The C. elegans even-skipped homologue, vab-7, specifies DB motoneurone identity and axon trajectory

Behrooz Esmaeili¹, Jennifer M. Ross²,*, Cara Neades¹, David M. Miller, Ill² and Julie Ahringer¹,†

¹Wellcome CRC Institute and Department of Genetics, University of Cambridge, Tennis Court Road, Cambridge CB2 1QR, UK
²Department of Cell Biology, Vanderbilt University Medical Center, Nashville, TN 37232-2175, USA
*Present address: University of Minnesota, 6-160 Jackson Hall, 321 Church Street SE, Minneapolis, MN 55455, USA
†Author for correspondence (e-mail: jaa@mole.bio.cam.ac.uk)

SUMMARY

Locomotory activity is defined by the specification of motoneurone subtypes. In the nematode, C. elegans, DA and DB motoneurones innervate dorsal muscles and function to induce movement in the backwards or forwards direction, respectively. These two neurone classes express separate sets of genes and extend axons with oppositely directed trajectories; anterior (DA) versus posterior (DB). The DA-specific homeoprotein UNC-4 interacts with UNC-37/Groucho to repress the DB gene, acr-5 (nicotinic acetylcholine receptor subunit). We show that the C. elegans even-skipped-like homeodomain protein, VAB-7, coordinate regulates different aspects of the DB motoneurone fate, in part by repressing unc-4. Wild-type DB motoneurones express VAB-7, have posteriorly directed axons, express ACR-5 and lack expression of the homeodomain protein UNC-4. In a vab-7 mutant, ectopic UNC-4 represses acr-5 and induces an anteriorly directed DB axon trajectory. Thus, vab-7 indirectly promotes DB-specific gene expression and posteriorly directed axon outgrowth by preventing UNC-4 repression of DB differentiation. Ectopic expression of VAB-7 also induces DB traits in an unc-4-independent manner, suggesting that VAB-7 can act through a parallel pathway. This work supports a model in which a complementary pair of homeodomain transcription factors (VAB-7 and UNC-4) specifies differences between DA and DB neurones through inhibition of the alternative fates. The recent findings that Even-skipped transcriptional repressor activity specifies neurone identity and axon guidance in the mouse and Drosophila motoneurone circuit points to an ancient origin for homeoprotein-dependent mechanisms of neuronal differentiation in the metazoan nerve cord.

Key words: C. elegans, vab-7, Motoneurone identity, Axons

INTRODUCTION

The nerve cords of organisms as diverse as nematodes, flies and humans embrace a common architecture with axial arrays of motoneurones distributed along midline bundles of neuronal processes (Hedgecock and Hall, 1990; Eisen, 1998; Jurata et al., 2000). In each case, locomotion depends on the coordinated activities of distinct classes of motoneurones, the interneurones that regulate them and the muscles that they innervate. The proper function of each neuronal subtype is defined by the adoption of appropriate axonal trajectory, neurotransmitter expression and synaptic connectivity. What are the mechanisms that regulate these specific traits? Recent evidence indicates that homeodomain (HD) proteins exercise key roles in the specification of cell type identity in the motoneurone circuit. Hierarchical cascades of interacting HD proteins segregate the vertebrate spinal cord into distinct progenitor domains (Briscoe et al., 2000; Muhr et al., 2001). Subsequently expressed HD proteins may induce the differentiation of specific interneurone and motoneurone subclasses within each of these regions (Tanabe et al., 1998; Jessell, 2000). The roles of many of these transcription factors appear to have been evolutionarily conserved. For example, the LIM class of HD proteins has been shown to govern axonal trajectory and targeting in both vertebrate and invertebrate motoneurone networks (Hobert and Westphal, 2000).

Three motoneurone subtypes, DA, DB and DD, are incorporated into the C. elegans ventral nerve cord (VNC) during embryonic development (Sulston, 1983; Sulston et al., 1983). Five additional subclasses of postembryonic motoneurones (VA, VB, VC, AS, VD) are added in the first larval stage (Sulston and Horvitz, 1977). These motoneurones are grouped on the basis of common morphological characteristics, neurotransmitter expression and presynaptic specificity (White et al., 1986). HD transcription factors that regulate subsets of these traits have been identified. The UNC-30 HD protein functions in DD and VD (A-class) motoneurones where it promotes expression of GABA pathway components and is required for normal neuronal morphogenesis (Jin et al., 1994). The LIM-HD protein, LIN-11, is expressed in postmitotic VC motoneurones and mediates VC motor axon fasciculation in the ventral nerve cord (VNC) (Hobert et al., 1998). The UNC-4 HD protein is expressed in A-class motoneurones (DA, VA) to prevent the adoption of B-
class (DB, VB) traits (Miller and Niemeyer, 1995). In unc-4 mutants, A-class motoneurones are morphologically normal but express a B-type nicotinic acetylcholine receptor (nACHR) subunit, act-5 (Winnier et al., 1999). In addition, mutations that disrupt unc-4 function result in the miswiring of VA motoneurones with presynaptic inputs normally reserved for B-class motoneurones (White et al., 1992). The dependence of these UNC-4 activities on physical interaction with the Groucho-like transcriptional co-repressor protein, UNC-37, indicates that UNC-4 is likely to function as a negative regulator of B-class genes (Pflugrad et al., 1997; Winnier et al., 1999).

