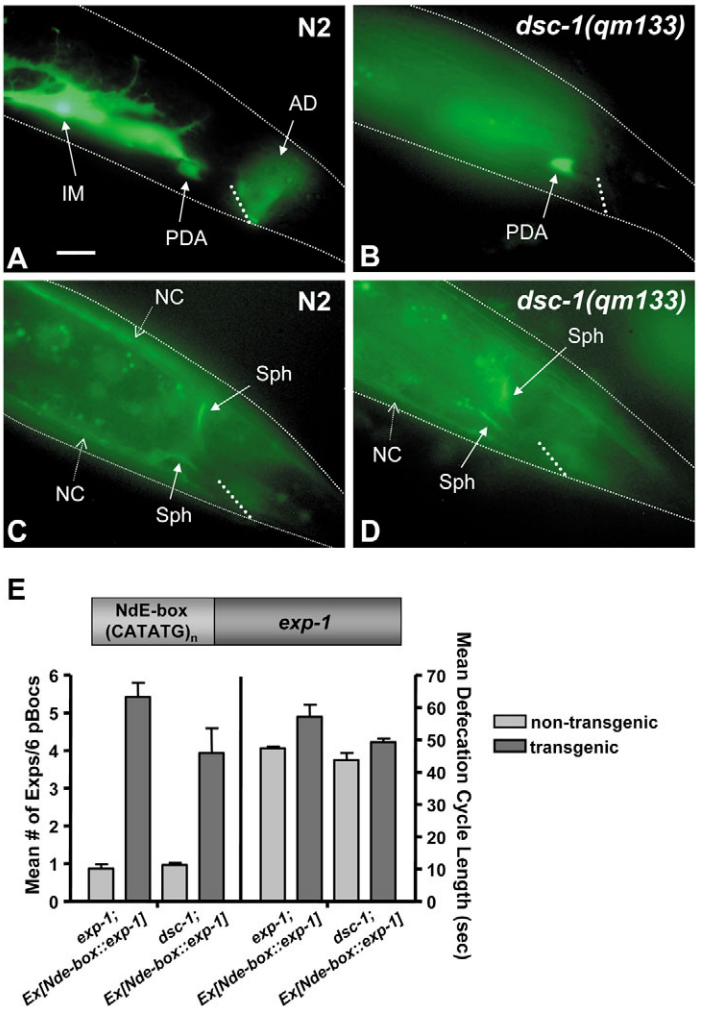


Fig. 6. The Exp defect of *dsc-1* is due to the lack of *exp-1* expression. (A,B) Expression of an *exp-1::gfp* transcriptional reporter clone (pAB04) (see Beg and Jorgensen, 2003) in wild-type and *dsc-1* mutant backgrounds. (The same extra-chromosomal array was expressed in all three backgrounds; the same results were obtained with >4 independent extra-chromosomal arrays.) (C,D) Expression of an integrated *unc-49B::gfp* translational reporter clone in wild-type and *dsc-1* mutant backgrounds. The same integrated array (*oxIs22*) (see Bamber et al., 1999) was expressed in all three backgrounds. Scale bar: 10 μm. NC, nerve chord; Sph, sphincter. (E) Expression of an *Nde-box::exp-1* fusion in *exp-1(sa6)* and *dsc-1(qm133)* backgrounds. For the left panel, the bars represent the average number of expulsions per six pBocs observed for three independent transgenic lines, and the error bars represent the standard deviations of the means obtained for each transgenic line. For the right panel, the bars represent that average defecation cycle length observed for three independent transgenic lines, and the error bars represent the standard deviations of the means obtained for each transgenic line. Twelve transgenic and 10 non-transgenic animals were scored per line.

The *dsc-1* expulsion defect is due to the absence of EXP-1

In order to test whether the lack of *exp-1* expression was responsible for the Exp defect of *dsc-1*, we used the Nde-box to drive *exp-1* expression in the IM and AD muscles (Fig. 6E). We found that this construct was able to fully rescue the Exp defect of *exp-1* mutants. We also found that this fusion was also able to rescue the Exp defect of *dsc-1*, which indicates that the expulsion defect of the *dsc-1* mutants is indeed due to the lack of *exp-1* expression. However, the rescue achieved with this construct was somewhat weaker in *dsc-1* than in *exp-1* and also weaker than the rescued achieved with *dsc-1(+)* expression (Fig. 5C). This suggests that *dsc-1* may also be

required for the expression of some positive regulators of *exp-1* or some other genes that contribute, but are not essential, for enteric muscle function.

