A neuron-specific enhancer of the *Drosophila* dopa decarboxylase gene

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At least two *cis*-regulatory elements are necessary for correct neuron-specific expression of the *Drosophila melanogaster* dopa decarboxylase gene, *Ddc*. In addition to a previously described proximal element located ~60 bp upstream of the mRNA start site, we have now characterized a distal ~600-bp DNA fragment, extending from -1019 to -1623 bp, which possesses enhancer-like properties and is essential for normal neuron-specific expression. Immunofluorescent labeling of neurons expressing deleted *Ddc* genes indicates that this region contains both general neuronal regulatory elements and cell-specific elements that selectively affect *Ddc* expression in either dopaminergic or serotonergic neurons. These selective effects can be correlated with the removal of sequence elements that are protected from DNase digestion by factors present in embryonic nuclear extracts. Several of these elements are also homologous to sequences located upstream of the evolutionarily diverged *Ddc* gene of *Drosophila virilis*. These results suggest that the neuron-specific expression of *Ddc* results from the combined action of several factors binding within this distal enhancer region.

[Key Words: DNA binding factors; transcriptional regulation; neurotransmitters]

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The *Drosophila melanogaster* *Ddc* gene encodes dopa decarboxylase, the last enzyme in the synthetic pathway leading to synthesis of the neurotransmitters dopamine and serotonin. *Ddc* is under tight cell-specific regulation, with expression limited to ~150 neurons in the central nervous system (CNS) (Beall and Hirsh 1987; Konrad and Marsh 1987). In addition, *Ddc* is expressed in the hypoderm, where the dopamine produced is used in pathways required for cuticular pigmentation and cross-linking (Lunan and Mitchell 1969; Wright 1987a).

The tissue-specific expression of *Ddc* is regulated both by *cis*-acting transcriptional elements (Scholnick et al. 1986; Bray et al. 1988) and by alternate splicing (Morgan et al. 1986). All *cis*-regulatory elements required to generate the normal pattern of neuronal *Ddc* expression must be contained within 2200 bp of *Ddc* 5'-flanking sequences, as this region can direct the expression of a heterologous gene in the normal ¹*Ddc*-containing neurons (Bray et al. 1988). The effects of deletions on *Ddc* expression demonstrate that only 200 bp of 5'-flanking DNA is required for correct temporal and tissue-specific expression in the hypoderm (Scholnick et al. 1986). Expression in the CNS, however, requires additional upstream sequences (Beall and Hirsh 1987). A proximal promoter element, element I, and its associated DNA-binding factor, termed Elf1 (Bray et al. 1988), appear to be essential for CNS expression of *Ddc*, but additional regulatory elements located between 760 and 2200 bp upstream of the mRNA start site are also required for normal neuron-specific expression (Beall and Hirsh

1987). *Ddc* genes lacking this distal regulatory region show an abnormal pattern of expression in the CNS, with little or no neuronal expression but increased expression in a set of glial cells that normally express *Ddc* at very low levels.

Here, we report the characterization of this *Ddc* distal regulatory region that functions as a neuron-specific enhancer. The ~600-bp distal enhancer fragment contains at least five binding sites for factors present in nuclear embryonic extracts. Progressive deletions of the distal enhancer suggest that some of these factors may differentially regulate *Ddc* expression in defined subsets of neurons, whereas others may be required for *Ddc* expression in all *Ddc*-containing neurons.

Results

Definition of a *Ddc* distal enhancer region

A wild-type third-instar larval CNS labeled by indirect immunofluorescence using a *Ddc* antiserum (Fig. 1A) shows scattered neuron clusters in the symmetrical brain lobes and a segmental pattern of labeling in the ventral ganglion (Beall and Hirsh 1987; Konrad and Marsh 1987). The stereotypical pattern of expression within the ventral ganglion consists of two symmetrical rows of doublet cells, referred to as the ventrolateral neurons, and a single row of midline cells, the medial neurons. Two additional symmetrical rows of single cells, the dorsolateral neurons, are not visible in this focal plane (for nomenclature, see Beall and Hirsh 1987). Experiments using a monoclonal antibody directed

¹*Ddc* refers to the gene *Ddc*; Ddc refers to the *Ddc* protein.