We show that the C. elegans Even-skipped homologue, VAB-7, is expressed in DB class motoneurones where it functions as a negative regulator of A-class traits. In vab-7 mutants, ectopic UNC-4 in DB motoneurones results in the adoption of DA type axonal trajectory and repression of the B-class act-5 gene. Ectopic expression experiments indicate that vab-7 may also promote expression of B-class genes through a parallel pathway that does not depend on unc-4 function. These findings indicate that the proper differentiation of DA and DB motoneurones depends on HD transcription factors and DB motoneurones with presynaptic inputs normally reserved for VA motoneurones. The dependence of proper differentiation of DA motoneurones on HD transcription factors and DB motoneurones with presynaptic inputs normally reserved for VA motoneurones depends on HD transcription factors and DB motoneurones with presynaptic inputs normally reserved for VA motoneurones. The dependence of proper differentiation of DA motoneurones on HD transcription factors and DB motoneurones with presynaptic inputs normally reserved for VA motoneurones.

**MATERIALS AND METHODS**

**Strains**

Strains were grown and maintained as described by (Brenner, 1974). The following strains were used in this study: vab-7(e1562), unc-4(e120), unc-37(e262); NC120, dpy-20(e1282); wds1[unc-4::lacZ dpy-20(+)]; JA1234, vab-7(e1562); evls82 [unc-129::gfp dpy-20(+)]; JA1236, unc-4(e120); vab-7(e1562); evls82; JA1201, unc-37(e262); evls82; JA1262, unc-37(e262); evls82; JA1276, unc-4(e120); evls82; JA1278, evls82; weEx43[unc-3::vab-7 rol-6 (d)]; JA1299, dpy-20(e1282); wds10[unc-3::vab-7 unc-17::gfp dpy-20(+)]; JA1301, unc-129(eve554); wds10; RM1872, pha-1(e12123); mdEx72[unc-17::gfp pha-1(+)]; JA1303, vab-7(e1562); mdEx72; JA1304, unc-4(e120); mdEx72; JA1305, wds10; JA1313, weEx52[acr-5::gfp::lacZ rol-6(d)]; JA1314, wds10[unc-5(e1562); JA1316, vab-7(e1562); weEx54[vab-7::gfp vab-7 rol-6 (d)]; NW1999, dpy-20(e1282); evls82; NC257, dpy-20(e1282); weEx6[unc-4::gfp dpy-20(+)]; NC244, vab-7(e1562); dpy-20(e1282); weEx6[acr-5::gfp dpy-20(+)]; NC241, unc-4(e120); vab-7(e1562); dpy-20(e1282); wds60; NC237, unc-37(e262); vab-7(e1562); dpy-20(e1282); wds60.

**Plasmid constructs**

To construct unc-3::vab-7, the vab-7 promoter was removed from the pJA17 vab-7-rescuing plasmid (Ahringer, 1996) by SacII and Spcl digestion. A 4.14kb DNA fragment containing putative unc-3 promoter to the beginning of exon 2 was amplified by PCR from the pPB6-1 plasmid (Prasad et al., 1998) using UNC-3P4 (5'-AAACTGACCCCAGGGCGATGGCCTGAGGTCGAC-3') and UNC-3P2 (5'-TTCTGGACAGCGCCACCTGAGAAGTATCTC-3') primers, cut with SacII and Spcl and cloned into pJA17/SacI/SphI to create plasmid pJA55. The UNC-3::VAB-7 protein product will contain the first 30 amino acids of UNC-3 upstream of VAB-7, and an extra glycine residue at the fusion site that was created to allow in-frame cloning. This unc-3::vab-7 construct rescues the forward movement defect of vab-7 mutants (not shown). The acr-5::gfp::lacZ plasmid pJA63 was constructed by subcloning a 4.2kb Spcl fragment containing the acr-5 promoter (from pJR7) (Winnier et al., 1999) into the gfp::lacZ expression plasmid pPD96.62.

**Generation of transgenic lines**

DNA microinjection experiments were performed as previously described (Mello and Fire, 1995). weEx43 was generated by injection of wild-type hermaphrodites with 100 μg/ml of pRF4 [rol-6(d)] (Mello et al., 1991) and 30 μg/ml of pJA55 (unc-3::vab-7). The weIs10 transgene was made by integration of an extrachromosomal array generated by injection of 30 μg/ml pJA55 (unc-3::vab-7) and 100 μg/ml pMH86 (dpy-20(+)); (Clark et al., 1995) into a dpy-20(e1282) background. The weIs10 line was integrated as described by Mello and Fire (Mello and Fire, 1995) and was out crossed three times before analysis. weEx52 (acr-5::gfp::lacZ + rol-6(d)) was generated by injection of 30 μg/ml pJA63 and 100 μg/ml pRF4. β-Galactosidase staining was performed as previously described (Fire et al., 1990).

**Generation of anti-VAB-7**

A fragment containing the entire vab-7-coding region was subcloned into the His tag vector pKSETB (Clontech) to create plasmid pJA18. Protein was purified on a nickel column after denaturation. A mouse was injected seven times over a period of 11 months with 15 μg of His-tagged VAB-7 in Freund’s complete adjuvant, followed by intravenous injection of 10 μg protein in phosphate-buffered saline (PBS). The animal was sacrificed 4 days later and the spleen frozen. After fusion of spleen cells, one monoclonal line (2C4) that gave bright staining with no background was obtained.

