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Lateral inhibition mediated by the *Drosophila* neurogenic gene Delta is enhanced by proneural proteins

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ABSTRACT **Cells in the neuroectoderm of *Drosophila* become either neural or epidermal progenitors. A critical threshold concentration of proneural gene products in a given cell causes it to develop as a neuroblast. The proteins encoded by the genes Delta (*Dl*) and Notch (*N*) act as the source and the receptor, respectively, of inhibitory signals sent by the neuroblast to neighboring cells that prevent these cells from also adopting the neural fate. We show here that proneural gene products activate transcription of Delta in the neuroectoderm by binding to specific sites in its promoter. This transcriptional activation enhances lateral inhibition and thus helps ensure that cells in the vicinity of prospective neuroblasts will themselves become epidermoblasts.**

Clusters of initially equivalent cells in the neuroectoderm of *Drosophila* have to decide between developing as neural or epidermal progenitors (1, 2). The fate of any given neuroectodermal cell to become either neuroblast or epidermoblast is determined by cellular interactions that involve a regulatory network containing products of the so-called proneural and neurogenic genes. The proneural genes promote neural development, and the neurogenic genes mediate signals between adjacent cells (3, 4). The proteins encoded by Notch (*N*) and Delta (*Dl*), two of the neurogenic genes, interact directly at the membranes of contiguous cells (5–7), passing a signal from the neuroblast to the neighboring cells that inhibits neural development (8–11). Delta protein is assumed to be the source (12, 13) and Notch protein is assumed to be the receptor (13–16) of this neural inhibitory signal. The relative signal strength is thought to determine whether, in a given cell, products of the proneural genes—for example, those of the achaete–scute gene complex (*ASC*) or of the Enhancer of split gene complex [*E(SPL)-C*]—functionally predominate. Predominance of ASC or E(SPL)-C products, all members of the basic helix–loop–helix family of transcriptional regulators (17–21), causes entry into the neural or epidermal pathway of development, respectively (3).

We show here that the basic helix–loop–helix proteins encoded by the *ASC* genes activate transcription of Delta within the neuroectoderm by binding to specific promoter sites. Activation of *Dl* enhances the neural inhibitory, epidermalizing signal sent by the neuroblast, as shown by minigenes carrying promoter fragments. Mutation of these binding sites reduces the amount of Delta transcripts in neuroectodermal cells and, as a consequence, decreases the efficacy of the "epidermalizing" signal sent by the neuroblast, thus allowing differentiation of excess neuroblasts.

MATERIALS AND METHODS

Strains and Preparation of Embryos. For experiments with minigenes, the amorphic mutation Dl^{F10} (22, 23) was used. Permanent strains were established with X- or second-chromosomal insertions of the minigenes, either in homozygosity or balanced, together with marker *439*, $Dl^{F10}/TM3$, Bd^S *lacZ*. The marker *439* is a *P–lacZ* element inserted in region 64A driven by a neural enhancer acting in all neuroblasts and sensory-organ mother cells (22, 24). This insertion was recombined into a chromosome carrying Dl^{F10} and balanced with *TM3*, Bd^S *lacZ*, which carries another *P-lacZ* element coupled to the 5′ region of the twist gene expressed at the blastoderm stage and thereafter (25). Dl^{F10} homozygotes can thus be unequivocally distinguished from heterozygous or *TM3*, Bd^S *lacZ* homozygous siblings at early embryogenic stages. Neural progenitors were visualized with an antibody against β-galactosidase, using a secondary antibody coupled to alkaline phosphatase; the embryos were also stained with an "anti-invected" antibody (secondary antibody coupled to horseradish peroxidase, brown reaction product, ref. 22) to label the metameric boundaries. Embryos were dissected and mounted flat to show the germ band in its entire extent. Whole-mount *in situ* hybridization was done on transgenic embryos carrying the construct *DlE1(-4.3)–lacZ* (24) and variants of it, with a 3-kb *Eco*RI digoxigenin-labeled fragment of pW-ATG-lacZ as probe (26). Cuticle preparations were made according to conventional techniques.