Figure 1. Labeling of *Ddc*-expressing neurons in the *D. melanogaster* larval CNS by indirect immunofluorescence using a *Ddc* antiserum. (A) Wild-type third-instar larval CNS. Labeled neurons are visible both in the symmetrical brain lobes (*top*) and the prominent ventral ganglion (*bottom*). (B) Schematic representation of wild-type larval CNS labeled with *Ddc* antiserum, adapted from Beall and Hirsh (1987). Segmentally repeated neurons within the ventral ganglion are labeled as DL (dorsolateral), VL (ventrolateral), and M (medial). The DL neurons are located more dorsal than the VL and M neurons and, therefore, are not visible in the focal plane shown in the photomicrographs in A and C. The fused ventral ganglion is also divided into subesophageal (Sb), thoracic (Th), and abdominal (Ab) segments. (C) *Ddc*^{DISinv(-360)} larval CNS labeled with *Ddc* antiserum.

against serotonin (5-HT; 5-hydroxytryptamine) have identified the ventrolateral neurons as serotonergic (Beall and Hirsh 1987), whereas the medial and dorsolateral cells are dopaminergic neurons (Budnik and White 1988). Because *Ddc* enzyme activity is essential for cuticle formation, the host strain used for P-element transformation with mutant *Ddc* genes contained a temperature-sensitive *Ddc* allele, *Ddc*^{ts2}. At nonpermissive temperatures (25°C), the host *Ddc* gene does not detectably contribute to *Ddc* immunoreactivity in the CNS, except for a small number of brightly labeled cells in the brain lobes and within the subesophageal ganglion. To avoid any confusion resulting from this background labeling, we have focused our analysis on the thoracic and abdominal segments of the ventral ganglion, which show essentially no background labeling in the *Ddc*^{ts2} host strain.

Ddc sequences upstream of -2200 bp are not required for normal neuronal expression but deletion to -760 bp causes a near total loss of neuron-specific expression with no effect on *Ddc* enzyme activity in the hypoderm (Beall and Hirsh 1987). We have established a more precise 5' limit of the neuron-specific distal regulatory region using transformant strains containing *Ddc*^{DIS(-760)}, which lacks all sequences upstream of -1623 bp (Fig. 2a). Expression of *Ddc*^{DIS(-760)} is normal, which, considered with previous results, identifies an 863-bp fragment extending from -760 to -1623 bp that contains essential distal regulatory elements. Moving the 863-bp fragment to two positions closer to the mRNA start site, as in *Ddc*^{DIS(-360)} and *Ddc*^{DIS(-209)} (Fig. 2b,c), results in a

normal expression pattern (data not shown; for comparable phenotype, see Fig. 1C). In addition to defining an 863-bp distal region, these results indicate that no essential sequences are located between -760 and -209 bp. A gene with the distal fragment inverted at -360 bp, *Ddc*^{DISinv(-360)} (Fig. 2d), also expresses normally (Fig. 1C). The ability of control elements within the distal fragment to function independently of position and orientation indicates that the region possesses basic enhancer properties.

Evolutionarily conserved sequences within the *Ddc* distal enhancer

The *Ddc* proximal promoter has been characterized extensively for sequence elements necessary for the temporal and tissue-specific expression of *Ddc*. Regulatory elements within the *Ddc* proximal promoter were identified initially (Bray and Hirsh 1986; Scholnick et al. 1986) as DNA sequences conserved between the *D. melanogaster* and *Drosophila virilis* *Ddc* genes. We have extended the sequence comparison to detect conserved elements located within the distal enhancer region.

Figure 3 shows the complete DNA sequence of the *D. melanogaster* distal enhancer extending from -1640 bp to -760 bp. A comparison of this sequence with DNA sequences located upstream of the *D. virilis* *Ddc* gene shows four regions of conservation displayed in boxes in Figure 3 labeled as elements A, B, C, and D. Element A shows the best sequence conservation with ~98% nucleotide sequence identity. Although the element A se-

Figure 2. Structure of *Ddc* genes used for analysis of distal region enhancer properties. The thick arrow represents the *Ddc* 5'-flanking *Cla*I–*Hind*III fragment extending from –1623 bp to –760 bp relative to the mRNA start site. (a) *Ddc*^{DIS(–760)}, contains the *Ddc* *Cla*I–*Hind*III fragment in its normal orientation and position at –760 bp. (b) *Ddc*^{DIS(–360)}, the *Ddc* *Cla*I–*Hind*III fragment in its normal orientation moved to –360 bp. (c) *Ddc*^{DIS(–209)}, the *Ddc* *Cla*I–*Hind*III fragment in its normal orientation moved to –209 bp. (d) *Ddc*^{DIS^{rev}(–360)}, contains the *Ddc* *Cla*I–*Hind*III fragment in reverse orientation moved to –360 bp.

quence from *D. virilis* exists as a nearly continuous stretch of 98 nucleotides, the corresponding sequence from *D. melanogaster* is interrupted by two short regions, suggesting that it may actually represent three smaller elements. This is represented in Figure 3 by labeling these elements in the *D. melanogaster* sequence as A1, A2, and A3.

