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Convergent genetic programs regulate similarities and differences between related motor neuron classes in *Caenorhabditis elegans*

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Abstract

How do genetic programs create features common to a specific cell or tissue type while generating modifications necessary for functional diversification? We have addressed this question using the nematode *Caenorhabditis elegans*. The dorsal D (DD) and ventral D (VD) motorneurons (mns), referred to collectively as the D mns, compose a cross-inhibitory network that contributes to the animal's sinuous locomotion. The D mns share a number of structural and functional features, but are distinguished from one another by their synaptic patterns and the expression of a neuropeptide gene. Our findings suggest that the similarities and differences are generated at the transcriptional level. UNC-30 contains a homeodomain and activates structural and functional genes expressed in both classes. UNC-55 is a nuclear receptor expressed in the VD mns that is necessary for generating features that distinguish the two classes of D mns from one another. In *unc-55* mutants, the VD mns adopt the DD mn synaptic pattern and peptide expression profile. Conversely, ectopic expression of *unc-55* in the DD mns causes them to adopt VD mn features. The promoter of the neuropeptide gene expressed in the DD mns contains putative binding sites for both UNC-30 and UNC-55; alteration of these sites suggests that UNC-55 represses the ability of UNC-30 to activate a subset of genes that are expressed in the DD mns but not in the VD mns. Thus UNC-55 acts as a switch for the features that distinguish these two functionally related classes of mns.

Keywords: Caenorhabditis elegans; Neural differentiation; Synaptic specificity and rearrangement; Nuclear hormone receptor; GABA; *unc-55* and *unc-30*

Introduction

Neurons share a number of unique characteristics that allow them to create a rapid communication system. In addition to these similarities, different neural classes express distinctive features and ultimately each exhibits a unique pattern of synapses. Using genetic approaches in the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans*, a variety of regulatory and structural genes have been identified that are critical to the process of neural differentiation. In virtually every case, the genes identified are members of homologous groups (gene families) whose products often perform related functions in other species. Although a number of genes responsible for morphology and neurochemistry have been identified, it has been difficult to identify mutations that specifically interfere with synaptic specificity, mainly because neural differentiation is a multi-step process and synaptic specification is often the last step. Thus, a number of permissive conditions must be met before synaptic specificity can be revealed. For example, it is difficult to distinguish between mutants that alter process guidance or the general processes of synaptogenesis from alterations in synaptic specificity.

C. elegans moves forward and backward using sinuous body waves. The dorsal D (DD) and ventral D (VD) motorneurons (mns) represent two classes of mns and together compose a cross-inhibitory network that preserves

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the proper phase relationship between waves of dorsal and ventral muscle contraction that propel the animal. The two classes of D mn cell bodies are the only GABAergic mns in the ventral nerve cord (McIntire et al., 1993). Both DD and VD mns have two longitudinal processes, one in the dorsal nerve cord and one in the ventral nerve cord and they are connected by a commissure that runs around the inner circumference of the cuticle. In adults, each of the six DD mns has a dorsal presynaptic process and a ventral postsynaptic process; thus, the DD mns conduct information from ventral to dorsal. Each of the thirteen VD mns has a ventral presynaptic process and a dorsal postsynaptic process; thus, the VD mns conduct information from dorsal to ventral (Walthall and Plunkett, 1995; White et al., 1978). The difference in the synaptic patterns is the primary functional characteristic that distinguishes the VD and DD mns from one another. It should be noted that this inhibitory network makes a larger contribution to backward movement than forward movement. This is based upon the observation that mutations that affect the D mns and ablations of the DD mns (Walthall et al., 1993), and DD and VD mns (McIntire et al., 1993) compromise backward movement to a greater degree than forward movement.

The DD mns are generated during embryonic development; by contrast, the VD mns are generated during postembryonic development (Sulston and Horvitz, 1977; Sulston et al., 1983). Furthermore, when the animal hatches, the DD mns receive input from dorsal excitatory mns and innervate ventral muscle, the synaptic pattern of the wild-type VD mns. Late in the first postembryonic stage (L1), the DD mns begin to respecify and adopt the adult synaptic pattern. The respecification is developmentally coincident but not dependent on the birth and differentiation of the VD mns (Gally and Bessereau, 2003; Hallam and Jin, 1998; Knobel et al., 1999; White et al., 1978). Starvation and mutations in heterochronic genes can affect the timing of the synaptic respecification (Hallam and Jin, 1998). The respecification of the DD mns requires minimally permissive conditions associated with growth and possibly instructive signals, although such signals have not been identified. The morphology of the DD and VD mns does not change once established. Thus, the DD and VD mns offer an ideal model for examining the relationship between neural differentiation, synaptic specificity, and plasticity because the latter two events occur after the completion of process guidance.

unc-30 acts as a master control gene for the differentiation of the DD and VD mns. The protein UNC-30 contains a homeodomain motif and is expressed shortly after the birth of cells in both classes. UNC-30 regulates several genes that are necessary for the D mn morphological features as well as the production and release of GABA (McIntire et al., 1993). Mutations in *unc-30* as well as target genes common to the DD and VD mns cause identical defects in both motorneuron classes. Furthermore, expression of UNC-30 in other neurons is sufficient to direct the expression of neurotransmitter genes normally expressed in the D mns (Eastman et al., 1999).

A second transcription factor, UNC-55, is necessary for the features that distinguish the D mns from one another. UNC-55 is a nuclear hormone receptor that is co-expressed transiently with UNC-30 in the VD mns at birth (Zhou and Walthall, 1998). In *unc-55* mutants, the VD mns adopt the synaptic pattern and function of the DD mns (Plunkett et al., 1995).

The expression of *unc-30* is necessary for the features shared by the DD and VD mns and for the DD mn synaptic pattern. UNC-55 modifies the UNC-30 genetic program and creates the VD mn synaptic pattern. Here, we investigate the role of these two transcription factors in determining the synaptic specificities between these two related classes of mns and investigate whether synapse specification is related to other aspects of differentiation that distinguish the DD mns from the VD mns.

