CNS and Hypoderm Regulatory Elements of the *Drosophila melanogaster* Dopa Decarboxylase Gene

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Expression of the dopa decarboxylase gene (*Ddc*) is regulated in a tissue- and developmental stage–specific manner throughout the life cycle of the fruit fly, *Drosophila melanogaster*. Essential *Ddc* regulatory elements lie within 208 base pairs upstream from the RNA start point. Functional elements within this 5′ flanking region were mapped by deletion analysis, which assayed expression in vivo after germline integration via P element vectors. One of the elements is essential for expression in both the larval and adult central nervous system, and at least two other elements are necessary for quantitatively normal expression in the hypoderm. Within each of the intervals that have regulatory effects are found sequence elements conserved between the *Ddc* genes of two distantly related species of flies. On the basis of this correlation, regulatory functions for these sequence elements can be postulated.

MANY GENES FOUND IN HIGHER organisms encode products used in metabolic pathways that are required in more than one tissue or at a number of times during development. The regulation necessary to produce differential tissue and/or temporal regulation can be achieved through the use of multiple promoters (*1*) or by using a single promoter that is subject to multiple regulatory inputs (*2*). The *Drosophila melanogaster* dopa decarboxylase gene, *Ddc,* is regulated via the latter mechanism. Dopa decarboxylase enzyme activity is found at high levels in two tissues in *D. melanogaster,* the hypodermal epithelium and the central nervous system (CNS) (*3*), yet the primary transcript in each tissue is identical (*4*). Although the *Ddc* gene produces an enzyme that is an essential component of the catecholamine biosynthetic pathway, the function of the pathway is different in each tissue. In the hypoderm, *Ddc* metabolites are precursors of quinones, which are involved in pigmentation and structural integrity of the exoskeleton (*5*), whereas in the CNS, *Ddc* metabolites are potential neurotransmitters (*6*). The temporal expression of the unique *Ddc* structural gene is different in the two tissues. In the hypoderm, *Ddc* transcripts and enzyme activity are induced 10- to 20-fold during late embryogenesis, pupariation, and adult eclosion compared to other times in the life cycle (*7*). In contrast, expression in the CNS remains nearly constant during at least two periods in which expression in the hypoderm changes dramatically (*3, 8*).

The *Ddc* genes of both *D. melanogaster* (*9, 10*) and the distantly related species *D. virilis* (*11*) are properly regulated after integration into the *D. melanogaster* genome via P element vectors. This indicates that *cis* regulatory elements are functionally conserved between the two *Ddc* genes. Analysis of the in vivo expression of deletion mutations of the *D. melanogaster* gene shows that regulatory elements lie within 208 bp upstream of the start of transcription and that no regulatory elements necessary for normal tissue-specific expression are located upstream of this region (*12*). This study also indicated that at least one element necessary for expression of *Ddc* in the CNS is located between 209 and 34 bp upstream from the RNA start point.

Comparison of the DNA sequences of the *D. melanogaster* and *D. virilis Ddc* genes shows that their structures are very similar, yet there is negligible sequence homology in most nontranslated regions (*11*). However, conserved sequence elements between the two species are clustered within the 5′ flanking DNA that is essential for proper expression of the *D. melanogaster Ddc* gene (Fig. 1). The following homologies are found: (i) An identical 7-bp sequence, CTCAGTT, flanking the RNA start point. This sequence matches the consensus sequence ATCA$^{G}_{T}$T$^{C}_{T}$ found at the RNA start point of a number of insect genes (*13*). (ii) An A·T–rich sequence GCTTTAAAAGC, that resembles a "TATA" box sequence, is perfectly conserved. (iii) A 9-bp sequence, homology region IV, that is represented once in *D. melanogaster,* but is found in two copies in *D. virilis*. The interspecies homology is, respectively, seven or eight out of nine bases. (iv) A 16-bp sequence, homology region I, that is perfectly conserved between the two species. (v) An 11-bp perfectly repeated sequence in *D. melanogaster,* homology region II, shown as regions IIA and IIB. This sequence is present once in the *D. virilis* flanking region, sharing 9 of 11 bases with the *D. melanogaster* sequence. (vi) A 9-bp sequence, homology region III, perfectly conserved between the two genes.