Elements B, C, and D show lesser but still significant degrees of conservation. Element B retains 72% nucleotide identity, whereas elements C and D retain 78% and 81%, respectively. Elements B and C are related to the same region of *D. virilis* sequence, suggesting that they may represent the duplication of a control element present in only one copy in *D. virilis*. Nonetheless, the four regions of homology show the same order and orientation relative to the mRNA start site, progressing from 5' to 3' in the order D–(C–B)–A. This conservation of relative position not only suggests a conservation of individual regulatory elements but also the more complex coordinated interactions between multiple elements. The distal conserved elements are also located at approximately the same distance from the mRNA start-point in both *D. melanogaster* and *D. virilis*. Our analysis detected no recognizable homology between the distal sequence elements and those identified previously in the proximal promoter (Bray and Hirsh 1986).

Multiple factor binding sites within the *Ddc* distal enhancer

Sequences within the distal enhancer that specifically bind soluble factors were detected using a DNase protection assay. Incubation of single-end-labeled DNA fragments from the distal region with embryonic nuclear extracts reveals at least eight footprints representing discrete regions that are protected from DNase digestion

(Fig. 4). Identified DNase footprints are associated with at least three of the four conserved sequence elements (Fig. 4A). Each region of DNase protection within the distal region was labeled according to its associated sequence element.

Conserved element D is associated with a factor, or factors, that weakly protects ~15 bp, referred to as binding site Df6, from –1474 to –1490 at the 5' end of the element. Strong hypersensitive bands are also apparent at either end of the Df6-binding site (Fig. 4B). Element C is bound by a factor that strongly protects sequences from –1382 to –1402 bp, represented by footprint Cf1. Footprint Bf2, a ~23-bp region of very strong protection from –1327 to –1350 bp, is associated with element B (Fig. 4B). At least five footprints were found unassociated with regions of sequence conservation (Fig. 4B,C,D). Footprints Uf3 (–1299 to –1317 bp), Uf7 (–1032 to –1046 bp), Uf8 (–990 to –1020 bp), Uf9 (–890 to –903 bp), and Uf10 (–834 to –846 bp) protect sequences primarily in the 3' half of the distal enhancer. We were unable to detect comparable regions of DNase protection within element A, the largest conserved region stretching over ~100 bp, although hypersensitive bands and very weak protections were observed over a ~15-bp region from –1207 to –1222 bp (data not shown).

Sequence elements necessary for *Ddc* expression in dopaminergic medial neurons

The functional significance of the identified factor binding sites was examined by analyzing the expression of *Ddc* genes containing progressive deletions of the distal enhancer region in P-element transformant strains. Figure 5 schematically depicts the extent of each deletion and the factor binding sites remaining. The 5'

Figure 3. Nucleotide sequence of the *Ddc* distal enhancer region. The sequence of the *D. melanogaster* *Ddc* gene 5'-flanking DNA is shown extending from -1639 to -760 bp, relative to the mRNA start site. Boxed sequences labeled A1-A3, B, C, and D show regions of significant nucleotide conservation with 5'-flanking sequences of the *D. virilis* *Ddc* gene. *D. virilis* sequences are aligned above the *D. melanogaster* sequences with mismatches marked by an asterisk (*). Complete sequences from both the *D. melanogaster* and *D. virilis* distal regions will be submitted to GenBank.

deletions retain sequences from -1623 to -2200 bp and progressively delete sequences from the 5' end of the distal enhancer region (-1623 bp to -760 bp).

Analysis of mutant phenotypes resulting from deletions of the 5' end of the distal enhancer suggest that the anatomical and pharmacological subsets of *Ddc*-expressing neurons may be regulated independently by the

coordinated interaction of specific binding factors. A gene containing a small deletion of 47 bp, *Ddc*^{EN5/Δ47}, expresses normally but results from *Ddc* genes with more extensive deletions that remove binding sites Df6 and Cf1, *Ddc*^{EN5/Δ199}, and *Ddc*^{EN5/Δ259}, respectively (Fig. 5B), suggest that at least one of these binding sites may specifically regulate *Ddc* expression in the dopaminergic

A.