Materials and methods

C. elegans strains were grown following procedures described in Brenner (1974). Genes and mutant alleles used in this study were *unc-55* (*e1170*, *jd4*, and *jd8*), *unc-30* (*e191*), and *lin-6* (*e1466*). Four transgenic strains carrying green fluorescent protein (*gfp*) constructs (Chalfie et al., 1994) served as starting points for constructing the strains analyzed in this study.

One strain (*jdIs101*) contains an integrated copy of a transgene that contains 1.8 kb of the upstream promoter of *unc-55*, fused to the *gfp* gene, *Punc-55::gfp*, and a plasmid containing a dominant *rol-6* mutation (*su1006*) (Zhou and Walthall, 1998). The second transgene is composed of 2.4 kb of the *unc-30* promoter fused to an *unc-55* cDNA and ligated into a promoterless *gfp* plasmid (pPD117.01). A third transgenic strain *juIs1* contains a transgene *Punc-25::*SNB::GFP (Hallam and Jin, 1998), and was provided to us by Mike Nonet. This transgenic line allowed the visualization of the presynaptic terminals of the VD and DD mns. A fourth strain (*NY1121*) is a stable line and contains the plasmid *Pflp-13::gfp*, it is composed of 2.3 kb upstream regulatory region of the neuropeptide gene *flp-13* fused to the *gfp* gene cassette. From this, a second transgene containing 1 kb of the upstream *flp-13* promoter was made *Pflp-13(1kb)::gfp*. To construct this, two primers were made, one complementary to sequence at 1 kb of the *flp-13* promoter (CAAAGGTTCTGGCGCCAGTG) and a second complementary to pPD117.01 *gfp* plasmid (CAAGTTGTTAGCGTATCCATCG). PCR was performed using *Pflp-13::gfp* (2.3 kb promoter, as a template). The product was 4.7 kb (1.0 kb flp-13 promoter, 3.7 kb pPD117.01). It was gel purified and used for injection.

Alterations in the flp-13 promoter

Separate modifications were made in which two putative cis-acting regulatory elements located in the *flp-13* promoter

were replaced. The original *flp-13* transgenic reporter contained 2.3 kb of the *flp-13* promoter. The primers FLP-13-5, GCATGCAATTGTGCCTCCTGATGCTG and FLP-13-3, ACCGGTATCATGTTCGAATCG were used to amplify this region. The resulting PCR product was inserted into the gfp vector pPD117.01, which had been digested with *Sph*I and *Age*I.

Six nucleotides GGATTA compose the UNC-30 binding site; they are located 154 bases upstream from the +1 site of the *flp-13* structural gene and were replaced with GGTACC to create *Pflp-13Δ-154::gfp*. To make this transgene, the primer described above, FLP-13-5, and a second primer GGTACCAAAGCTTAAATCTTGCTCATTC were used to amplify the 2.15 kb region upstream of nucleotide 154. Primer FLP-13-3 and a second primer GGTACCTAAAA-GATGCATGATTTTGCAAC were used to amplify the 150 bp downstream fragment. The two fragments were digested with *Kpn*I, ligated, and inserted into the gfp vector pPD117.01.

Six nucleotides TGACCT compose a putative UNC-55 half-binding site; this half-site is located 183 base pairs upstream from the *flp-13* +1 site. These were replaced with the nucleotides GGTACC to create *Pflp-13Δ-183::gfp* transgene. To make this transgene, FLP-13-5 and a second primer GGTACCGTTGCAAAATCATCGCATCTTT were used to amplify the 2.1 kb fragment upstream of nucleotide 183. Primers FLP-13-3 and GGTACCCCTAATTCCTGATGGC-CAAT were used to amplify the 180 bp fragment downstream of nucleotide 183. The two fragments were then digested with *Kpn*I, ligated, and inserted into the gfp vector pPD 117.01. Each transgene construct was confirmed by sequencing.

mRNA was isolated from wild-type and mutant strains of *C. elegans*. Populations of RNA were reverse transcribed into cDNAs and primers designed to amplify selected cDNAs, which were then sequenced. The Georgia State University Molecular Core Facility performed the nucleotide sequencing on an ABI 3100 Genetic Analyzer.

Lines of transgenic animals were obtained by microinjection of transgenes into the gonads of adult hermaphrodites following a protocol described in Mello et al. (1991). Transgenic plasmids were injected at concentrations ranging from 25 to 50 μg/ml. The pattern of GFP expression for each transgene was observed in a minimum of three independent lines. The expression patterns were consistent within the ventral nerve cord cell bodies and the data and images reported came from stable lines.

Immunohistochemistry

Animals were harvested from the plates, collected in 1.5-ml plastic tubes and fixed overnight at 4°C in 4% paraformaldehyde and 0.5% glutaraldehyde diluted in 0.1 M sodium cacodylate buffer (pH 7.4). Following three washes in cacodylate buffer, animals were processed through a two-step permeabilization procedure. First, we incubated overnight in 5% 2-mercaptoethanol, 1% Triton X-100 in 0.1 M Tris–HCl pH 7.2. The animals were washed in phosphate buffered saline (PBS) and in step 2 incubated for approximately 8 h at 37°C in 900 units/ml of collagenase (Sigma, Type IV), 1 mM $CaCl_2$ in 0.1 M Tris–HCl (pH 7.2). The incubation time for the enzyme digestion is critical and exact times varied. Small aliquots of animals were examined under a dissecting scope, and the digestion was stopped and permeabilization was considered adequate when approximately 10% of the animals were broken in half. Animals were then washed in PBS and prepared for double-label immunohistochemistry. Liquid transfers were performed under a dissecting microscope to limit the loss of animals.

