Repression and Activation of the *Drosophila* Dopa Decarboxylase Gene in Glia

GRANT S. MASTICK AND STEVEN B. SCHOLNICK[†]*

Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, Pennsylvania 15213

Received 5 May 1992/Returned for modification 15 July 1992/Accepted 17 September 1992

Glial expression of the *Drosophila* dopa decarboxylase gene (*Ddc*) is repressed by a regulatory region located approximately 1 kb upstream of the transcriptional start site. We have used in vitro mutagenesis and germ line transformation to determine which elements within the *Ddc* promoter mediate repression. Our evidence suggests that the hypodermal cell activator elements IIA and IIB play a major role in the transcriptional regulation of *Ddc* in glial cells. A variety of mutations demonstrate that element IIA is a strong glial activator element and that element IIB is necessary for glial repression. Although these two regulatory elements are nearly identical in sequence, our data suggest that they are not redundant. Altering the wild-type number and spacing of elements IIA and IIB indicates that the wild-type arrangement of this repeat is critical for repression. We conclude that these key elements of the *Ddc* promoter regulate both activation and repression in glia.

The development of cell types with distinct structural and functional properties requires the expression of specific genes in particular spatial and temporal patterns. The mechanisms regulating these patterns can be combinatorial in nature, involving both positively and negatively acting factors and long- or short-range interactions (3, 17). Spatially or temporally restricted negative regulation can add specificity to the action of otherwise ubiquitous transcriptional activators (1, 13, 20).

Transcriptional repression plays a role in the cell-type-specific expression of the dopa decarboxylase (DDC) gene (*Ddc*) in the central nervous system (CNS) of *Drosophila melanogaster* (2, 12). In neurons, *Ddc* encodes an enzyme essential for the synthesis of the neurotransmitters dopamine and serotonin and is expressed at high levels in a specific set of dopamine- and serotonin-containing neurons (2, 6, 8, 18, 28). In contrast, *Ddc* is expressed at low levels in a subset of glia on the ventral and lateral surfaces of the third instar CNS, where its function is unknown (2). *Ddc* expression within both cell types is regulated by sequences lying approximately 1 kb upstream of the transcriptional start site. *Ddc* transgenes which retain the entire 2.5 kb of 5'-flanking sequence are properly regulated (2, 23, 25). Deletion of transgene sequences upstream of position -210 causes derepression in the subset of glial cells that normally express *Ddc* at low levels and eliminates expression in neurons (2). A portion of this upstream region extending from positions -760 to -1623, referred to here as the distal regulatory region, can restore the wild-type neuronal and glial patterns and levels of expression in a distance- and orientation-independent manner (12). The distal regulatory region, therefore, has basic properties that are silencer-like in glia and enhancer-like in neurons. The wild-type pattern of CNS expression results, in part, from cell-type-specific repression of strong glial activator elements in the promoter.

The experiments described in this paper examine the role of promoter elements in the regulation of expression of *Ddc* in glia. We have used in vitro mutagenesis and germ line

transformation to map the glial activation-repression region of the promoter to the promoter elements IIA and IIB (4, 23). These two elements are each composed of two repeats of a hexamer consensus sequence common to all of the known *Ddc* promoter elements (C(T/A)GCGG [4, 23]). Our data suggest that element IIA mediates glial activation and that element IIB is necessary for glial repression.

MATERIALS AND METHODS

DNA constructions. Standard methods were used for recombinant DNA manipulations (19). All deletion and duplication endpoints, except where noted below, were joined by 8-bp *Xba*I linkers (New England Biolabs). Deletion endpoints are designated by the first and last base pairs missing from the construct, while duplication endpoints are designated by the first and last base pairs included in the duplication. All endpoints were verified by dideoxynucleotide sequencing with Sequenase (U.S. Biochemicals).

Mutants lacking various amounts of the 5'-flanking region, 5'Δ-173, 5'Δ-145, and 5'Δ-126, were constructed by Howard Bomze (Carnegie Mellon University) from a set of *Bal* 31 nuclease-generated deletions by removing various portions of the flanking DNA around position -210 (22a). The 5'-truncation derivatives were generated by using a *Bgl*II linker to replace the *Ddc* DNA between the *Bgl*II site at position -2300 and the *Xba*I linkers marking the original deletion endpoints. The truncated flanking DNA and the *Ddc* gene were transferred as *Bgl*II-*Pst*I fragments to p1298, a derivative of the *Adh*⁺ P-element vector p1108 (23), in which the *Ddc* and *Adh* genes are transcribed in the same right to left orientation, preventing interference from the *Adh* transcriptional controls, which occurs when *Ddc* has very little 5'-flanking region (9).

