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CNS midline enhancers of the *Drosophila slit* and *Toll* genes

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The Drosophila CNS midline cells comprise a small, well-characterized group of neurons and glia in which the transcriptional control of CNS development can be studied. Using germ-line transformation of *lacZ* fusion constructs, we have dissected putative regulatory regions of the *slit* and *Toll* genes to identify CNS midline-restricted transcriptional enhancers. This analysis has uncovered DNA regions able to drive *lacZ* expression in most tissues in which embryonic *slit* and *Toll* are expressed, including three separable CNS midline-conferring regions: one in the *Toll* gene which is expressed early in all of the CNS midline precursors, and two in the *slit* gene which are expressed later in the midline glia (MG).

***slit*; *Toll*; Gene regulation; *Drosophila*; CNS midline development**

Introduction

Central nervous system (CNS) development results in the stereotypic arrangement of diverse cell types into a highly ordered structure. One major issue in CNS development concerns how cells acquire their unique identities and are able to express different sets of genes. The complexity of the CNS makes this a difficult question to address. However, one attractive system involves studying the development and control of gene expression in a small, discrete set of cells that lie along the midline of the *Drosophila* embryonic CNS (Nambu et al., 1992). The CNS midline cells consist of about 30 neurons and glia per segment which play an important role in the overall development of the CNS. Furthermore, their cell lineage is well described, and a large number of cytological markers exist which allow identification of the different cell types during development (Klämbt et al., 1991; Crews et al., 1992). We are interested in understanding control of gene expression throughout the development of the CNS midline precursor cells into their neuronal and glial progeny. Thus, it becomes possible to address issues such as what controls whether a cell becomes midline vs. lateral CNS, how neurons and glia are distinguished, and what controls neural identity.

Several transcription factors have been identified which function in CNS midline development or are expressed prominently in these cells. The *single-minded* (*sim*) gene is expressed at the beginning of CNS midline development, and genetic studies have shown that it is required for the proper development of this lineage (Thomas et al., 1988; Nambu et al., 1991). Function of the *sim* gene is also required for transcription of most or all CNS midline-expressed genes (Nambu et al., 1990). The *sim* gene encodes a protein of the helix-loop-helix class of DNA binding transcription factors (Nambu et al., 1991), and is expressed throughout CNS midline development. It is likely to directly regulate genes whose CNS midline expression is activated early in neurogenesis, although later roles are suggested by its continued expression in midline glia and weakly in a subset of ventral neurons. Several additional transcription factors are expressed later in CNS midline cells, often in subsets of midline cells, including the *Cut* (Blochlinger et al., 1990) and *ocelliless* (Finkelstein et al., 1990) homeobox proteins, the *Cf1A* POU-box protein (Johnson and Hirsh, 1990), and the *Krüppel* zinc-finger protein (Hoch et al., 1990). These proteins may control the expression of genes involved in cell-specific functions of the midline cells. In order to understand this process, it is important to identify genomic DNA sequences which possess CNS midline enhancer activity.

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The purpose of this paper is to identify transcriptional control elements of two previously characterized genes which have prominent CNS midline expression. The *Toll* gene, which encodes a transmembrane receptor, is expressed in all of the CNS midline precursor cells about 15–30 min after Sim protein is first detected (Fig. 1; Hashimoto et al., 1991). The *slit* gene is also expressed in CNS midline precursor cells shortly after the appearance of Sim protein and continues expression in the midline glia later in development (Fig. 1; Rothberg et al., 1988, 1990). In this paper, using P-element mediated germline transformation of *lacZ* fusion genes, we identify regions of DNA within the *slit* and *Toll* genes which drive *lacZ* expression in CNS midline cells, as well as other cell types that express *slit* and *Toll* during embryogenesis. These experiments provide the foundation for biochemical and mutational analysis of how regional CNS midline and cell type-specific gene expression is controlled within the developing nervous system.