Animals were incubated for 30 min in a blocking solution of 5% normal donkey serum (Jackson Labs), 1% Triton X-100, and 0.1% BSA diluted in 0.1 M Tris–HCl (pH 7.2) buffer. The blocking solution was removed and replaced with a solution containing the primary antisera. A guinea pig polyclonal anti-GABA antiserum was purchased from Protos (New Jersey), and a rabbit polyclonal anti-GFP antiserum was purchased from Clontech. Each of the antisera was diluted 1:400 and incubation volumes were 50 μl/tube. Following the incubation, animals were washed three times in PBS. A fluorescein-conjugated donkey anti-guinea pig antiserum and a rhodamine-conjugated donkey anti-rabbit antiserum were each diluted 1:40 in 0.1 M Tris–HCl (pH 7.2) and used as the secondary antibodies (both were purchased from Jackson Immuno Labs). Incubations in secondary antisera were for 2 h at room temperature in the dark. Following the incubation, animals were washed three times in PBS and prepared for observation by mounting on agar pads on microscope slides in a solution of 70% glycerol/30%PBS with 1 mg/ml *p*-phenylenediamine and covered with a 1.0 cover slip.

Microscopy

Preparations were viewed on a Zeiss LSM 510 laser scanning confocal microscope and image stacks were collected using Zeiss 510 software (version 2.8). For preparations in which GFP expression was viewed directly, living animals were immobilized on 4% agar pads containing 1 mM sodium azide. Micrographs composed of visible and fluorescent images were made by collapsing fluorescent image stacks and then superimposing this composite image onto the image from a single differential interference contrast slice taken from the center of the stack. Colocalization determinations were performed using depth coding of individual images that composed the stacks. This was done by using the colocalization function of the Zeiss 510 software and insuring that the output histograms of the rhodamine and gfp channels were aligned for each section of the stack. Images of the immunocytochemistry presented are projections of stacks from individual animals in which the *Z*-axis was collapsed. Images were exported into Adobe Photoshop for final modifications.

Table 1
Summary of results of imaged animals

Transgenic strains	No. of animals	Genetic background	Stage of development[a]	Pattern of GFP expression
Punc-55::gfp	>25	Wild type	L1/L2	VD & AS mns
	>25	*unc-30(e191)*	L1/L2	VD & AS mns
Punc-30::unc-55a::gfp	10	Wild type	L1	6 DD mns
			Post L2	6 DD & >9 VD mns[b]
Punc-25::snb::gfp[c]	5	*lin-6(e1466)*	L1	DD mns (ventral processes)
			Post L2	DD mns (dorsal processes)
Punc-30::unc-55a::gfp & Punc-25::snb::gfp	5 + 2 mosaics[d]	*lin-6(e1466)*	Post L2	6 DD mns (ventral processes)
Pflp-13(2.3kb)::GFP-IR & GABA-IR	11	Wild type	Post L2	GABA & FLP-13-IR co-expressed in 6 DD mns
	8	*unc-30(e191)*	Post L2	80% reduction in # of DD mns expressing GABA & FLP-13
	10	*unc-55(e1170)*	Post L2	GABA & FLP-13 expressed in 6 DDmns & >9 VD mns[b]
Pflp-13(1kb)::gfp	>15	Wild type	Post L2	6 DD mns
Pflp-13 Δ-154::gfp	15	Wild type	Post L2	No expression in vnc
Pflp-13 Δ-183::gfp	10	Wild type	Post L2	GFP expressed in 6 DDmns & >9 VD mns[b]

[a] L1/L2 refers to the developmental period flanking the first molt. Post L2 refers to developmental periods following DD mn respecification.
[b] VD mns 1 and 2 in the retrovesicular ganglion and 12 and 13 in the preanal ganglion could not be unequivocally identified.
[c] First reported in Hallam and Jin (1998).
[d] See text for details regarding mosaicism among this transgenic strain.

Table 1 summarizes the types of transgenic strains, the numbers, the genetic backgrounds, the developmental stage, and a brief summary of the outcomes for the animals imaged in the confocal microscope.

Results

The expression of unc-55 in the VD mns does not depend upon UNC-30

unc-30 and *unc-55* both encode transcription factors that are expressed in the VD mns (Jin et al., 1994; Zhou and Walthall, 1998). Because UNC-30 has been shown to regulate the expression of a number of genes expressed in the D mns, we asked whether UNC-30 regulated the expression of *unc-55* in the VD mns. The expression patterns of the transgenic reporter *Punc-55::gfp* were compared in an *unc-30 (+)* background and an *unc-30 (e191)* mutant background. The spatial and temporal pattern of *Punc55::gfp* expression was identical in the two strains. As reported previously (Zhou and Walthall, 1998), the AS-VD mn doublets express GFP sequentially beginning with the anterior pair, which is born first, and progressing incrementally to the posterior pair (Figs. 1A and B). Expression in the *unc-30 (+)* background (Fig. 1A) has not progressed as far posterior as in the *unc-30 (e191)* background (Fig. 1B). The spatial and temporal activation pattern of *Punc-55::gfp* expression in the VD mns therefore, is independent of UNC-30; thus, the expression of these two transcription factors is regulated independently but converges in the VD mns.

The ectopic expression of unc-55 causes the DD mns to adopt the VD mn synaptic pattern

In *unc-55* mutants, the VD mns adopt the synaptic pattern and function of the DD mns (Walthall and Plunkett 1995); thus, the presence of UNC-30 and UNC-55 in the VD mns is required for establishing the proper VD mn synaptic pattern. To determine whether the VD mn synaptic pattern would result if UNC-30 was present with UNC-55 in the DD mns, the *unc-30* promoter was fused to an *unc-55* cDNA that was capable of restoring locomotion in *unc-55* mutants (*Punc-30::unc-55a::gfp*). A variety of embryonic

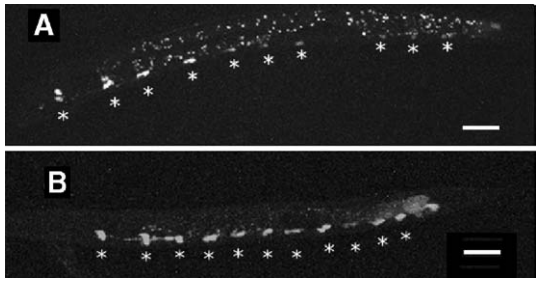

Fig. 1. Expression of a *Punc-55::gfp* reporter in the presence and absence of UNC-30. (A) *Punc-55::gfp* expression viewed directly in an *unc-30(+)* background. The VD and AS mn doublets (indicated by asterisks) are seen and form a linear sequence of paired cell bodies in the ventral nerve cord (Zhou and Walthall, 1998). Expression begins in the anterior most pair and progresses toward the posterior. (B) The spatial and temporal pattern of expression of the *Punc-55::gfp* was identical in the *unc-30(e191)* mutant background. *Punc-55::gfp* expression has progressed further posterior because the animal is at a slightly later developmental stage.