Double mutants with a deletion of the distal regulatory region and additional internal deletions in the promoter were constructed from the promoter mutants Δ-166, -60, Δ-186, -60, and Δ-91, -60 (24) by cutting at the *Eco*RV site at position -209, attaching *Bgl*II linkers, and then isolating the *Bgl*II-*Pst*I fragment containing *Ddc*. These constructs were then transferred (by Mohanish Deshmukh [Carnegie Mellon University]) to p1298 to make 5'Δ-210(Δ-166, -60), 5'Δ-210(Δ-186, -60), and 5'Δ-210(Δ-91, -60).

* Corresponding author.

[†] Present address: Department of Otolaryngology, Washington University Medical School, St. Louis, MO 63110.

The deletion of element IIB, Δ IIB, was made by Cathy Mauro (Carnegie Mellon University) using fragments from *Bal* 31 nuclease digestions initiated at either upstream (position -209) or downstream (position -26) sites. The appropriate fragments were joined by the *Xba*I linkers inserted at the deletion endpoints, and the resulting deletion removes sequences from positions -98 to -83. Point mutations in element IIB were made by using oligonucleotide-directed mutagenesis (16) of an *Eco*RI-*Nco*I subclone of the *Ddc* promoter with a 24-mer oligonucleotide containing four substitutions. This changes the normal sequence of element IIB (underlined), ATTCGAAGTTCAGCGGCTGCGGACTGCGA, to ATTCGAAGTTAAGCTTATGCGGACTGCGA (mutation designated IIB*). These point mutations create a new *Hind*III site and disrupt the hexamer repeat shared by elements IIB and III (4). The IIB* promoter fragment was introduced into an intact *Ddc* gene via a three-part ligation, including a fragment extending downstream from the *Bgl*II site at the 5' end of *Ddc* to the *Eco*RI site at position -380, the mutant *Eco*RI-*Nco*I promoter fragment, and a fragment extending from the *Nco*I site at position -25 through the 3' end of the wild-type clone and the vector sequences. Both Δ IIB and IIB* genes were transferred as *Bgl*II-*Pst*I fragments to p1108. In p1108, *Ddc* and *Adh* are divergently transcribed.

The duplication of element IIA, dupIIA, was created by replacing the *Xba*I-*Sal*I fragment (positions -82 through +1106) of Δ (-98, -83) with the corresponding fragment of Δ (-209, -124) (i.e., positions -123 through +1106) (23), resulting in a tandem duplication of the sequence from positions -123 to -99. Mutation dupIIA/III, which duplicates IIA, III, and most of element IIB (i.e., duplication of sequences from positions -120 to -83) was made by a three-part ligation. This reaction included a fragment extending from the 5' *Bgl*II site to an *Xba*I site at position -83, a fragment extending from an *Xba*I site at position -120 through the rest of the promoter and gene to the *Pst*I site at the 3' end of the wild-type clone, and *Bgl*II-*Pst*I-digested p1108. The complete duplication of elements IIA, III, and IIB, dupIIA/III/IIB, also was made by a three-part ligation, resulting in the duplication of sequences from positions -120 to -74. Initially, a new *Xba*I site was created at position -74 by using oligonucleotide-directed mutagenesis (16). Three fragments were then ligated: a fragment extending from the 5' *Bgl*II site (approximately at position -2300) to the *Eco*RI site at position -380 of the wild-type clone, a fragment from this *Eco*RI site to the newly created *Xba*I site at position -74, and the fragment extending from an *Xba*I site at position -120 through the 3' *Pst*I of the gene (including vector sequences). The dupIIA/III/IIB *Bgl*II-*Pst*I fragment from the resulting plasmid was then transferred to *Bgl*II-*Pst*I-digested p1108.

Insertions of 10 and 13 bp utilized the *Nsp*V site at position -98, between elements IIA and IIB. An *Eco*RI-*Nco*I subclone was digested with *Nsp*V, end filled with the Klenow fragment of *Escherichia coli* DNA polymerase, and then ligated to unphosphorylated *Xba*I linkers, resulting in a single-linker insertion. We used the following protocol (26): 2.5 μ g of unphosphorylated, annealed octomer *Xba*I linkers (New England Biolabs) were ligated overnight at 4°C to 5 μ g of phenol-extracted end-filled DNA in a volume of 20 μ l containing 30 mM NaCl, 30 mM Tris (pH 7.5), 8 mM MgCl₂, 0.25 mM rATP, 2 mM dithiothreitol, 0.2 mM EDTA, 1 mM spermidine, 0.1 mg of bovine serum albumin (BSA) per ml, and 2 U of T4 ligase (Boehringer Mannheim). The ligation mixture was heated to 90°C, quick-chilled, and gel-purified in

low-melting-temperature agarose. The gel fragment was melted, diluted, and slowly cooled before transformation. Sequencing confirmed the insertion of 10 bp, and that subsequent end filling and religation of the *Xba*I linker created a 13-bp insertion. These mutant promoter fragments were introduced into an otherwise wild-type 7.5-kb *Ddc* fragment and transferred as *Bgl*II-*Pst*I fragments to p1108.

