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Coordinated Transcriptional Regulation of the *unc-25* Glutamic Acid Decarboxylase and the *unc-47* GABA Vesicular Transporter by the *Caenorhabditis elegans* UNC-30 Homeodomain Protein

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An important aspect of the specification of neuronal fate is the choice of neurotransmitter. In *Caenorhabditis elegans* the neurotransmitter GABA is synthesized by the UNC-25 glutamic acid decarboxylase (GAD) and packaged into synaptic vesicles by the UNC-47 transporter. Both *unc-25* and *unc-47* are expressed in 26 GABAergic neurons of five different types. Previously, we have identified that the *unc-30* homeobox gene controls the fate of 19 type D GABAergic neurons. We report here that the UNC-30 homeodomain protein transcriptionally regulates the expression of *unc-25* and *unc-47* in the 19 type D neurons. UNC-30 bound to the *unc-25* and *unc-47* promoters sequence-specifically. Mutations in the UNC-30 binding sites of the *unc-25* and *unc-47* promoters abolished the expression of reporter genes in the D neurons. The ectopic expression of UNC-30 induced the ectopic expression of reporter genes driven by the wild-type *unc-25* and *unc-47* promoters. Our data establish a mechanism for cell type-specific transcriptional coregulation of genes required for the synthesis and packaging of the neurotransmitter GABA.

Key words: C. elegans*; homeodomain; transcription; GABA; GAD; GABA transporter*

The function of a neuron depends critically on which neurotransmitter it uses. Once a neuron chooses a neurotransmitter, the neurotransmitter must be packaged into synaptic vesicles to be released. Thus, the synthesis and packaging of a neurotransmitter could be regulated coordinately. However, very few regulators that control the choice of neurotransmitter have been identified, and even less is known regarding how the coordination of neurotransmitter synthesis and packaging is achieved. For example, the temporal and spatial expression patterns of the members of the Phox2 homeodomain protein family in mouse and chick suggest that these proteins may regulate the expression of dopamine β-hydroxylase, the synthetic enzyme for dopamine (Valarche et al., 1993; Groves et al., 1995), and the rat Phox2 has been shown to stimulate transcription of dopamine β-hydroxylase (Swanson et al., 1997). The *Drosophila* gene *islet* is required for the synthesis of dopamine and serotonin in a subset of ventral cord neurons (Thor and Thomas, 1997); however, it is unknown whether *islet* regulates the biosynthetic enzymes for dopamine and serotonin directly. Neurotransmitter synthesis and packaging can be regulated coordinately, as in *Caenorhabditis elegans, Drosophila*, and mammals; the genes encoding choline acetyltransferase and the acetylcholine vesicular transporter share common promoter sequences (Rand, 1989; Eiden, 1998; Kitamoto et al., 1998). However, this mechanism cannot be universal, because genes encoding neurotransmitter synthetases and vesicular transporters generally do not appear to be clustered. For example, in both *C. elegans* and mammals the genes that encode glutamic acid decarboxylase (GAD), which synthesizes the neurotransmitter GABA, and the GABA transporter gene are not closely linked (Brenner, 1974; Bu et al., 1992; McIntire et al., 1997; Jin et al., 1999).

In *C. elegans*, 26 neurons of five different classes express GABA (McIntire et al., 1993b). Nineteen of these GABAergic neurons, known as the type D neurons, are required for normal locomotion by providing dorsoventral cross-inhibition to body wall muscles (White et al., 1986; McIntire et al., 1993b). Wild-type animals move smoothly backward in a sinusoidal manner in response to a gentle touch on the head. Animals in which all 19 D neurons are killed by laser ablation simultaneously contract dorsal and ventral body wall muscles in response to a touch on the head, resulting in a phenotype known as "shrinker" (McIntire et al., 1993b). Loss-of-function mutations in several genes result in a shrinker phenotype, suggesting that they are required for the development and/or function of the D neurons (Hodgkin, 1983; McIntire et al., 1993a). Two such genes have been shown to be involved in GABA synthesis and packaging. In *unc-25* mutants none of the 26 GABAergic neurons expresses GABA (McIntire et al., 1993a), and *unc-25* encodes GAD (Jin et al., 1999). In *unc-47* mutants, GABAergic neurons contain high levels of GABA (McIntire et al., 1993a), and *unc-47* encodes the vesicular transporter for GABA (McIntire et al., 1997).

Mutations in a third gene, *unc-30*, result in a shrinker phenotype and a lack of GABA in only the 19 type D neurons (McIntire et al., 1993a). In *unc-30* mutants the D neurons also exhibit

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defects in axonal pathfinding and synaptic connections (J. White, personal communication; our unpublished observations). *unc-30* encodes a homeodomain transcription factor that is expressed in the type D neurons (Jin et al., 1994). Moreover, the ectopic expression of *unc-30* can alter axonal projection patterns of other types of neurons and can induce many cells to express GABA. These observations suggest that *unc-30* is essential for determining the fate of type D neurons.

The UNC-30 protein is the founding member of a new group of homeodomain proteins, which includes Ptx1 (Lamonerie et al., 1996), RIEG/Ptx2/Brx1 (Semina et al., 1996; Gage and Camper, 1997; Kitamura et al., 1997), Pitx2 (Logan et al., 1998; Piedra et al., 1998; Yoshioka et al., 1998), and Crx (Chen et al., 1997; Furukawa et al., 1997). These proteins are expressed widely in both neuronal and non-neuronal tissues. Their functions are diverse, from participating in vertebrate embryonic left–right asymmetry (Logan et al., 1998; Piedra et al., 1998; Yoshioka et al., 1998) to determining photoreceptor cell fate in murine (Chen et al., 1997; Freund et al., 1997; Furukawa et al., 1997). A common feature of these UNC-30-related homeodomains is that they contain a lysine residue at position 50 in the homeodomain, as does *Drosophila bicoid* (Driever and Nusslein-Volhard, 1989). The amino acid at this position determines the DNA binding specificity of the homeodomain (Desplan et al., 1988; Hanes and Brent, 1989; Treisman et al., 1989) so that *bicoid* binds core sequences containing TAATCC with highest affinity, whereas *ftz*, which contains glutamine at position 50, has highest affinity to core sequences containing TAATTG. Two of the UNC-30 related proteins, Crx and Ptx1, bind DNA sequences with TAATCC *in vitro* and can transactivate reporter genes driven by promoters containing such a sequence (Lamonerie et al., 1996; Chen et al., 1997). However, whether they regulate transcription from these promoters *in vivo* remains to be tested.

The mutant phenotypes and gene products of *unc-30*, *unc-25*, and *unc-47* suggest that these genes may be involved in a regulated pathway for GABA expression and packaging in the type D neurons. Here we present evidence that the UNC-30 homeodomain binds the *unc-25* and *unc-47* promoters *in vitro* and that *unc-30* regulates the transcription of *unc-25* and *unc-47 in vivo*. This regulation requires both the UNC-30 binding sites in the *unc-25* and *unc-47* promoters and the UNC-30 homeodomain. We conclude that UNC-30 controls the specification of the GABAergic neurotransmitter phenotype of the D neurons by directly regulating the expression of *unc-25* and *unc-47*.

MATERIALS AND METHODS

C. elegans *genetics.* Strains were maintained on agar plates as described by Brenner (1974). Transgenes of green fluorescent protein (GFP) reporter constructs were introduced into *unc-30* mutants as follows. Males that carried the transgene were generated by mating wild-type males with hermaphrodites from the transgenic strains. Then these males were mated with hermaphrodites of either genotype *unc-30* or *unc-30; lin-15(n765ts). unc-30* animals are uncoordinated (Unc) (Brenner, 1974), and *lin-15(n765ts)* animals are multivulva at 22.5°C (Clark et al., 1994). Unc animals were selected in subsequent generations, and the presence of the transgene was recognized by examining the GFP expression with fluorescent microscopy and/or by suppressing the Lin-15 multivulva phenotype. Mutations used included *unc-30(e191), unc-30(e2327), unc-30(e646),* and *lin-15(n765ts)* (Brenner, 1974; Clark et al., 1994).

