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

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Received for publication 14 July 2004, revised 27 January 2005, accepted 31 January 2005

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.

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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...
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 DD 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 motoneuron 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 co-expresses 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 julsl1 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 (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, GCATGCAATTGTGCCCTCGATGCTG and FLP-13-3, ACCGGTATCATGTTGAATCG were used to amplify this region. The resulting PCR product was inserted into the gfp vector pPD117.01, which had been digested with SpaI and AgeI.

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 GGTACCAAGCTTAAATCTTGCTCATTC were used to amplify the 2.15 kb region upstream of nucleotide 154. Primer FLP-13-3 and a second primer GGTACCTAAA-GATGCAATTGTGCCTCCTGATGCTG were used to amplify the 150 bp downstream fragment. The two fragments were digested with KpnI, 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 +1 site of the flp-13 +1 site. These were replaced with the nucleotides GGTACC to create Pflp-13Δ-183::gfp transgene. To make this transgene, FLPI-13-5 and a second primer GGTACCGTTCATCATGCAATTC were used to amplify the 2.1 kb fragment upstream of nucleotide 183. Primers FLP-13-3 and GGTACCGTTCATCATGCAATTC were used to amplify the 180 bp fragment downstream of nucleotide 183. The two fragments were then digested with KpnI, ligated, and inserted into the gfp vector pPD117.01. 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 CaCl2 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 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 Punc-55::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

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**Table 1**

<table>
<thead>
<tr>
<th>Transgenic strains</th>
<th>No. of animals</th>
<th>Genetic background</th>
<th>Stage of development</th>
<th>Pattern of GFP expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Punc-55::gfp</td>
<td>&gt;25</td>
<td>Wild type</td>
<td>L1/L2</td>
<td>VD &amp; AS mns</td>
</tr>
<tr>
<td></td>
<td>&gt;25</td>
<td>unc-30(e191)</td>
<td>L1/L2</td>
<td>VD &amp; AS mns</td>
</tr>
<tr>
<td>Punc-30::unc-55a::gfp</td>
<td>10</td>
<td>Wild type</td>
<td>L1</td>
<td>6 DD mns</td>
</tr>
<tr>
<td>Punc-30::unc-55a::gfp &amp;</td>
<td>5 + 2 mosaics</td>
<td>lin-6(e1466)</td>
<td>Post L2</td>
<td>6 DD mns (ventral processes)</td>
</tr>
<tr>
<td>Punc-25::snb::gfp</td>
<td>5</td>
<td>lin-6(e1466)</td>
<td>Post L2</td>
<td>6 DD mns (dorsal processes)</td>
</tr>
<tr>
<td>Pflp-13(2.3kb)::GFP-IR &amp;</td>
<td>11</td>
<td>Wild type</td>
<td>Post L2</td>
<td>GABA &amp; FLP-13-IR co-expressed in 6 DD mns &amp; 80% reduction in # of DD mns expressing GABA &amp; FLP-13</td>
</tr>
<tr>
<td>Pflp-13(1kb)::gfp</td>
<td>&gt;15</td>
<td>Wild type</td>
<td>Post L2</td>
<td>6 DD mns</td>
</tr>
<tr>
<td>Pflp-13 A-154::gfp</td>
<td>15</td>
<td>Wild type</td>
<td>Post L2</td>
<td>No expression in vnc</td>
</tr>
<tr>
<td>Pflp-13 A-183::gfp</td>
<td>10</td>
<td>Wild type</td>
<td>Post L2</td>
<td>GFP expressed in 6 DDmns &amp; &gt;9 VD mns</td>
</tr>
</tbody>
</table>

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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.
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 nerve cord indicates the DD mns did not undergo respecification. 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.
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 (Walshall 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 DM 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 (red) and GFP (green). Within the ventral nerve cord mns, four of eight animals 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. 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 DD mns. Thus, Pflp-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 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,
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 allele (e191) 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, DD mns 1 and 2 are located in the preanl ganglion where cell identification is difficult to confirm. DD mns 12 and 13 are located in the preanl 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 palindromic 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-13A-154::gfp) or an altered UNC-55 binding site (Pflp-13A-183::gfp).

If the UNC-30 enhancer is necessary for the expression of flp-13, then we would not expect expression of Pflp-13A-154::gfp in the ventral nerve cord. This prediction proved true (Fig. 4C). Pflp-13A-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 motoneurons 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-13A-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
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(e170) 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

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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.
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 fjz and the nuclear receptor gene fjz-fl1 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 neuromorphic 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 LXXLL 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.

Acknowledgments

We thank Jung A. Kim, Benton Lawson, Vincent Rehder, Robert Simmons, and H. Mimi Zhou for discussion and comments on previous drafts of this manuscript and Mike Nonet for providing the strain juls1 containing Punc-23::snb::gfp. Some of the strains were obtained from the Caenorhabditis Genetics Center, which is supported by a grant from the National Institutes of Health. Some of this work was supported by NSF Grants IBN-9808861 and IBN-9973581, and NIH Grants K02AG00708 and NS42459 (CL).
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