Results

Mapping and expression of maternal and zygotic tissue-specific Toll regulatory regions

During oogenesis, maternal *Toll* mRNA is deposited within the growing oocyte by germ-cell derived nurse cells. *Toll* immunoreactivity appears at the cellu-

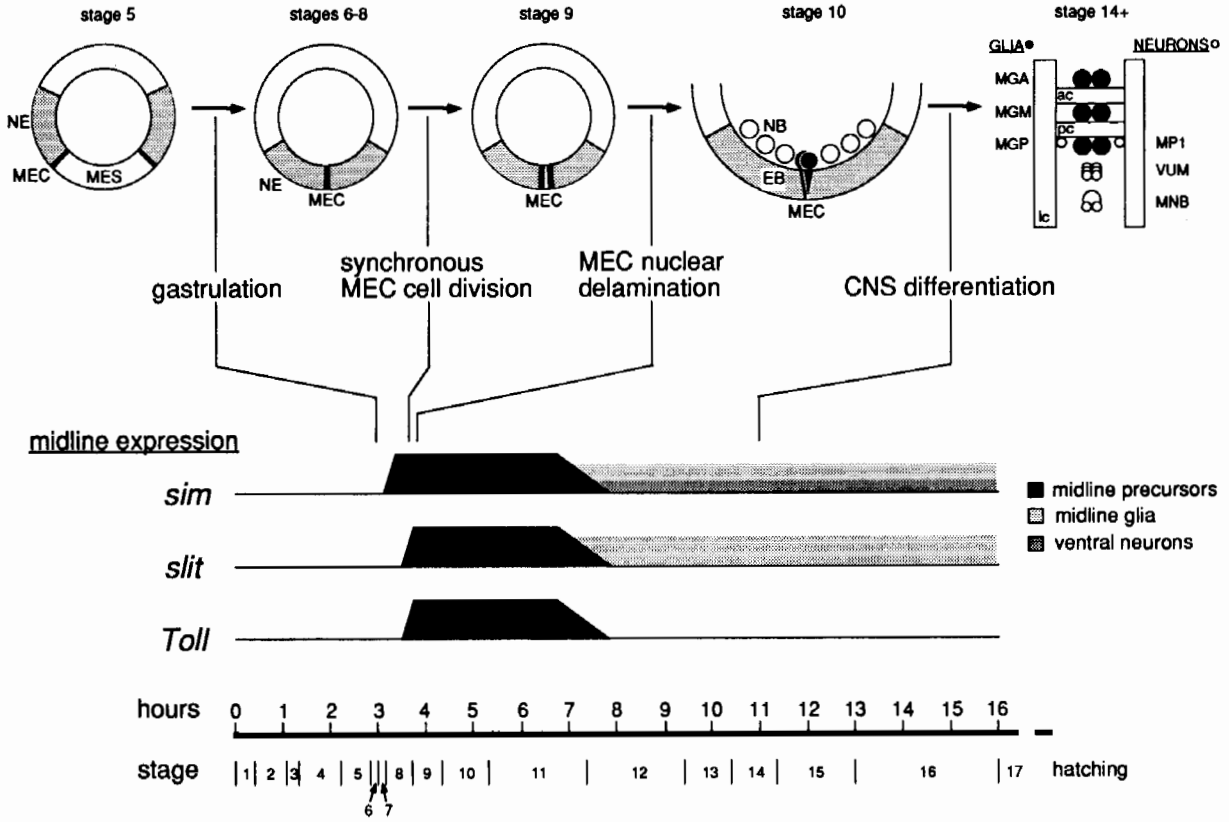


Fig. 1. Summary of CNS midline development and expression of *single-minded*, *slit* and *Toll*. Schematic cross-sections of embryos at selected stages, and CNS midline expression of *sim*, *slit*, and *Toll* are depicted above a developmental timeline of *Drosophila* embryogenesis (stages according to Campos-Ortega and Hartenstein, 1985). (Stage 5) Cellular blastoderm. The mesectodermal cells (MEC, black stripe), lying between the presumptive mesoderm (MES) and lateral neurogenic ectoderm (NE, shade), give rise to the CNS midline cells. (Stages 6–8) Gastrulation brings the MES into the interior of the embryo and the two rows of MEC to the ventral midline of the ectoderm. (Stage 9) The MEC cells undergo a synchronous cell division (Foe, 1989) which is complete by the beginning of stage 9. (Stage 10) The ventral half of the germ-band extended embryo is depicted. The MEC cell nuclei move into neuroblast (NB) cell layer (white circles) but maintain a cytoplasmic connection to the epidermoblasts (EB, light shade). (Stage 14) The mature larval CNS midline cells consist of midline glia (MG) (black circles) and neurons (white circles). Schematized here is a ventral view of one segment of midline neurons and glia relative to the axon scaffold. The axon scaffold consists of anterior and posterior commissures (ac and pc) and paired longitudinal connectives (lc). Three pairs of MG, the anterior, median, and posterior (MGA, MGM, and MGP, respectively), ensheath the ac and pc. Identified neurons include the