**Immunostaining of embryos, larvae and adults**

Immunostaining experiments were performed as follows: embryos isolated by hypochlorite treatment were placed on a poly-lysine coated slide, squashed under a coverslip and frozen on dry ice for 10 minutes. After freezing, the coverslip was flicked off, and 100 μl of 5% formaldehyde in PBS placed on the sample for 20 minutes in a humid chamber. After incubation, the slides were immediately placed in 100% methanol for 4 minutes, in PBS with 0.2% Tween (PBST) for 4 minutes, blocked in 1% non-fat milk in PBST for 10 minutes, then placed in PBS for 10 minutes. Monoclonal anti-VAB-7 primary antibody (from cell culture supernatant) or anti-β-galactosidase antibody (Cappel) was incubated overnight at 4°C; secondary antibodies (FITC anti-mouse or Texas Red Amersham) were incubated for 1-2 hours at room temperature. Samples were mounted using mowiol. For staining of larvae and adults, worms were washed four times in 15 ml distilled water in 15 ml centrifuge tubes before they were placed on poly-lysine-coated slides for adhesion, and then treated as above.

**Identification of neurons in the VNC**

Identities of neurons in the ventral cord were assigned based on the position of their nuclei in the VNC, their commissures and their axonal processes in the DVC (Sulston, 1983; Sulston et al., 1983; White et al., 1986). Identification of postembryonic neurons expressing VAB-7 was aided by the unc-129::gfp marker, which is expressed in DA and DB motoneurones (Calavita et al., 1998). Non-
DB motoneurones were identified by comparing DAPI and unc-129::gfp staining data with that of White et al. (White et al., 1986). For identification of neurones with ectopic ACR-5::GFPLACZ in wels10;weEx52 (acr-5::gfp lacZ) animals, the unc-17::gfp neuronal marker which is expressed in DA, DB, AS, VA, VB and VC motoneurones in the VNC was used (Lickteig et al., 2001) (Rand et al., 2000).

RESULTS

VAB-7 is expressed in DB motoneurones

vab-7 mutants exhibit defects in forward locomotion, as well as in the patterning of posterior muscle and epidermal cells (Ahringer, 1996). vab-7 mutant L1 larvae are virtually immobile. Beginning with the second larval stage, after post-embryonically derived motoneurones have been added to the ventral cord circuit, vab-7 mutants show normal backwards movement but curl ventrally when induced to move forwards. These findings indicate that vab-7 may be important for embryonic motoneurone development. However, previous work using a vab-7::lacZ reporter gene did not detect expression in the nervous system; only posterior muscle and epidermal expression was seen (Ahringer, 1996).

To discover whether VAB-7 is expressed in the nervous system, a mouse monoclonal antibody was generated against VAB-7 recombinant protein. Staining embryos with this anti-VAB-7 antibody confirmed that VAB-7 is expressed in posterior muscle and epidermal cells (Fig. 1A,B). However, we also discovered a second phase of VAB-7 expression in embryonic ventral nerve cord (VNC) neurones, beginning at the 1.5-fold stage (Fig. 1C). At the threefold stage VAB-7 is expressed in nine neuronal nuclei (seven in the VNC and two nuclei in the head), and in the five most posterior epidermal nuclei, which form the hyp8 to hyp11 cells (Fig. 1C-E). The vab-7(e1562) allele [which introduces an early stop codon (Ahringer, 1996)] appears to be null, as VAB-7 is not detectable in these embryos (data not shown).

Three classes of motoneurones (DA, DB and DD) are present in the embryonic VNC; DAs and DBs are excitatory cholinergic motoneurones that stimulate dorsal muscles (Fig. 2A). The DDs are GABAergic neurones (McIntire et al., 1993) that are believed to coordinate body bending by inhibiting bodywall muscles on the side opposite to a region of cholinergic motoneurone excitation (i.e. dorsal versus ventral). All three of these motoneurone classes have cell bodies and processes in the VNC, commissures that travel around the body to the dorsal side, and processes in the dorsal nerve cord (DNC) (White et al., 1986). To identify the VAB-7-expressing neurones in the VNC, we compared VAB-7 antibody staining with that of two neuronal markers, unc-25::lacZ and unc-4::lacZ, that are expressed in DD and DA motoneurones, respectively (Miller and Niemeyer, 1995; Jin et al., 1999). These experiments showed that VAB-7 is expressed in the seven DB class motoneurones (Fig. 1D and data not shown); VAB-7 staining is also seen in two additional head neurones that have not been identified. DB motoneurones are required for normal forward movement (Chalifie et al., 1985); therefore, the lack of vab-7 expression in the DBs could explain the forward locomotory defect of vab-7 mutants.

DB neurones express VAB-7 throughout development (Fig. 1 and data not shown). In addition, we found that VAB-7 is expressed continuously in the hypodermal syncitium hyp10, and from the L1 stage in four unidentified neurones in the tail. Finally, at the adult stage, VAB-7 is detected in three VC neurones: VC1, VC2 and VC6 (data not shown).