Drosophila manipulations. P-element-mediated transformation of host strain *Adh*^{fn23} *Ddc*^{ts2} was carried out as described previously (22, 25). Transformed lines were identified by screening for ethanol resistance [Δ IIB, dupIIA, dupIIA/III, the 10- and 13-bp insertions, 5' Δ -210(Δ -166, -60), 5' Δ -210(Δ -186, -60)] or wild-type *Ddc* pupal pigmentation at 25°C (IIB*, dupIIA/III/IIB, and the rest of the 5' Δ strains). All transgenic lines identified as ethanol-resistant had wild-type pupal pigmentation and vice versa. Repeated intercrosses led to lines that were mostly homozygotes. In some cases, independent strains were made homozygous and isogenic by using crosses involving the second and third chromosome balancer strain *CyO*^{NB} *MKRS/T(2,3) ap*^{Xa}. Southern analysis using a probe to detect the 3' junction of the P-element vector with the genome revealed that all of the transformant lines described in this work contain single-copy inserts. A number of independent transformed lines were analyzed for each construct: 5' Δ -173 (four lines), 5' Δ -145 (three lines), 5' Δ -126 (two lines), 5' Δ -210(Δ -91, -60) (three lines), 5' Δ -210(Δ -166, -60) (one line), 5' Δ -210(Δ -186, -60) (four lines), Δ IIB (four lines), IIB* (five lines), the 10- and 13-bp insertions (three and five lines, respectively), dupIIA (three lines), dupIIA/III (two lines), and dupIIA/III/IIB (three lines).

Affinity purification of DDC antisera and immunofluorescence. The generation and properties of the affinity-purified DDC antiserum are described by Scholnick et al. (24). The affinity-purified antibodies give the same patterns and levels of DDC staining of Canton S (wild-type) CNSs as antisera used in other reports (2, 14). The transformation host *Adh*^{fn23} *Ddc*^{ts2} has a very low background level of staining when grown at 25°C, with bright staining of only a few neurons and no detectable glial staining.

Indirect immunofluorescent detection of DDC protein was carried out with dissected CNSs of late third instar larvae (2, 24). CNSs were dissected in ice-cold Ca-free Ringer's solution on a cold stage by using forceps, rinsed in cold Ringer's in batches of 5 to 10 CNSs, and then fixed immediately in 0.5-ml Eppendorf tubes at room temperature in fresh 4% formaldehyde-phosphate-buffered saline (PBS) for 30 min. Prompt fixation was found to be critical both for the preservation of glial morphology and for consistent levels of glial staining. The CNSs were washed two times with PBS and two times with PBT (PBS plus 0.1% [wt/vol] BSA plus 0.2% [vol/vol] Triton X-100) for 5 min per wash. After a final 1-h PBT wash on a rotating platform (Labquake Shaker; Labindustries, Inc.), the CNSs were incubated with affinity-purified primary antibodies, at a 1/200 dilution in PBT, for from 4 h to overnight with rotation. The CNSs were washed four times (5 min per wash) with PBT, with a final wash lasting several hours. The CNSs were then incubated for from 4 h to overnight with a 1/200 dilution of a rhodamine-labeled, affinity-purified goat anti-rabbit secondary antibody (Cappel) and washed again with PBT. The CNSs were dehydrated through a glycerol series and mounted in 90% glycerol-2 mM sodium bicarbonate (pH 9)-1-mg/ml 1,2-phenylenediamine (ortho, free base; Aldrich) to prevent photobleaching. Independent transformed lines for each construct were examined at least three times each, and then

FIG. 1. Diagram of the promoter deletion mutations used to localize glial activator elements. The top line represents the 7.3-kb *Bg/III-PstI* *Ddc* genomic clone, with exons represented as shaded boxes. The distal regulatory region containing the glial silencer and neuronal enhancer activities extends from positions -1623 to -760 (12). The promoter region is shown at an expanded scale in the middle part of the figure, with the transcription start site (position +1) depicted by the large arrow. The known regulatory elements are represented by labeled boxes above the promoter coordinates (base pairs upstream of the start site). Transgenes designed to localize glial activator sequences are shown below the expanded promoter, with the strain designations to the right. The first three constructs are missing all sequences upstream of the deletion endpoint, including the distal regulatory region. The next three constructs are truncated at the *EcoRV* site at position -209, and also contain internal deletions extending upstream from position -60, as indicated by breaks in the line. Eight-base pair *XbaI* linkers join the internal deletion endpoints and are not shown. Two key constructs from previous work are shown at the bottom of the figure for reference (24). The table on the right side of the figure summarizes levels of *Ddc* expression in glia and neurons of the late third instar CNS, as assayed by indirect immunofluorescence (Materials and Methods). Glial staining levels were scored on a scale ranging from wild-type (very low, but detectable; +/ -), to very high levels typical of 5'Δ-210 strains (+++). All of the transgenic strains had no detectable neuronal expression above the level of the injection host, *Adh^{tr23} Ddc^{ts2}* (not shown), with the exception of those carrying the 5'Δ-210(Δ-91, -60) transgene. These strains had low levels of expression in class 1 neurons, a pattern of expression observed in some promoters lacking element I (24).

representative strains of each construct were compared with control strains in parallel staining reactions. Specimens were photographed on Hypertech film (Microfluor Inc.) with a Zeiss Axiophot microscope. Identical exposures were used for Canton S and mutant strains.