and postembryonic neurons express *unc-30* (Jin et al., 1994) and transgenic progeny showed phenotypes that included embryonic lethality as well as morphological and functional defects. We restricted our analysis to newly hatched (L1) animals that exhibited wild-type locomotion and expressed *Punc-30::unc-55a::gfp* in the six DD mns. In one experiment, 25 GFP-positive L1 progeny were observed and seven animals met the criteria described above. Fig. 2A shows a projection of the L1 stage of one of the transgenic animals analyzed. The triangles in the micrograph indicate GFP expression in cell bodies that correspond in position to DD mns 1–5. The locomotion of this animal and the other six was reexamined after 24 h (this allowed the animals to recover from anesthesia and proceed through the stages when the VD mns are born and the DD mns complete synaptic respecification). In the seven animals examined, forward movement was normal (data not shown); however, the backward movement of all seven was asymmetrical (Fig. 2B). Shown is a second-by-second analysis of an animal attempting backward movement. The circle indicates the dorsal side of the animal. As the frames progress, the animal coils with the dorsal side central. This was in contrast to the sinusoidal backward movement observed in non-GFP expressing siblings and wild-type animals.

If the presence of UNC-55 in the DD mns compromised their function, then the resulting absence of dorsal inhibition would result in the dorsal asymmetric pattern of locomotion. An interesting possibility is that the presence of UNC-55 in the DD mns prevented their synaptic rearrangement and caused the presynaptic processes to remain in the ventral nerve cord. A transgenic strain carrying the fusion gene *Punc-25::snb::gfp* is expressed almost exclusively in the presynaptic processes of the VD and DD mns due to the *unc-25* promoter and the signal sequence on the SNB. The presence of the transgene can be visualized because of the GFP (Hallam and Jin, 1998; Nonet, 1999). *lin-6 (e1466)* blocks postembryonic lineages including the ones that generate the VD mns (Horvitz and Sulston, 1980); thus, the expression of *Punc-25::snb::gfp* in the *lin-6* mutant background allows SNB::GFP to be viewed in the presynaptic processes of the DD mns in the absence of the VD mns. Fig. 2C shows such the dorsal presynaptic process of a respecified DD mns in a late stage *lin-6* mutant. If the presence of UNC-30 and UNC-55 blocks the synaptic respecification of the DD mns, then SNB::GFP should accumulate along the ventral nerve cord instead of the dorsal nerve cord. Fig. 2D shows a *lin-6* mutant expressing both transgenes, *Punc-30::unc-55a::gfp* and *Punc-25::snb::gfp*. Seven animals were examined that expressed both transgenes. The localization of the SNB along the ventral cord indicates that the DD mns failed to respecify their synaptic pattern in the presence of the ectopic UNC-55. Two of the seven animals showed alternate ventral and dorsal presynaptic processes (SNB::GFP). We believe that these are mosaic animals that lost the *Punc-30::unc-55a::gfp* transgene in one of the two DD mn lineages.

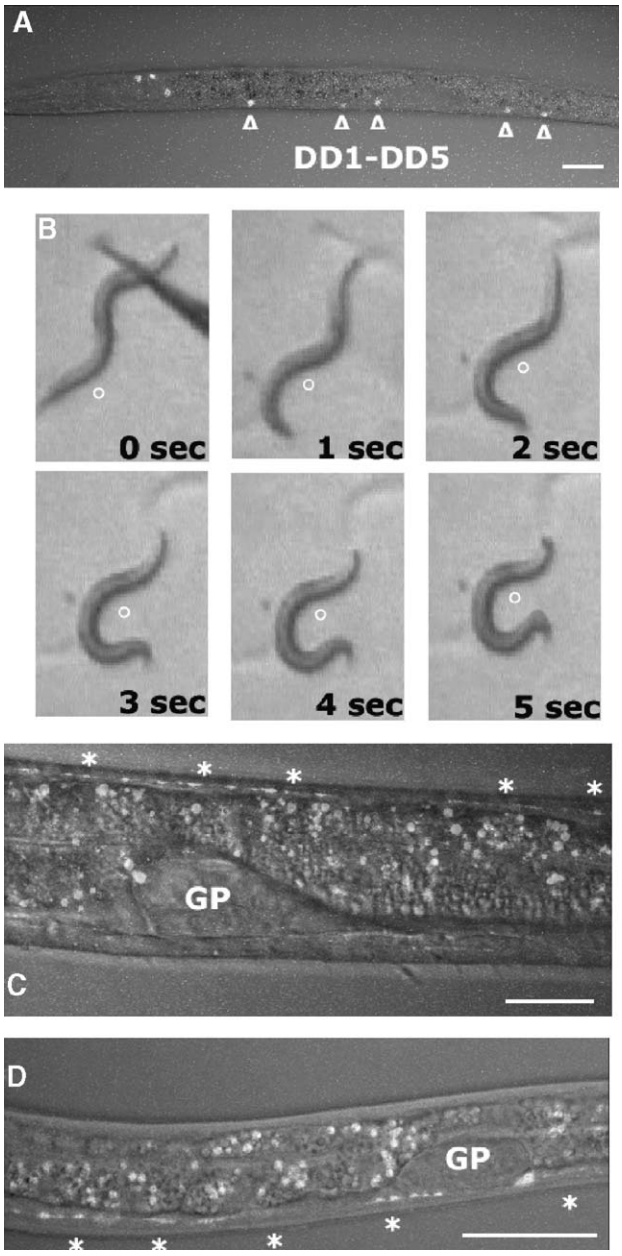

Fig. 2. Ectopic expression of a functional *unc-55::gfp* in the DD mns. (A) An L1 transgenic animal expressing the *Punc-30::unc-55A::gfp* in the DD mns. The open triangles indicate the positions of the DD mn cell bodies. (B) Animal shown in panel A 12 h later. The symmetrical backward movement observed throughout the L1 stage (data not shown) has shifted to a dorsal asymmetric pattern of backward movement. The open circle represents the dorsal side of the animal. (C) The expression of *Punc-25::snb::gfp* in the dorsal nerve cord (*) of the DD mns following respecification (see text for additional details). (D) The expression of *Punc-25::snb::gfp* (*) in the ventral nerve cord of DD mns that had expressed ectopic *unc-55a::gfp*. The SNB::GFP remains in the ventral nerve cord indicating that the DD mns did not undergo respecification.