Regions I, II, and III are related, since the *D. virilis* homology regions I and II overlap, and the *D. melanogaster* regions IIB and III overlap. A sequence represented by the consensus C$^{A}_{T}$GCG$^{G}_{A}$ is found associated with each of these elements. Furthermore, element II consists of two copies of this consensus sequence.

A set of deletions was constructed in the *D. melanogaster Ddc* gene to examine functional elements in the 5′ flanking region. The deletions were constructed with a common upstream end point at −208 and retained another 2.3 kb of normal upstream sequences (Fig. 2). This upstream DNA was included as a buffer against influence from vector sequences, since genes lacking the normal upstream flanking sequences can be influenced significantly by vector sequences (*12*).

The deleted *Ddc* genes were inserted into an *Adh*$^{+}$ P element vector and integrated into the germline of *Ddc*^{ts2}*Adh*^{fn23} flies by microinjection of early embryos in the presence of a helper P element. Homozygous lines were established from strains carrying the reintegrated genes, and *Ddc* enzyme activity was assayed as a function of development (Fig. 3). These data show a gradual

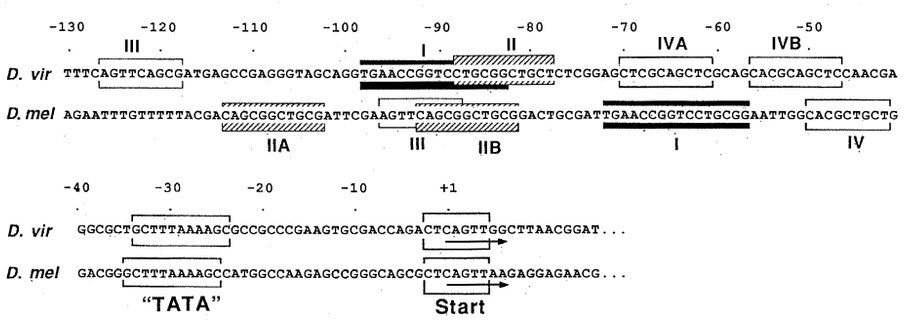

Fig. 1. Sequences flanking the *D. melanogaster* and *D. virilis Ddc* genes [adapted from (*11*)]. The position of the major *D. melanogaster* RNA start point is indicated by the beginning of the arrow within the "Start" box (*12*). The *D. virilis* start point has been determined by alignment of the "TATA" and start point sequences (*11*). This assignment of the start point is consistent with a primer extension analysis (*21*). The homologous sequences have been numbered with roman numerals in decreasing order of their interstrain relatedness. Intrastrain repetitive sequences are designated by A and B suffixes.

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reduction in expression as more 5′ flanking DNA is deleted, from near-normal expression in the $Ddc^{\Delta(-208,-106)}$ strains, to expression near background levels in the $Ddc^{\Delta(-208,-38)}$ strains. In some of the latter strains *Ddc* is still induced at pupariation and adult eclosion, although the level of expression is greatly reduced and varies between strains. Normal induction of *Ddc* at 16 to 18 hours of embryogenesis is observed in all of the deletion strains assayed. As with postembryonic expression, there is a gradual decrease in the level of expression during embryogenesis as a function of the extent of deletion.

The first altered gene to show detectable abnormalities in developmental expression is $Ddc^{\Delta(-208,-106)}$. Strains containing this gene show rather large interstrain variability in expression, presumably caused by effects of chromosomal position. These effects are not consistent over development, since a strain that shows a significant reduction in expression at one developmental time may show normal expression at another.

The quantitative effects of the deletions on whole animal expression are shown more clearly in Fig. 4. Data from all transformant lines at the two stages of maximal postembryonic expression, pupariation (Fig. 4A), and adult eclosion (Fig. 4B) are shown. Since 90 to 95% of *Ddc* enzyme activity in whole animals is located in the hypoderm (*9*), whole animal measurements will primarily reflect expression in this tissue. The $Ddc^{\Delta(-208,-83)}$ strains are the first to show a consistent reduction in expression, with expression more severely reduced at pupariation than at adult eclosion. Deleting to −59 has no additional effect on expression at pupariation, but leads to a two- to threefold decrease in expression at adult eclosion. Deleting to −38 causes a further decrease in expression, although two of the three strains examined show significant induction over background. Finally, deleting to −33, just within the TATA box sequence, essentially abolishes expression.