Construction of Plasmids for DNA-Binding Assays. For construction of expression vectors, the coding region of achaete (*ac*), beginning at position +24 upstream of the translation start site, was amplified by PCR, using the following primers: sense, 5′-AATCATGGATCCAGTTTTCAACGACGACGAGGAGTCA-3′; antisense, 5′-CGGTCAGGATCCCAGGTCGTCCTGCCAGAGTGATATATA-3′. The amplified product was cloned into the *Bam*HI site of the pET3a expression vector (27). lethal of scute expression plasmids were generated from a full-length cDNA, provided by J. Modolell (CBM, Madrid), by cloning a *Pvu* II site (+44)–*Eco*RV fragment into the filled-in *Bam*HI site of the pET3c expression vector. The construct for expression of daughterless protein was provided by Harald Vässin (Columbus, Ohio).

DNA-Binding Assays. Protein expression and purification was essentially as described in ref. 28. For DNA-binding studies, double-stranded oligonucleotides (Table 1) and appropriate restriction fragments (see Fig. 1, fragment I: *Eco*RI–*Eco*RV, 298 bp; fragment II: *Eco*RV–*Bgl* II, 302 bp; fragment III: *Bgl* II–*Hin*dIII, 310 bp) were labeled at both ends by filling-in with Klenow fragment in the presence of [α-^{32}P]dATP. Labeling, binding reactions, and footprint experiments were done as described (28).

***In Vitro* Mutagenesis.** For *in vitro* mutagenesis, the following *Dl* genomic fragments were cloned in Bluescript KS(+) (see Fig. 1): (*i*) *Eco*RI–*Hin*dIII fragment, (*ii*) *Eco*RV–*Pst* I fragment, (*iii*) *Pst* I–*Xba* I fragment, (*iv*) *Xba* I–*Xba* I. Mutations were introduced into the genomic fragments by

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Abbreviations: *ASC*, achaete–scute gene complex; *E(SPL)-C*, Enhancer of split gene complex; mg*XX*, minigene *XX*.

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Table 1. Binding of proneural proteins to specific sites in *Dl* promoter

Binding sites	Oligonucleotide (genomic) sequence	*da/da*	*ac/ac*	*l'sc/l'sc*	*da/l'sc*	*da/ac*
I*	CTGCATCTAACAACTGGCAAAGGA	Medium	–	–	–	–
II*	ATTAACTAGCCAGCTGTTGTTGTTGTG	Strong	–	–	++	++
III*	GATTGGAAAGCAGCTGTTTTCGAGCT	Strong	Weak	Weak	+++	+++
IV†	TTCGACACATCCACATGTACCTGCTGATGAGAGTCTG	Medium	–	–	+	+
V†	CTGTGGGAAGCGCACATGTTCGTCCTTCG	Medium	–	–	+	+
VI†‡	GTTCTTGTTACATTTGATCTAATATGG	Strong	–	–	–	–
VII†	GTTACATTTGCATCTGTGTGGGAAAA	Strong	–	–	+	+
VIII†	TCCCGGACCGCACCTGCCTTGCAAAA	Strong	–	–	–	+
IX	AACCGGTTTACAGGTGCTAACTTGGC	ND	ND	ND	+++	+++
X	TATTGCCATTACAGTTGGACATCTTT	ND	ND	ND	(+)	(+)
XI	ATGTGGCGTGTGCACGTGTAGCTTGCAA	ND	ND	ND	++	++
XIIII	ATTGCAAAACCATGTGCGACAAGTA	ND	ND	ND	+	+

Strong, medium, and weak refers to degree of protection in DNase I assays. –, No protection. *l'sc*, lethal of scute (*l(1)sc*). The E-boxes are underlined. In most cases, 1 or 2 nt flanking the E-boxes were also protected. For site IV, 18 consecutive nt (underlined and in boldface type) were protected. Number of pluses indicates relative binding affinity; +, very low affinity; –, no binding; ND, not determined. All oligonucleotides were synthesized in a Cyclon Plus (Millipore) DNA synthesizer.
*Fragment I, *Eco*RI–*Eco*RV.
†Fragment II, *Eco*RV–*Bgl* II.
‡Because boxes VI and VII are very near each other, oligonucleotide VI was deliberately changed to mutate E-box VII. E-box VI was not mutated in oligonucleotide VII because no binding of heterodimers to E-box VI was found.