Consistent with the finding that the GABA-gated EXP-1 channel is responsible for the *dsc-1* phenotype, neither *exp-1* nor *dsc-1* mutants can be rescued by exogenous GABA (data not shown).

dsc-1 regulates the defecation cycle length

We have described two defecation defects for the *dsc-1* mutant: an expulsion defect and a cycle length defect, both of which result from a lack of *dsc-1(+)* expression in the IM and AD. One possibility is that the shortened defecation cycle length is in fact a secondary effect of the Exp defect. It has been shown for other Exp mutants, such as the *unc-43(gf)* mutant, that a lack of enteric muscle contractions leads to a progressive shortening of the cycle as the intestine fills with bacterial debris. This eventually leads to a forceful expulsion of the gut contents, which is generally followed by one or two longer cycles (Reiner et al., 1999). We also observed a similar oscillation in cycle length with *dsc-1*, albeit over a very small range (see legend of Fig. 7), suggesting that the Exp phenotype could contribute to the shortened cycle length of *dsc-1*, but only in a minor way. Consistent with this, we found that the *Nde-box::exp-1* construct, which rescues the Exp defect of *exp-1* and *dsc-1*, fully rescued the cycle length defect of *exp-1*, but only partially that of *dsc-1* (Fig. 6E).

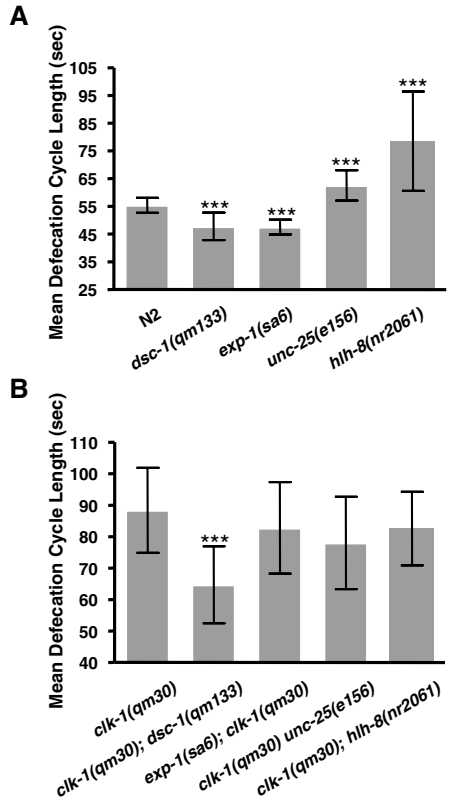


Fig. 7. The effect of Exp mutations on the defecation cycle length in wild-type and *clk-1* mutant backgrounds. The bars represent the mean of the mean cycle lengths of *n* animals that had each been scored for five consecutive defecation cycles; the error bars represent the standard deviation of the animal means ($n>10$ animals). (A) In a wild-type (N2) background, both *dsc-1(qm133)* and *exp-1(sa6)* significantly shorten the defecation cycle; *unc-25(e156)* and *hlh-8(nr2061)* significantly lengthen the cycle. All the Exp mutants show an oscillation in cycle length, with the cycles getting progressively shorter as the worm becomes increasingly constipated (Con), which eventually produces a forceful expulsion, and is then followed by one or two longer cycles. This phenotype is revealed by the variability of the cycle length in individual animals scored for five cycles. This can be expressed as a per animal standard deviation. The average per animal standard deviations are as follows: N2: 0.89, *dsc-1:* 1.90, *exp-1:* 3.43, *unc-25:* 3.92 and *hlh-8*: 11.68. (B) Of the four Exp mutants surveyed, only the *dsc-1(qm133)* mutation can significantly suppress the lengthened defecation cycle of *clk-1(qm30)* mutants. The asterisks indicate the data that are significantly different from N2 (A) or *clk-1* (B). All significant differences detected were at a level of $P<0.0001$.

Although the cycle length of *dsc-1* mutants is affected by the absence of the expulsion step, several observations suggest that *dsc-1* has a role in cycle length regulation that is independent of its effect on the expulsion step of the cycle. The first observation relates to the fact that *dsc-1(qm133)* was identified in a screen for suppressors of the slow defecation cycle phenotype of *clk-1* mutants, in which we did not recover any other Exp mutant, although the screen may have begun to reach saturation (Branicky et al., 2001).