Strains expressing less than 15 to 20% of normal levels of *Ddc* enzyme activity at pupariation form abnormally pigmented pupal cases (*9, 14*). The pupal case pigmentation of all strains carrying genes with flanking sequence deletions was consistent with the expected phenotype. Strains containing genes retaining 58 or more bases of normal 5′ flanking sequences had pigmentation indistinguishable from that of wild type. More severe deletions lead to a progressive loss of normal pigmentation in a manner consistent with the reduced expression at pupariation. The pigmentation phenotype of strains retaining at least 58 bp of normal flanking sequences suggests that the remaining enzyme activity must still reside within the hypoderm. Furthermore, this expression is largely confined to the hypoderm (*15*).

We also examined the effects of the deletions on expression in the third instar larval CNS (Fig. 4A) and adult brain (Fig. 4B). Although these stages are not precisely the same as those used for whole animal measurements, this is not critical because levels of *Ddc* activity in CNS do not change significantly over the intervals between early third instar and pupariation, and between newly eclosed and older adults (*3, 8*).

All genes containing at least 83 bp of normal 5′ flanking sequences show CNS expression within a factor of 2 of wild-type levels. Deleting further to −59 essentially abolishes expression in both the larval CNS and adult brain. This striking loss of larval CNS expression occurs with no change in the whole animal expression at pupariation (Fig. 4A). In adults, deleting from −83 to −59 leads to a two- to threefold loss in whole animal expression; however, the decrease in adult brain expression is at least 50-fold. Thus, deleting across a small segment of 5′ flanking sequences between −59 and −83 has selective effects on CNS *Ddc* expression at two different developmental stages.

The CNS specific effect of the Δ(−208,−59) deletion is not due to the loss of a CNS-specific transcription start site upstream from the site used by the hypodermal RNA. The CNS *Ddc* RNA start site was determined by primer extension analysis, by