RESULTS

Definition of glial activator elements. Beall and Hirsh (2) provided evidence for a distal negative regulatory region by demonstrating that truncation of *Ddc* upstream sequences at position -210 (5'Δ-210) greatly derepresses *Ddc* expression in a subset of the glia. DDC enzyme activity in these transgenic CNSs can reach 300% of that found in both neurons and glia of their Canton S counterparts (9). In addition, promoter deletions extending from positions -209 to -60 (Δ-209, -60) abolish all CNS expression (2, 23), implying that potent glial activators lie in the position -209 to -60 interval. Several sequences have been identified in the *Ddc* promoter (Fig. 1) which are both highly conserved during evolution (4) and functionally important in vivo (2, 5, 23). These include the repeated promoter elements IIA and IIB, which are required for hypodermal expression, the neuronal activator element I, and element IV, a weak general activator. Elements IIA and IIB are nearly identical (11 of 12 bp conserved) and consist of tandem repeats of a hexamer sequence common to many of the proximal *Ddc* regulatory elements [C(A/T)GCGG] (4). An additional evo-

lutionarily conserved sequence, element III, overlaps the 5' hexamer of element IIB (4). The two elements have not been functionally separated. Neuronal activator element I (positions -73 to -58) also appears to be a glial activator, since Δ-209, -84 strains have high levels of glial expression, while Δ-209, -60 strains have neither glial nor neuronal expression (2, 23). Since the Δ-209, -84 mutation removes elements IIA, IIB, and III, element I must be able to drive glial expression in the absence of these promoter elements. The element IIA/IIB region contains additional glial activators which can function in the absence of element I. Transgenes with deletions of both the distal regulatory region and element I, 5'Δ-210(Δ-83, -60), express at high levels in glia, while a further deletion of elements IIA, IIB, and III, 5'Δ-210(Δ-136, -60), greatly reduces expression (24) (Fig. 1). These data suggest that element I and the IIA/III/IIB complex may have redundant activating functions in glia. They also suggest that no strong glial activators lie 3' of element I.

We have refined the localization of these glial elements and dissected their functions with additional mutant transgenes. A series of 5' truncations, 5'Δ-173, 5'Δ-145, and 5'Δ-126 (Fig. 1), was used to investigate whether additional glial activators lie between position -209 and the 5' end of element IIA (position -110). P-element-mediated transformation was used to establish several independent transgenic lines. We then used an affinity-purified *Ddc* antibody and indirect immunofluorescence to examine transgene expres-

FIG. 2. Indirect immunofluorescent detection of DDC protein in third instar CNSs of wild-type strain and strains carrying silencer deletions. Expression levels and patterns of *Ddc* were examined by using an affinity-purified anti-DDC serum and rhodamine-labelled secondary antibody. The anterior of the CNS is oriented toward the top of each figure, with the ventral ganglia extending downward to the lower left-hand corner. (A) Canton S wild-type (WT) control. High levels of staining are observed in a reproducible subset of neurons. Glial expression appears as weak staining of a fibrous meshwork which covers much of the surface of the CNS. (B) CNS from a 5'Δ-126 strain in which all sequences upstream of position -125 are deleted. These strains show a cell-type-specific derepression of *Ddc* expression in glia, with a much higher intensity of staining than in wild-type strains. The high levels of expression appear to be in the same glial cells as those of the wild type, although specific glial cells have not been identified. (C) 5'Δ-210(Δ-186,-60) strain showing low levels of glial expression. This transgene has a deletion within the promoter in addition to its 5' truncation. No neuronal expression is detectable above the background of the injection host, *Adh^{ts2} Ddc^{ts2}*.

sion in the late third instar larval CNS (Materials and Methods).

In the wild-type CNS, only neuronal cell bodies and their processes stain strongly (Fig. 2A). There is also weaker staining of a meshwork of glial fibers which cover the ventral and lateral surfaces of the CNS (2). There is no detectable glial *Ddc* staining in wild-type optic lobes. In contrast, transformed lines carrying either 5'Δ-173, 5'Δ-145, or 5'Δ-126 constructs express very high levels of *Ddc* in glia (Fig. 2B). Parallel staining reactions demonstrated that these new transgenes are expressed at levels comparable to those in 5'Δ-210 controls (not shown). We, therefore, conclude that the essential glial activators are located downstream of position -126 in the region of the already mapped neuronal and hypodermal elements. Individual DDC⁺ cells have not been identified and mapped within the wild-type glial network. However, the elevated expression of these 5'Δ transgenes closely matches the spatial organization of wild-type glial expression, strongly suggesting that expression is taking place within normally DDC⁺ cells. It should be noted that weak staining of fibrous structures in the optic lobe region of the cerebral hemispheres was consistently observed for all 5'Δ strains. It is not yet clear whether this represents ectopic expression or elevation of expression in glia which normally express very low levels of *Ddc*. Neuronal expression was not observed in the 5'Δ strains, consistent with the deletion of the distal regulatory region and the retention of a normal promoter (2, 12, 24).