The six DD mns are generated from symmetrical lineages, such that DD mns 1, 3, and 5 have a common grandmother, as do DD mns 2, 4, 6 (Sulston et al., 1983). If the *Punc-30::unc-55a::gfp* is absent in one of the two lineages, then three of the DD mns would be expected to respecify and

three would not and this would be expected to occur in alternate DD mns. Taken together, the results from transgenic and transgenic mosaic animals suggest that the presence of UNC-55 and UNC-30 in either the VD or the DD mns is sufficient to create the VD mn synaptic pattern.

In *unc-30* mutants, the DD and VD mns exhibit similar structural and functional defects (McIntire et al., 1993). *unc-55* is necessary for the synaptic pattern that distinguishes the VD mns from the DD mns (Walthall and Plunkett, 1995), and in mutants, both classes adopt the DD mn synaptic pattern; thus, the DD mn synaptic pattern appears to represent the default condition. Ectopic expression of *unc-55* blocks the respecification of the DD mns so that both D mn classes adopt the VD mn synaptic pattern. The simplest explanation is that UNC-55 suppresses a subset of genes normally activated by UNC-30 in the DD mns. A target gene expressed in the DD mns but not in the VD mns would provide a test of this prediction.

The neuropeptide gene flp-13 is expressed in the DD but not the VD mns

The *flp* gene family in *C. elegans* encodes neuropeptides of the FMRFamide class. Thus far, 23 members of this family have been identified (Kim and Li, 2004; Nelson et al., 1998). Transgenic animals carrying a *gfp* reporter gene fused to the promoter region of *flp-13 (Pflp-13::gfp)* (strain *NY1121*) show GFP expression in six cell bodies in the ventral nerve cord. Their positions correspond to that of the six DD mns. To confirm their identities, *Pflp-13::gfp* animals were simultaneously stained with antisera against GABA (red) and GFP (green). Within the ventral nerve cord, we consistently observed six neurons that colocalized GABA and the *Pflp-13::*GFP (yellow) (Fig. 3A). These are the cell bodies of the six DD mns. Thirteen additional mns were stained by the GABA antibody (red) but not by the antibody to GFP in *Pflp-13::gfp* animals. These correspond in position and number to the VD mns. Thus, *flp-13::gfp* reporter represents a gene that is expressed in the DD mns but not in the VD mns.

Although a mutant phenotype for *flp-13* in *C. elegans* has not been determined, the function of the FLP-13 neuropeptide has been addressed in *Ascaris suum*, a large nematode that is suitable for physiological and biochemical manipulations. Injection of FLP-13 into *A. suum* caused the animal to elongate (muscle relaxation) and to stop generating contractile waves (Reinetz et al., 2000). Both of these effects are consistent with the physiological role for FLP-13 in the DD mns of inhibiting muscle contraction.

UNC-30 is necessary for the expression of Pflp-13::gfp in the DD mns

To determine whether the expression of *Pflp-13::gfp* in the DD mns was dependent on a wild-type copy of *unc-30*, we compared the expression pattern of *Pflp-13::gfp* and

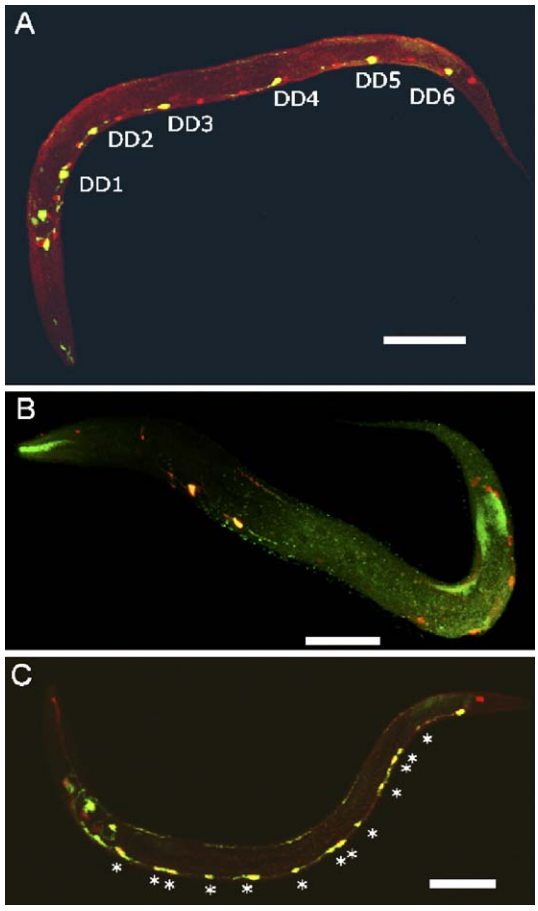

Fig. 3. Regulatory interactions at the cellular level between *unc-30* and *unc-55* as revealed through simultaneous visualization using antisera to *Pflp-13::*GFP (green) and GABA (red). (A) Image shows an animal in which the *Pflp-13::*GFP-IR and GABA-IR reveal the DD mn specific expression of *Pflp-13::gfp* in wild-type background. DD mns are labeled, the red cell bodies (GABA-IR positive but *Pflp-13::*GFP negative) are the VD mns. (B) The reduction of *Pflp-13::*GFP and GABA-IR mns in an *unc-30 (e191)* animal reveals the requirement of *unc-30(+)* for the normal expression of FLP-13 in the DD mns. This prep was unusual in that seven ventral cord neurons expressed GABA and two mns expressed both *Pflp-13::gfp* and GABA (see text for details). (C) VD and DD mn cell bodies in the ventral nerve cord (indicated by asterisks) express *Pflp-13::*GFP and GABA-IR in an *unc-55 (e1170)* mutant background. Each animal is in either the L3 or L4 developmental stage and is oriented with anterior to the left and ventral down. The scale bars represent 50 μm.