using the oligonucleotide-directed *in vitro* mutagenesis system, version 2 (Amersham Buchler) and the following oligonucleotides with mutated sense-strand sequences (boldface and underlined). (*i*) TGGCAAAGGATTAACTAGC**GATATC**TTGTTGTTGTGCGAAATCC (box II); (*ii*) TACACGAAAAGATTGGAAA**TAATCC**TTTTCGAGCTCCCTGGGAA (box III); (*iii*) AGGACACATCCACATGTAC**ATCATTAAT**AGAGTCTGTGGGAAGCGCAC (box IV); (*iv*) GCCGACAGCCAACCGGTTTA**GATATC**CTAACTTGGCTTTAAAAGTCGG (box IX); (*v*) TTGTTTAAATATTGCCATTA**GATATC**GACATCTTTATAGTGAATT (box X); (*vi*) GCAACAAAAAATGTGGCGTGTA**GATATC**TAGCTTGCAACAACGAC (box XI). The mutated fragments were used for footprint analysis, as well as to replace the corresponding fragments in the *Dl–lacZ* promoter construct *DlE1(-4.3)–lacZ* (24) and for germ-line transformation, as described (24). The recipient strain for germ-line transformation was w^{1118}.

Minigenes. The minigenes used were minigene 39 (mg39) and two mutated variants of it, mg24, which has two mutant boxes (III and IV), and mg48, which has six mutated boxes (II, III, IV, IX, X, and XI). mg39 (described in ref. 24) consists of (*i*) a 9.2-kb *Eco*RI–*Xba* I fragment (from −9.2 kb to −369 bp) of the 5′ genomic DNA, (*ii*) a full-length cDNA clone, and (*iii*) the genomic trailer, cloned in vector PW5N (a modification of the CaSper vector with the polylinker of PW8, from *Eco*RI to *Pst* I). The cDNA clone was a fusion between a *Bgl* II fragment from clone cG6.1 (from +234 to +1661) and a *Bgl* II–*Spe* I fragment from clone c3.2 (23). The genomic 3′ region used was a 1.6-kb *Spe* I fragment fused to the *Spe* I site (+4947 bp) of the cDNA.

RESULTS

***ASC* Regulates *Dl* Transcription.** Using *Dl* promoter–*lacZ* reporter fusions, we previously showed that a genomic fragment containing 4.3 kb of sequence immediately upstream of the transcription start site, the untranslated leader, the first exon, and the first intron (referred to as the 4.3-kb promoter fragment, Fig. 1) generates a striking regular neuroectodermal expression pattern consisting of two clusters of cells per hemisegment (ref. 24; Fig. 2*A*). The pattern, which is visible immediately preceding segregation of the S1 neuroblasts (2, 3), is striking for two reasons: (*i*) it is not distinguishable in the distribution of endogenous Delta RNA and (*ii*) it is reminiscent of the transcription pattern of the *ASC* genes at this stage. Indeed, the neuroectodermal clusters are not found in embryos homozygous for $Df(1)sc^{19}$, which lack three of the four *ASC* genes—namely, achaete, scute, and lethal of scute. Each one of these genes controls expression of *Dl–lacZ* in a particular domain of the clusters, as shown by studying the promoter–*lacZ* fragment in the background of deletions affecting individual genes. In addition, the transcription pattern of the endogenous gene, which is normally expressed in all cells of the neuroectoderm, is also changed in mutants lacking *ASC*, exhibiting conspicuous gaps that correlate with the position of the clusters of Delta–lacZ RNA (24). All these data thus suggest that the proteins encoded by achaete, scute, and lethal of scute genes are directly or indirectly responsible for activation of transcription of *Dl* in the neuroectodermal cell clusters.

Proneural Proteins Bind to Specific Sites in the *Dl* Promoter. To substantiate further the relationships between proneural genes and *Dl*, we used bacterially expressed achaete, lethal