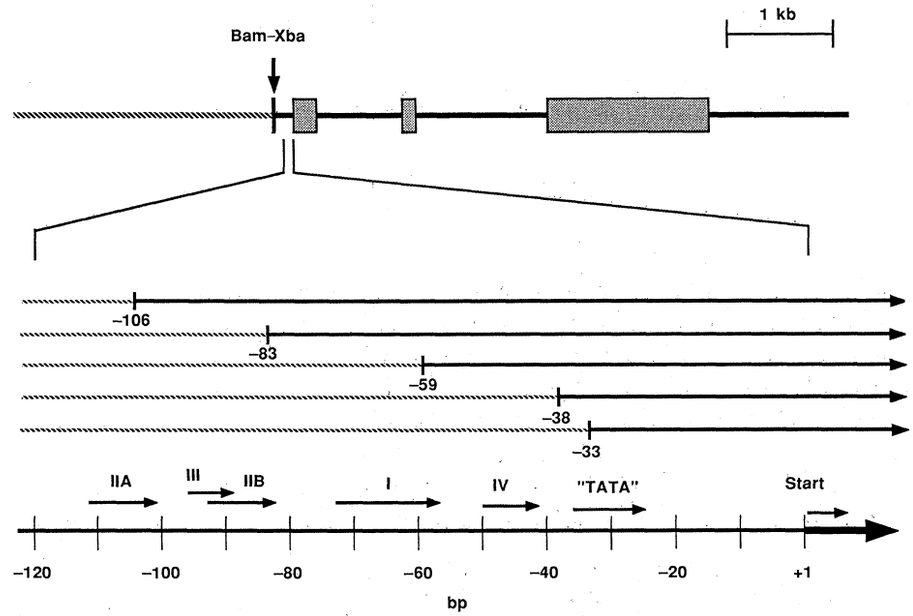

Fig. 2. Schematic diagram of the *D. melanogaster Ddc* gene and some of the deletion derivatives. Exons of the major *Ddc* transcript are drawn as gray rectangles. Bam-Xba represents the Bam HI–Xba I linker that is the upstream end point of all deletions shown. Sequences upstream of this linker are represented by a striped line. The elements identified in Fig. 1 are drawn at the bottom. All nucleic acid manipulations were as previously described (*12, 22*). Enzymes and linkers were obtained from New England Biolabs. The mutations were constructed in a 7.5-kb Pst I *Ddc* fragment (*9, 11*) that had been modified by the insertion of synthetic Bam HI and Xba I linkers into the Eco RV site 209 bp upstream of the major RNA start site. This Xba I site is unique in all of the plasmids described below. The resulting plasmid, p1210, was digested with Xba I, and the linear molecules were digested with Bal 31 nuclease under conditions that resulted in the removal of approximately 25 bp from each end per minute. The ends of the digested molecules were repaired with the Klenow fragment of DNA polymerase I, ligated to synthetic Xba I linkers, recircularized, and transformed into *Escherichia. coli* JM83 (*22, 23*). Plasmids carrying deletions of the desired size were identified after electrophoresis of Eco RI-Xba I fragments on 2% agarose gels. Xba I–Sal I fragments extending from the deletion end point to the unique Sal I site within *Ddc* were used to replace the corresponding fragment of p1210. This resulted in a nested set of deletions, each having an upstream end point at −208 bp and extending variable distances towards the transcription start site. The deletion end points were precisely localized by dideoxynucleotide sequencing of duplex templates with a synthetic oligonucleotide primer complementary to a sequence 100 bp downstream of the RNA start site. Sequence analysis revealed the presence of a Bam HI linker adjacent to the inserted Xba I linker, apparently the result of contamination within the Xba I linker preparation. P element transformation vectors were prepared by inserting the 7.1- to 7.3-kb Bgl II–Pst I *Ddc* inserts from the relevant plasmids into the unique Bgl II–Pst I sites of p1108, an Adh^{+} vector (*11*) based on the defective P element vector psxΔ1 (obtained from J. Posakony). These *Ddc* segments retain at least 2 kb of 5′ flanking DNA, and 1 kb of 3′ flanking DNA. The orientation of the *Adh* and *Ddc* genes in the resulting vectors is such that transcription from the genes is divergent. Embryo injections, identification of transformants, and establishment of homozygous lines were carried out as described (*9, 11*). Southern blots of genomic DNA were performed to confirm the identity of each *Ddc* deletion.

means of a primer complementary to *Ddc* RNA sequences 100 bases downstream from the normal start point. The hypodermal, larval CNS, and adult head transcripts all initiate at the same start sites (Fig. 5). Although adult heads are not a pure CNS tissue source, the majority of *Ddc* enzyme activity in the head is in the brain (*8*).

The homology elements of the 5′ flanking sequence found by comparing the *D. melanogaster* and *D. virilis Ddc* genes are drawn in alignment with the deletion end points in Figs. 3 and 4. The only homology element in the segment between −83 and −59 associated with the dramatic loss of CNS expression in both larvae and adults is element I. This 16-bp sequence, which is perfectly conserved between the two *Ddc* genes, is the most likely candidate for the functional element required for *Ddc* expression in the CNS.

Whether this element or another closely related element is associated with other genes expressed in the CNS is unknown. Computer-based searches have so far failed to reveal any significant homologies with other neurally expressed genes. However, the number of such genes sequenced is still rather small. Furthermore, only in the case of the *D. melanogaster* gene *fushi tarazu* have sequences responsible for neural expression been localized to any extent (*16*).

The deletion of each interval defined by the end points between −106 and −33 has an effect on hypodermal expression of *Ddc*. We cannot eliminate the possibility that the graded decrease in hypodermal expression observed as deletions progressively remove this region may be caused by interference with hypodermal expression by sequences at the distal end point of the deletions. However, the finding of homology elements within each of these intervals and the differing effects of these deletions on expression in the CNS indicate that a majority of the effects are probably due to loss of these elements.

Deletion of the first interval, defined by deletions with proximal end points at −106 and −83, leads to a decrease in hypodermal expression of approximately threefold at pupariation, and approximately twofold at adult eclosion. This region has selective effects on hypodermal *Ddc* expression, since *Ddc* expression in the larval CNS or adult brain is unaffected by loss of this region. Within this interval are the overlapping homology elements IIB and III. One or both of these elements are likely to be functional elements necessary for normal hypodermal expression.

The next interval, between −83 and −59, contains element I, the presumptive CNS element. Deletion of this interval has no effect on hypodermal expression at pupariation, but results in a two- to threefold decrease in expression at adult eclosion.