In a second series of constructs, we made double mutants lacking all sequences upstream of position -210 as well as various portions of the remaining 5'-flanking sequence. The *Ddc*-flanking sequence remaining between position -209 and the 5' endpoint of the deletion shields the remaining promoter elements from spurious interactions with the vector. Such artifacts have been observed with *Ddc* transgenes with extremely short flanking sequences (9). Promoter mutation 5'Δ-210(Δ-91,-60) deletes elements I, IIB, and III but leaves element IIA intact (Fig. 1). This transgene is

expressed at high levels in glia, although these levels are lower than those of 5'Δ-210 (not shown). 5'Δ-210(Δ-91,-60) strains also show weak neuronal expression that appears to correspond to ectopic expression in class I neurons (24). This neuronal pattern is consistent with the behavior of the Δ-91,-60 transgene which retains the distal regulatory region (24). Strains carrying the 5'Δ-210(Δ-166,-60) and 5'Δ-210(Δ-186,-60) transgenes (Fig. 1) express *Ddc* at low, approximately wild-type levels in glia (Fig. 2C). This is consistent with the phenotype of 5'Δ-210(Δ-136,-60) strains (24) and supports the conclusion that strong glial activators lie between positions -136 and -60, the region containing elements IIA, III, IIB, and I (positions -114 to -58). Since the high level of glial expression of 5'Δ-210(Δ-91,-60) strains is reduced by further deletion to position -136, element IIA is a candidate for the activator. These data also suggest that element IIB is not required for glial activation in the presence of element IIA. Residual glial expression appears to be the result of weak activators downstream of position -60, such as element IV, or as yet unidentified activators downstream of the transcription start site.

Repeated promoter elements necessary for glial repression.

Our results suggest that element IIA is functional in glial cells and can act as a glial activator. Since elements IIA and IIB have an 11-bp sequence identity, element IIB may also have a role in glia. We tested the potential regulatory functions of element IIB with two mutations which disrupted its sequence (Fig. 3; Materials and Methods). A deletion of element IIB, ΔIIB, specifically deletes sequences from positions -98 to -83 while leaving the adjacent promoter elements IIA and I intact (Fig. 3). A second promoter variant, IIB*, introduces a cluster of four single-base mutations into the sequence shared by elements IIB and III (Fig. 3). These point mutations alter the *Ddc* hexamer consensus sequences of element IIB without changing the spacing of any promoter elements. The two altered promoter fragments were substituted into otherwise wild-type *Ddc* genes with 2.5

FIG. 3. Mutants of promoter elements IIA and IIB and summary of their CNS expression levels. Mutations affecting elements IIA and IIB are diagrammed below the expanded promoter, with the strain designations to the right. These transgenes contain the rest of the wild-type DNA of the original *BgIII-PstI* clone, including the distal regulatory region. Deletions are indicated by breaks in the line, point mutations are indicated by asterisks, insertions are indicated by labeled boxes, and duplications are indicated by overlaps. The precise sequence alterations are described in Results and Materials and Methods. Eight-base-pair *XbaI* linkers join the deletion and duplication endpoints, and are not shown. A summary of *Ddc* expression levels in glia and neurons as assayed by indirect immunofluorescence is given in the table on the right. Glial staining levels were estimated on a scale ranging from those of the wild type (very low, but detectable: +/-) to very high levels typical of element II promoter mutations (+++). Neuronal staining levels were at wild-type levels (++), except for about half of the Δ IIB and IIB* strains, which had reduced levels of neuronal staining (+).

kb of 5'-flanking sequences, including the distal regulatory region.

All of the independent strains carrying single copies of either Δ IIB or IIB* transgenes show a dramatic derepression of *Ddc* in glia (Fig. 4A) compared with Canton S wild-type CNSs (Fig. 2A). Overexpression is at the same levels as that of 5' Δ -210 strains (2, 19a; data not shown). These results

indicate that proper glial repression requires element IIB, even in the presence of the distal regulatory region. Since point mutations in IIB have the same phenotype as the deletion of IIB, the derepression observed in Δ IIB strains is probably not the result of the creation of a novel glial activator by juxtaposition of novel sequences or by changing the spacing of other control elements. Despite having a