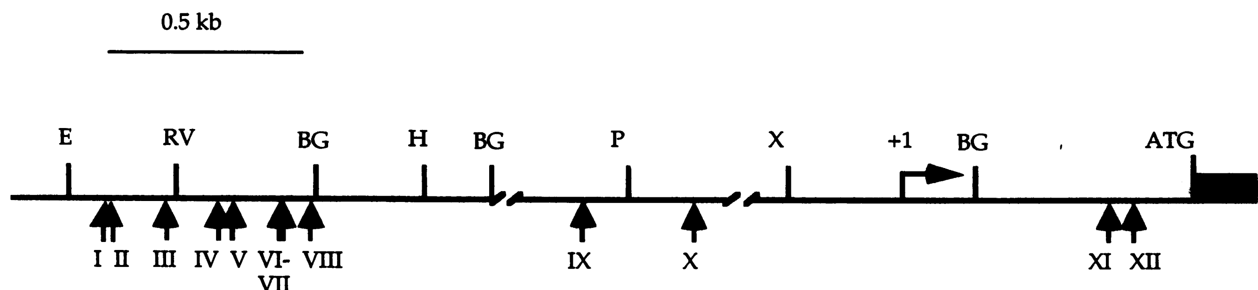

FIG. 1. Positions of binding sites for proneural proteins (indicated by Roman numerals) on the 4.3-kb promoter fragment. E, *Eco*RI; RV, *Eco*RV; BG, *Bgl* II; H, *Hin*dIII; P, *Pst* I; X, *Xba* I. Start site and direction of Delta transcription are indicated by the bent arrow.

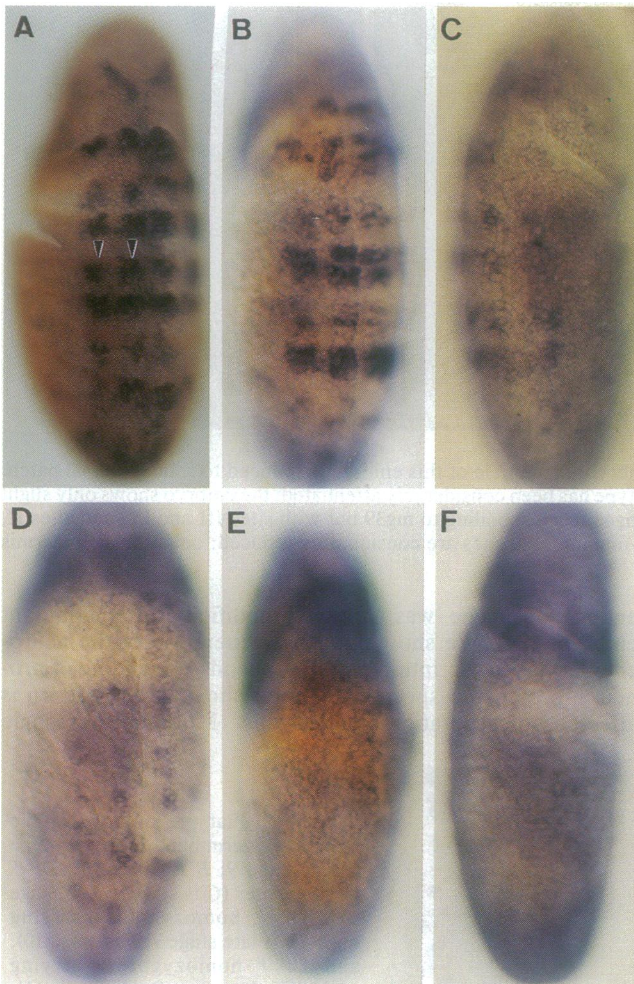

FIG. 2. *In situ* hybridizations with digoxigenin-labeled *lacZ* probes on transgenic stage-8 embryos. (*A*) Embryo carries the *DlE2(−4.3)* construct (24), which consists of the *Dl* wild-type 4.3-kb promoter fragment and *lacZ* reporter gene. Notice the neuroectodermal clusters of expression (arrowheads). In *B–F* the embryo carries the same transgene with different boxes mutated. (*B*) Embryo carries construct M2, with a mutated IV box. (*C*) Embryo carries construct M1 with box III mutated. (*D*) Embryo carries construct M5 with mutated boxes IX, X, and XI. (*E*) Embryo carries construct M3 with boxes III and IV mutated. (*F*) Construct M6 carried mutations in boxes II, III, IV, IX, X, and XI. Embryos carrying construct M4, with boxes II, III, and IV mutated (data not shown), exhibit the same expression as those shown in *E* and *F*. (Anterior is at top in all photographs; ×125.)

of scute, and daughterless proteins for electrophoretic mobility shift and DNase I protection assays. daughterless gene (*da*) is ubiquitously expressed and encodes a basic helix–loop–helix protein (29, 30) capable of forming heterodimers with proteins encoded by *ASC* that bind to DNA with high affinity (31). We find high-affinity binding of daughterless/lethal of scute and daughterless/achaete heterodimers, as well as low-affinity binding of the corresponding homodimers, to a number of E-boxes (CANNTG) and other sites within the 4.3-kb promoter fragment (Fig. 1, Table 1). The highest binding affinity for both heterodimers was found at sites III and IX; sites II and XI show slightly lower affinities, and all the remaining sites show much lower affinities. One binding site (IV) consisted of 18 nt (CACATGTACCTGCTTATG) that were protected in DNase I assays, including one conventional and two modified E-boxes; all the other binding sites detected were conventional E-boxes, although 1 or 2 nt flanking most E-boxes were also protected in DNase I protection assays.