Construction of GFP reporter genes. pSC117, 96, 88, and 86 were generated by inserting *unc-25* genomic DNA from pSC67, the plasmid containing the complete *unc-25* gene (Jin et al., 1999), into the GFP vector TU#62 at the appropriate restriction enzyme sites (Chalfie et al., 1994). pCZ137 was generated by subcloning into pPD95.75 (A. Fire, personal communication) at the *Bam*HI–*Apa*I fragment from the *unc-47*-GFP construct created by K. Schuske and E. Jorgensen (McIntire et al., 1997). Deletion constructs of *unc-25*-GFP and *unc-47*-GFP reporter gene constructs were generated either by the use of convenient restriction enzyme sites or by Exonuclease III deletion (Sambrook et al., 1989). pSC319, 320, 321, and other GFP reporter constructs that contain mutations in the homeodomain binding sites were generated first by using PCR to generate mutations in the *unc-25* and *unc-47* promoters (see below) and then by subcloning the mutant promoters into TU#61 (for *unc-25*) and pCZ137 (for *unc-47*). The sequences of the 5′ ends of the deletion constructs were determined by using either the fmol sequencing kit (Promega, Madison, WI) or the ABI sequencing kit (Applied Biosystems, Foster City, CA), according to each manufacturer's instructions.

Generation of homeodomain binding site mutations. Mutations in the homeodomain binding sites in the *unc-25* promoter were generated by several rounds of PCR. For example, to mutate the homeodomain binding (HD) sites to GC-rich sequences, we first performed three separate PCR reactions, using pSC86 as a template with different primer pairs. PCR reaction A used two primers complementary to the sequences around each of the HD sites but that contained GC-rich sequences in place of the HD sites; this reaction generated the *unc-25* promoter sequences between the two HD sites (−124 to −38), with each HD site replaced by GC sequences. PCR reaction B used one primer matching the sequences that are 50 bp upstream of the 5′ end of the *unc-25* promoter and another primer complementary to the 5′ 15 nucleotides of the primer used in PCR reaction A containing GC-rich mutations in HD1; this reaction generated the *unc-25* DNA from the 5′ end of the *unc-25* promoter to the GC-mutated HD1 site (−180 to −109). PCR reaction C used one primer complementary to the sequences that are 50 bp downstream of the 3′ end of the *unc-25* promoter and another primer matching the sequences of the 5′ 15 nucleotides of the primer used in PCR reaction A containing the GC-rich mutations in HD2; this reaction generated the *unc-25* promoter sequences from HD2 to the transcriptional start site of *unc-25* (−53 to +1). Thus, the DNA fragments resulting from PCR reaction A share 15 bp with those from PCR reaction B at the 5′ end and 15 bp with those from PCR reaction C at the 3′ end. Then the products of these three PCR reactions were purified and mixed together as templates in a final round of PCR, using the two primers flanking the 5′ and 3′ ends of the *unc-25* promoter to generate a full-length *unc-25* promoter that contained GC-rich mutations in both HD sites. This fragment subsequently was subcloned either into TU#61 to drive GFP expression or into pBluescript for use in gel shift experiments.

To generate *unc-25* promoters that contained a GC-rich mutation in only one of the HD sites, we used one primer that contained GC-rich mutations in the desired HD site in conjunction with a primer complementary to the wild-type sequences of the other HD site in PCR reaction A. Similarly, *unc-25* promoters that contained *ftz*-like HD site mutations were generated by using primers containing TAATTG in place of TAATCC.

Mutations in the homeodomain binding site in the *unc-47* promoter were generated similarly. The *unc-47* promoter DNA including −230 to −167 was generated by using one primer upstream of the 5′ end of *unc-47* and one primer complementary to the sequences around the HD site but that contains GC sequences in place of the HD sequence. The *unc-47* promoter DNA including −180 to +1 was generated by using a primer downstream of the *unc-47* initiation codon ATG with a primer that matches the 15 nucleotides of the primer containing the HD mutation. These two DNA fragments overlapped by 14 bp and together span the entire *unc-47* promoter. Then these DNA fragments were purified and mixed together as templates for another round of PCR by using the primer upstream and the primer downstream of the *unc-47* promoter to generate the full-length *unc-47* promoter containing the mutated HD site.

All of the *unc-25* and *unc-47* DNA sequences in the final constructs were determined to confirm that no other mutations had been introduced.

Germline transformation and analysis of GFP expression. lin-15(*n765ts*) mutant animals were transformed with 50 ng/μl of the *lin-15*(+)-rescuing plasmid, p*lin-15*(EK) (Clark et al., 1994), and 50–100 ng/ml of GFP reporter constructs by following standard procedures (Mello et al., 1991). At the nonpermissive temperature (22.5°C) *lin-15(n765)* animals were multivulva, and transformants were identified as non-Muv animals. GFP reporter genes were maintained as extrachromosomal arrays. Transformed adult hermaphrodites in the F1 and subsequent generations were examined for GFP expression by a Zeiss Axioskop with an HQ-FITC