GABA in the wild-type *unc-30(+)* background using immunocytochemistry to simultaneously visualize GABA and GFP (Fig. 3A) and an *unc-30(e191)* mutant background (Fig. 3B). Eight *unc-30 (e191)* mutants were examined for GABA-IR and *Pflp-13::gfp* IR and we observed a dramatic reduction in the number of GABA and *Pflp-13::gfp* labeled ventral cord mns. Four of eight *unc-30* mutants showed no GABA-IR or *Pflp-13::gfp* IR in the ventral nerve cord. Three showed from two to six ventral cord neurons that expressed both GABA-IR and *Pflp-13::*GFP-IR, a DD mn profile. The eighth animal shown in Fig. 3B is exceptional in that this *unc-30* mutant showed two anterior ventral cord mns that colocalized the two antibodies, the DD mn profile,

and five posterior cells that exhibited GABA-IR without *Pflp-13::*GFP-IR, a VD mn profile.

McIntire et al. (1993) did not report the occasional presence of GABA in subsets of D mns in *unc-30* mutants. Detection of GABA in these mns in the *e191* mutants may be due to an improvement of sensitivity in detection protocols for immunohistochemistry. We compared the sequence of three *unc-30(e191)* cDNA isolates and two N2 cDNA isolates to the *unc-30* sequence reported in the *C. elegans* database. The only difference found was a change from C to G in codon 16 that creates an amber stop codon (UAG) in the mutant mRNA. The occasional GABA and *Pflp-13::gfp* expression are likely due to the occasional read through the translational stop codon of the UNC-30 nascent peptide; alternatively, it may reveal a parallel pathway that contributes to the activation of D mn differentiation (see below).

UNC-55 suppresses the expression of Pflp-13::gfp in the VD mns

Since *Pflp-13::gfp* is normally expressed in DD mns but not VD mns, we hypothesized that UNC-55 suppresses *Pflp-13::gfp* in the VD mns of wild-type animals. Mutations in *unc-55* would result in *Pflp-13::gfp* expression in the VD mns. *C. elegans* strains homozygous for the *unc-55* alleles (*e1170* and *jd4*) and carrying the *Pflp-13::gfp* reporter were constructed. In contrast to the six GFP-positive DD mns that normally express *Pflp-13::gfp* in the ventral nerve cord (Fig. 3A), additional mn cell bodies, all of which showed GABA-IR, were observed (Fig. 3C). The additional mns correspond in number to the VD mns. There are 13 VD mns, however, VD mns 1 and 2 are located in the retrovesicular ganglion of the head and their identity is difficult to confirm. VD mns 12 and 13 are located in the preanal ganglion where cell identification is also difficult. Our counts ranged from 15 to 19 with a mean number of 17 cell bodies (n = 10). Neurons that expressed only GABA-IR (red) or *Pflp-13::GFP*-IR (green) were present in the head and/or tail in each of the ten animals observed. Thus, UNC-55(+) either directly or indirectly suppresses *flp-13* expression in the VD mns.

Identification of cis-acting elements for UNC-30 and UNC-55 in the flp-13 promoter

The *Pflp-13::gfp* reporter construct contains 2.3 kb of the upstream promoter of the *flp-13* gene. Examination of this sequence revealed a consensus UNC-30 enhancer site GGATTA. This site had previously been identified in other genes and shown to bind with UNC-30 to regulate the expression of two genes involved in the synthesis and packaging of GABA (Eastman et al., 1999). The sequence in the *flp-13* regulatory region was found at nucleotide −154, a location that coincides closely with that found in *unc-47* (Eastman et al., 1999). The site is also found in the *flp-13* promoters in *Caenorhabditis briggsae* (−159) and *remanei* (−149).

The *D. melanogaster* gene *seven-up* and *unc-55* share a great deal of similarity in the two Zn finger domains (the P-box and D-box) (Zhou and Walthall, 1998). The enhancer recognized by SVP is a direct repeat of the sequence TGACCT, or the palindrome AGGTCA, separated by one or a few nucleotides, each is referred to as a half-site (Zelhof et al., 1995). This half-site appears four times in the region upstream to the *flp-13* coding region at positions −2575, −1902, −1168, and −183 (Fig. 4A); however, in none of these cases do we see two half-sites in close proximity. Sites corresponding to the putative UNC-55 half-site at −183 are found at (−193) and (−180) in the *flp-13* homologues in *C. briggsae* and *C. remanei*, respectively.

We tested whether the UNC-30 enhancer site (−154) and the putative UNC-55 enhancer half-site located nearest the *unc-30* binding site (−183) are necessary for the regulation of *Pflp-13::gfp* expression by changing the combination of six conserved nucleotides that compose each of the sites (Fig. 4A). Strains of *C. elegans* were generated with transgenes having either an altered UNC-30 binding site (*Pflp-13Δ-154::gfp*) or an altered UNC-55 binding site (*Pflp-13Δ -183::gfp*).

If the UNC-30 enhancer is necessary for the expression of *flp-13*, then we would not expect expression of *Pflp-13Δ-154::gfp* in the ventral nerve cord. This prediction proved true (Fig. 4C). *Pflp-13Δ-154::gfp* expression was observed in the head neurons, UNC-30 independent expression, but there was no expression in the ventral nerve cord. The complete absence of GFP expression among these motorneurons was observed in the 15 animals examined; thus, the UNC-30 cis-acting element is required for the expression of *Pflp-13::gfp*. This result also suggests that the occasional presence of GABA-IR and *flp-13::*GFP-*IR* in the cell bodies of ventral mns in the *unc-30 (e191)* mutant (Fig. 3B) was due to read through the translational stop codon.