Proneural Proteins Activate *Dl* Transcription. Oligonucleotide-directed mutagenesis was used to modify binding sites II, III, IV, IX, X, and XI in the *Dl* 4.3-kb promoter fragment. Six constructs with different mutated boxes (construct M1 with box III mutated; construct M2 with a mutated box IV; construct M3 with boxes III and IV mutated; construct M4 with boxes II, III, and IV mutated; construct M5 with mutated boxes IX, X, and XI; and construct M6 with mutations in boxes II, III, IV, IX, X, and XI; Figs. 1 and 2) were made. Footprinting analysis using mixtures of daughterless, lethal of scute and/or achaete proteins gave negative results for binding to the fragment carrying the mutated sites II, III, and IV. Because after mutagenesis sites IX, X, and XI have the same sequence as mutated site II, we assume that binding of proneural proteins to these sites is also abolished. It is worth mentioning that mutation of site IV (CACATGTACCTGCTTATG) to CACATGTACATCATTAAT abolished its binding abilities, although the resulting sequence still included a conventional (CACATG) E-box, indicating that this E-box is not sufficient for DNA binding.

To test whether binding of ASC and daughterless proteins is responsible for generation of the neuroectodermal clusters of Delta–lacZ transcripts, the six mutated 4.3-kb promoter fragments were cloned in front of the *lacZ* reporter gene and used for germ-line transformation. To make sure that all embryos carried the mutated transgene, only homozygous or hemizygous viable insertions of each construct were studied; between two and six independent insertions, depending on the construct, were used. Staining conditions were the same in all cases. The pattern of Delta–lacZ transcription was altered in embryos carrying any of the mutated 4.3-kb promoter–*lacZ* fragments (Fig. 2), in that the amount of RNA detected by *in situ* hybridization using a digoxigenin-labeled *lacZ* probe was reduced in all cases. Mutagenesis of box III produces far less detectable Delta–lacZ RNA than does alteration of site IV (Fig. 2 *B* and *C*), in agreement with the higher binding affinity of box III. Mutation of boxes III and IV together leads to virtually complete loss of transcriptional activity of the Delta–lacZ constructs within the neuroectoderm (Fig. 2 *D–F*). The present material did not allow us to conclude whether mutation of particular binding sites reduces Delta–lacZ transcripts in defined regions of the neuroectoderm.

Epidermal-Differentiating Activity Depends on Transcriptional Activation by Proneural Proteins. A minigene containing the entire wild-type coding region of *Dl* driven by a −9.2-kb fragment of wild-type 5′ genomic DNA (mg39; ref. 24) can almost completely restore *Dl* function during early neurogenesis and, consequently, rescues the neurogenic phenotype of embryos homozygous for the amorphic mutation Dl^{F10} (refs. 22–24; see Figs. 3 and 4). Accordingly, we used variants of this minigene to test the function of the mutated promoter fragments. Positional effects were controlled by studying three to four independent insertions of each minigene. The ability of mg39 to rescue the neurogenic phenotype of the loss-of-function of *Dl* was considerably reduced by mutation of binding sites for basic helix–loop–helix proneural proteins. Transgenic Dl^{F10} embryos carrying either mg24, which corresponds to mg39 with two mutant sites (III and IV), or mg48, which has six mutated sites (II, III, IV, IX, X, and XI) in the same fragment, exhibited a strongly neuralized neuroectoderm, which attained an intermediate phenotypic degree (Figs. 3 *D* and *E* and 4 *G* and *H*).