DB motoneurones exhibit reversed axonal polarity in vab-7 mutants

To determine if the lack of vab-7 expression perturbs the generation of DB motoneurones, we viewed DB neurones using unc-129::gfp, a promoter fusion that drives GFP expression in DA and DB cell bodies and processes (Colavita et al., 1998). We found that all DB neurones are generated in vab-7 mutants (compare Fig. 2B with 2C), indicating that vab-7 is not required for the production of these cells. In addition, two other DB characteristics are normal in vab-7 mutants. First, each DB commissure (White et al., 1986) travels around the body on the correct side (i.e. right or left) to reach the dorsal nerve cord (data not shown). Second, as in wild-type, DB neurones express unc-17/cha-1::gfp (see Fig. 6B,D and data not shown), a marker for acetylcholine production (Lickteig et al., 2001) (Rand et al., 2000).

The above findings showed that DB neurones are present in vab-7 mutants and that they retain at least some of the key characteristics of excitatory motoneurones. We next examined their axonal polarity, a DB-specific fate. In wild-type animals, DB neurones extend posteriorly directed processes in the VNC and in the DNC; DA neurones adopt a similar structure, but send out axons (DNC) and dendrites (VNC) with the opposite or anteriorly directed trajectory (Fig. 2A,D; Table 1) (White et al., 1986). Strikingly, in vab-7 mutants, the axons of DB neurones, like the DAs, turn anterior rather than posterior when

![Fig. 1. VAB-7 is expressed in DB motoneurones, as well as in posterior mesodermal and epidermal precursors. Immunostaining of VAB-7 in (A) 100 cell stage embryo and (B) bean stage embryo is in mesodermal and epidermal precursors. (C) Two-fold embryo. VAB-7 is found in five posterior epidermal cells (hyp8-hyp11; arrowhead), seven DB motoneurones in the ventral nerve cord (arrow) and two unidentified neurones in the head. One DB and the two neurones in the head are not visible in this image. (D) A threefold embryo showing VAB-7 (green) and unc-25::lacZ, a DD motoneurone marker (red). (E) An L1 larva showing expression of VAB-7 in all seven DB motoneurones. Anterior is towards the left. Scale bar: 10 μm.](image-url)
they enter the DNC (Fig. 2E; Table 1). DA motor axon trajectories are not perturbed. Therefore, vab-7 is required for proper (posterior) DB axonal polarity.

The congruent polarities of DA and DB motor axons in the dorsal nerve cord of vab-7(e1562) animals caused us to examine the exit trajectories of commissures emanating from DA and DB soma in the ventral nerve cord. In the wild type, DA commissures exit the soma in a posterior direction, whereas DB commissures project anteriorly from the cell body (White et al., 1986). In vab-7(e1562) mutant animals, most of the DB commissures adopt the posterior trajectory normally reserved for DA motor axons in the ventral nerve cord (Table 2). Thus, DB neurones assume both the posterior exit trajectory of DA commissures in the ventral nerve cord as well as the anterior polarity of DA motor axons in the dorsal nerve cord.

The reversed axonal polarity of the DBs is due to ectopic UNC-4 expression

The finding that DB motoneurones adopt the axonal morphology of DA motoneurones in vab-7 mutants indicated that DBs might also adopt other DA-specific traits. We investigated this possibility by examining the expression of unc-4::gfp in vab-7(e1562) mutants. unc-4 encodes a homeodomain protein (Miller et al., 1992) that is expressed in the DAs embryonically (Fig. 3A), and in two other classes of ventral cord motoneurones (VA and VC) post-embryonically (Miller and Niemeyer, 1995; Lickteig et al., 2001). Examination of vab-7(e1562) animals showed ectopic expression of unc-4::gfp in the DBs (Fig. 3B; Table 3). This result indicates that vab-7 represses unc-4 in the DBs, either directly or indirectly. By contrast, unc-4 does not appear to regulate vab-7 as VAB-7 expression is normal in unc-4 mutants (data not shown).

Is ectopic unc-4 expression in the DBs responsible for the reversal of DB axonal polarity in vab-7 mutants? If it is, then the removal of unc-4 activity from a vab-7 mutant background
vab-7 specifies motoneurone identity

should restore normal polarity. To test this model, we examined DB polarity in unc-4; vab-7 double mutants. In this mutant background, DB motoneurones adopt the wild-type (posterior) polarity (Fig. 2F; Table 1). Therefore, ectopic expression of unc-4 in the DBs reverses their axonal polarity from posterior to anterior in vab-7 mutants.

UNC-4 function depends on physical interaction with UNC-37, a ubiquitously expressed Groucho-like transcriptional co-repressor (Miller et al., 1993; Pflugrad et al., 1997). Furthermore, the UNC-4/UNC-37 complex is known to function as a negative regulator of DB and VB motoneurone-specific genes (Winnier et al., 1999). We used the missense allele, unc-37(e262), to determine whether the UNC-4-induced reversal of DB axonal polarity also requires wild-type UNC-37 activity. As shown Fig. 2G, DB axonal polarity is restored to its normal posterior trajectory in unc-37(e262); vab-7(e1562) mutant animals (Table 1). This finding indicates that the UNC-4 is likely to function as a negative regulator of DB genes that direct posterior axonal outgrowth and that this repression is sufficient to impose an anterior trajectory in vab-7 mutant animals.

vab-7 is required for acr-5 expression in DB motoneurones

acr-5 encodes an acetylcholine receptor subunit that is normally expressed in B-class (DB, VB) but not in A-class (DA, VA) motoneurones (Winnier et al., 1999) (Fig. 4A). In