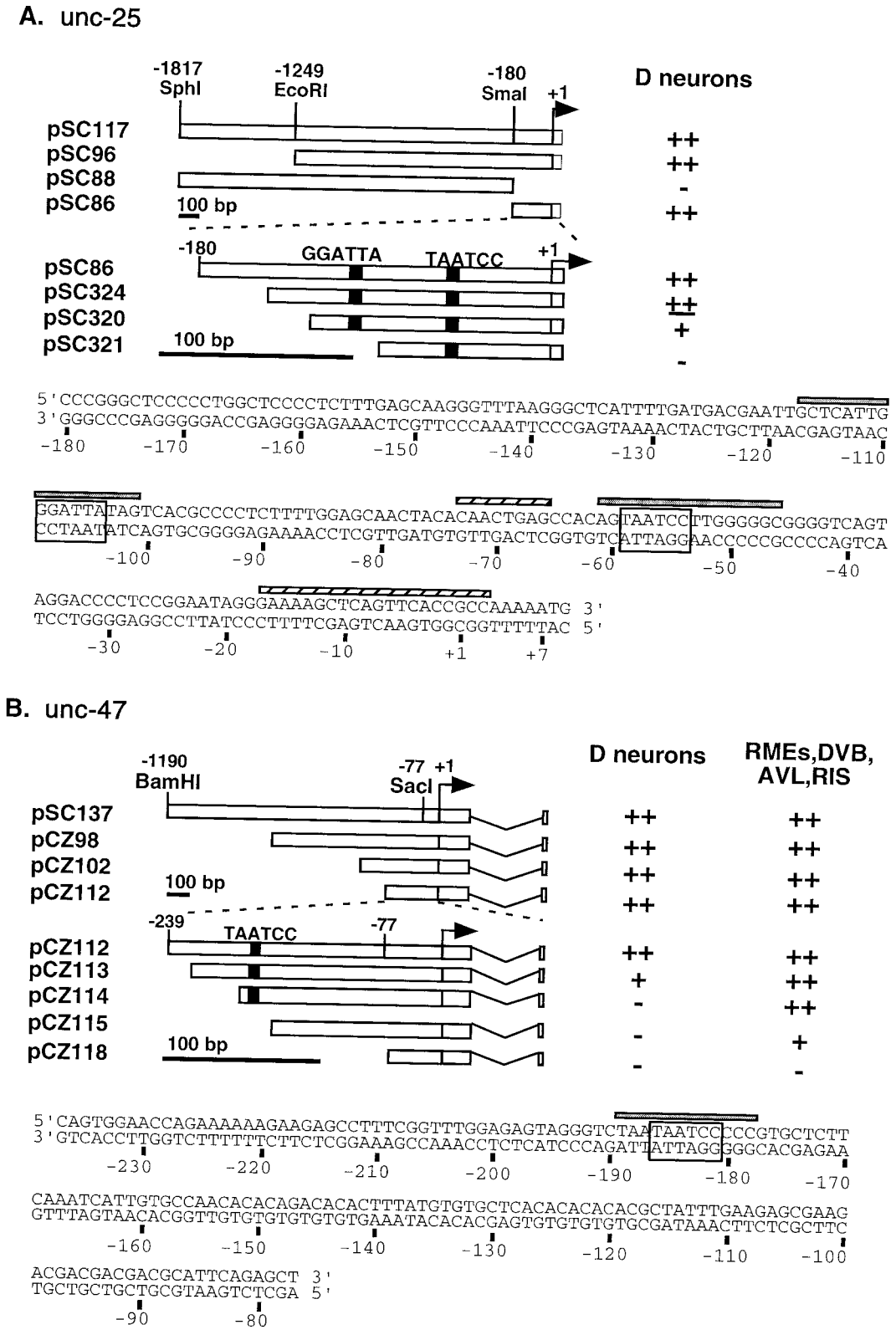

Figure 1. The minimal *unc-25* and *unc-47* promoters required for expression in the type D neurons contain consensus homeodomain binding sequences. *A*, Identification of the *unc-25* promoter required for expression in the type D neurons. *B*, Identification of the *unc-47* promoter required for expression in the type D neurons. Selected restriction enzyme sites are shown; *+1* indicates the transcription start site. The consensus homeodomain binding sites are shown as *black boxes* and are *boxed* in the DNA sequences. *Gray bars above* the DNA sequence indicate sequences protected by the UNC-30 homeodomain in wild-type promoters, but not in *unc-25* or *unc-47* mutant promoters (see Fig. 5). *Hatched bars* indicate sequences protected in both wild-type and *unc-25* mutant promoters. ++, Strong GFP expression; $\pm\pm$, a slightly weaker GFP expression in the indicated neurons both in the F1 and subsequent transgenic progeny; +, moderate GFP expression in the indicated neurons in the F1 but weak expression in the F2 and later transgenic progeny; −, no GFP expression in the indicated neurons in transgenic animals.

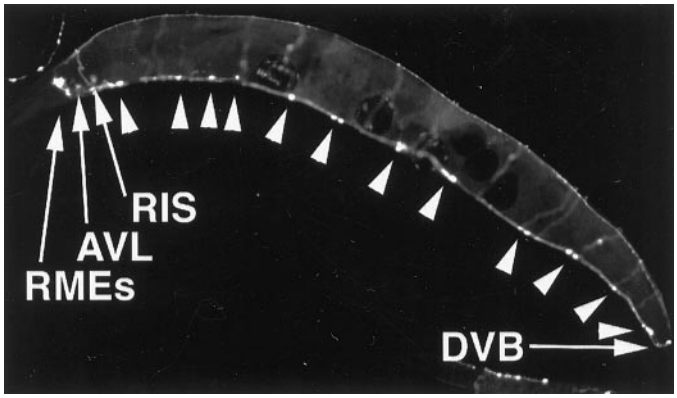

Figure 2. *unc-47*-GFP is expressed in all GABAergic neurons. Adult hermaphrodite of the genotype *lin-15(n765ts); Ex[*P_{unc-47}-GFP(pCZ137), *lin-15(+)]*, viewed with fluorescence microscopy. *Arrows* point to the non-D-type GABAergic neurons; there are four *RMEs*. *Arrowheads* point to type D neurons (only some of the D neurons are indicated). The expression of P_{unc-25}-GFP was identical to the expression of P_{unc-47}-GFP (data not shown).

filter (Chroma Technology, Brattleboro, VT). F1 transformants (15–50) for each reporter construct were scored. Animals (15–30) from at least three independently established lines were examined and scored individually. Both the number of neurons expressing GFP and the relative intensity of GFP expression were scored. Strong GFP expression in the neurons of interest in the F1 and in subsequent transgenic progeny was scored as ++. Moderate GFP expression in the neurons of interest in the F1 but weak expression in the F2 and later transgenic progeny was scored as +.

Electromobility shift assays. A region of the *unc-30* cDNA containing the entire homeodomain and 43 flanking amino acids (18 N-terminal and 25 C-terminal to the homeodomain) was amplified first by PCR and then fused in-frame to glutathione *S*-transferase (GST) in pGEX-5X (Pharmacia, Piscataway, NJ). Protein was induced by the addition of 10 mM isopropyl β-D-thiogalactoside (IPTG) and purified over glutathione–agarose columns (Sigma, St. Louis, MO). GST and GST-UNC-30 proteins were eluted with 50 mM NaCl and 10 mM reduced glutathione. Protein concentrations were quantitated by comparison with known amounts of bovine serum album by Coomassie blue staining after SDS-PAGE.

A 161 bp *Hin*DIII–*Sac*I *unc-47* promoter fragment from pCZ112 was labeled with α-^{32}P-dATP, using the Klenow enzyme, and was gel-purified essentially as described (Sambrook et al., 1989). A 227 bp *Bam*HI–*Xho*I *unc-25* promoter fragment from pSC 86 was labeled similarly.

Wild-type competitor oligonucleotides were formed by annealing 5′-GGGATTACTGCA-3′ and 5′-GTAATCCCTGCA-3′. Mutant competitor oligo nucleotides were formed by annealing 5′-GCGCGCGCTGCA-3′ and 5′-GCGCGCGCTGCA-3′.

Approximately 20,000 cpm of the labeled DNA was used in each binding reaction. This level of activity was calculated to correspond to 5.4 fmol of all *unc-25* probes and 10.8 fmol of all *unc-47* probes. Binding reactions contained (in mM) 20 Tris-HCl, pH 7.5, 1 EDTA, 5 $MgCl_2$, 0.03–0.05 dithiothreitol, and 1.7–2.5 KCl plus 20 μg/BSA, 10% glycerol, 0.1% Igepal (Sigma), and 0.02–0.04 mg/ml sonicated salmon sperm DNA. Reactions were incubated at 30°C for 20 min or as stated in each experiment. Products were separated on 6% native polyacrylamide gels; the gels were dried and exposed to films at −80°C overnight.

DNaseI footprint analyses. GST-UNC-30 proteins were prepared as described above. The minimal wild-type and mutant *unc-25* promoters were end-labeled at their 3′ ends. The minimal wild-type and mutant *unc-47* promoters were end-labeled at their 5′ ends. Footprint reactions were performed essentially as described (Desplan et al., 1988), with the following exceptions. All reactions were performed at room temperature in the same binding buffer as that for the electromobility shift assay. Before brief incubation with 0.1–0.3 U of DNaseI, HEPES, pH 7.5, was added to 40 mM, $MgCl_2$ was added to 20 mM, and $CaCl_2$ was added to 5 mM. Reactions were stopped by the addition of 100 mM EDTA, pH 8, and 60 mM Tris, pH 8. Identical DNA fragments were treated chemically with dimethyl sulfate, which preferentially cleaves after guanine, to produce a ladder as marker (Sambrook et al., 1989). DNA samples were analyzed by using 6% denaturing polyacrylamide gels; the gels were dried and exposed to film at room temperature.