Secondly, we examined the defecation cycle lengths of other Exp mutants in both *clk-1(+)* and *clk-1(qm30)* backgrounds (Fig. 7). Our survey included *unc-25*, a mutant of GAD (Jin et al., 1999), *hlh-8*, a mutant that affects the development of the enteric muscles (Corsi et al., 2000), and *exp-1*, which, as described above, affects the enteric muscle contraction in response to GABA (Beg and Jorgensen, 2003). All mutants showed the oscillation in cycle length characteristic of Exp mutants (see legend of Fig. 7). We found that only *exp-1,* like *dsc-1,* significantly shortened the average cycle length in a wild-type background. The others (*unc-25* and *hlh-8*) significantly increased the average cycle length (Fig. 7A), which suggests that an expulsion defect per se does not necessarily lead to an average shortening of the defecation cycle length. Moreover, we found that, by contrast to what is observed with *dsc-1* mutants, none of the Exp mutants, including *exp-1*, could suppress the lengthened defecation cycle of *clk-1* mutants (Fig. 7B).

Thirdly, we observed that overexpression of *dsc-1* can significantly increase the defecation cycle length. The transgenic lines that express *dsc-1*, either under the control of the *dsc-1* promoter or the Nde-box, which presumably leads to overexpression, have significantly longer defecation cycle lengths than the wild type (Fig. 5C; Fig. 1B). As a loss of *dsc-1* activity results in a shortened defecation cycle length, this suggests that the level of *dsc-1(+)* expression is somehow involved in setting the defecation rate. A similar observation has been made with the *itr-1* gene, except that the effect is reversed (loss-of-function mutations slow down, whereas overexpression speeds up, the cycle) (Dal Santo et al., 1999).

Fourthly, we found that *dsc-1* can also significantly shorten the lengthened defecation cycle of the *itr-1(sa73)* mutant (data not shown). This again supports a role for *dsc-1* in cycle length regulation, and suggests that *dsc-1* affects the cycle length by a mechanism that is independent of the regulation that depends

on calcium signaling in the gut. However, given that *itr-1(sa73)* is not a null allele, potential interactions between *dsc-1* and *itr-1* need to be further investigated.

Discussion

The developmental roles of Paired-like homeobox genes

The Paired class of homeobox genes, which are related to the *Drosophila* gene *paired* (*prd*), can be divided into three sub-classes according to the residue at position 50 of the HD. The Pax or Prd-type genes are characterized by a serine at position 50 and also contain a second DNA-binding domain, the so-called Paired domain (PD). The other two Prd-like sub-classes lack the PD and have either a lysine (K_{50}) or a glutamine (Q_{50}) at position 50 (reviewed by Galliot et al., 1999). A number of orthologous gene families have been identified within each sub-class. For example, more than 15 orthologous families of K50 or Q50 Prd-like genes have been identified. In *C. elegans*, there are 13 Prd-like genes that fall into only eight of these families.

Caenorhabditis elegans Prd-like genes have mostly been studied for their roles in the nervous system and, with one exception, have all been shown to have roles in specifying neuron sub-type identity: that is, particular aspects of neuronal function such as choice of neurotransmitter synthesis, connectivity or sensory abilities. Briefly, *unc-30*, the worm Rx family ortholog, specifies the identities of 19 GABAergic neurons (Eastman et al., 1999; Jin et al., 1999). The worm Otx family orthologs *ttx-1, ceh-36* and *ceh-37* specify the identities of the ASH, the AWB and AWC, and the ASE sensory neurons, respectively (Lanjuin et al., 2003; Satterlee et al., 2001). *ceh-10* specifies the identity of the AIY interneuron (Altun-Gultekin et al., 2001; Svendsen and McGhee, 1995). *unc-4* specifies the identities of the VA motoneurons (Miller et al., 1992; White et al., 1992). *ceh-17*, a worm Prx family member, also functions in a subset of neurons, but appears to be required for axonal outgrowth rather than subtype identity (Pujol et al., 2000).