MP1 neurons, ventral unpaired median (VUM) cells, and the median neuroblast and its progeny (MNB). (Midline expression) Sim protein first appears in CNS midline nuclei at the beginning of stage 8, while *slit* and *Toll* CNS midline expression starts approximately 15–30 min later during early stage 9. *Toll* expression fades in the CNS midline during stage 12 (Hashimoto et al., 1991), while *sim* and *slit* continue expression in the MG, with *sim* weakly staining some ventral neurons (Thomas et al., 1988; Rothberg et al., 1989 and 1990). CNS midline expression of *slit* and *Toll* is absent in *sim*- embryos (Nambu et al., 1990).

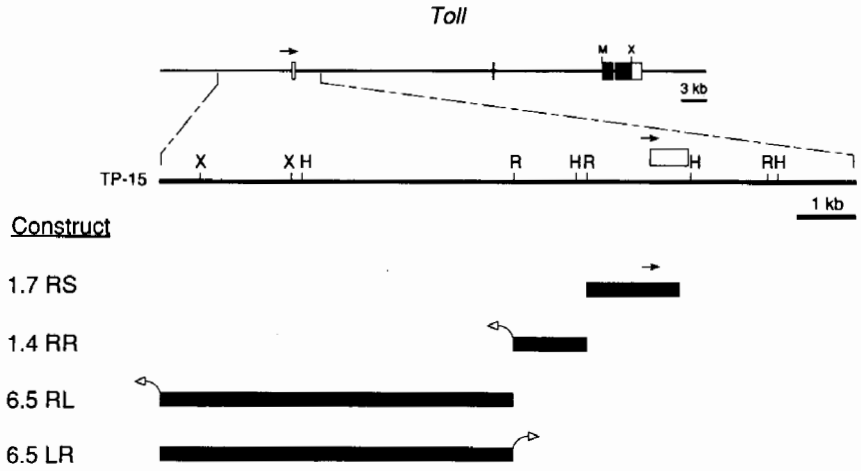


Fig. 2. Genomic structure of *Toll* gene and primary *Toll-lacZ* constructs. The *Toll* gene is comprised of 4 exons which span approximately 44 kb as shown in the schematic at the top of the figure. Each exon is denoted by a box, with coding sequences filled and non-coding sequences unfilled. Start and stop translational codons are marked with an M and X, respectively. The start of transcription is denoted by a rightward arrow preceding exon 1. The region surrounding exon 1 is contained within genomic phage clone TP15, shown expanded below the gene map. The regions of this phage clone used to make transformation constructs are indicated below phage map. Construct nomenclature is generally in the format 'x YZ', where x is the length of the fragment, and Y and Z are the flanking restriction sites. Constructs which use a weak heterologous promoter fused to *lacZ*, and their orientation, are designated by the curved arrow, while the construct which uses the endogenous *Toll* promoter driving *AUG-lacZ* (1.7 RS) is designated by a straight arrow above the fragment. Restriction sites: X = *Xho*I; R = *Eco*RI; H = *Hin*dIII.