DISCUSSION

The proteins encoded by the group of proneural genes, to which *ASC* belongs, are known to render neuroectodermal cells competent for a neural fate (3, 4). Our data show that proteins encoded by *ASC* genes and *da* also directly activate Delta transcription by binding to specific sites in the *Dl*

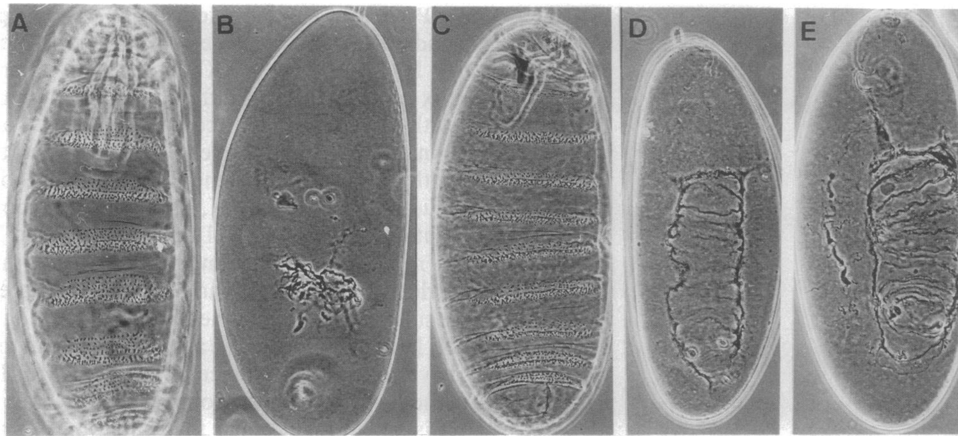

FIG. 3. Cuticle preparations. (*A*) Wild type. (*B*) Homozygous Dl^{Fx10} embryo; the cuticle of this embryo is reduced to a small dorsal patch. (*C*) Homozygous Dl^{Fx10} embryo transgenic for mg39 (24). The mutant phenotype has been considerably attenuated; the embryo shows only slight head defects. (*D* and *E*) Homozygous Dl^{Fx10} embryos carrying mg24 and mg48, corresponding to mg39 but with sites III and IV, and sites II, III, IV, IX, X, and XI, respectively, mutated. The rescuing abilities of the mutant minigenes are considerably reduced: only dorsal epidermis develops. (×125.)

promoter. The clusters of Delta–lacZ RNA-containing neuroectodermal cells expressed by the 4.3-kb promoter fragment had been reported absent in embryos lacking all *ASC* genes and strongly modified in those lacking individual *ASC* genes; also the neuroectodermal transcription pattern of the endogenous gene was strongly modified in such mutant embryos (24). We show here that generation of the neuroectodermal transcription pattern depends on binding of proneural proteins to specific promoter sites, as this pattern is affected upon mutation of these sites. Mutation of a single