Remarkably, the function of some of these genes is precisely conserved in vertebrates. For example, vertebrate orthologs of *unc-30*, *ceh-36* and *ceh-37*, and *ceh-17* (Pitx2, Otx1 and Phox2b, respectively) are all expressed in cell types similar to those in which they are expressed in the worm, and can complement the mutant worm genes (Lanjuin et al., 2003; Pujol et al., 2000; Westmoreland et al., 2001). However, this does not mean that they are not involved in other processes as well. For example, Pitx2 is also involved in the development of the pituitary gland, craniofacial region, eyes, heart, abdominal viscera and limbs (Gage et al., 1999; Kitamura et al., 1999; Lin et al., 1999; Liu et al., 2001).

dsc-1 encodes a Q50 Prd-like protein, which does not appear to have any clear ortholog in any species but *C. briggsae*. It is expressed in a few sensory neurons (Fig. 4), as well as in the non-striated enteric muscles. We have determined that *dsc-1* is required in three of these muscles for the expression of *exp-1*, a GABA-gated cation channel. Our analysis suggests that, as in other systems, Prd-like genes in *C. elegans* may play key developmental roles in non-neuronal tissues. We speculate that other Prd-like genes in *C. elegans* might also be involved in specifying neurotransmitter sensitivity. Indeed, *unc-42*, another Prd-like gene in *C. elegans* that is without a clear vertebrate ortholog, appears to be required for regulating glutamate receptor expression in the nervous system (Baran et al., 1999).

Enteric muscle development and function in *C. elegans*

Muscles in worms are of two broad types. The main class of muscles is the striated muscles, the so-called 'body-wall muscles', which attach to the outer body wall and contract longitudinally for locomotion. The second class is the non-striated muscles, which are a diverse group of single-sarcomere muscles. These muscles include the pharyngeal muscles, which are used for feeding, the uterine and vulval muscles, which are used for egg-laying, the male-tail associated muscles, which are used for mating, and the enteric muscles we have discussed here (the AD, IM and sphincter), which are required for the expulsion part of the defecation behavior (www.wormatlas.org).

Only very few genes required for the development of the enteric muscles have been identified so far. The most extensively characterized is *hlh-8*, the *C. elegans* homolog of Twist, a basic helix-loop-helix (bHLH) transcription factor that has an evolutionarily conserved role in mesodermal patterning (Corsi et al., 2000; Harfe et al., 1998). *hlh-8* mutants have defects in the patterning of the post-embryonic mesodermal lineages, and in the development of the IM and AD muscles, which develop embryonically. One downstream target of *hlh-8* in the AD muscle is the NK-2 class homeobox gene *ceh-24*, whose expression is dependant on *hlh-8(+)* activity. However, *ceh-24* mutants do not appear to have expulsion defects (Harfe and Fire, 1998), suggesting that the activity of *ceh-24* is not necessary for the main aspects of muscle function. *hlh-8* is not required for the development of the sphincter muscle (Corsi et al., 2000). In fact, although a number of genes have been reported to be expressed in the sphincter (WormBase Release WS147, www.wormbase.org), none has been shown to be required for its development.

Here we have shown that *dsc-1* is required for an aspect of terminal differentiation of the AD and IM muscles; that is, the specification of their sensitivity to GABA. Like *hlh-8*, *dsc-1* does not appear to have a role in the sphincter, although it does contain some elements in its promoter that can drive its expression there. Indeed, the transcriptional *dsc-1::gfp* reporter is expressed exclusively in the sphincter (Fig. 4C); conversely, the translational reporter is expressed only in the AD and the IM (Fig. 4F). This suggests that there are elements in the *dsc-1* promoter that direct expression to the sphincter, while there are elements in the intronic and/or exonic regions that prevent expression in the sphincter but instead promote expression in the AD and IM. We speculate that *dsc-1* participates in a combinatorial code of expression that identifies all muscles that are involved in defecation as a group, but also ensures that *exp-1* is only expressed in the muscles that need to contract during defecation.