Figure 4. DNase I footprinting of the *Ddc* distal enhancer region. (A) Correlation of DNase-protected regions with conserved sequence elements. The solid line represents *Ddc* distal enhancer sequences. Regions of DNase protection are represented by solid shapes labeled above the line (see text for nomenclature). The representation of footprints by similar shapes should not necessarily be interpreted as suggestive of structural or functional relationships between footprints. Evolutionarily conserved sequence elements are shown as open boxes labeled as elements A–D (see text). (B) DNase protection of the distal enhancer region extending 5' from the *Ava*II site at –1130 bp. A schematic representation of nucleotide position and protected regions is shown to the right of each panel. Sites that show hypersensitivity to DNase are indicated by arrows. The 860-bp *Cla*I–*Hind*III fragment was subcloned into pUC18 and then was labeled with 32 P using polynucleotide kinase at the *Ava*II site at –1130 bp. DNase I footprinting assays were performed as described in Experimental procedures. Autoradiograms of 7% buffer gradient sequencing gels are shown. The amount of extract used (μ l/50- μ l reaction) is indicated at top of each lane. (G/A) Lanes containing Maxam-Gilbert purine cleavage fragments. (C) DNase protection of the distal region extending 3' from the *Ava*II site at –1130 bp. The subcloned *Cla*I–*Hind*III fragment was labeled with 32 P using polynucleotide kinase at the *Ava*II site at –1130 bp. (D) DNase protection of the distal region extending 5' from the *Hind*III site at –760. The subcloned *Cla*I–*Hind*III fragment was labeled with 32 P using polynucleotide kinase at the unique *Hind*III site.

medial neurons. Larval CNS from transformant strains of *Ddc*^{EN5'Δ199} and *Ddc*^{EN5'Δ259} display very similar phenotypes in which *Ddc* immunoreactivity in the medial neurons is either absent or severely diminished (Fig. 6B). Genes with very large deletions, *Ddc*^{EN5'Δ395} and *Ddc*^{EN5'Δ435}, removing 395 and 435 bp, respectively, also lack binding sites Bf2 and Uf3 (Fig. 5B) and result in little, if any, neuronal *Ddc* expression (data not shown, for comparable phenotype, see Fig. 6C). Although the results from these deletions imply that sequences essential for neuronal *Ddc* expression lie between –1364 and –1228 bp, relatively large deletions such as these should be interpreted with caution, because the removal of internal binding sites such as Bf2 and Uf3 occurs in the context of binding sites lying farther upstream also being absent.

Deletion of sequences from the 3' end of the distal enhancer region demonstrates that a number of the bound factors are nonessential for normal *Ddc* expression and also identifies a more precise 3' limit for the minimally functional enhancer. These deletions were based on the DIS(–360) construct (Fig. 2b), which contains a deletion

extending from –760 bp to –360 bp and expresses a wild-type phenotype. *Ddc*^{EN3'Δ87} and *Ddc*^{EN3'Δ259}, which contain deletions from the 3' end of the distal fragment of 87 and 259 bp, respectively, express in a wild-type pattern. Thus the minimal distal enhancer is contained within, at most ~604 bp, extending from –1019 bp to –1623 bp. Further deletion to 371 bp, as in *Ddc*^{EN3'Δ371}, causes a complete loss of neuron-specific expression (Fig. 6C), suggesting the presence of essential elements between these deletion end points. The results from 5' and 3' deletions of the distal enhancer show that essential regulatory elements are removed by both of the non-overlapping deletion series, thereby implying that no individual element alone is sufficient for enhancer function.

A region required for Ddc expression in serotonergic neurons

Data presented above define a minimally functional distal enhancer located between –1623 and –1019 bp. Additional small deletions, however, localize regulatory

A.

B. 250 bp

C. 25 bp

elements near the 5' end of the minimal distal enhancer, which specifically affect *Ddc* expression in serotonergic neurons. Figure 5C depicts a series of small deletions that remove sequences from a ~60-bp region centered on the 5' end of the identified minimal distal enhancer fragment at -1623 bp. As described above, *Ddc*^{DIS(-760)} (Fig. 2a), which contains a deletion of all sequences upstream of -1623 bp, expresses normally. In addition, *Ddc*^{EN5'Δ47} (Figs. 5B,C), which lacks sequences from -1623 to -1576 bp while retaining sequences upstream of -1623 bp, also expresses normally (data not shown). However, deletions extending in both directions from -1623 bp, as in *Ddc*^{Δ-1636-1576} and *Ddc*^{Δ-1636-1596}, display a phenotype in which *Ddc* immunoreactivity in the serotonergic ventrolateral neurons is either absent or severely diminished (Fig. 6D). These results can be explained most simply by the presence of two redundant regulatory elements on either side of -1623 bp, either of which can function to allow *Ddc* expression in the sero-