FIG. 4. CNS expression of *Ddc* in transgenic strains carrying mutations in elements IIA and IIB. (A) IIB* strain carrying clustered point mutations in element IIB resulting in very high levels of expression in glia. (B and C) 10-bp-insertion-containing and dupIIA strains, carrying transgenes with a 10-bp insertion between elements IIA and IIB, and a duplication of element IIA, respectively, resulting in very high levels of expression in glia. The micrograph in panel C underrepresents glial staining in portions of the ventral ganglia because it was taken on a slightly different focal plane than the other panels. Neuronal expression is at wild-type levels in all of these strains.

nearly identical sequence, the single remaining element IIA is not sufficient to replace the function of IIB: either elements IIA and IIB are not redundant or element III plays a role in repression. The large increase in expression is specific for glia, since neuronal staining levels are actually reduced in about half of the Δ IIB and IIB* lines (not shown). Element IIB may have a minor function in determining neuronal expression levels, or mutations in IIB may interfere with the function of element I, the major neuronal activator.

The Δ IIB and IIB* derepression phenotypes are consistent with the hypothesis that element IIB is a negative regulatory site; a promoter without a functional element IIB overexpresses *Ddc* in glia because of the loss of an essential negative regulator. We examined two related potential mechanisms for repression by element IIB. (i) IIB interacts with the glial activator element IIA in a spatially constrained manner. (ii) The two elements form a paired unit involved in repression.

Elements IIA and IIB are separated by two helical turns (21 bp) which places them on the same face of the DNA helix. This arrangement is reminiscent of the repeated heat shock elements of the *Drosophila hsp70* gene which display both cooperativity (27) and dependence on the periodic spacing between the heat shock elements for activation of transcription (7). We tested for dependence on periodic spacing by introducing insertions of 10 and 13 bp at a point (position -98) between elements IIA and IIB (Fig. 3; Materials and Methods). The 10-bp insertion preserves the elements' helical arrangement, but the 13-bp insertion moves them out of register. If helical alignment is the only critical parameter, then only the 13-bp mutation should disrupt repression. However, if the wild-type spacing or precise sequence between elements IIA and IIB is required, then both spacing mutations should disrupt repression.

Both the 10- and 13-bp constructs are expressed at very high levels in glia (Fig. 4B); the levels are similar to those seen with Δ IIB and IIB* strains. These results suggest that glial repression requires the strict wild-type spacing of elements IIA and IIB or that a glial repressor sequence exists between IIA and IIB which is disrupted by the insertions.

Since both of the spacing mutations result in glial derepression, the two elements may form a paired unit with spatially constrained interactions necessary for repression. In this case, an additional copy of element IIA might drive high levels of glial expression even in the presence of a wild-type IIA/IIB pair. This model further predicts that duplicating an extra copy of the entire IIA/III/IIB complex would restore repression, provided that any other elements involved in repression, such as the distal regulatory region, are capable of interacting with more than one complex. Similarly, addition of element III alone to the duplicated copy of IIA could potentially separate the functions of elements III and IIB.

We combined the appropriate restriction fragments from various deletion mutations to duplicate sequences from positions -123 to -99 (duplication of element IIA alone, designated dupIIA), -120 to -83 (IIA and III, designated dupIIA/III), and -120 to -74 (IIA/III/IIB, designated dupIIA/III/IIB). The structure of these transgenes is diagrammed in Fig. 3.

The strains carrying any of the duplication constructs all express *Ddc* at very high levels in glia (Fig. 3 and 4C), with the same levels of glial derepression caused by the loss of element IIB or the spacing mutations. As predicted, dupli-

cation of IIA alone does disrupt glial repression, consistent with its role as a glial activator element. However, the larger duplications, dupIIA/III and dupIIA/III/IIB, are also derepressed. This is striking given the retention of the wild-type spacing and stoichiometry within the fully duplicated IIA/III/IIB complex. Taken together with the data obtained from the insertion mutations, our experiments show that both the wild-type number and the spacing of elements IIA and IIB are required for glial repression and that a paired IIA/IIB unit is not sufficient for that repression. However, repression cannot simply be the result of the negative regulation of element IIA by IIB or III since it appears that the stoichiometry or position of these elements relative to other regulatory elements is also important. These data do rule out the possibility that glial activation is solely controlled by a negative regulatory sequence lying between IIA and III/IIB because these sequences are included in the dupIIA/III/IIB construct.

DISCUSSION

Glial *Ddc* expression is mediated by a negatively acting distal regulatory region and positive and negative elements more proximal to the transcription start site (2, 12; this study). Deletion of the distal regulatory region causes a dramatic derepression of transgene expression in glia (2). Previous studies also demonstrated that strong glial activators lie within the position -210 to -60 interval, and that element I or the element IIA/III/IIB region can drive high levels of glial expression (2, 24). In this study, we have used in vitro mutagenesis and in vivo expression of transgenes to further characterize the proximal elements which respond to the distal regulatory region and to probe the mechanism(s) of repression.