If the conserved UNC-55 half-site at −183 kb mediates the suppression of *Pflp-13::gfp* in the VD mns, then altering the half-site would be expected to result in the expression of the transgene in the six DD mns and the 13 VD mns. A transgenic animal with *Pflp-13Δ -183::gfp* is shown in Fig. 4D and has additional mns that express the transgene. In nine additional animals, we observed a range from 15 to 19 cell bodies. These corresponded to a combination of six DD mns and 13 VD mns. Evidence supporting the identification of these cells as D mns was obtained by crossing the transgene into an *unc-30 (e191)* background. In the absence of UNC-30, GFP-positive cell bodies were not observed in the ventral nerve cord (data not shown; $n > 30$ animals).

There are four putative UNC-55 half-sites within 2.6 kb upstream of the first codon of *flp-13* in *C. elegans*. A second *Pflp-13::gfp* transgene was constructed that included only 1 kb of the promoter (*Pflp-13(1.0 kb)::gfp*) and expressed. The removal of the three more distant sites had

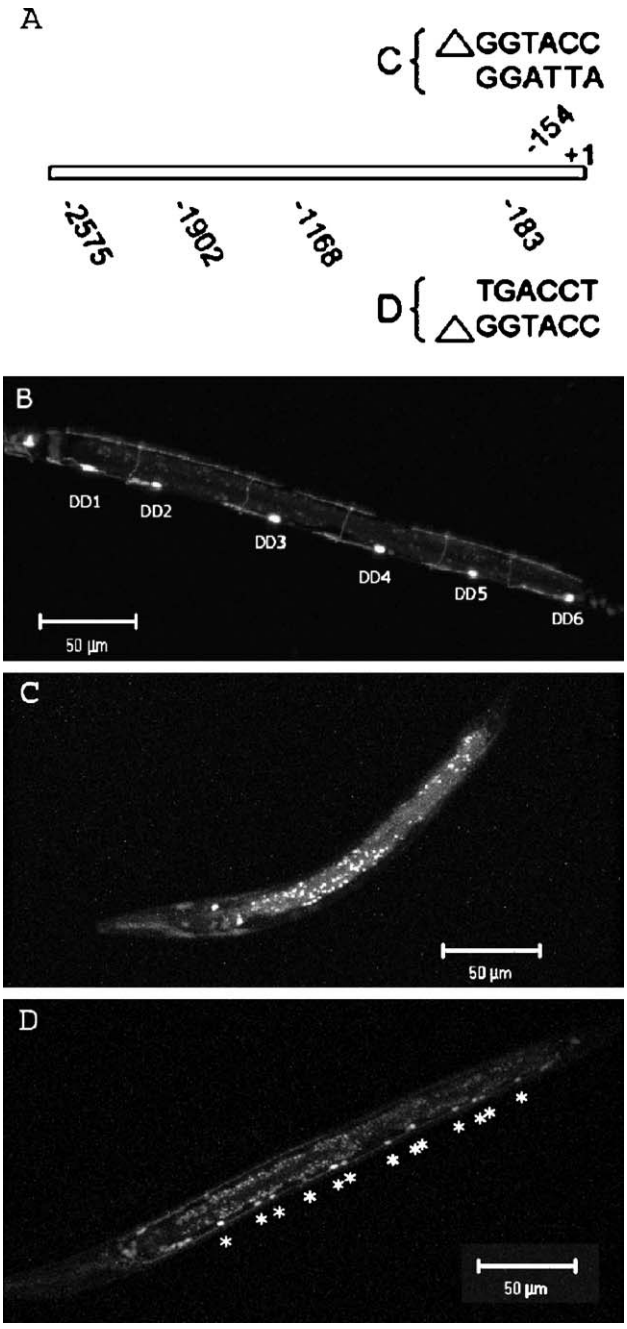

Fig. 4. Identification of the cis-acting sites (enhancers) for UNC-30 and UNC-55. (A) Illustrated are the four UNC-55 binding sites (below the line; nucleotides −2575, −1902, −1168, and −183) relative to the start codon (AUG) for the neuropeptide gene *flp-13* and the UNC-30 binding site (above the line; nucleotide −154). Also shown are the binding site modifications to create *Pflp-13Δ -154::gfp* and *Pflp-13Δ -183::gfp* transgenes used in C and D. (B) Image of a transgenic animal expressing *Pflp-13(1.0 kb)::gfp* in the head and the six DD mn cell bodies. (C) Image of a transgenic animal in which the UNC-30 binding site was changed from GGATTA to GGTACC. *Pflp-13Δ -154::gfp* expression in this and 14 other animals was restricted to neurons in the nerve ring. (D) Image of a transgenic animal in which the UNC-55 binding site was changed from TGACCT to GGTACC. *Pflp-13Δ -183::gfp* expression in this and other animals was observed in a combination of DD and VD mns. Asterisks indicate the four DD mn cell bodies and nine VD mns located between the anterior retrovesicular ganglion and the posterior preanal ganglion.

no effect on the expression of *Pflp-13::gfp* in the ventral nerve cord (Fig. 4B). The shortened 1.0 kb *flp-13* promoter was crossed into *unc-30(e191)* and *unc-55(e1170)* mutants and expression patterns were identical to those observed using the 2.3 kb *flp-13* promoter.

The results presented in Figs. 3 and 4 illustrate the necessity of the cis-elements and trans-acting factors in mediating features shared by the two classes of D mns as well as features that distinguish the two classes from one another.

Discussion

Nuclear receptor and homeobox genes represent two ancient transcription factor gene families that function in the differentiation of the nervous systems of both vertebrates and invertebrates. In *C. elegans*, a genetic program initiated by the homeobox gene *unc-30* in the embryonic DD mns and the postembryonic VD mns is modified in the VD mns by the expression of the nuclear receptor gene *unc-55*. The expression of *unc-55* in the VD mns is necessary for their distinctive synaptic pattern and prevents the expression of *Pflp-13::gfp* and presumably the neuropeptide FLP-13. Furthermore, ectopic expression of *unc-55* in the DD mns blocks the expression of *Pflp-13::gfp* and results in the adoption of the VD mn synaptic pattern. These results support a model in which UNC-55 functions in the D mns as a developmental switch between two differentiated states. Both D mn classes have the potential to adopt either fate. UNC-55 could accomplish this by acting autonomously within the VD mns and suppressing the genes that normally destabilize the initial synaptic pattern that accompanies the DD mn respecification. Alternatively, UNC-55 could act by suppressing genes whose products are involved in the reception and transduction of an extracellular signal that normally triggers the DD mn synaptic rearrangement. Our current data do not distinguish between these alternatives.