The roles of *dsc-1* in defecation

Two types of defecation mutants have been identified in *C. elegans*: those that affect the aBoc and/or Exp step, and those that affect periodicity. In general, these two classes of mutants are distinct, although a few mutants fall into both classes (e.g. the *flr* mutants and the *unc-43(gf)* mutant) (Reiner et al., 1999;

Take-Uchi et al., 1998; Take-Uchi et al., 2005). Mutants that affect both the aBoc and Exp steps (defining the *aex* genes) have general synaptic transmission defects (Doi and Iwasaki, 2002; Iwasaki et al., 1997). Similarly, there is a class of dominant Exp mutants, also egg-laying defective, that affect another general property, which is muscle excitability (Reiner et al., 1995). Most of the recessive Exp mutants reported are defective in either the function of the GABAergic DVB neuron (Hobert et al., 1999), the synthesis or transport of GABA (McIntire et al., 1993a; McIntire et al., 1993b), or the response of the enteric muscles to GABA (Bamber et al., 1999; Beg and Jorgensen, 2003). Consistently, we find that *dsc-1* is required for an aspect of GABA neurotransmission; that is, the expression of the appropriate GABA receptor in the IM and AD.

As described in the Introduction, a number of genes affecting the periodicity of the defecation cycle have been identified: in particular, *itr-1* (*dec-4/lef-1*), *elo-2*, *dsc-4*, *flr-1* and *flr-4*. Although these genes appear to affect diverse processes, all affect defecation by acting in the gut, and presumably by altering some of its properties (Dal Santo et al., 1999; Kniazeva et al., 2003; Shibata et al., 2003; Take-Uchi et al., 1998; Take-Uchi et al., 2005). By contrast, we have found that *dsc-1* modulates the defecation cycle length by acting in the enteric muscles. As the gut appears to control the rhythm of muscle contraction in this system, we speculate that *dsc-1* is required for a property of these muscles that allows them to act in a feedback mechanism from the muscles to the gut. One type of feedback mechanism that is operating in this system works through gut distention. Indeed, a lack of enteric muscle contractions leads to a progressive shortening of the defecation cycle until the contents of the gut are forcefully expulsed by pressure alone, and is then followed by a few longer cycles. We speculate that *dsc-1* is necessary for a feedback from the muscles that may involve a humoral signal that acts to coordinate the calcium signal generated in the gut with the timed contractions of the various muscles types. In this context, it is interesting to note that the *flr-1* and *flr-4* mutants, which have a shortened defecation cycle length, are also Exp. Furthermore, expression of both genes in the intestine alone is sufficient to rescue both the cycle length and the Exp defects. Possibly these genes could somehow be involved in transducing the putative humoral signal from the muscle to the gut.

Interestingly, we have found that *dsc-1* suppresses the lengthened defecation cycle of *clk-1(qm30)* mutants. Although *exp-1* is a target of *dsc-1* in the IM and AD muscle for the expulsion step of the defecation behavior, *dsc-1* probably acts through different targets for cycle length regulation. Indeed mutation of *exp-1* cannot suppress the lengthened defecation cycle of *clk-1*. The identification of the downstream effectors of *dsc-1* are likely to give new insights into the slow defecation phenotype of *clk-1* mutants and, more generally, into mechanisms that regulate the defecation cycle length in *C. elegans*.

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References

Altun-Gultekin, Z., Andachi, Y., Tsalik, E. L., Pilgrim, D., Kohara, Y. and Hobert, O. (2001). A regulatory cascade of three homeobox genes, *ceh-10*, *ttx-3* and *ceh-23*, controls cell fate specification of a defined interneuron class in *C. elegans*. *Development* **128**, 1951-1969.

Bamber, B. A., Beg, A. A., Twyman, R. E. and Jorgensen, E. M. (1999). The *Caenorhabditis elegans unc-49* locus encodes multiple subunits of a heteromultimeric GABA receptor. *J. Neurosci.* **19**, 5348-5359.

Baran, R., Aronoff, R. and Garriga, G. (1999). The *C. elegans* homeodomain gene *unc-42* regulates chemosensory and glutamate receptor expression. *Development* **126**, 2241-2251.

Beg, A. A. and Jorgensen, E. M. (2003). EXP-1 is an excitatory GABA-gated cation channel. *Nat. Neurosci.* **6**, 1145-1152.

Branicky, R., Shibata, Y., Feng, J. and Hekimi, S. (2001). Phenotypic and suppressor analysis of defecation in *clk-1* mutants reveals that reaction to changes in temperature is an active process in *Caenorhabditis elegans*. *Genetics* **159**, 997-1006.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* **77**, 71-94.