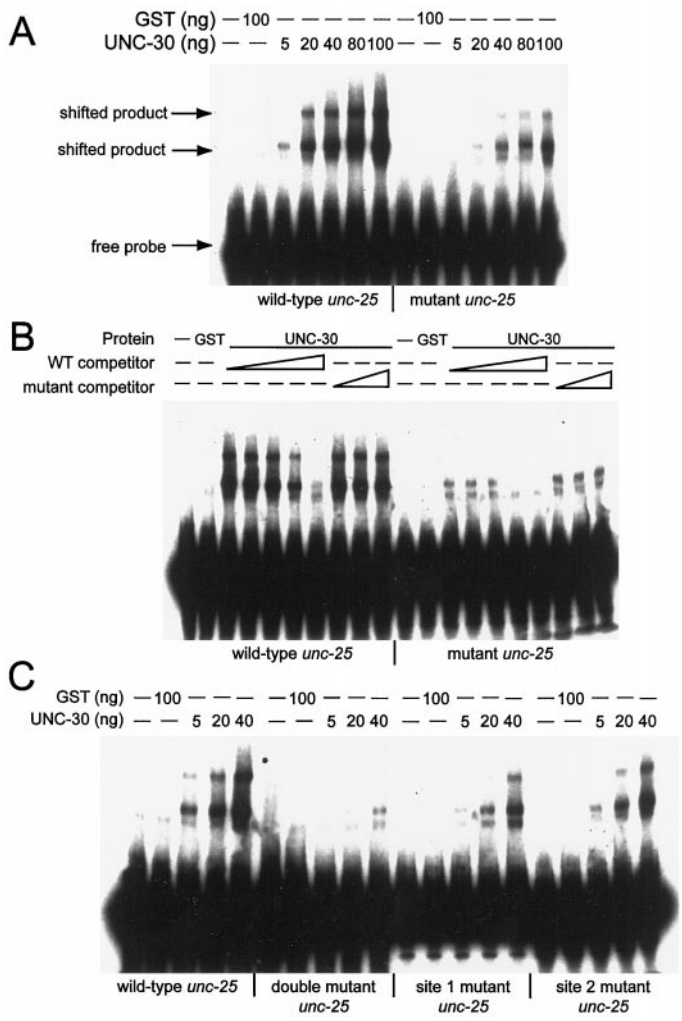

Figure 3. The UNC-30 homeodomain binds the *unc-25* promoter. *A*, UNC-30 binds the wild-type *unc-25* promoter. The wild-type *unc-25* promoter that was used was from pSC86. The *unc-25* mutant promoter that was used contained GCGCGC in place of each of the consensus homeodomain binding sequences TAATCC. −, No protein added. *B*, Sequence-specific competition of the binding of UNC-30 to the *unc-25* promoter. GST (100 ng) or GST-UNC-30 (40 ng) was used in the binding reactions; −, no protein added. The wild-type competitor contained TAATCC, and the mutant competitor contained GCGCGC. *Triangles* indicate increasing amounts of competitor added to the binding reactions: the excesses of WT competitor were 1×-, 1.8×-, 4.4×-, and 17.6×-fold; the excesses of mutant competitor were 1×-, 1.8×-, and 4.4×-fold. −, No competitor added. *C*, UNC-30 binds to both consensus homeodomain binding sites in the *unc-25* promoter. The double mutant *unc-25* promoter contained GCGCGC at each of the homeodomain binding sites. Site 1 mutant *unc-25* and site 2 mutant *unc-25* promoters contained GCGCGC in place of the first or second consensus homeodomain binding sequence, respectively. −, No protein added.

Northern blot analysis. Poly(A^+) RNA was isolated from mixed-stage N2 and *unc-30(e191)* worms. Poly(A^+) RNA (1 μg) was loaded on a denaturing agarose gel. Gel electrophoresis and probing were performed by following standard procedures (Sambrook et al., 1989). The rDNA plasmid was a gift of M. Koelle (personal communication) and was used as a loading standard.

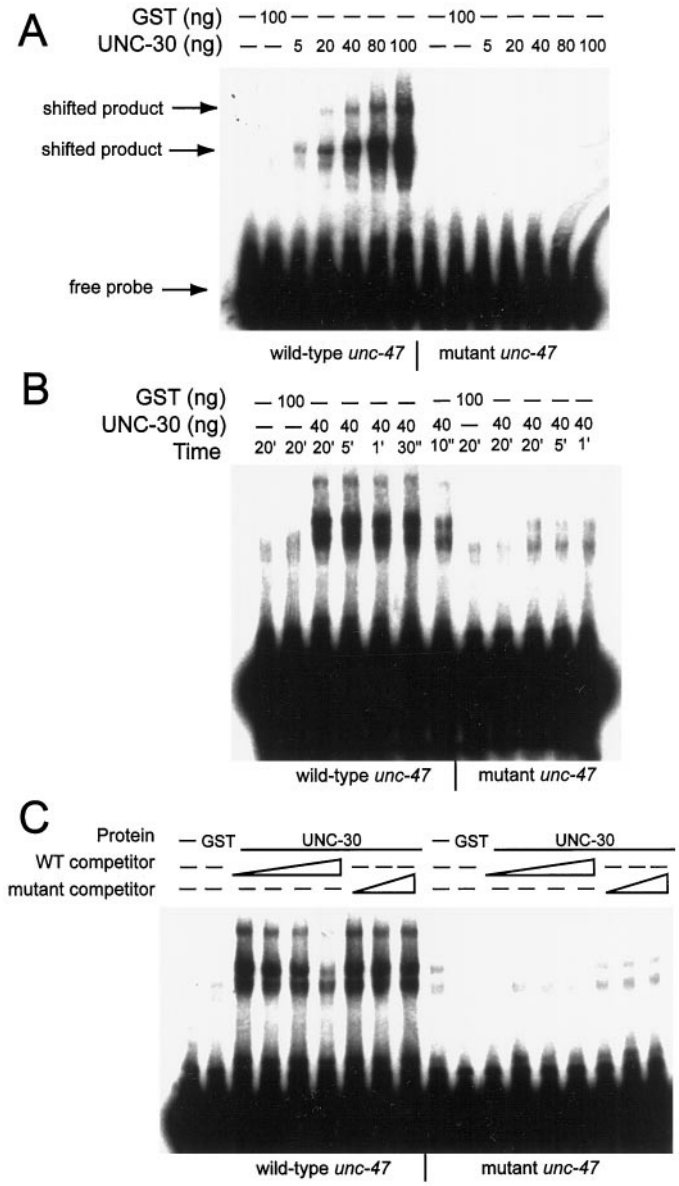

Figure 4. The UNC-30 homeodomain binds the *unc-47* promoter. *A*, UNC-30 binds the wild-type *unc-47* promoter. The wild-type *unc-47* promoter that was used was from pCZ112. The *unc-47* mutant promoter that was used contained GGGGCC in place of the consensus homeodomain binding sequence TAATCC. −, No protein added. *B*, A time course of UNC-30 binding to the *unc-47* promoter. −, No protein added. *C*, Sequence-specific competition of the binding of UNC-30 to the *unc-47* minimal promoter. GST (100 ng) or GST-UNC-30 (40 ng) was used in the binding reactions. −, No protein added. The wild-type competitor and the mutant competitor are the same as in Figure 3*B*. *Triangles* indicate increasing amounts of competitor added: the excesses of WT competitor were 0.5×-, 1×-, and 2.2×-fold; the excesses of mutant competitor were 0.5×-, 1×-, and 2.2×-fold. −, No competitor added.

RESULTS

The minimal *unc-25* and *unc-47* promoters contain consensus homeodomain binding sequences specific for *bicoid* class homeodomain proteins

To examine the transcriptional regulation of *unc-25* and *unc-47* in the type D neurons, we created transgenes in which 5′ upstream sequences from each gene were fused to the GFP (Chalfie et al., 1994). pSC117 contains 1.8 kb of the *unc-25* upstream sequence, and pCZ137 contains 1.2 kb of the *unc-47* upstream sequence (Fig. 1). Both reporter genes were expressed in all GABAergic neurons, which include the 19 D neurons in the ventral cord, the four RMEs, AVL, and RIS in the head, and DVB in the tail (Fig. 2). These expression patterns were identical to those for GABA (McIntire et al., 1993b), *unc-47* (McIntire et al., 1997), and *unc-25* (Jin et al., 1999).