Conceivably, the VD and DD mns could express identical genetic programs in which UNC-30 regulates *unc-55* expression and differences in the synaptic patterns arise from extrinsic factors that correlate with developmental timing, for example, the presence or absence of a hormone. A single genetic program activated in six mechanosensory neurons creates touch-sensitive neurons that detect light touch in *C. elegans* (Duggan et al., 1998; Xue et al., 1993). Two distinct neural circuits composed of different sets of interneurons and mns are activated by the anterior and posterior mechanosensory neurons. The synaptic specificity of the mechanosensory cells results from differences in the positions of the cells within the animal and not differences in the genetic programs expressed in the six mechanosensory neurons (Chalfie et al., 1985; Walthall and Chalfie, 1988; Way and Chalfie 1988). However, the expression of *Punc-55::gfp* in the VD mns of *unc-30*

mutants indicates that *unc-55* is not regulated by UNC-30; thus, the two classes of D mns are the products of differential gene expression. Whether UNC-55 regulates the expression of *unc-30* in the VD mns has not been addressed using molecular approaches; however, two lines of evidence argue against this interaction. *First*, the VD and DD mns in *unc-30(+); unc-55(e1170)* strains exhibit the differentiated characteristics and function of the DD mns; *second*, the locomotion of *unc-30(e191)* mutants was compared to that of the *unc-30(e191); unc-55(e1170)* double mutant and the locomotion defect of the double mutant was indistinguishable from that of the *unc-30* mutant (Walthall and Plunkett, 1995). These results suggest that these two transcription factors are activated independently of one another in the D mns.

Transcriptional interactions had been described previously between the homeobox gene *ftz* and the nuclear receptor gene *ftz-f1* in *D. melanogaster*. FTZ and FTZ-F1 act as cofactors and are involved in embryonic segmentation (Guichet et al., 1997; Yu et al., 1997). These two studies showed that the heterodimer increased the affinity of FTZ for its binding sites, thus broadening the number of binding sites and genes regulated by FTZ.

UNC-55 acts in the VD mns to limit the number of genes activated by UNC-30. This is also true of two other genes that share sequence similarity with *unc-55*, *seven-up* (*svp*) in *D. melanogaster* and the mammalian form of *chicken ovalbumin upstream promoter-transcription factor* (*coup-tf*) (Leng et al., 1996). Based upon molecular taxonomy, *unc-55*, *svp*, and *coup-tf* are classified in the second of six subfamilies of the nuclear hormone receptor super family. This entire group is composed of orphan receptors and all are involved in the development of the nervous system (Giguere, 1999). Additional similarities exist between the roles played by *unc-55* and *svp* in *D. melanogaster*. Hypomorphic alleles of *svp* cause photoreceptors 3, 4, 1, and 6 to adopt the differentiated features of photoreceptor 7 (Kramer et al., 1995). Hypomorphic alleles of *unc-55* cause the VD mns to adopt the synaptic pattern and neuropeptide profile of the DD mns. The sequence similarity between the two factors is greatest in their two zinc-finger domains and, like SVP and COUP-TF, UNC-55 binds to a set of six conserved nucleotides in the promoter of a targeted gene (*flp-13*; Fig. 4). The cis-acting element is composed of TGACCT/AGGTCA. For SVP and COUP-TF targets, this site is found as a direct repeat, separated by one to four nucleotides. The six nucleotides are defined as a half-site (Zelhof et al., 1995; reviewed in Van Gilst et al., 2002).

The expression of UNC-55 in the VD mns repressed the ability of UNC-30 to activate the *Pflp-13;;gfp* reporter and by implication genes necessary for synaptic rearrangement in the VD mns. This suppression required both the trans-acting nuclear receptor and the conserved cis-acting enhancer site present in the *Pflp-13* promoter. The absence of either resulted in the expression of *Pflp-13::gfp* and the DD mn synaptic pattern in the VD mns (Figs. 3C and 4D). Although our data do not address whether UNC-55 and UNC-30 interact directly, it is clear that UNC-55 requires its DNA binding site to suppress the activation of *flp-13* by UNC-30. Genes in the VD mns activated by UNC-55 have not been identified. There is a core L*XX*LL motif located in the 3′ activation domain of UNC-55. This motif is required for binding transcriptional co-activators to nuclear receptors (Heery et al., 2001).

The genome of *C. elegans* contains 270 potential nuclear hormone receptor gene family members; however, mutant phenotypes have been established for only a few. Those members characterized thus far contribute to a variety of activities, including neural development (Miyabashi et al., 1999; Much et al., 2000), sex determination (Carmi et al., 1998), and growth and dauer formation (Antebi et al., 2000). Most of these genes are expressed widely and *unc-55* is no exception. In addition to the VD mns, *unc-55* is expressed transiently in a pair of chemosensory neurons and interneurons in hermaphrodites and a set of epithelial cells and a neuron in males (Shan and Walthall, manuscript in preparation). However, co-expression with *unc-30* is restricted to the VD mns.

Mice have three genes that share extended nucleotide and amino acid similarity with *unc-30: pitx1*, *2*, and *3*. One of these, *pitx2*, is involved in regulating GABA synthesis in the mammalian brain and has retained the ability to interact with the binding sites of UNC-30 targets in *C. elegans* (Westmoreland et al., 2001). The convergence of the two transcription factors UNC-30 and UNC-55 in the VD mns creates the functional features that distinguish the VD mns from the DD mns. A binary switch utilizing transcriptional suppression provides an efficient means for modifying an existing genetic program to create novel features among functionally related classes of neurons.

Intrinsic and extrinsic regulation of synaptic diversity and specificity is fundamental to the ability of nervous systems to generate behaviors and behavioral adaptations that are necessary for survival. Here we show that a genetic program conserved in nematodes and mammals can be modified in nematodes by a second highly conserved transcription factor to generate a distinct set of features in a related class of mns.

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