Figure 5. Deletion analysis of the *Ddc* distal enhancer. (A) Factor binding sites within the *Ddc* distal enhancer region. DNase footprints within the distal enhancer region are represented as open shapes labeled as described in the text. The use of similar shapes to indicate two different footprints is not intended to suggest any structural or functional relationships between the various footprints. Slashes denote deviation from scale relative to the distal enhancer region. (B) *Ddc* genes containing progressive 5' and 3' deletions of the distal enhancer. Sketches of the distal enhancer region show sequences and corresponding factor binding sites retained after 5' deletions initiating from the *Clai* site at -1623 bp (see Experimental procedures). All 3' deletions also lack sequences between -760 and -360 bp, in addition to the indicated deletions from the distal region. These sequences were shown to be nonessential for normal *Ddc* expression (see text). (Right) Effects of deletions on in vivo levels of *Ddc* immunoreactivity. (+) *Ddc* immunoreactivity present at near wild-type levels (>50%); (-) *Ddc* immunoreactivity present at <10% wild-type levels; (VL) ventrolateral neurons; (M) medial neurons; (DL) dorsolateral neurons. (C) Deletions with end points used to define serotonergic neuron-specific regulatory elements. Diagonal slashes represent the continuation of sequences extending 3' to include all downstream *Ddc* sequences and 5' to -2200 bp. The open box labeled *XbaI* represents an 8-bp *XbaI* linker sequence inserted by blunt-end ligation to Klenow-repaired fragment ends.

tonergic neurons. Transformant strains of *Ddc*^{Δ-1636-1576} and *Ddc*^{Δ-1636-1596}, still express normally in the dopaminergic dorsolateral and medial neurons indicating that these small deletions specifically affect *Ddc* expression in the serotonergic neurons. The insertion of an *XbaI* linker at -1623 bp, as in *Ddc*^{XbaI,-1623} (Fig. 5C), has no effect on *Ddc* expression (data not shown), suggesting the presence of two separate but redundant control elements located in the 40-bp region between -1636 and -1596 bp.

Discussion

The remarkable complexity of the CNS is based on the unique characteristics of individual neurons that may be morphologically similar but anatomically, pharmacologically, and functionally distinct. How this complexity is generated during early development is not well understood but may involve cell-cell interactions (Hafen et

Figure 6. Effect of progressive deletion of the distal enhancer region on in vivo *Ddc* expression. Dissected third-instar larval CNS showing high magnification of ventral ganglion labeled by indirect immunofluorescence with *Ddc* antiserum. Although all *Ddc*-immunoreactive neurons in whole-mounted CNS are not located in the same focal plane, photomicrographs shown were taken in the same focal plane for purposes of comparison. (A) Wild-type (Canton-S) ventral ganglion shows *Ddc* immunoreactivity in segmentally patterned neurons within the ventral ganglion (for nomenclature, see Fig. 1 and text). Black arrows identify labeled neurons of a single segment as ventrolateral (vl) and medial (m). (B) *Ddc*^{EN5'Δ199} mutant ventral ganglion shows severely diminished levels of *Ddc* immunoreactivity in dopaminergic medial neurons but near wild-type levels remaining in serotonergic ventrolateral neurons. *Ddc* immunoreactivity in network of putative glial cells is also increased relative to wild-type (see text). (C) *Ddc*^{EN3'Δ371} mutant ventral ganglion shows no neuronal *Ddc* immunoreactivity but high levels in glial network. (D) *Ddc*(Δ-1636-1596) mutant ventral ganglion shows normal levels of *Ddc* immunoreactivity in dopaminergic medial neurons but severely diminished levels in serotonergic ventrolateral neurons. Slight alterations in the spatial arrangement of the medial neurons are unique to individual CNS and are not a characteristic of the mutant phenotype.

al. 1987; Harrelson and Goodman 1988; Reinke and Zipursky 1988), hormonal regulation by various soluble growth factors or peptides (Laufer and Changeux 1987; Hofer and Barde 1988), or early commitment to specific cell lineages (Furst and Mahowald 1985; Truman and Bate 1988). Regardless of the actual developmental processes that specify functional and pharmacological identity, the eventual result is a program of temporal and

cell-specific gene regulation necessary for proper neuronal differentiation. In other tissues such as the pituitary gland (Bodner et al. 1988; Ingraham et al. 1988), the liver (Courtois et al. 1987), and circulating lymphocytes (Lenardo et al. 1987; Müller et al. 1988; Scheidereit et al. 1988), such regulation has been shown to be mediated by transcription factors that interact with specific DNA sequences as well as with each other to cause transcriptional activation or repression.