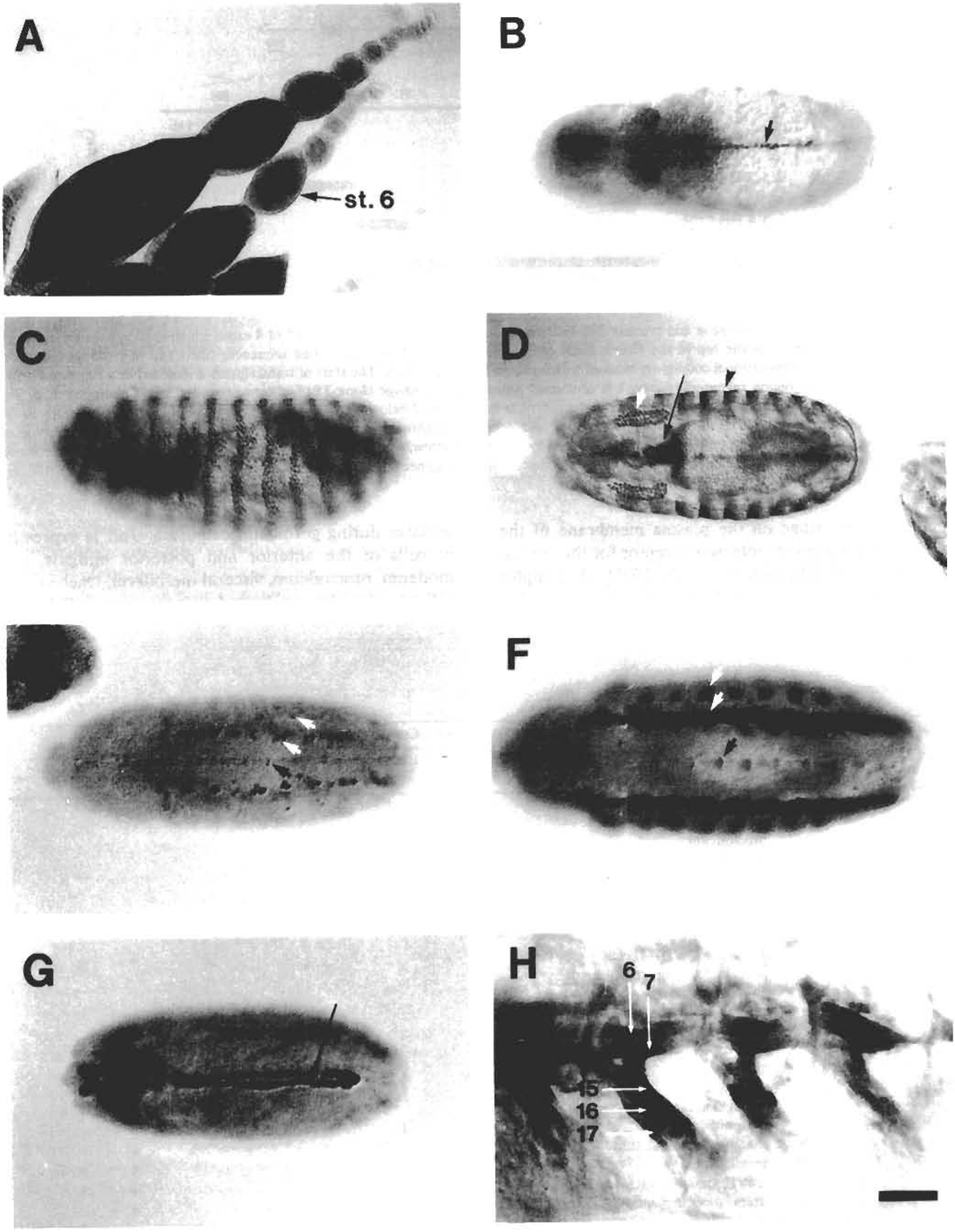
lar blastoderm stage on the plasma membrane of the embryo, supporting its role as a receptor for the ventral polarity signal (Hashimoto et al., 1991). A complex spatial and temporal pattern of zygotic *Toll* expression initiates during germband extension: *Toll* is expressed in cells of the anterior and posterior midguts, stomodeum, proctodeum, visceral mesoderm, tracheal and salivary placodes, epidermal stripes, and in CNS mid-