We have refined the localization of the glial activator elements by creating 5'-truncation mutations as well as double mutants lacking both the distal regulatory region and portions of the promoter region. Those promoter deletions which remove critical glial activators suppress the derepression phenotype of the distal regulatory region deletion and result in lower levels of glial expression. Our data demonstrate that no essential activators exist upstream of position -126, since 5' Δ -173, 5' Δ -145, and 5' Δ -126 all have as high levels as 5' Δ -210 (Fig. 1 and 2B and data not shown, respectively). Element IIA is a glial activator, as demonstrated by reduced glial expression from 5' Δ -210(Δ -136, -60) transgenes relative to their 5' Δ -210(Δ -91, -60) counterparts (24; this study) (Fig. 1). Element IIB is apparently not required for high levels of expression in the presence of element IIA, because the phenotypes of the 5' Δ -210(Δ -83, -60) and 5' Δ -210(Δ -91, -60) transgenes are similar (24; this study) (Fig. 1). Further experiments will be required to determine whether element IIB functions as an activator in the absence of both element IIA and I. This might be expected because elements IIA and IIB share an 11-bp sequence identity.

Our results clearly indicate that the sequence containing overlapping elements IIB and III is necessary for glial repression, since both deletion and clustered point mutations in their shared sequence cause glial derepression (Fig. 3 and 4A). The fact that IIB* strains have the same phenotype as Δ IIB strains suggests that the clustered point mutations do indeed create a III/IIB null mutation. This similarity argues against the phenotype of Δ IIB being an artifact of changing the spacing of promoter elements or the juxtaposition of

novel sequences to create activator sites. Because transacting factors binding to element IIB have not yet been identified, the molecular mechanism of repression is unclear. IIB may be a binding site for a repressor that directly inhibits the transcription complex or interferes with the function of transcriptional activators bound to other promoter elements. The observation that both dupIIA and dupIIA/III/IIB duplications cause derepression indicates that the negative role of IIB is not simply to repress activation by IIA. The nearly identical sequences of elements IIA and IIB suggest that element IIA could act as a negative regulator. By this reasoning, point mutations in element IIA should also disrupt glial repression.

Glial repression requires the presence of several different negative regulatory elements, and the loss of any one of these elements results in the same very high levels of expression in glia. We conclude that the elements involved in repression cannot compensate for each other's loss, and therefore seem to function cooperatively rather than independently. This suggests the existence of a single mechanism of repression involving several elements functioning in concert. The distal regulatory region and promoter elements may form a repression complex, perhaps by DNA looping to allow interactions between the distal and proximal elements, as has been proposed for the silencers that flank the *Saccharomyces cerevisiae* mating type locus *HML* (10). The disruption of repression by the duplication mutations, from which no promoter sequences are deleted, implies that the process of cooperative repression is sensitive to the specific structure of the element IIA/IIB repeat, not simply to the presence of the elements. The distal regulatory region appears to interact with the promoter region in an inflexible manner, perhaps with a fixed stoichiometry with respect to the repeat.

The *Ddc* promoter has a complex role in regulation, in that it contains elements which mediate positive and negative regulation of transcription in a particular cell type. The sequence similarity of elements IIA and IIB suggests that the same or similar regulatory factors may bind to these elements. Demonstration of their equivalence will require data from experiments that are the reciprocal of those reported here. However, precedents for dual functions of regulatory factors can be found in studies of the regulation of a number of genes. The different functions have been proposed to result from different contexts of binding sites, alterations in the number and positioning of binding sites, or protein-protein interactions which regulate the mode of action (17, 21). In *Drosophila melanogaster*, a number of transcriptional activator proteins also act as repressors (11, 15, 20). Determination of which specific mechanisms are utilized for *Ddc* will require the identification of the regulatory protein(s) that bind to elements IIA and IIB in glial cells and testing how they act in the process of repression.

ACKNOWLEDGMENTS

We thank Cathy Mauro for construction of the Δ IIB plasmid; Howard Bomze and Mohanish Deshmukh for helping make the 5' Δ constructs; Nancy Ruffings, Sarah Gaffen, Henry Lue, and Joanne Klemencic for technical assistance; and Javier Lopez, Vaidyanathan Subramaniam, Cynthia Cain, and Peggy Weidman for comments on the manuscript. G.S.M. thanks Javier Lopez for providing space and support during the completion of this work.

This study was supported by PHS grant 1RO1GM34776 and NSF grant DCB-8802713 to S.B.S.