To identify the DNA sequence elements required for the expression of *unc-25* and *unc-47* in the type D neurons, we generated a series of nested deletion GFP reporter gene constructs and analyzed the expression of GFP in transgenic animals. The 5′ boundaries of these two promoters needed to drive robust GFP expression in the type D neurons were at position −180 for *unc-25* (Fig. 1*A*,*B*) and at position −239 for *unc-47* (Fig. 1*C*,*D*). For simplicity, we use the term "minimal promoter" in the text to refer to the *unc-25* and *unc-47* DNA fragments in pSC86 and pCZ112, respectively. By inspection we determined that the minimal *unc-25* and *unc-47* promoters contain DNA sequences (TAATCC) that match the high-affinity binding sites for *bicoid* (Driever and Nusslein-Volhard, 1989). The *unc-47* promoter contains one copy of this sequence at nucleotide positions −186 to −181; the *unc-25* promoter contains two copies of this sequence at nucleotide positions −109 to −103 and nucleotide positions −58 to −53 (Fig. 1*B*,*D*).

UNC-30 binds the *unc-25* and *unc-47* promoters in a sequence-specific manner

The presence of homeodomain binding sites in the *unc-25* and *unc-47* minimal promoters suggests that the UNC-30 homeodomain may bind these promoters. We tested this possibility by performing electromobility gel shift assays, using bacterially produced recombinant UNC-30 protein and the minimal *unc-25* and *unc-47* promoter fragments. Incubation of as little as 5 ng of GST-UNC-30 with either the wild-type *unc-25* or *unc-47* minimal promoters resulted in products shifted in their gel mobilities (Figs. 3*A*, 4*A*). Formation of the gel mobility-shifted products was rapid. For example, they appeared within 30 sec of incubation of GST-UNC-30 with the *unc-47* promoter (Fig. 4*B*).

To determine the sequence specificity of these interactions, we performed the following two experiments. First, we mutated the homeodomain consensus sequences to GC-rich sequences in both the *unc-25* and *unc-47* minimal promoters (see Materials and Methods) and found that the gel mobility-shifted products either were reduced substantially in amount (Fig. 3*A*) or were never formed (Fig. 4*A*) when increasing amounts of GST-UNC-30 proteins were added. Second, we added cold competitor oligo nucleotides to the binding reactions and found that the formation of gel mobility-shifted products was reduced significantly, whereas little effect was seen with the addition of cold GC-rich oligo nucleotides (Figs. 3*B*, 4*C*).

Because there are two copies of the homeodomain consensus binding site in the *unc-25* promoter, we examined whether both sites could be bound by UNC-30. We performed the binding reactions, using mutated *unc-25* promoters in which only one of the core homeodomain binding sites was mutated to a GC-rich sequence (see Materials and Methods). The gel mobility-shifted products were formed on the singly mutated *unc-25* promoters, although the formation of these gel-shifted products required a slightly higher amount of GST-UNC-30 proteins than did the

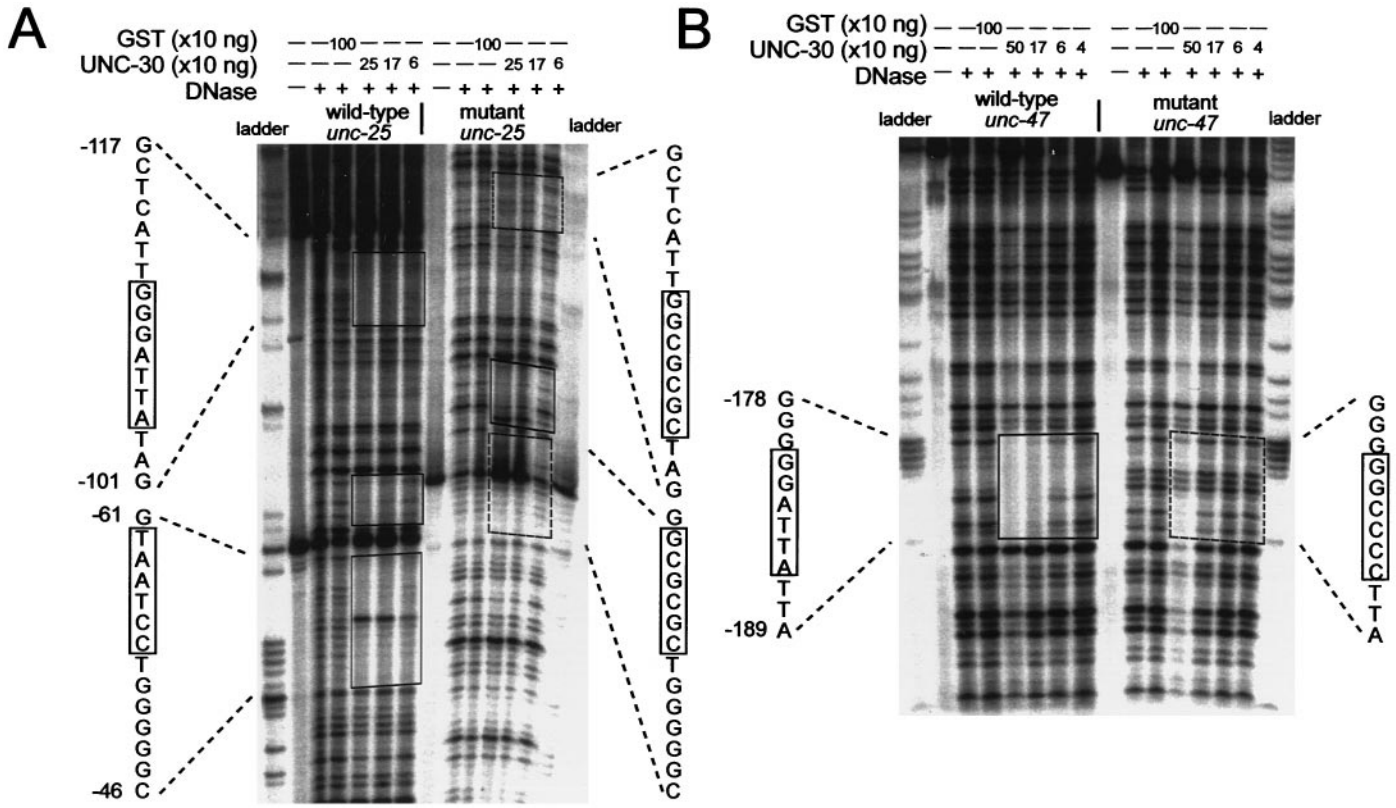

Figure 5. The UNC-30 homeodomain protects the core HD binding sites in the *unc-25* and *unc-47* promoters. *A*, Autoradiographs of footprint analyses of recombinant UNC-30 protein on the wild-type and mutant *unc-25* promoters. UNC-30 protected primarily the consensus homeodomain binding sites in the wild-type *unc-25* minimal promoter, but not in the mutant *unc-25* promoter in which both of the homeodomain binding sites were changed to GCGCGC. *B*, Autoradiographs of the footprint analyses of UNC-30 protein on the wild-type and mutant *unc-47* promoters. UNC-30 protected the consensus homeodomain binding sequence in the wild-type minimal *unc-47* promoter, but not in the mutant *unc-47* promoter in which the homeodomain binding site was changed to GGGGCC. *Ladders* represent cleavage products after guanines for the wild-type and mutant promoter probes, respectively. *Solid boxes* indicate sequences protected in the wild-type promoters, but not in the mutant promoters. *Dashed boxes* in *A* indicate sequences partially protected in both the wild-type and the mutant *unc-25* promoters; these footprints do not overlap with the consensus binding sequences. −, No protein added/no DNaseI added; +, 1 U of DNaseI added (*A*) or 3 U of DNaseI added (*B*).

wild-type promoter (Fig. 3*C*). From these experiments we conclude that UNC-30 can bind the *unc-25* and *unc-47* promoters *in vitro* in a sequence-dependent manner.