Ddc encodes an essential enzyme in the synthesis of the neurotransmitters dopamine and serotonin. As expected, CNS expression of *Ddc* occurs primarily in serotonergic and dopaminergic neurons. These neurons are distributed in a bilaterally symmetrical pattern that is repeated segmentally in the ventral ganglion. A wild-type CNS labeled by indirect immunofluorescence with *Ddc* antiserum (Fig. 1A) shows *Ddc* immunoreactivity in ~150 unique neurons surrounded by 10⁵–10⁶ nonexpressing neurons. Therefore, the in vivo conditions dictating the presence or absence of transcription factors regulating the expression of *Ddc* are also unique, differing substantially between adjacent neurons. We have defined a distal enhancer region which, in conjunction with the proximally located Element I, appears to interact with a number of specific enhancer-binding factors to regulate the correct neuron-specific expression of *Ddc* in the *Drosophila* CNS. Our characterization of the *Ddc* distal enhancer is based on an analysis of P-element transformant strains expressing *Ddc* genes containing deletions of the distal enhancer, the results of which are summarized in Figure 7. Progressive deletion of the *Ddc* distal enhancer reveals two regions (–1364 to –1228 bp and –1131 to –1019 bp), which may contain general regulatory elements necessary for all neuronal *Ddc* expression (Fig. 7). In addition, we have identified two regions that have selective effects on subsets of *Ddc* immunoreactive neurons, suggesting that ana-

Figure 7. Neuron-specific regulatory functions within the *Ddc* distal enhancer region. At least two redundant serotonergic (5HT) neuron-specific regulatory elements are depicted near the 5' end of the minimal *Ddc* distal enhancer, one of which is just outside but within 13 bp of the minimal enhancer. At least one regulatory element specific for one set of dopaminergic (DA) cells, the medial DA neurons, must lie between –1576 and –1364 bp. In addition, results suggest that regulatory elements required for normal expression in all *Ddc*-expressing neurons are located between –1364 and –1228 bp as well as between –1131 and –1019 bp.

tomical and pharmacological subsets are regulated independently. Deletions removing sequences from the 5' end of the distal enhancer but retaining sequences from -1623 to -2200 bp, *Ddc*^{EN5'Δ199} and *Ddc*^{EN5'Δ259} (Fig. 5B), selectively abolish *Ddc* immunoreactivity in the medial dopaminergic neurons (Fig. 6B), suggesting that a factor responsible for footprint Df6 may be a medial/dopaminergic-specific regulatory factor. The identical phenotypes of *Ddc*^{EN5'Δ199} and *Ddc*^{EN5'Δ259} strains prevent any conclusions concerning the significance of the Cfl-binding site; however, preliminary results from point mutations within Cfl suggest that both Df6 and Cfl may bind medial/dopaminergic-specific factors (W. Johnson, in prep.). *Ddc*^{EN5'Δ199} and *Ddc*^{EN5'Δ259} strains retain *Ddc* expression in serotonergic neurons, as well as in the dopaminergic dorsolateral neurons. The retention of *Ddc* expression in a subset of dopaminergic neurons indicates that these elements do not simply make a distinction between serotonergic and dopaminergic *Ddc* expression but define additional anatomical or functional subsets of dopaminergic neurons as well. These strains also overexpress *Ddc* in a subset of glial cells, as seen previously in mutants deleting the entire distal enhancer (Beall and Hirsh 1987).