TABLE 1

Toll-lacZ expression summary

Stage	Tissue/structure	Construct		
		1.7 RS	1.4 RR	6.5 RL 6.5 LR
6 (oogenesis)	nurse cells	+		
5	cellular blastoderm	+		
8	proctodeum		+	
	stomodeum		+	
9	CNS midline precursors		+	
	anterior/posterior midgut			
10	epidermal stripes	+	+	
	salivary gland placodes		+	
	dorsal median (DM) cells			+
11	lateral sensory organ precursor	+		
	muscle precursors		+	
	cardioblasts			+
13	proventriculus		+	
	pharynx	+	+	
	hindgut		+	
	esophagus		+	
	malpighian tubules		+	
	epidermis	+1	+2	
	pharyngeal musculature			+
14–17	dorsal vessel			+
	ventral somatic muscles 6, 7, 15, 16, 17			+
	lateral somatic muscles 21, 22, 23			+

Key: +, initiates β-galactosidase expression in tissue or structure at this stage; 1, stains anterior margin of segmental groove; 2, stains posterior margin of segmental groove.


A
st. 6
B
C
D
F
G
H
6
7
15
16
17

line precursors (Gertulla et al., 1988; Hashimoto et al., 1991). Expression continues in many of these tissues throughout the rest of embryogenesis, with prominent expression in the dorsal vessel, a subset of ventral-lateral somatic muscles (Nose et al., 1992), epidermal stripes, and throughout the gut and Malpighian tubules. The function of zygotic *Toll* expression is unclear, as zygotic *Toll* mutants die as larvae without apparent anatomic defects (Gertulla et al., 1988).

The four exons comprising the *Toll* locus span approximately 44 kb, with introns one and two spanning 25 and 14 kb, respectively (Hudson, 1989). In order to identify regulatory regions that control *Toll* transcription, we transformed *lacZ* fusion genes of the region immediately 5′ of *Toll* exon 1. A summary of the *Toll* genomic map, phage clone TP15 which encompasses the region around *Toll* exon 1, and initial constructs made are illustrated in Fig. 2. One construct, 1.7 RS, was designed to use the endogenous *Toll* promoter to drive *AUG-lacZ*, while the remaining constructs used genomic fragments fused to weak heterologous promoters driving *lacZ*. A summary of the β-galactosidase expression pattern of each construct is shown in Table 1.

Construct 1.7 RS contains most of exon 1 and contains approximately 1.0 kb upstream of the start of transcripton (Hudson, 1989). β-Galactosidase expression is first observed during stage 6 of oogenesis (Fig. 3A; stages according to King, 1970), accumulating in the nurse cells just prior to extrusion of nurse cell cytoplasm into the oocyte. Expression of this construct remains in the blastoderm, and weakly in various tissues throughout embryogenesis, including epidermal stripes and the pharynx.

Construct 1.4 RR contains 1.4 kb immediately upstream of the 1.7 kb fragment used to make 1.7 RS, and was fused to a weak, heterologous (P-element) promoter in enhancer tester vector CPLZ (see Materials and Methods). This construct reveals patterns of *lacZ* expression which strongly mimic *Toll* expression in many tissues. During germband extension, this expression includes the anterior and posterior midguts, proctodeum, stomodeum, CNS midline precursors, salivary gland placodes, and epidermal stripes (Fig 3B). Later expression of this construct is prominent throughout the gut and Malpighian tubules, in the salivary gland, and in the epidermis just posterior to the segmental grooves (Fig. 3C,D).