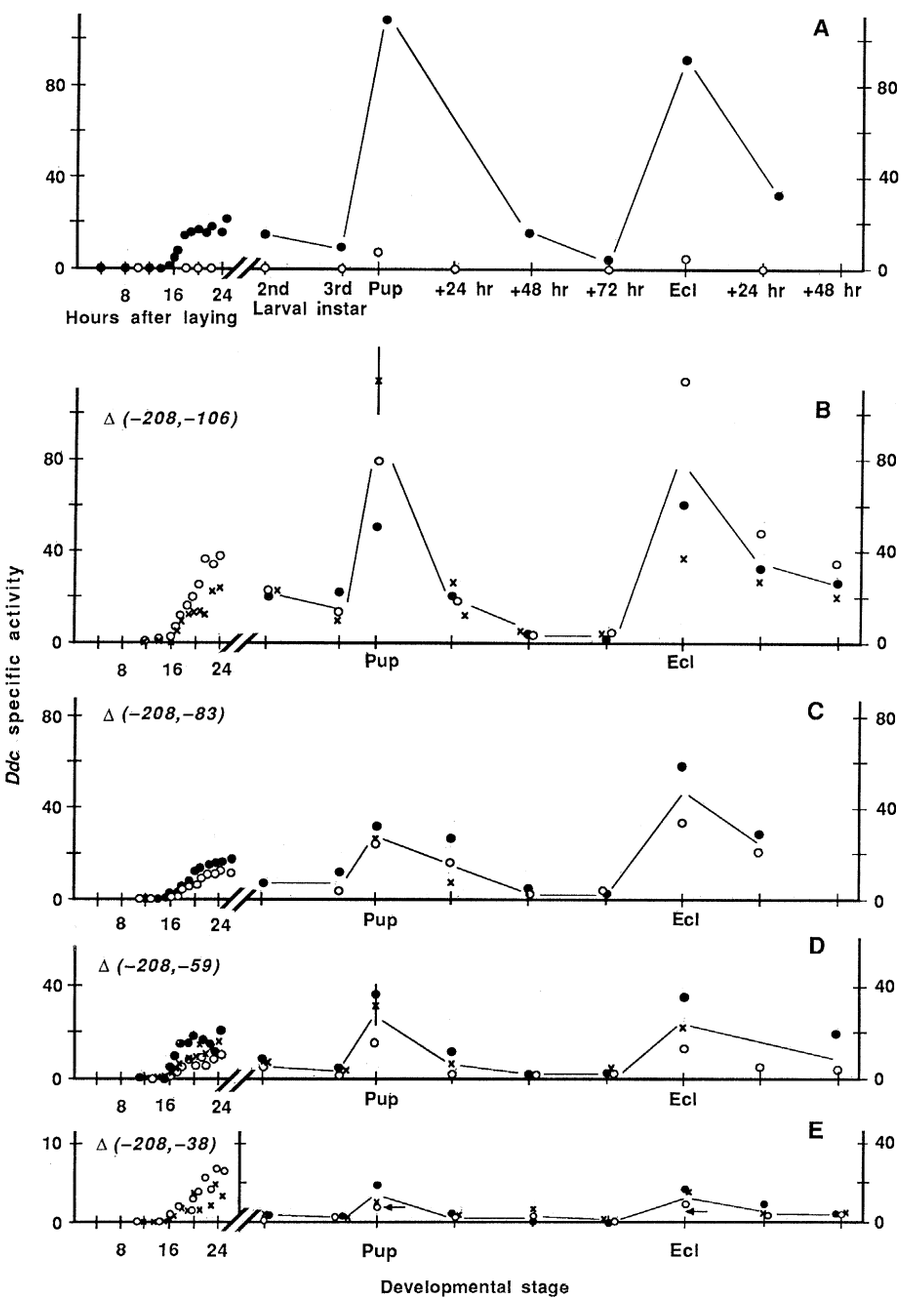

Fig. 3. Developmental profiles of *Ddc* enzyme expression in wild-type and representative deletion strains. The ages shown for the embryonic time points are the mean ages from embryos collected over a 2-hour interval. All strains hatched between 19 and 21 hours. Postembryonic data points represent the means of three to five assays. Standard errors are indicated when larger than 10% of the mean. All strains are homozygous for single *Ddc* mutant genes unless indicated. The nomenclature used, $\Delta(-x, -y)$, indicates the bases $-x$ through $-y$ have been deleted. Pup, pupariation; Ecl, adult eclosion. (A) Wild-type (Canton-S) strain, ●; injection host strain ($Ddc^{ts2}Adh^{fn23}$), ○. In B through F, different symbols indicate different strains with deletions as described. (B) $Ddc^{\Delta(-208,-106)}$ strains. (C) $Ddc^{\Delta(-208,-83)}$ strains. (D) $Ddc^{\Delta(-208,-59)}$ strains. The strain designated by filled circles contains two inserts. (E) $Ddc^{\Delta(-208,-38)}$ strains. Note the fourfold change in scale for the embryonic data. The horizontal arrows in (E) indicate the expression from the injection host strain. *Ddc* enzyme assays were performed as described (*11*). One specific activity unit of enzyme leads to formation of 1 nmol dopamine per 20 minute incubation at 30°C per milligram of protein. *Ddc* specific activity in larval CNS and adult brains was determined on hand-dissected preparations. A twofold variation in specific activities of CNS extracts was observed in two different lots of assay mix, even though whole-animal *Ddc* specific activity levels were constant. This is presumably due to variation in stability of *Ddc* at the low protein concentrations present in the CNS extracts. Assays of wild-type CNS extracts were included to control for this variation.