### Table 1. Percentage of neurones with wild-type axonal polarity

<table>
<thead>
<tr>
<th>Genotype</th>
<th>DA (anterior)</th>
<th>DB (posterior)</th>
<th>AS (anterior)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>99% (247)</td>
<td>100% (207)</td>
<td>100% (60)</td>
<td>50</td>
</tr>
<tr>
<td>vab-7(e1562)</td>
<td>99% (200)</td>
<td>18% (161)</td>
<td>100% (59)</td>
<td>65</td>
</tr>
<tr>
<td>unc-4(e120)</td>
<td>100% (50)</td>
<td>100% (50)</td>
<td>100% (44)</td>
<td>22</td>
</tr>
<tr>
<td>vab-7(e1562); unc-4(e120)</td>
<td>100% (77)</td>
<td>100% (67)</td>
<td>nd</td>
<td>21</td>
</tr>
<tr>
<td>unc-37(e262)</td>
<td>100% (50)</td>
<td>100% (50)</td>
<td>nd</td>
<td>10</td>
</tr>
<tr>
<td>vab-7(e1562); unc-37(e262)</td>
<td>100% (150)</td>
<td>100% (150)</td>
<td>nd</td>
<td>30</td>
</tr>
<tr>
<td>wels10 (unc-3; vab-7)</td>
<td>22% (91)</td>
<td>99% (80)</td>
<td>6% (102)</td>
<td>25</td>
</tr>
<tr>
<td>vab-7(e1562)</td>
<td>nd</td>
<td>100% (30)</td>
<td>nd</td>
<td>15</td>
</tr>
<tr>
<td>weIs10 (unc-3::vab-7)</td>
<td>nd</td>
<td>100% (30)</td>
<td>nd</td>
<td>15</td>
</tr>
<tr>
<td>weIs10 (unc-3::vab-7)</td>
<td>nd</td>
<td>100% (30)</td>
<td>nd</td>
<td>15</td>
</tr>
</tbody>
</table>

Percentages refer to wild-type axonal polarities; the remainder had the opposite polarity (e.g. 82% of DB neurones of vab-7(e1562) animals had anterior, rather than posterior polarity).

n, the number of animals examined; the numbers of neurones scored are in parentheses; nd, not determined.

Dorsal axonal polarities were visualized with either evIs82 (unc-129::gfp) (DA and DB axons) or mdEx72 (unc-17::gfp) (DA, DB and AS axons). wels10 refers to the integrated unc-3::vab-7; unc-17::gfp strain.

### Table 2. DB motor axons adopt DA axonal polarity in the ventral nerve cord

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Motoneurone</th>
<th>Wild type</th>
<th>vab-7(e1562)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DA</td>
<td>100% (100/100)</td>
<td>100% (100/100)</td>
<td></td>
</tr>
<tr>
<td>DB</td>
<td>100% (80/80)</td>
<td>7% (3/43)</td>
<td></td>
</tr>
<tr>
<td>DB</td>
<td>0% (80/80)</td>
<td>93% (40/43)</td>
<td></td>
</tr>
</tbody>
</table>

DA and DB axonal phenotypes were scored in L3 and L4 larvae with the unc-129::gfp marker.

Schematic drawings show the polarity of axonal exits in the VNC as well as trajectories of axonal processes in the DNC.

In the wild type, commissures tend to exit posteriorly from the DA cell body, whereas commissures exit anteriorly from the DB soma.

DA and DB motoneurones for which the exit trajectory could not be reliably scored are depicted as motoneurone soma with vertically projecting commissures.

Twenty animals were scored in each case.

Numbers in parentheses refer to number of neurones with the indicated morphology/number of neurones examined.

### Fig. 3. vab-7 represses unc-4 in DB motoneurones. (A) Wild-type expression of unc-4::GFP in DA motoneurones of an L1 larva. (B) unc-4::gfp is ectopically expressed in the DBs in a vab-7(e1562) background. (C) Wild-type expression of unc-4::lacZ in DAs of an L1 larva (D) unc-4::lacZ is repressed in DAs of an L1 larva by ectopic VAB-7 expression from unc-3::vab-7 transgene (wels10). β-Galactosidase staining is retained in a cluster of neurones at the anterior end of the VNC in which unc-3::vab-7 is not expressed. Anterior is towards the left in all panels. Scale bars: in A, 10 μm in A,B; in C, 2.5 μm in C,D.
vab-7(e1562) mutants, however, we found that acr-5::gfp expression is specifically lost from the DBs (Fig. 4B; Table 4).

It has previously been shown that acr-5::gfp is negatively regulated by UNC-4 and its co-factor UNC-37 in DA and VA motoneurones (Winnier et al., 1999) (Fig. 4C). Given that unc-4 is derepressed in the DBs of vab-7 mutants (Fig. 3B), loss of acr-5::gfp expression could be due to the ectopic expression of unc-4. This is indeed the case, as acr-5::gfp expression is restored to the DBs in unc-4; vab-7 double mutants (Fig. 4D; Table 4). Repression of acr-5::gfp by ectopic UNC-4 also depends on unc-37 as DB motoneurones express acr-5::gfp in unc-37; vab-7 animals (Table 4). Therefore, in DB motoneurones, vab-7 effectively promotes acr-5::gfp expression by repressing UNC-4 repressor activity.