Brockie, P. J., Madsen, D. M., Zheng, Y., Mellem, J. and Maricq, A. V. (2001). Differential expression of glutamate receptor subunits in the nervous system of *Caenorhabditis elegans* and their regulation by the homeodomain protein UNC-42. *J. Neurosci.* **21**, 1510-1522.

Bulow, H. E., Berry, K. L., Topper, L. H., Peles, E. and Hobert, O. (2002). Heparan sulfate proteoglycan-dependent induction of axon branching and axon misrouting by the Kallmann syndrome gene *kal-1*. *Proc. Natl. Acad. Sci. USA* **99**, 6346-6351.

Corsi, A. K., Kostas, S. A., Fire, A. and Krause, M. (2000). *Caenorhabditis elegans* twist plays an essential role in non-striated muscle development. *Development* **127**, 2041-2051.

Dal Santo, P., Logan, M. A., Chisholm, A. D. and Jorgensen, E. M. (1999). The inositol trisphosphate receptor regulates a 50-second behavioral rhythm in *C. elegans*. *Cell* **98**, 757-767.

Dattani, M. T., Martinez-Barbera, J. P., Thomas, P. Q., Brickman, J. M., Gupta, R., Martensson, I. L., Toresson, H., Fox, M., Wales, J. K., Hindmarsh, P. C. et al. (1998). Mutations in the homeobox gene HESX1/Hesx1 associated with septo-optic dysplasia in human and mouse. *Nat. Genet.* **19**, 125-133.

Doi, M. and Iwasaki, K. (2002). Regulation of retrograde signaling at neuromuscular junctions by the novel C2 domain protein AEX-1. *Neuron* **33**, 249-259.

Eastman, C., Horvitz, H. R. and Jin, Y. (1999). Coordinated transcriptional regulation of the *unc-25* glutamic acid decarboxylase and the *unc-47* GABA vesicular transporter by the *Caenorhabditis elegans* UNC-30 homeodomain protein. *J. Neurosci.* **19**, 6225-6234.

Ewbank, J. J., Barnes, T. M., Lakowski, B., Lussier, M., Bussey, H. and Hekimi, S. (1997). Structural and functional conservation of the *Caenorhabditis elegans* timing gene *clk-1*. *Science* **275**, 980-983.

Ferda Percin, E., Ploder, L. A., Yu, J. J., Arici, K., Horsford, D. J., Rutherford, A., Bapat, B., Cox, D. W., Duncan, A. M., Kalnins, V. I. et al. (2000). Human microphthalmia associated with mutations in the retinal homeobox gene CHX10. *Nat. Genet.* **25**, 397-401.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E. and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**, 806-811.

Gage, P. J., Suh, H. and Camper, S. A. (1999). Dosage requirement of Pitx2 for development of multiple organs. *Development* **126**, 4643-4651.

Galliot, B., de Vargas, C. and Miller, D. (1999). Evolution of homeobox genes: Q50 Paired-like genes founded the Paired class. *Dev. Genes Evol.* **209**, 186-197.

Harfe, B. D. and Fire, A. (1998). Muscle and nerve-specific regulation of a novel NK-2 class homeodomain factor in *Caenorhabditis elegans*. *Development* **125**, 421-429.

Harfe, B. D., Vaz Gomes, A., Kenyon, C., Liu, J., Krause, M. and Fire, A.

(1998). Analysis of a Caenorhabditis elegans Twist homolog identifies conserved and divergent aspects of mesodermal patterning. *Genes Dev*. **12**, 2623-2635.

Hobert, O. (2002). PCR fusion-based approach to create reporter gene constructs for expression analysis in transgenic C. elegans. *Biotechniques* **32**, 728-730.

Hobert, O., Tessmar, K. and Ruvkun, G. (1999). The *Caenorhabditis elegans lim-6* LIM homeobox gene regulates neurite outgrowth and function of particular GABAergic neurons. *Development* **126**, 1547-1562.

Iwasaki, K., Liu, D. W. and Thomas, J. H. (1995). Genes that control a temperature-compensated ultradian clock in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* **92**, 10317-10321.

Iwasaki, K., Staunton, J., Saifee, O., Nonet, M. and Thomas, J. H. (1997). aex-3 encodes a novel regulator of presynaptic activity in *C. elegans*. *Neuron* **18**, 613-622.