A complementary phenotype selectively reducing *Ddc* expression in serotonergic neurons is seen after the deletion of a 40-bp sequence between -1636 and -1596 bp. These strains express *Ddc* normally in dopaminergic neurons but show an absence or severe reduction of *Ddc* expression in serotonergic neurons. We suggest that this 40-bp region contains two redundant control elements, as deletions removing all sequences upstream of -1623 bp, as well as internal deletions removing sequences from the 5' end of the identified distal enhancer but retaining sequences upstream of -1623 bp, all express *Ddc* normally (Fig. 5C). As long as one of the two elements is retained, as in *Ddc*^{DIS(-760)} or *Ddc*^{EN5'Δ47}, *Ddc* is expressed normally. However, the deletion of both elements, as in *Ddc*^(Δ-1636-1576) and *Ddc*^(Δ-1636-1596), results in the specific loss of *Ddc* expression in serotonergic neurons (Fig. 6D). An alternative explanation might be the presence of additional functional elements located farther 5' of -1623 bp which are brought into close proximity of the *Ddc* distal enhancer by the introduced deletions. This possibility should certainly be considered because *Ddc* lies within a chromosomal gene cluster with an identified transcription unit, *1(2)37Cc*, located immediately upstream of *Ddc* (for review, see Wright 1987b). We consider this situation unlikely, however, given the small size of the deletions in *Ddc*^(Δ-1636-1576) and *Ddc*^(Δ-1636-1596) and given that the inserted *Xba*I linker restores a portion of the wild-type spacing. In addition, the *1(2)37Cc* transcription unit is not functional in the transformed *Ddc* genes since these constructs contain only the extreme 3' end of *1(2)37Cc*. We have been unable to detect any sequences that are conserved in *D. virilis* within this 40-bp region, and preliminary experiments show, at most, very weak protection from DNase digestion by embryonic nuclear extracts.

The specific effects of progressive deletions indicate that the *Ddc* distal enhancer is constructed of numerous

individual elements, each responsible for selected aspects of the overall *Ddc* pattern of expression, as well as general elements that are essential in all *Ddc*-expressing neurons (Fig. 7). Thus, no single element alone is sufficient for normal activity. We have identified possible regulatory elements by a correlation between *Ddc* enhancer sequences conserved within the *D. virilis* *Ddc* gene and sequences protected from DNase digestion by embryonic nuclear extracts. These experiments reveal at least five footprints within the minimal distal enhancer (Fig. 4). Three of these footprints correspond to sequence elements identified as regions of conservation (Fig. 4A). Our inability to detect more than very weak DNase protection within conserved element A was surprising considering its remarkable degree of sequence conservation. These results, however, may be explained by one of three possibilities. First, element A may not be functionally relevant in vivo. Because element A is located near the center of the minimal distal enhancer, the results of our progressive deletions yield no information concerning its function. Nonetheless, we consider this possibility unlikely. Alternatively, element A may have an important, but as yet uncharacterized, role in *Ddc* regulation but may function by a mechanism that does not involve the binding of specific DNA-binding factors. The third, and most likely, explanation is that the in vivo function of element A is mediated by the binding of specific protein factors, but these factors are either not detectable in our assay system or are not present in late (10–22 hr) embryos. This potential lack of correlation between in vivo function and in vitro factor binding to element A may result from limitations imposed by our assay system. It has not been feasible to produce active nuclear extracts from larval nuclei, thus necessitating the exclusive use of embryonic nuclear extracts in the in vitro factor binding assay. Yet our in vivo functional analysis has relied on the effect of *Ddc* mutations on expression in the third-instar larval CNS. The dissection and immunohistochemical labeling of the larval CNS is technically easier, as well as more reliable, than labeling of the embryonic CNS. We feel that our suggestion of relationships between the binding of embryonic DNA-binding factors and larval *Ddc* expression is nonetheless valid because the fundamental identity of *Ddc*-containing neurons is established in late embryos and maintained in the larval CNS (Budnik and White 1988). However, regulatory factors displaying unique patterns of temporal expression may not be detected in our system.

Although we have attempted to correlate results from deletion analysis with individual factor binding sites, our results do not allow specific in vivo effects to be attributed conclusively to individual sequence elements. However, preliminary results indicate that neuron-specific effects can be duplicated by point mutations disrupting individual factor binding sites (W. Johnson, in prep.). We conclude that the expression of *Ddc* in any single neuron requires the interaction of at least three regulatory elements. Element I, located in the proximal promoter, is essential for all neuronal expression, whereas at least two elements within the distal enhancer, ~ 1000 bp distant, are necessary as well. Which

elements of the distal enhancer are functional depends on the particular neuron and its spatial location. This requirement for the interaction of at least two enhancer binding motifs in the determination of cell specificity is similar to results reported for the prototype enhancer from SV40 (Fromental et al. 1988).

The regulatory model alluded to here would suggest that the pattern of *Ddc* CNS expression results from the overlapping patterns of expression of several regulatory factors, a situation not unlike that suggested for the interaction of genes involved in pattern formation during early embryonic development in *Drosophila* (Ingham 1988). A number of these genes require large regulatory regions to generate a complex pattern of developmental expression (Bender et al. 1983; Laughon et al. 1986; Peifer and Bender 1986). It is clear, however, that the generation of a cell-specific pattern of gene expression does not necessarily require such massive expanses of regulatory DNA. Indeed, the generation of a defined pattern of neuronal *Ddc* expression would appear to be somewhat analogous to the segmentation gene *fushi tarazu* (*ftz*). The segmental pattern of *ftz* expression is produced by a relatively short 5'-flanking sequence of ~6.1 kb (Hiromi et al. 1985; Hiromi and Gehring 1987). These sequences include a much smaller 'neurogenic element' responsible for *ftz* expression in specific neurons of the ventral nervous system, which is independent of elements necessary for epidermal *ftz* expression.