Ectopic VAB-7 expression induces DB characteristics in embryonic and post-embryonic neurones

The anteriorly directed trajectory of DB motor axons in vab-7(e1562) mutants indicates that vab-7 function is necessary to specify posterior axonal outgrowth in the DBs. To determine if vab-7 function is also sufficient to specify a posterior axonal trajectory in other classes of motoneurones, we expressed VAB-7 ectopically under the control of the unc-3 promoter. The C. elegans unc-3 gene encodes a homologue of the O/E family of mammalian transcription factors. In the ventral nerve cord, unc-3 is expressed in embryonic DA, DB neurones and in the post-embryonic VA, VB and AS motoneurones (Prasad et al., 1998) (T. Starich and J. Shaw, personal communication).

Animals carrying the unc-3::vab-7 transgene wels10 can move forwards, but exhibit a strong backwards movement defect (data not shown). This striking UNC-4-like phenotype indicates that A-type motoneurones (DA and VA) may be affected. If the ectopically expressed VAB-7 protein retains wild-type function, then unc-4 should be repressed in the DA and VA motoneurones. This is indeed the case: unc-4::lacZ expression is lost from A-type motoneurones in the unc-

### Table 3. Expression of unc-4::gfp in vab-7(e1562)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>DA</th>
<th>DB</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>89% (144/162)</td>
<td>0% (0/135)</td>
<td>27</td>
</tr>
<tr>
<td>vab-7(e1562)</td>
<td>99% (143/144)</td>
<td>88% (105/120)</td>
<td>24</td>
</tr>
</tbody>
</table>

n, number of animals examined at the L1 stage; the numbers of neurones examined are given in parentheses.

Only DA and DB embryonic motoneurones in the ventral nerve cord were scored (five DBs, six DAs).

The integrated transgene, wds14, was used to detect unc-4::gfp expression.

### Table 4. Embryonic motor neuron expression of acr-5::gfp in mutant backgrounds

<table>
<thead>
<tr>
<th>Genotype</th>
<th>DA</th>
<th>DB</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>1%</td>
<td>92%</td>
<td>23</td>
</tr>
<tr>
<td>unc-4(e120)</td>
<td>91%</td>
<td>98%</td>
<td>22</td>
</tr>
<tr>
<td>vab-7(e1562)</td>
<td>8%</td>
<td>9%</td>
<td>25</td>
</tr>
<tr>
<td>vab-7(e1562);unc-4(e120)</td>
<td>83%</td>
<td>87%</td>
<td>24</td>
</tr>
<tr>
<td>unc-37(e262)</td>
<td>94%</td>
<td>96%</td>
<td>27</td>
</tr>
<tr>
<td>vab-7(e1562);unc-37(e262)</td>
<td>80%</td>
<td>90%</td>
<td>27</td>
</tr>
</tbody>
</table>

n, number of animals examined.

Only the DA and DB embryonic motoneurones in the ventral nerve cord were scored at the L1 stage (five DBs, six DAs).

Animals carrying the unc-3::vab-7 transgene wels10 can move forwards, but exhibit a strong backwards movement defect (data not shown). This striking UNC-4-like phenotype indicates that A-type motoneurones (DA and VA) may be affected. If the ectopically expressed VAB-7 protein retains wild-type function, then unc-4 should be repressed in the DA and VA motoneurones. This is indeed the case: unc-4::lacZ expression is lost from A-type motoneurones in the unc-

---

**Fig. 4.** acr-5 expression in DB neurones is induced by VAB-7 repression of unc-4. (A-D) acr-5::gfp in lateral views of L1 larvae. (A) acr-5::gfp is expressed in wild-type DB motoneurones but is absent (B) from DBs in a vab-7(e1562) mutant. (C) acr-5::gfp is ectopically expressed in DA neurones of an unc-4(e120) mutant. (D) acr-5::gfp expression is restored to the DBs by genetic removal of unc-4 in the unc-4(e120);vab-7(e1562) double mutant. (E) acr-5::gfp::lacZ detected by anti-β-galactosidase antibody in an adult hermaphrodite expressing ectopic VAB-7 from wels10. acr-5::gfp::lacZ, which is normally only in DBs and VBs, is induced in DAs, VAs and ASs. (F) unc-17::gfp expression of animal in E used to identify the neurones. Anterior is towards the left. Scale bar: 10 μm.
Fig. 5. Ectopic VAB-7 is sufficient to promote posterior axonal outgrowth in cholinergic motoneurones. Dorsal views of unc-17:cha-1:gfp staining of DA, DB and AS axons entering the DNC of adult animals. (A) DA and AS motor axons project anteriorly in the wild type; DB axons are posteriorly directed. (B) An unc-3::vab-7 transgene (wels10) drives ectopic VAB-7 expression in ventral cord motoneurones and redirects AS and DA axons to adopt a posterior trajectory. Yellow arrow points to DNC. Anterior is towards the left. Scale bar: 15 μm.

Table 5. Ectopic vab-7 activates acr-5::lacZ

<table>
<thead>
<tr>
<th>Genotype</th>
<th>DA</th>
<th>VA</th>
<th>DB</th>
<th>VB</th>
<th>AS</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (Exacr-5::lacZ)</td>
<td>0% (66/66)</td>
<td>0% (132/132)</td>
<td>100% (88/88)</td>
<td>100% (176/176)</td>
<td>0% (154/154)</td>
<td>22</td>
</tr>
<tr>
<td>weIs10:Exacr-5::lacZ</td>
<td>70% (29/41)</td>
<td>85% (51/60)</td>
<td>100% (52/52)</td>
<td>91% (62/68)</td>
<td>93% (63/68)</td>
<td>47</td>
</tr>
</tbody>
</table>

n, number of adult hermaphrodites examined.