Jiang, G., Zhuang, L., Miyauchi, S., Miyake, K., Fei, Y. J. and Ganapathy, V. (2004). A Na+/Cl–coupled GABA transporter, GAT-1, from *Caenorhabditis elegans*: Structural and functional features, specific expression in GABAergic neurons, and involvement in muscle function. *J. Biol. Chem.* **280**, 2065-2077.

Jin, Y., Hoskins, R. and Horvitz, H. R. (1994). Control of type-D GABAergic neuron differentiation by *C. elegans* UNC-30 homeodomain protein. *Nature* **372**, 780-783.

Jin, Y., Jorgensen, E., Hartwieg, E. and Horvitz, H. R. (1999). The *Caenorhabditis elegans* gene *unc-25* encodes glutamic acid decarboxylase and is required for synaptic transmission but not synaptic development. *J. Neurosci.* **19**, 539-548.

Katsura, I., Kondo, K., Amano, T., Ishihara, T. and Kawakami, M. (1994). Isolation, characterization and epistasis of fluoride-resistant mutants of *Caenorhabditis elegans*. *Genetics* **136**, 145-154.

Kissinger, C. R., Liu, B. S., Martin-Blanco, E., Kornberg, T. B. and Pabo, C. O. (1990). Crystal structure of an engrailed homeodomain-DNA complex at 2.8 A resolution: a framework for understanding homeodomain-DNA interactions. *Cell* **63**, 579-590.

Kitamura, K., Miura, H., Miyagawa-Tomita, S., Yanazawa, M., Katoh-Fukui, Y., Suzuki, R., Ohuchi, H., Suehiro, A., Motegi, Y., Nakahara, Y. et al. (1999). Mouse Pitx2 deficiency leads to anomalies of the ventral body wall, heart, extra- and periocular mesoderm and right pulmonary isomerism. *Development* **126**, 5749-5758.

Kniazeva, M., Sieber, M., McCauley, S., Zhang, K., Watts, J. L. and Han, M. (2003). Suppression of the ELO-2 FA elongation activity results in alterations of the fatty acid composition and multiple physiological defects, including abnormal ultradian rhythms, in *Caenorhabditis elegans*. *Genetics* **163**, 159-169.

Lanjuin, A., VanHoven, M. K., Bargmann, C. I., Thompson, J. K. and Sengupta, P. (2003). *Otx/otd* homeobox genes specify distinct sensory neuron identities in *C. elegans*. *Dev. Cell* **5**, 621-633.

Lin, C. R., Kioussi, C., O'Connell, S., Briata, P., Szeto, D., Liu, F., Izpisua-Belmonte, J. C. and Rosenfeld, M. G. (1999). Pitx2 regulates lung asymmetry, cardiac positioning and pituitary and tooth morphogenesis. *Nature* **401**, 279-282.

Liu, C., Liu, W., Lu, M. F., Brown, N. A. and Martin, J. F. (2001). Regulation of left-right asymmetry by thresholds of Pitx2c activity. *Development* **128**, 2039-2048.

Liu, D. W. and Thomas, J. H. (1994). Regulation of a periodic motor program in *C. elegans*. *J. Neurosci.* **14**, 1953-1962.

Liu, L. X., Spoerke, J. M., Mulligan, E. L., Chen, J., Reardon, B., Westlund, B., Sun, L., Abel, K., Armstrong, B., Hardiman, G. et al. (1999). High-throughput isolation of Caenorhabditis elegans deletion mutants. *Genome Res.* **9**, 859-867.

Mathews, E. A., Garcia, E., Santi, C. M., Mullen, G. P., Thacker, C., Moerman, D. G. and Snutch, T. P. (2003). Critical residues of the *Caenorhabditis elegans unc-2* voltage-gated calcium channel that affect behavioral and physiological properties. *J. Neurosci.* **23**, 6537-6545.

McIntire, S. L., Jorgensen, E. and Horvitz, H. R. (1993a). Genes required for GABA function in *Caenorhabditis elegans*. *Nature* **364**, 334-337.

McIntire, S. L., Jorgensen, E., Kaplan, J. and Horvitz, H. R. (1993b). The GABAergic nervous system of *Caenorhabditis elegans*. *Nature* **364**, 337-341.

McIntire, S. L., Reimer, R. J., Schuske, K., Edwards, R. H. and Jorgensen, E. M. (1997). Identification and characterization of the vesicular GABA transporter. *Nature* **389**, 870-876.