Constructs 6.5 RL and 6.5 LR contain additional DNA upstream of 1.4 RR fused to a weak heterologous (*hsp70*) promoter in enhancer tester vector HZ50PL (Hiromi and Gehring, 1987) in both transcriptional orientations. Insertions of both constructs show identical patterns of expression. Beginning at stage 10 of germ band extension, two cells per segment stain along the ventral midline. These cells stain throughout germband retraction (Fig. 3E) and are identified as the midline muscle pioneer cells, or dorsal median (DM) cells (Fig. 3F), previously identified by staining with neuroglian (Bieber et al., 1989) and basement membrane components laminin (Montell and Goodman, 1989) and glutactin (Olson et al., 1990). The paired DM cells extend lateral processes which span the dorsal aspect of the ventral nerve cord to terminate at the apodemes or muscle attachment sites. Constructs 6.5 RL and 6.5 LR also show *lacZ* expression during stage 11, in four segmentally repeated clusters of mesodermal cells that lie in two parallel rows on either side of the ventral midline (Fig. 3E,F). These cells likely represent a subset of muscle precursor cells, as they eventually form ventral-lateral somatic muscles 6, 7, 15, 16, and 17, as shown in Fig. 3H. (Muscle nomenclature is according to Crossley, 1978) Muscles 21, 22, and 23 also stain to a lesser extent (data not shown). This pattern of muscle expression in the ventral muscle group is similar to the pattern of *Toll* muscle expression using *Toll* antisera, except that the ventral-most oblique muscle (no. 17), which does not stain with *Toll*

Fig. 3. *Toll-lacZ* expression patterns during oogenesis and embryogenesis. Shown are the β-galactosidase staining patterns of *Toll-lacZ* constructs (A) 1.7 RS, (B–D) 1.4 RR, (E–H) 6.5 LR or 6.5 RL (both stain identically). All panels show expression in whole-mount preparations as detected by either either X-gal histochemistry (A) or anti-β-galactosidase monoclonal antibody immunocytochemistry (B–H). Anterior is to the left in all embryos, and panels C and H have dorsal facing the top of the page. (A) X-gal stain of ovaries of 1.7 RS transformant flies. Staining initiates in stage 6 of oogenesis, stages 1 through 10b depicted in the upper ovariole, with development proceeding from upper-right to lower-left (stages according to King, 1970). Staining is found in germ-cell derived nurse cells beginning at stage 6 (arrow) and accumulates within the oocyte throughout oogenesis due to extrusion of nurse-cell cytoplasm into the oocyte. (B–D) Construct 1.4 RR. (B) Ventral view of a stage 11 embryo. Note staining in CNS midline precursors (black arrow) and paired salivary gland placodes (white arrow) in segment T2. (C) Parasaggital view of a stage 16 embryo showing epidermal stripes, salivary gland, and hindgut. (D) Mid-ventral view of a stage 16 embryo with salivary gland (white arrow), proventriculus (black arrow), and epidermal staining posterior to each segmental groove (arrowhead). (E–H) Constructs 6.5 RL and 6.5 LR. (E) Ventral view of a stage 12 embryo with weak midline β-galactosidase staining in two cells/segment. (F) Stage 14 embryo with the stained pair of midline cells lying (DM cells; see text) on the dorsal surface of the CNS extending processes to the muscle attachment sites along the body wall. Subsets of muscle precursor cells also express β-galactosidase at stage 12 (E). There are four segmentally repeated cell clusters (two on each side of the midline) each consisting of one to two cells (white arrows). These precursor cells give rise to β-galactosidase-expressing ventral and lateral muscles (F, white arrows). The ventral-most muscles that stain can be seen in (H) at high power: 6, 7 (ventral internal longitudinal), 15, 16, 17 (ventral external oblique). (G) Dorsal view of a stage 16 embryo with β-galactosidase expression in the cardioblasts (black arrow) and pharyngeal musculature (arrowhead). Scale bar: (A) 125 mm; (B–G) 64 mm; (H) 20 mm.