Whether this is because of a secondary function for this element, a second unidentified element, or effects of sequences on the distal side of the deletion cannot be discerned from our present analysis. In any case, the effect on hypodermal expression is small compared to the approximately 50-fold effect on CNS expression.

The other interval with effects on hypodermal expression of *Ddc* is −59 to −33. Although deletion of this interval leads to a clear loss of expression, it is less clear whether the majority of this loss results from deletion of the sequences between −59 and −38 or −38 and −33 since the expression of different $Ddc^{\Delta(-208,-38)}$ strains is variable. Because the element necessary for CNS expression has already been deleted by less extensive deletions, it is not possible to determine whether this interval contains tissue-specific regulatory elements, or nonspecific promoter elements. Element IV and the TATA element are found within this interval.

The elements with quantitative effects on expression of *Ddc* in the hypoderm may not be responsible for the complex temporal regulation of *Ddc*. The severely deleted $Ddc^{\Delta(-208,-38)}$ gene still shows induction at both pupariation and adult eclosion, although expression is quantitatively reduced from normal levels and shows variation in different strains. All of the upstream elements except the TATA element have been removed by this deletion. This leaves open the possibility that not all of the *cis* regulatory sequences are contained in the 5′ flanking region. An alternative possibility is that redundant sequences upstream of −208 are