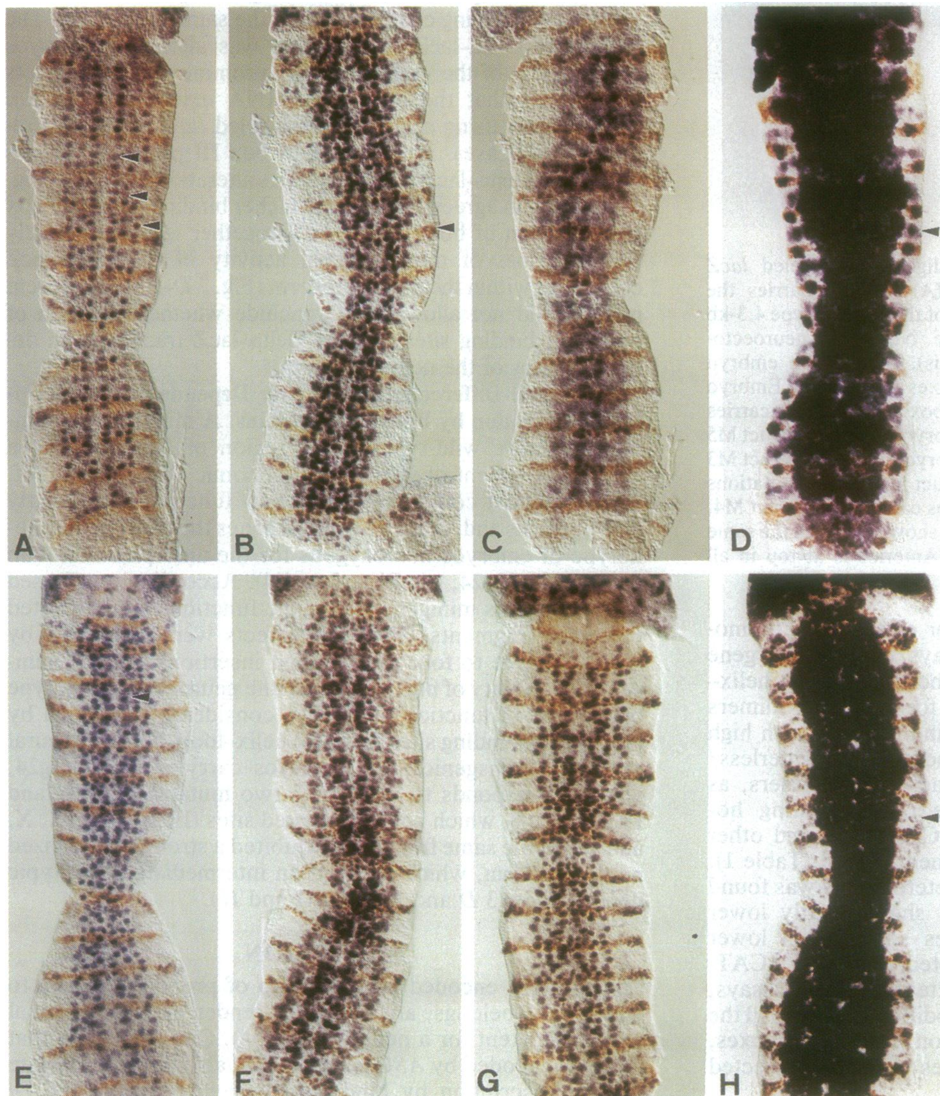

FIG. 4. Flat preparations of the embryonic germ band from wild-type (*A*) is stage 9; *B* is stage 10), Dl^{F10} homozygotes (*C* is stage 9; *D* is stage 11), Dl^{F10} homozygotes carrying mg39 (*E* is late stage 9; *F* is stage 10), and Dl^{F10} homozygotes carrying mg24 (*G* is late stage 9; *H* is stage 11). Neuroblasts (arrowheads in *A*) and sensory-organ mother cells (arrowhead in *B*) were visualized with an antibody against β-galactosidase and a secondary antibody coupled to alkaline phosphatase (violet reaction product); β-galactosidase activity in these cells was driven by a neural enhancer. All embryos were also stained with secondary antibody coupled to horseradish peroxidase, brown reaction product (22). The number of neuroblasts increases between stages 9 and 11 in the wild type (*A–B*), and the neuralization of the neuroectoderm and proneural clusters that give rise to sensory organs (arrowhead in *D*) follows a similar course in the mutants (*C–D*). The mutant phenotype is considerably weakened by a copy of mg39. The pattern of neuroblasts in *E* resembles that of wild type (*A* and *B*). However, more neuroblasts than normal are detected in older mutant embryos (*F*), which may account for the slight cuticular defects. Initial stages of neurogenesis are essentially normal in Dl^{F10} homozygotes that carry mg24, corresponding to mg39 but with boxes III and IV mutated (*G*). However, Delta activity supplied by mg24 is insufficient at later stages, and the final neuralization seen in these embryos is considerable (*H*). (Anterior is at top in all photographs; ×125.)

high-affinity binding site, such as site III, leads to strong reduction of the Delta–lacZ transcripts in the neuroectodermal cell clusters; mutation of site III along with mutation of the low-affinity site IV abolishes transcriptional activity of the Delta–lacZ construct in the neuroectoderm. Furthermore, our results show that the influence toward an epidermal fate of the Delta protein on neighboring cells depends on activation of Delta transcription mediated by binding of the proneural proteins: mg39 loses its ability to rescue the neurogenic phenotype of Dl^{F10}-homozygous embryos after mutation of two or more of the binding sites for proneural proteins.

These findings thus suggest the following mechanism for segregation of the neuroblast from an initially equivalent group of cells. It is assumed that neuroblasts, like sensory-organ mother cells (32, 33), are singled out from proneural clusters because they express proneural proteins at levels exceeding a critical threshold. Note that proneural gene products activate transcription of their own genes (34, 35). Activation of Delta transcription by proneural proteins enhances the ability of the prospective neuroblast to send an inhibitory signal to neighboring cells. Within an array of mutually interacting cells, fluctuations in proneural proteins could increase inhibitory strength by one of the cells, thus lowering the probability that any surrounding cells adopt the neural fate. This differentiation, in turn, reduces their efficacy as sources of lateral inhibition and eventually leads to a reinforcement of the neural-fate decision of the neuroblast.

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