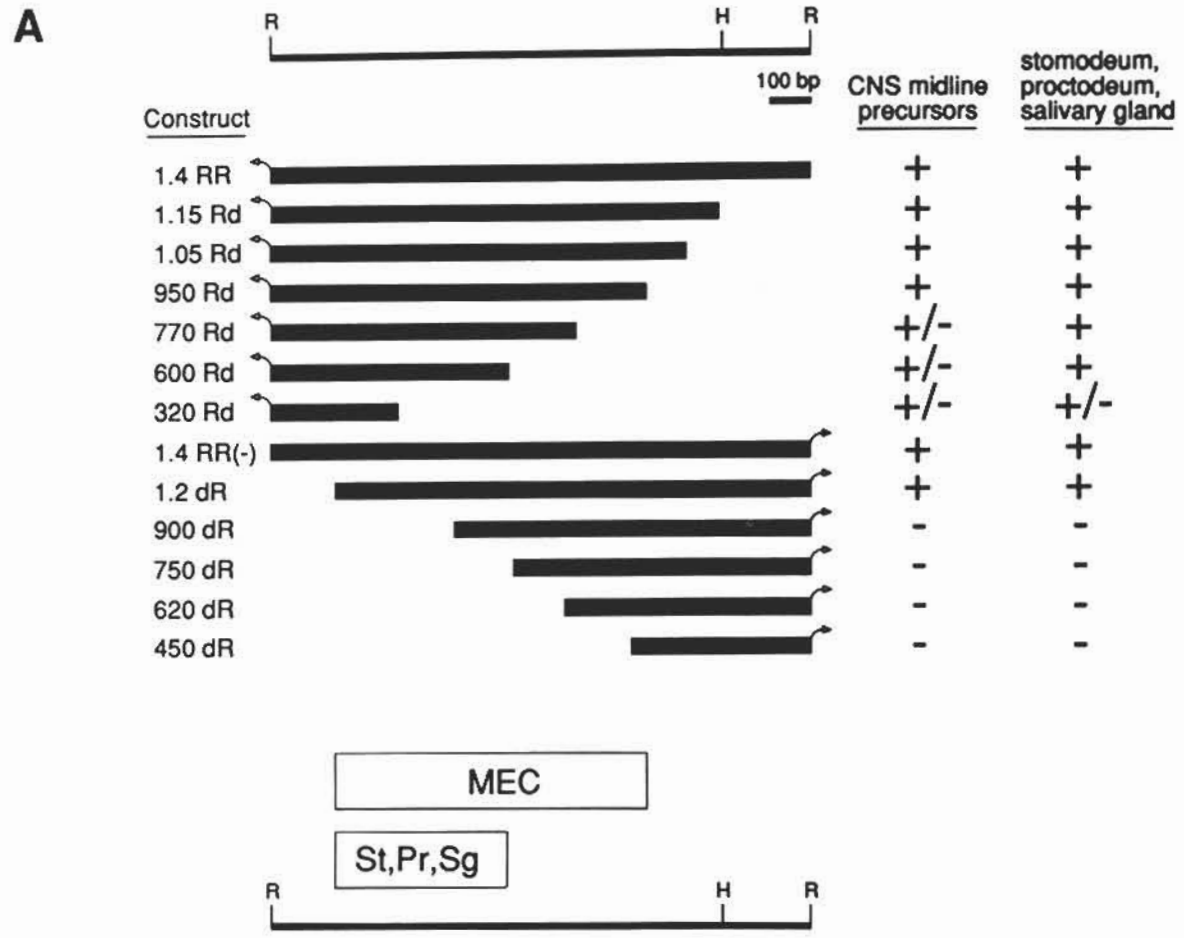


Fig. 4. Deletions of *Toll* fragment 1.4 RR and the CNS midline regulatory region. (A) Summary of 1.4 RR fragment deletion constructs and tissue specific staining. Deletion fragments and their transcriptional orientation relative to a weak, heterologous promoter-*lacZ* are depicted below expanded map of fragment 1.4RR. CNS midline precursor staining is present at high levels in stage 9–10 embryos in constructs designated with a '+'; it is expressed weakly beginning late in stage 10 in constructs with a '+/−'; and is absent in constructs marked with a '−'. Expression in the stomodeum, proctodeum, and salivary gland placode is present in constructs with a '+', weakly in '+/−'; and absent in '−'. The boundaries of these regions are summarized by the labelled boxes below the constructs: MEC = mesectoderm/midline precursors; St, Pr, Sg = stomodeum, proctodeum, and salivary gland. Restriction sites: R = *Eco*RI; H = *Hin*dIII. (B) Sagittal view of construct 1.4 RR in stage 11 embryo. Note staining in the stomodeum and proctodeum, midline precursors (black arrow), and slightly out of focus salivary placode (arrowhead). Deletion constructs 1.15 Rd, 1.05 Rd, 950 Rd, 1.4 RR(−), and 1.2 dR all stain identically to 1.4 RR. (C) Saggital view of construct 600 Rd in stage 11 embryo. Note specific absence of staining in the MEC while staining remains in the stomodeum, proctodeum, and salivary placode (arrowhead). Construct 770 Rd stains identically to 600 Rd, while 320 Rd stains the same tissues much more weakly. Scale bar: (B,C) 64 mm.

antisera, stains with these *lacZ* lines (Nose et al., 1992). These constructs also stain the pharyngeal musculature and the cardioblasts, which, during germband retraction, migrate dorsally to fuse along the dorsal midline, forming the dorsal aorta (Fig. 3G).