LaC expression was detected by antibody staining.

Expression of the unc-17::gfp marker in the weIs10 (unc-3::vab-7 unc-17::gfp) strain and DAPI staining aided in the identification acr-5::lacZ-positive postembryonic neurons (VA, VB, AS). In parentheses are the number of neurones with acr-5::lacZ expression/number of neurones examined.

to the DAs with anterior axonal projections (White et al., 1986). The unc-3::vab-7 transgene, however, reverses AS axonal polarity (Fig. 5B; Table 1). AS motoneurones also show ectopic expression of acr-5::gfp (Fig. 4E; Table 5) and unc-129::gfp (data not shown), which indicates that these postembryonic motoneurones have assumed other aspects of DB fate in addition to the posteriorly directed axonal trajectory. Therefore, ectopic expression of VAB-7 is sufficient to induce a spectrum of DB-like traits in both embryonic and postembryonic motoneurones.

vab-7 function is necessary for fasciculation of dorsal as well as ventral nerve cords

In addition to the axonal polarity reversals of DB motoneurones, vab-7(e1562) mutants also show significant disorganization of neuronal processes in both the dorsal and ventral nerve cords (Fig. 6B,D). Although the polarity reversal of vab-7 mutants is rescued by removal of unc-4 activity, this defasciculation defect remains (Fig. 2F), indicating that abnormal polarity is not the cause of defasciculation. In addition, abnormal expression of unc-4 in DB motoneurones does not account for the disrupted fascicular organization of the axial nerve cords. We conclude that vab-7 must mediate some other DB-trait that in turn is necessary for proper process placement in both the dorsal and ventral nerve cords. To test this idea, we examined nerve cord fasciculation in vab-7 mutants ectopically expressing VAB-7 from the unc-3::vab-7 transgene. We found that the unc-3::vab-7 transgene rescues both the fascication (Fig. 6E) and forward movement defects of vab-7 mutants (data not shown). Interestingly, although the DNCs and VNCs of vab-7 mutants appear defasciculated when viewed using the unc-17::gfp reporter (expressed in all cholinergic neurones; Fig. 6B,D), fasciation appears normal in the VNC but not the DNC when viewed with unc-129::gfp, a reporter expressed only in DA and DB neurones (Fig. 6F). This suggests that neurones other than DAs and DBs are defasciculated in the VNC. Our results indicate that DB neurones might have an important role in bundling in the nerve cords.

DISCUSSION

Coordinated movement depends on the integration of distinct functions provided by separate classes of motoneurones. In the nematode, C. elegans, DA and DB class motoneurones innervate dorsal muscles but adopt axonal trajectories of opposite polarity (DA, anterior; DB, posterior) and express separate sets of genes. We have shown that the differentiation of these motoneurone subclasses depends on the antagonistic actions of the VAB-7 and UNC-4 homeodomain proteins.
The DB motoneurone fate

VAB-7 appears to have two roles in DB fate determination. First, VAB-7 blocks expression of the A-class gene, unc-4, in DBs (Fig. 7). In vab-7 mutants, ectopic expression of UNC-4 represses B-class genes and induces DB motoneurones to adopt the anteriorly directed axonal trajectory of DA motoneurones. Second, VAB-7 promotes DB characteristics independently of UNC-4 repression. Ectopic expression of VAB-7 in cholinergic motoneurone classes that do not express UNC-4 is sufficient to induce expression of the B-class genes (i.e. acr-5, unc-129) and to impose the posterior polarity characteristic of DB motor axons. Posterior DB polarity also appears to be controlled by another, as yet unknown pathway, as this trait is normal in unc-4; vab-7 double mutants. Finally, as discussed below, the defasciculation defects observed in unc-4; vab-7 animals reveal an independent vab-7 function that is necessary for proper bundling of processes in both the dorsal and ventral nerve cords.

General models of neuronal fate determination

vab-7 directs the DB motoneurone fate. Are there functional counterparts of vab-7 for other motoneurone classes? Two genes with properties in common with vab-7 are unc-4 and unc-30. These genes encode paired-class homeodomain proteins important for A-type and D-type motoneurone fate, respectively (Miller et al., 1992; Jin et al., 1994). unc-4 is predominately expressed in DA and VA motoneurones, and is required for proper VA synaptic inputs and for repression of acr-5 in these neurones (White et al., 1992; Winnier et al., 1999). unc-30 controls GABA expression, axonal pathfinding and synaptic connections in DD and VD motoneurones (Jin et al., 1994). It is not yet known whether unc-4 or unc-30, like vab-7, are sufficient for a range of A-type or D-type fates, respectively, although UNC-30 has been shown to control genes for both the synthesis and packaging of the D-type neurotransmitter GABA (Eastman et al., 1999). It is interesting that unc-4 is not required for the maintenance the anterior polarity of A-type motor axons (White et al., 1992; Miller and Niemeyer, 1995). Our finding that ectopic unc-4 can reverse the polarity of DB neurones argues that UNC-4 may have additional A-type specifying roles that may be masked in unc-4 loss-of-function mutants by partial redundancy with an as yet unknown factor. Because VAB-7, UNC-4 and UNC-30 control the fates of different motoneurone classes, but do not control their production, these proteins are likely to function largely in postmitotic cells to define subsets of neurone-specific traits.