UNC-30 footprints the core homeodomain binding sequences in the *unc-25* and *unc-47* promoters

Although the minimal *unc-47* promoter and the singly mutated *unc-25* promoters each contain only one consensus homeodomain binding site, several gel-shifted products were observed in our binding reactions. These observations raised the possibility that the UNC-30 homeodomain might bind to other sites in addition to the consensus homeodomain binding sites. To determine which sequences in the *unc-25* and *unc-47* promoters were bound by UNC-30, we performed DNaseI footprinting analysis, using recombinant UNC-30 protein (see Materials and Methods). The wild-type *unc-25* promoter was strongly protected at two regions, −101 to −117 and −46 to −61, corresponding precisely to the core homeodomain binding sites (Figs. 5*A*, 2*B*). Both the wild-type and mutant *unc-25* promoters also were weakly protected at two additional regions, −66 to −73 and −17 to +3. Similarly, the wild-type *unc-47* promoter was protected from −178 to −189, corresponding to the core binding site (Figs. 5*B*, 2*D*). Thus, our data indicate that UNC-30 binds primarily to the consensus homeodomain binding sequences in the *unc-25* and *unc-47* promoters *in vitro*. The multiple gel-shifted products seen in Figures 3 and 4 may be the result of dimer formation mediated by the GST portion in GST-UNC-30, because GST is known to form dimers (Tudyka and Skerra, 1997) and/or the generation of different forms of a single gel-shifted product during electrophoresis.

UNC-30 positively regulates *unc-25* and *unc-47* in the type D neurons

The UNC-30 protein binds the *unc-25* and *unc-47* promoters *in vitro*. Is *unc-30* required for the expression of *unc-25* and *unc-47* in the type D neurons? To examine whether *unc-30* transcriptionally regulates *unc-25* and *unc-47* in the type D neurons, we crossed transgenic arrays containing pSC117 and its derivatives or pCZ137 and its derivatives into *unc-30(e2327)* and *unc-30(e191)* animals, respectively. These *unc-30* animals are genetic and molecular null mutants (Jin et al., 1994). In these *unc-30* mutants, GFP from these transgenic arrays were expressed in RMEs, AVL, RIS, and DVB, but not in the D neurons (Fig. 6*A*). Moreover, using Northern blots, we detected an ~10-fold reduction of *unc-25* mRNA in *unc-30* mutant animals (Fig. 6*B*).

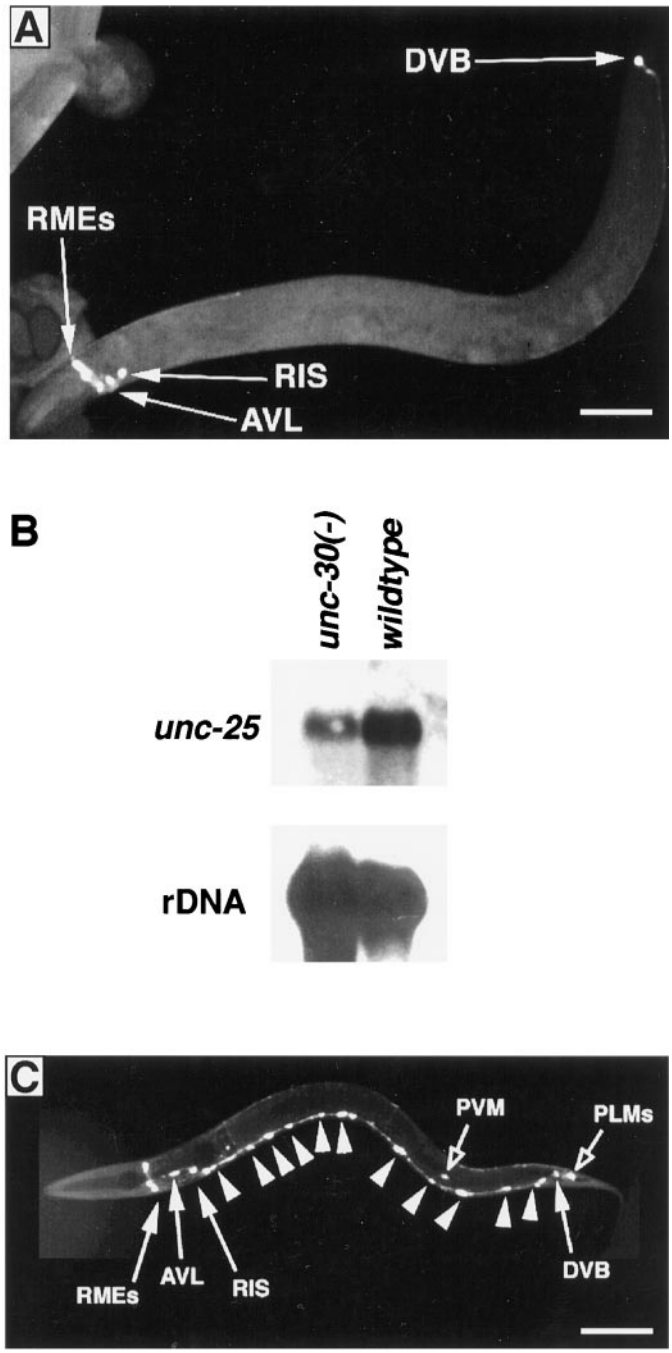

Figure 6. *unc-30* regulates the expression of *unc-25* and *unc-47 in vivo*. *A*, *unc-30* is required for the expression of *unc-47*-GFP in the type D neurons. Shown is an adult worm of the genotype *unc-30(e191); lin-15(n765ts); Ex[*P_{unc-47}-GFP(pCZ137), *lin-15(+)]*. GFP is completely absent in the D neurons but is present in other GABAergic neurons (*arrows*). The expression of *unc-25*-GFP in this background was indistinguishable from that of *unc-47*-GFP. *B*, *unc-25* mRNA is greatly reduced in *unc-30* mutants. A Northern blot containing poly(A^+) RNA from mix-staged wild-type (N2) and *unc-30(e191)* animals was probed with labeled *unc-25* cDNA. Ribosomal DNA (*rDNA*) was used as a loading control; although overexposed, essentially equal amounts of RNA were present in both lanes. *C*, Ectopic UNC-30 can drive ectopic expression of *unc-47*-GFP. Shown is an adult worm of the genotype *lin-15(n765ts); Ex[*P_{mec-7}*(unc-30wt);* P_{unc-47}-GFP; *lin-15(+)]*. Ectopic expression of P_{unc-47}-GFP was seen in the touch neurons *PVM* and *PLM* (*open arrows*). Ectopic UNC-30 similarly induced the ectopic expression of P_{unc-25}-GFP (data not shown).

UNC-30 thus is specifically required for the expression of *unc-25* and *unc-47* in the D neurons.

The core homeodomain binding sequences in the *unc-25* and *unc-47* promoters are required for regulation by UNC-30 *in vivo*

The UNC-30 protein binds the consensus homeodomain binding sequences in the *unc-25* and *unc-47* promoters *in vitro*. To determine the importance of these binding sequences *in vivo*, we analyzed the expression of GFP driven by mutant *unc-25* and *unc-47* promoters (see Materials and Methods). When either one of the two consensus sites in the *unc-25* minimal promoter was mutated to either a GC-rich sequence or to a consensus binding sequence specific for *ftz* class homeodomains, the expression level of GFP in the D neurons was reduced slightly (Fig. 7*A*). When both sites were mutated, GFP no longer was expressed in the type D neurons (Fig. 7*A*). Similarly, mutations in the single binding sequence in the *unc-47* promoter completely abolished GFP expression in the D neurons (Fig. 7*C*). The intensity of GFP expression in the other non-D-type GABAergic neurons also was decreased by ~50%, and GFP expression in these neurons was more mosaic than was expression from the wild-type *unc-47*-GFP construct. This effect might be a consequence of interference by the mutations with enhancer elements for the other GABAergic neurons. These results indicate that the homeodomain consensus sites in the *unc-25* and *unc-47* promoters are required specifically for the expression of these genes in the type D neurons.