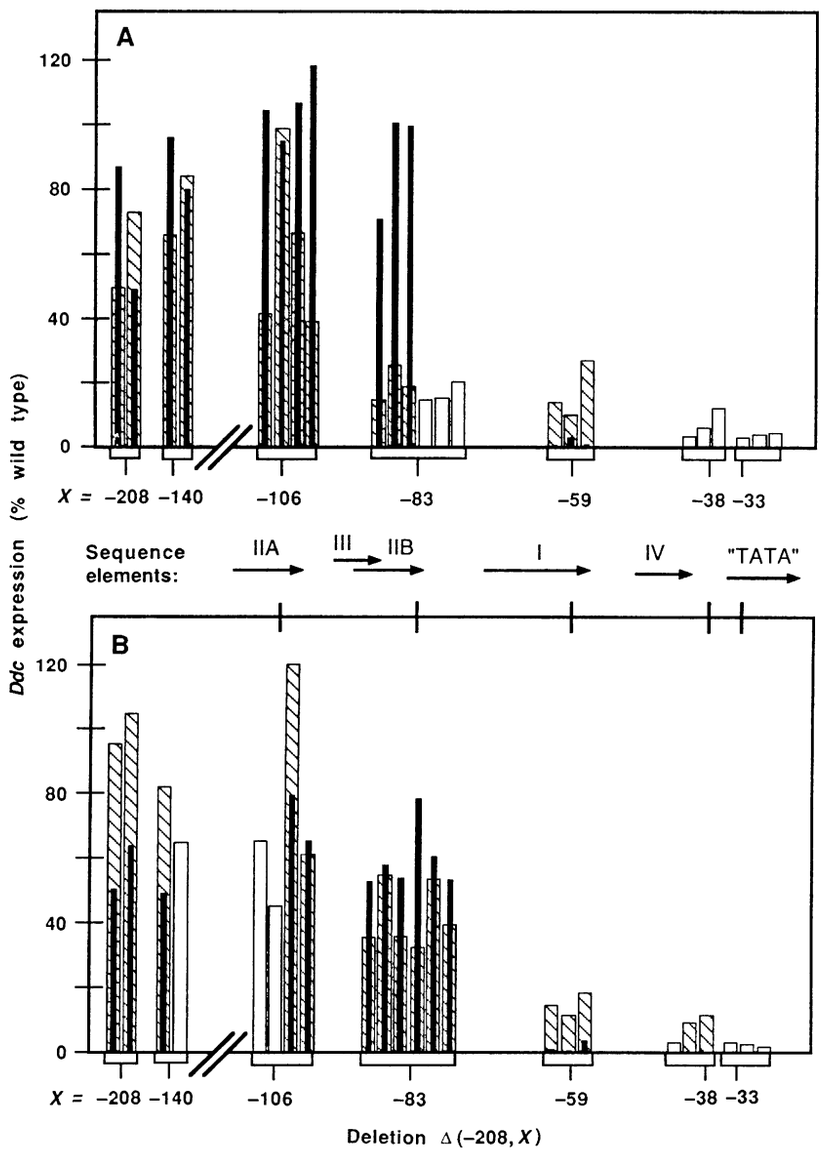

Fig. 4. *Ddc* expression as a function of deletion end point, measured (A) in whole puparia and in larval CNS or (B) in whole eclosed adults and in adult brains. The alignment of the sequence homology elements to the deletion end points is shown between the two panels. The data are plotted relative to *Ddc* expression in a Canton-S wild-type strain. Whole animal expression, open or cross-hatched bars; CNS expression, black bars. Strains for which assays of CNS activity were not performed are indicated by the open bars. Each pair of nested bars represents the level of expression in a given strain, corrected for multiple insertions where necessary. Data from individual strains are presented in the same order in (A) and (B). *Ddc* enzyme activity from the Ddc^{ts2} genes of the injection host has been subtracted from the values shown. The *Ddc* gene indicated as Δ(−208,−208) contains a Bam-Xba linker inserted at −208 (Fig. 2, top). The level of expression in wild-type CNS tissue is 56 units per milligram of protein in larval CNS, and 92 units per milligram of protein in adult brain. Standard errors were generally less than 10% of the mean.

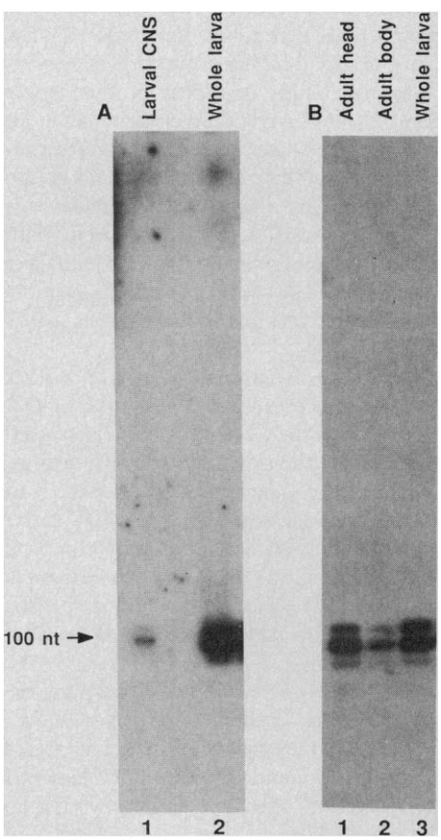

Fig. 5. The *Ddc* RNA start points in larval and adult CNS. We analyzed the RNA start points by primer extension analysis, priming reverse transcription from a synthetic oligonucleotide primer complementary to RNA 100 bp downstream of the major start point (*12*). The minor bands are start points ±3 bp from the two adjacent major start points, which in this figure migrate as a single band. (A) RNA isolated from larval CNS, consisting of brain lobes and ventral ganglia, or whole third instar larvae. (B) RNA isolated from adult heads, adult bodies, or whole third instar larvae. Unlabeled complementary DNA was synthesized from *D. melanogaster* polyadenylated RNA with avian reverse transcriptase, primed by a 15-base synthetic oligonucleotide complementary to *Ddc* RNA sequences 100 bases from the start point. The cDNA was fractionated on 6% polyacrylamide–6*M* urea sequencing gels and electroblotted onto Genescreen (New England Nuclear). Extension products were detected by hybridization with a ^{32}P-labeled probe encompassing the *Ddc* first exon. Primer extension assays are described in (*11*). nt, Nucleotide.