Miller, D. M., Shen, M. M., Shamu, C. E., Burglin, T. R., Ruvkun, G., Dubois, M. L., Ghee, M. and Wilson, L. (1992). *C. elegans unc-4* gene encodes a homeodomain protein that determines the pattern of synaptic input to specific motor neurons. *Nature* **355**, 841-845.

Pujol, N., Torregrossa, P., Ewbank, J. J. and Brunet, J. F. (2000). The homeodomain protein CePHOX2/CEH-17 controls antero-posterior axonal growth in *C. elegans*. *Development* **127**, 3361-3371.

Rankin, C. H. (2002). From gene to identified neuron to behaviour in *Caenorhabditis elegans*. *Nat. Rev. Genet.* **3**, 622-630.

Reiner, D. J. and Thomas, J. H. (1995). Reversal of a muscle response to GABA during *C. elegans* male development. *J. Neurosci.* **15**, 6094-6102.

Reiner, D. J., Weinshenker, D. and Thomas, J. H. (1995). Analysis of dominant mutations affecting muscle excitation in *Caenorhabditis elegans*. *Genetics* **141**, 961-976.

Reiner, D. J., Newton, E. M., Tian, H. and Thomas, J. H. (1999). Diverse behavioural defects caused by mutations in *Caenorhabditis elegans unc-43* CaM kinase II. *Nature* **402**, 199-203.

Satterlee, J. S., Sasakura, H., Kuhara, A., Berkeley, M., Mori, I. and Sengupta, P. (2001). Specification of thermosensory neuron fate in C. elegans requires *ttx-1*, a homolog of *otd/Otx*. *Neuron* **31**, 943-956.

Shibata, Y., Branicky, R., Landaverde, I. O. and Hekimi, S. (2003). Redox regulation of germline and vulval development in *Caenorhabditis elegans*. *Science* **302**, 1779-1782.

Svendsen, P. C. and McGhee, J. D. (1995). The *C. elegans* neuronally expressed homeobox gene *ceh-10* is closely related to genes expressed in the vertebrate eye. *Development* **121**, 1253-1262.

Take-Uchi, M., Kawakami, M., Ishihara, T., Amano, T., Kondo, K. and Katsura, I. (1998). An ion channel of the degenerin/epithelial sodium channel superfamily controls the defecation rhythm in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* **95**, 11775-11780.

Take-Uchi, M., Kobayashi, Y., Kimura, K. D., Ishihara, T. and Katsura, I. (2005). FLR-4, a Novel Serine/Threonine Protein Kinase, Regulates Defecation Rhythm in *Caenorhabditis elegans*. *Mol. Biol. Cell.* **3**, 1355-1365

Thomas, J. H. (1990). Genetic analysis of defecation in *Caenorhabditis elegans*. *Genetics* **124**, 855-872.

Westmoreland, J. J., McEwen, J., Moore, B. A., Jin, Y. and Condie, B. G. (2001). Conserved function of Caenorhabditis elegans UNC-30 and mouse Pitx2 in controlling GABAergic neuron differentiation. *J. Neurosci.* **21**, 6810-6819.

White, J. G., Southgate, E. and Thomson, J. N. (1992). Mutations in the *Caenorhabditis elegans unc-4* gene alter the synaptic input to ventral cord motor neurons. *Nature* **355**, 838-841.

Whittaker, A. J. and Sternberg, P. W. (2004). Sensory processing by neural circuits in Caenorhabditis elegans. *Curr. Opin. Neurobiol.* **14**, 450-456.

Wilson, D. S., Guenther, B., Desplan, C. and Kuriyan, J. (1995). High resolution crystal structure of a paired (Pax) class cooperative homeodomain dimer on DNA. *Cell* **82**, 709-719.

Winnier, A. R., Meir, J. Y., Ross, J. M., Tavernarakis, N., Driscoll, M., Ishihara, T., Katsura, I. and Miller, D. M., 3rd (1999). UNC-4/UNC-37-dependent repression of motor neuron-specific genes controls synaptic choice in *Caenorhabditis elegans*. *Genes Dev.* **13**, 2774-2786.

Wong, A., Boutis, P. and Hekimi, S. (1995). Mutations in the *clk-1* gene of *Caenorhabditis elegans* affect developmental and behavioral timing. *Genetics* **139**, 1247-1259.