Ectopic UNC-30 can induce ectopic expression of *unc-25*-GFP and *unc-47*-GFP

Previously, we showed that ectopic expression of the UNC-30 protein was sufficient to induce ectopic expression of GABA (Jin et al., 1994). We now asked whether ectopic expression of UNC-30 was sufficient to induce ectopic expression of *unc-25* and *unc-47*. We generated transgenes containing *unc-25*-GFP or *unc-47*-GFP and a *mec-7* promoter-driven *unc-30* cDNA (P_{mec-7}*unc-30*) (see Materials and Methods). The *mec-7* promoter drives gene expression in the six touch neurons (PLMs, PVM, ALMs, and AVM) (Hamelin et al., 1992). Approximately 80% of animals carrying these transgenes expressed GFP in some of the touch cells in addition to all GABAergic neurons (Figs. 6*C*, 7*B*,*D*).

To test whether such ectopically induced expression requires the consensus homeodomain binding sites in the *unc-25* and *unc-47* promoters, we generated transgenic animals containing the P_{mec-7}*unc-30* construct with the *unc-25*-GFP and *unc-47*-GFP constructs in which the homeodomain binding sites were mutated. GFP expression in the touch neurons was diminished (Fig. 7*B*,*D*), indicating that *unc-30*-induced ectopic expression of *unc-25*-GFP and *unc-47*-GFP required these sites. We further examined whether the DNA binding specificity of UNC-30 is required for the ectopic expression of *unc-25*-GFP and *unc-47*-GFP. We created a mutant *unc-30* cDNA in which the lysine at position 50 of the homeodomain was replaced with a glutamine [*unc-30* (*K50Q*)]. Homeodomains containing glutamine at this position are predicted to bind the sequence TAATTG (Desplan et al., 1988; Treisman et al., 1989). In transgenic animals containing P_{mec-7}*unc-30* (*K50Q*) and wild-type *unc-25*-GFP or *unc-47*-GFP, respectively, we observed that GFP was expressed in all GABAergic neurons but never in the touch neurons (Fig. 7*B*,*D*). This result also indicated that the ectopic expression of GFP in

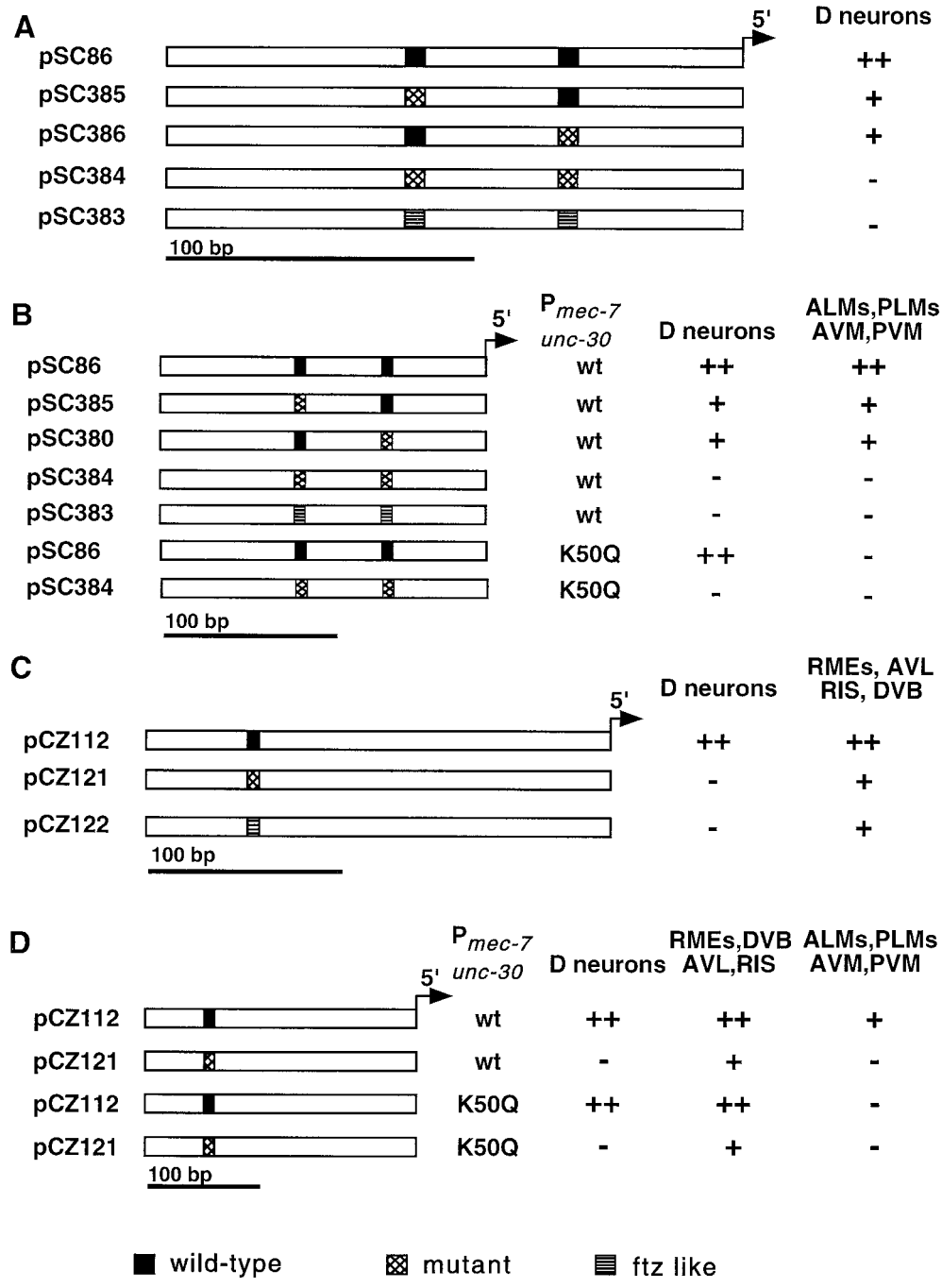

Figure 7. Both the UNC-30 homeodomain and the consensus homeodomain binding sites are required for the expression of P_{unc-25}-GFP and P_{unc-47}-GFP. *A*, *C*, Analysis of GFP expression from the transgenes in which GFP was driven by the wild-type or mutant *unc-25* promoters (*A*) or by the wild-type or mutant *unc-47* promoters (*C*). Homeodomain consensus binding sites, indicated by the *black boxes*, are required for P_{unc-25}-GFP and P_{unc-47}-GFP expression in the type D neurons. *B*, *D*, Analysis of ectopic GFP expression in touch neurons from transgenes containing P_{unc-25}-GFP (*B*) or P_{unc-47}-GFP (*D*) with P_{mec-7}*unc-30* or P_{mec-7}*unc-30* (*K50Q*). Both homeodomain consensus binding sequences in the *unc-25* and *unc-47* promoters and the UNC-30 homeodomain were required for ectopic expression of P_{unc-25}-GFP or P_{unc-47}-GFP driven by ectopic UNC-30. *Black boxes*, Wild-type consensus binding sequence (TAATCC); *cross-hatched boxes*, GCGCGC in *A* and *B* and GGGGCC in *C* and *D*. In the P_{mec-7}*unc-30* column, *wt* indicates the full-length wild-type *unc-30* cDNA driven by the *mec-7* promoter, and *K50Q* indicates the full-length *unc-30* cDNA in which the lysine at position 50 in the homeodomain was mutated to glutamine. ++, Strong GFP expression in both the F1 and subsequent transgenic progeny; +, weak GFP expression in the indicated neurons in the F1 and F2; −, no GFP expression in the indicated neurons in transgenic animals.

touch neurons is not attributable to trans-splicing from the array created by the cotransformation of the P_{mec-7}*unc-30* (*K50Q*) constructs but rather via an *unc-30*-mediated mechanism. Thus, taken together, these *in vivo* analyses support the conclusion that the core consensus homeodomain binding sequences in the *unc-25* and *unc-47* promoters and the wild-type UNC-30 homeodomain are both required for regulation of *unc-25* and *unc-47* by UNC-30 in the type D neurons.