partially capable of compensating for the loss of *Ddc* regulatory elements. However, no regulatory elements necessary for normal tissue-specific expression are located upstream of −208 (*12*), nor have we found any normal flanking sequences duplicated within 200 bp upstream of this point. Supporting the possibility of intragenic regulatory elements is the finding (*12*) that severely deleted *Ddc* genes retaining only 24 bp of upstream flanking sequences appear to show some components of normally regulated expression when stimulated by elements from adjacent vector sequences.

Our results precisely localize specific elements that determine the tissue-specific expression of a higher eukaryotic gene. Previous studies have implicated both enhancer elements (*17*) and other elements without detectable enhancer activity (*18*) as determinants of tissue-specific expression. Although protein-binding assays have detected factors binding to specific sequences within both the immunoglobulin and insulin enhancer regions (*19*), the precise identification of the functional elements within these regions is unknown.

Our deletions provide tools for further investigating the physiological role of *Ddc* expression in the CNS. We have constructed genes with phenotypically normal expression in the hypoderm that are not expressed at detectable levels in the CNS. Flies carrying these genes will allow a more complete assessment of the role of potential neurotransmitters synthesized by the *Ddc* gene product in normal behavior and learning (*6*).

Note added in proof: Recent immunohistochemical experiments (*20*) have shown that at least one other previously undetected element is required in addition to element I for correct cell-specific expression of *Ddc* in the larval CNS.

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From Stimulation to Undulation: A Neuronal Pathway for the Control of Swimming in the Leech

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Initiation and performance of the swimming movement in the leech (*Hirudo medicinalis*) are controlled by neurons organized at at least four functional levels—sensory neurons, gating neurons, oscillator neurons, and motor neurons. A paired neuron, designated as Tr1, in the subesophageal ganglion of the leech has now been shown to define a fifth level, interposed between sensory and gating neurons. Cell Tr1 is activated by pressure and nociceptive mechanosensory neurons, which mediate body-wall stimulus–evoked swimming activity in intact leeches. In the isolated leech nervous system, brief stimulation of cell Tr1 elicits sustained activation of the gating neurons and triggers the onset of swimming activity. The synaptic interactions between all five levels of control are direct. Discovery of the Tr1 cells thus completes the identification of a synaptic pathway by which mechanosensory stimulation leads to the swimming movements of the leech.

RHYTHMIC MOVEMENTS OF ANIMALS are generated and regulated by neurons organized at several interdependent functional levels, including sensory input, two levels of control neurons ("trigger" and "gating"), oscillator neurons, and motor neuron output (*1*). The central level is that of the oscillator, which consists of neuronal networks interconnected to generate rhythmic timing cues (*2*). The oscillator provides phasic excitatory and inhibitory output to effector muscles via motor neurons. The expression of rhythmic movement is controlled by excitatory or inhibitory inputs to the central oscillator from two types of neurons: trigger neurons, that when transiently activated initiate prolonged rhythmic output, and gating neurons, which elicit rhythmic motor patterns only while they are active (*1, 3*). Input to these trigger or gating neurons can arise from many sources, including sensory neurons. Although progress has been made in describing many of these functional levels, especially in various invertebrate species, the neuronal elements that form and link all of the functional levels have not been identified for any rhythmic movement in any species (*4*).

For the neuronal circuits that underlie swimming movements in the medicinal leech (*Hirudo medicinalis*), four functional levels—sensory, gating, oscillator, and motor neurons—have been identified (*5*). Here we describe a paired neuron, cell Tr1, which defines a fifth, or trigger, functional level.

The Tr1 cell pair is located in the most anterior neuromere of the leech subesophageal ganglion (*6*). The neurite of either Tr1 cell crosses the midline of the subesophageal ganglion and projects caudally in the contralateral connective nerve to the posterior

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