Fasciculation and eve in Drosophila and C. elegans

In vab-7 mutants, both dorsal and ventral nerve cords are defasciculated. This defect is not rescued by restoring proper posterior polarity of DB neurones (by removing unc-4), but is...
rescued by ectopic VAB-7 expression, suggesting that vab-7, and possibly DB neurones promote process bundling. Interestingly, Even-skipped in Drosophila also has a role in fasciculation (Landgraf et al., 1999). Axonal growth of Eve-expressing neurones (aCC and RP2) in the ISN nerve trunk and their subsequent innervation of dorsal muscles is dependent on Even-skipped. Furthermore, ectopic expression of Even-skipped in the nervous system promotes SN and ISN nerve trunk fasciculation. Landgraf et al. (Landgraf et al., 1999) have provided indirect evidence that eve activity is required for expression of an unknown neuronal adhesion molecule. Mutations in a number of genes are known to cause nerve bundle defasciculation in C. elegans (McIntire et al., 1992; Wightman et al., 1997; Bloom and Horvitz, 1997). One candidate for a downstream target of vab-7 is the α-integrin INA-1, which is expressed in DB (and other) neurones and is required for nerve bundle fasciculation (Baum and Garriga, 1997).

Conservation of Even-skipped function

Genetic studies in C. elegans, Drosophila, and the mouse have shown that Even-skipped homologues function to distinguish alternative fates in the motoneurone circuit. In each case, Eve prevents one class of neurone from adopting traits normally reserved for another. In Drosophila, Eve is expressed in motoneurones that project along the ISN nerve to innervate dorsal muscles. In eve mutants, these motoneurones adopt the axonal trajectory of a different class of ISN motoneurones that synapse onto ventral muscles (Landgraf et al., 1999). Similarly, in vab-7 mutants in C. elegans, DB motoneurones reverse their normal posterior axonal polarity and instead assume the anteriorly directed trajectory of DA motor axons (this work). In the spinal cord of mouse Evx1 mutants, V0 interneurones are apparently transformed into V1 interneurones (Moran-Rivard et al., 2001). At least in C. elegans and in Drosophila, ectopic expression of Even-skipped is also sufficient to impose axonal trajectories normally associated with eve-expressing motoneurones (Landgraf et al., 1999) (this work).

A common element of Eve function in all three species is the repression of a downstream HD protein, which is normally expressed in the alternative neurone. In C. elegans, vab-7 prevents expression of the DA gene, unc-4, in DB motoneurones. Evx1 functions in mouse V0 neurones as a negative regulator of the engrailed homologue, En1, a marker for V1 cells (Saueressig et al., 1999; Moran-Rivard et al., 2001). In Drosophila, ectopic expression of Eve is sufficient to inhibit Islet in ISN motoneurones (Landgraf et al., 1999).

In addition to these similarities in Eve function, our work shows that VAB-7 functions within a reciprocally inhibitory network: VAB-7 inhibits the DA fate and UNC-4 inhibits the DB fate. Thus, one way that VAB-7 promotes DB differentiation is by blocking expression of a HD transcription factor that antagonizes DB traits. By extension, we propose that HD transcription factors in Drosophila and mouse are likely to antagonize fate promoted by Eve. For example, EN1 might exert a negative effect on V0 interneurone differentiation when ectopically expressed in V1 cells in Evx1 mutants just as UNC-4 inhibits DB fates in vab-7 mutant animals. In this case, normal V0 cell migration and axonal trajectory might be restored in Evx1 En1 double mutant mice (Saueressig et al., 1999; Moran-Rivard et al., 2001). Both UNC-4 and EN1 include EH-1 domains that have been shown to recruit the transcriptional co-repressor protein, Groucho (Jimenez et al., 1997; Winnier et al., 1999). In the case of UNC-4, interactions with the nematode Groucho homologue, UNC-37, repress B-class motoneurone traits (Pflugrad et al., 1997; Winnier et al., 1999). Reciprocal inhibition by EH-1-containing HD proteins that recruit Groucho might be common, as recent work has revealed that such a mechanism in the vertebrate spinal cord defines distinct domains of neural progenitor cells (Muhr et al., 2001). Thus, our work demonstrates that important elements of both the logic and molecular mechanisms employed by HD proteins in the specification of neuronal fates in the motor circuit have been preserved in evolution from nematodes to mammals.

We thank D. Frisby and J. Rand for unc-17/cha-1::gfp, B. Prasad and R. Reed for the unc-3 promoter, J. Culotti for unc-129::gfp, A. Fire for gfp expression vectors, and R. Smith for monoclonal antibody help. We are also grateful to members of the Ahringer and Miller laboratories for helpful discussions. Some strains used in this study were obtained from the Caenorhabditis Genetics Center (University of Minnesota, St Paul) which is funded by the NIH NCRR. B. E was supported by the BBSRC; C. N. and J. A. were supported by a Wellcome Trust Career Development Award (No. 045515) and a Senior Research Fellowship (No. 054523) to J. A.; J. R. was supported by an HHMI predoctoral fellowship; and D. M. was funded by NIH (NS 26115).

REFERENCES