DISCUSSION

unc-30 directly controls the expression in the type D neurons of GAD and the GABA vesicular transporter

We previously reported that the UNC-30 homeodomain protein is necessary for GABA expression in the type D neurons and can induce many cells to express GABA when it is expressed ectopically (Jin et al., 1994). In this paper we identified two genes, the *unc-25* GAD and the *unc-47* GABA vesicular transporter, as *in vivo* targets for UNC-30. We found that the promoters required for *unc-25* and *unc-47* expression in the type D neurons contain DNA sequences that were bound *in vitro* by the UNC-30 protein. These UNC-30 binding sites were necessary for the expression of reporter genes driven by the *unc-25* and *unc-47* promoters in the type D neurons *in vivo*. Moreover, the reporter gene expression driven by the *unc-25* and *unc-47* promoters in the type D neurons was abolished specifically in *unc-30* null mutants. Ectopically expressed *unc-30* could induce the ectopic expression of reporter genes driven by the *unc-25* and *unc-47* promoters. We further demonstrated that this *in vivo* interaction among *unc-30* and *unc-25* and *unc-47* required both intact UNC-30 binding sites in the *unc-25* and *unc-47* promoters and the intact UNC-30 homeodomain. Our data indicate that *unc-30* coregulates the transcription of *unc-25* and *unc-47* in the type D neurons by binding directly to their promoters.

Our studies raised several interesting questions about the regulation of *unc-25* and *unc-47* in other neurons. Both *unc-25* and *unc-47* are expressed in seven non-D-type GABAergic neurons that do not express *unc-30* (McIntire et al., 1997; Jin et al., 1999). We have identified an *unc-47* promoter fragment that is sufficient for driving GFP expression in the seven non-D-type GABAergic neurons but that does not interact with UNC-30 protein. Perhaps there are other transcription factors that express specifically in those neurons and play similar roles as *unc-30* in the type D neurons. At present, such candidates have not been identified. Moreover, *unc-30* normally is expressed in six neurons that do not express *unc-25* and *unc-47* (Jin et al., 1994). Although we have shown that *unc-30* is able to induce GFP expression ectopically from *unc-25* and *unc-47* promoters, we do not think that *unc-30* is able to do so in all types of cells. We have envisaged that in the six non-GABAergic neurons there are proteins that act to inhibit *unc-30* function (Jin et al., 1994). The fact that *unc-30* can induce *unc-25* and *unc-47* expression in touch neurons suggests that the touch neurons and D neurons have common factors that act in concert with UNC-30. These common factors may play a general role in transcriptional activation, whereas UNC-30 confers a D-type cell-specific regulation, and other transcriptional factors, namely UNC-86 and MEC-3, confer a touch neuron-specific regulation (Duggan et al., 1998).

In mammalian cells the level of GAD mRNA can be altered by many factors, such as *fos* and retinoic acid (Bain et al., 1993; Bowers et al., 1998). However, little is known about how GAD and the GABA transporter are activated in a cell-specific manner. Our findings reveal that GAD and the GABA transporter can be coactivated transcriptionally by a single homeodomain protein in a specific subtype of GABAergic neurons.

Mechanisms of coregulation of neurotransmitter synthetases and vesicular transporters

All classical neurotransmitters and many peptide neurotransmitters must be packaged into synaptic vesicles to be released. The coupling of neurotransmitter synthesis and packaging would provide a mechanism to ensure neurotransmitter function. Indeed, many neurotransmitter synthetases, although they are cytosolic proteins, are found predominantly in association with synaptic vesicles (D'Amelio et al., 1987; Ueda et al., 1987; Erlander et al., 1991). Another mechanism for coupling neurotransmitter synthesis and packaging has been shown for acetylcholine. From *C. elegans* and *Drosophila* to mammals the choline acetyltransferase gene and the acetylcholine transporter gene share common first exon and upstream regulatory regions and hence can be coregulated transcriptionally (Rand, 1989; Misawa et al., 1995; Eiden, 1998; Kitamoto et al., 1998).

GABA is the major inhibitory neurotransmitter in both vertebrate and invertebrate nervous systems (Cooper et al., 1991). The protein and gene structures of GAD are highly conserved between worms and mammals (Jin et al., 1999), as is the protein structure of GABA transporters (McIntire et al., 1997). However, *unc-25* and *unc-47* are >15 map units apart (Brenner, 1974; McIntire et al., 1997; Jin et al., 1999), and GAD and GABA transporters in other organisms do not appear closely linked either (Bu et al., 1992; McIntire et al., 1997). In *C. elegans* the expression of *unc-25* and *unc-47* is regulated by the same transcriptional factor in the type D neurons. It appears that an early duplication of certain promoter elements may have been maintained by selective pressure to coordinate the expression of two genes that function in the same process. Whether this mechanism is conserved in other organisms remains to be seen.

Proteins with UNC-30-like homeodomains have diverse developmental functions

The UNC-30 homeodomain is the founding member of a novel group of homeodomain proteins. The UNC-30 homeodomain is 85% identical to its closest relative, that of murine Ptx1 and Ptx2. Members of this family play important roles in many developmental processes. The human *crx* gene is required for photoreceptor development (Chen et al., 1997; Freund et al., 1997; Furukawa et al., 1997). Mutations in the human *RIEG* gene lead to Rieger syndrome, which involves learning disabilities and other defects (Semina et al., 1996). The murine RIEG homolog RIEG/Ptx-2/Brx1 is expressed in the eye and several anterior brain structures (Semina et al., 1996; Gage and Camper, 1997; Kitamura et al., 1997). Murine Ptx1 is expressed in the stomodeum during embryogenesis and regulates the transcription of the pro-opiomelanocortin gene in the pituitary gland (Lamonerie et al., 1996; Lanctot et al., 1997). Mutations in human Ptx1 may cause Treacher Collins' syndrome (Crawford et al., 1997), in which craniofacial tissues are malformed. Pitx2 participates in the determination of left–right asymmetry in the mouse and chicken (Logan et al., 1998; Piedra et al., 1998; Yoshioka et al., 1998). Of these homeodomain proteins, only Crx and Ptx1 have candidate target genes.

In addition to controlling GABA function, *unc-30* may regulate target genes with other functions. In *unc-30* mutants the type D neurons display axonal pathfinding defects and make few synaptic connections when encountering muscle targets (J. White, personal communication; our unpublished observations). However, the type D neurons in *unc-25* or *unc-47* single mutants and in *unc-25 unc-47* double mutants have normal neuronal morphology and synaptic connectivity (McIntire et al., 1993b; Jin et al., 1999; C. E. and Y. J., unpublished results), suggesting that other target genes regulated by *unc-30* function in these other aspects of type D neuron differentiation. UNC-30 also is expressed in several non-D and non-GABAergic neurons. However, defects in these neurons in *unc-30* mutants have not been well characterized. It is

not yet known whether any of the vertebrate UNC-30-related homeodomain proteins function in GABAergic neurons. It thus remains to be seen if such proteins include functional UNC-30 homologs and whether they regulate similar sets of target genes in other organisms.

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