REFERENCES

1. Atchinson, M. L. 1988. Enhancers: mechanisms of action and cell specificity. *Annu. Rev. Cell Biol.* 4:127-153.
2. Beall, C. J., and J. Hirsh. 1987. Regulation of the *Drosophila* dopa decarboxylase gene in neurons and glial cells. *Genes Dev.* 1:510-520.
3. Brand, A. H., L. Breeden, J. Abraham, R. Sternglanz, and K. Nasmyth. 1984. Characterization of a "silencer" in yeast: a DNA sequence with properties opposite to those of a transcriptional enhancer. *Cell* 41:41-48.
4. Bray, S. J., and J. Hirsh. 1986. The *Drosophila virilis* dopa decarboxylase gene is developmentally regulated when integrated into *Drosophila melanogaster*. *EMBO J.* 5:2305-2311.
5. Bray, S. J., W. A. Johnson, and J. Hirsh. 1988. A cis-acting sequence and associated binding factor required for CNS expression of the *Drosophila melanogaster* dopa decarboxylase gene. *EMBO J.* 7:177-188.
6. Budnick, V., and K. White. 1988. Catecholamine-containing neurons in *Drosophila melanogaster*: distribution and development. *J. Comp. Neurol.* 268:400-413.
7. Cohen, R. S., and M. Meselson. 1988. Periodic interactions of heat shock transcriptional elements. *Nature (London)* 332:856-858.
8. Dewhurst, S. A., S. G. Croker, K. Ikeda, and R. E. McCamen. 1972. Metabolism of biogenic amines in *Drosophila* nervous tissue. *Comp. Biochem. Physiol.* 43:975-981.
9. Hirsh, J., B. A. Morgan, and S. B. Scholnick. 1986. Delimiting regulatory sequences of the *Drosophila melanogaster* *Ddc* gene. *Mol. Cell. Biol.* 6:4548-4557.
10. Hofmann, J. F.-X., T. Laroche, A. H. Brand, and S. M. Gasser. 1989. RAP-1 factor is necessary for DNA loop formation in vitro at the silent mating type locus *HML*. *Cell* 57:725-737.
11. Jiang, J., D. Kosman, T. Y. Ip, and M. Levine. 1991. The dorsal morphogen gradient regulates the mesoderm determinant *twist* in early *Drosophila* embryos. *Genes Dev.* 5:1881-1891.
12. Johnson, W. A., C. A. McCormick, S. B. Bray, and J. Hirsh. 1989. A neuron-specific enhancer of the *Drosophila* dopa decarboxylase gene. *Genes Dev.* 3:676-686.
13. Kadesch, T. R., P. Zervos, and D. Ruezinsky. 1986. Functional analysis of the murine IgH enhancer: evidence for negative control of cell-type specificity. *Nucleic Acids Res.* 14:8209-8221.
14. Konrad, K. D., and J. L. Marsh. 1987. Developmental expression and spatial distribution of dopa decarboxylase in *Drosophila*. *Dev. Biol.* 122:172-185.
15. Krasnow, M. A., E. E. Saffman, K. Kornfeld, and D. S. Hogness. 1989. Transcriptional activation and repression by *Ultrathorax* proteins in cultured *Drosophila* cells. *Cell* 57:1031-1043.
16. Kunkel, T. A., J. D. Roberts, and R. A. Zakour. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* 154:367.
17. Levine, M., and J. Manley. 1990. Transcriptional repression of eukaryotic promoters. *Cell* 59:405-408.
18. Livingstone, M. S., and B. L. Tempel. 1983. Genetic dissection of monoamine neurotransmitter synthesis in *Drosophila*. *Nature (London)* 303:67-70.
19. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- 19a. Mastick, G. S. Unpublished observations.
20. Pankratz, M. J., and H. Jackle. 1990. Making stripes in the *Drosophila* embryo. *Trends Genet.* 6:287-292.
21. Renkawitz, R. 1990. Transcriptional repression in eukaryotes. *Trends Genet.* 6:192-197.
22. Rubin, G. M., and A. C. Spradling. 1982. Genetic transformation of *Drosophila* with transposable element vectors. *Science* 218:348-353.
- 22a. Scholnick, S. B. Unpublished observations.
23. Scholnick, S. B., S. J. Bray, B. A. Morgan, C. A. McCormick, and J. Hirsh. 1986. CNS and hypoderm regulatory elements of the *Drosophila melanogaster* dopa decarboxylase gene. *Science*

- 234:998-1002.
24. Scholnick, S. B., P. A. Caruso, J. Klemencic, G. S. Mastick, C. Mauro, and M. Rotenberg. 1991. Mutations within the *Ddc* promoter alter its neuron-specific pattern of expression. *Dev. Biol.* **146**:423-437.
 25. Scholnick, S. B., B. A. Morgan, and J. Hirsh. 1983. The cloned dopa decarboxylase gene is developmentally regulated when integrated into the *Drosophila* genome. *Cell* **34**:37-45.
 26. Seth, A. 1986. A new method for linker ligation. *Resolutions* (FMC Bioproducts) **2**:1-3.
 27. Topol, J., D. M. Ruden, and C. S. Parker. 1985. Sequences required for *in vitro* transcriptional activation of a *Drosophila hsp70* gene. *Cell* **42**:527-537.
 28. Valles, A. M., and K. White. 1988. Serotonin-containing neurons in *Drosophila melanogaster*: distribution and development. *J. Comp. Neurol.* **268**:414-428.