The complex neuron-specific regulation of *Ddc* might be exploited as a means of investigating the neurological functions of the neurotransmitters dopamine and serotonin. The essential metabolic role of the *Ddc* enzyme presents the possibility that the identification of separable dopaminergic and serotonergic *Ddc* regulatory elements could be used to regulate independently the levels of each metabolite in the CNS, thus providing a non-pharmacological, nondestructive, but highly specific, alteration of *in vivo* neurochemistry. The background *Ddc^{ts2}* allele present in transformant strains described here produces sufficient *Ddc* enzyme activity, even at nonpermissive temperatures (25°C), to yield relatively high levels of serotonin in CNS neurons (White and Valles 1985; B. Morgan and J. Hirsh, unpubl.). This prevents the direct use of these strains in such experiments until transformed chromosomes have been placed in a *Ddc* null background.

We have yet to determine with certainty whether all of the detected footprints represent the binding of separate and distinct factors or if multiple sites are present for a single factor, or conversely, multiple factors competing for a single binding site. Purification of individual factors by DNA-affinity chromatography will help to answer these questions, as well as determine whether the cell-specific activity of identified control elements can be correlated with the presence of corresponding binding factors in selected neurons.

Experimental procedures

Sequence analysis

DNA sequences were determined by dideoxy nucleotide se-

quencing techniques, using modified T7 DNA polymerase (Tabor and Richardson 1987). Relevant DNA fragments were subcloned from the cloned *Ddc* gene of the wild-type Canton-S strain of *D. melanogaster* (Hirsh and Davidson 1981) and from *D. virilis* (Bray and Hirsh 1986) into M13 vectors for sequencing on both strands. Homology analyses were performed using Intelligenetics, Inc., programs accessed via BIONET. *D. melanogaster* and *D. virilis* *Ddc* 5'-flanking sequences used in the analysis will be submitted to GenBank.

P-element vector construction and transformation

Nucleic acid manipulations were largely as described in Maniatis et al. (1982), except where indicated. Restriction enzymes were purchased from New England Biolabs or Boehringer-Mannheim Biochemicals and used as suggested by the supplier. Progressive 5' deletions were generated by *Bal31* deletion from the unique *Clal* site at -1623 bp, followed by insertion of *XbaI* linkers. Constructs containing 3' deletions of the distal region were produced by creating unique *BamHI* sites in the subcloned *Clal-EcoRI* fragment (-1623 to -360 bp), using oligonucleotide directed *in vitro* mutagenesis (Taylor et al. 1985; Amersham). Deleted *Ddc* genes were cloned into a P-element vector containing the *Drosophila Adh* gene as a selectable marker. Methods for P-element integration were essentially as described previously (Rubin and Spradling 1982; Scholnick et al. 1983). The host strain was *Adh^{ts23}Ddc^{ts2}*. Transformant flies were identified by screening for wild-type *Ddc* pupal phenotype at 25°C. Two to five independent transformant strains were established for each transforming vector. Strains were confirmed to contain single-copy inserts of the appropriate P-element vector by Southern blot analysis of genomic DNA.

DNase I footprinting

Nuclear extracts were prepared from 10- to 22-hr embryos and fractionated on heparin-agarose (Heberlein and Tjian 1988). Footprinting reactions were performed as described previously (Heberlein et al. 1985), using proteins eluted from heparin-agarose between 0.1 and 0.4 M KCl. One microliter of synthetic poly[d(I-C)] (Pharmacia; 10 U/ml) was added to each reaction as nonspecific competitor. Probes were end-labeled at a single 5', end using polynucleotide kinase as indicated in the figure legends. Digested fragments were analyzed on 7% polyacrylamide sequencing gels.

Immunofluorescence

Anti-*Ddc* antiserum and protocols for labeling of isolated third-instar larval CNS were as described by Beall and Hirsh (1987). CNS were labeled in groups of four to eight per strain, repeated at least three times with similar results. Although variations in labeling intensity of as much as twofold were observed between transformant strains containing identical mutant *Ddc* genes, no position effects on overall pattern were observed.

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