In summary, DNA 9 kb upstream of *Toll* exon 1 contains regulatory elements which are capable of mimicking endogenous *Toll* expression in many tissues, with the exception of mesoderm and tracheal system expression. It is unclear whether a transcriptional mesodermal element actually exists, because selective degradation of maternal *Toll* mRNA in nonmesodermal tissues following gastrulation could account for accumulation of *Toll* mRNA and protein in the mesoderm (Gertulla et al., 1988; Hashimoto et al., 1991).

Deletions of Toll fragment 1.4RR and the CNS midline regulatory region

The previous experiments indicated that the 1.4 kb RR fragment contained a CNS midline enhancer. In order to narrow the boundaries of this enhancer, bidirectional exonuclease deletions of the 1.4 kb RR fragment were generated, cloned into enhancer-tester vector C4PLZ, and transformed into flies. A summary of the staining patterns of these deletions is shown in Fig. 4A.

Constructs 1.15 Rd, 1.05 Rd, and 950 Rd, which represent progressive deletions from the 3′ end of this fragment, all stain identically to construct 1.4 RR. These constructs show expression of β-galactosidase in the CNS midline precursors, gut, and salivary gland placode in a stage 11 embryo (Fig. 4B). However, β-galactosidase staining in deletions 770 Rd and 600 Rd is severely reduced in the CNS midline relative to other tissues (gut and salivary gland) as shown in Fig. 4C. A low level of CNS midline staining is seen in these constructs after stage 10, and only in a subset of cells. Deletion 320 Rd stains most of the same tissues as 600 Rd, including the weak CNS midline expression, but at lower levels. These deletions separate the region responsible for early CNS midline expression from those regions which confer gut, epidermal, and salivary gland expression, placing its 3' boundary approximately 400 bp upstream of the 3′ *Eco*RI site and about 1.4 kb upstream of *Toll* exon 1. Analogous deletions from the 5' end of the 1.4 RR fragment place the 5′ boundary of both CNS midline and gut/salivary gland regions approximately 200 bp from the 5' *Eco*RI site.

These results demonstrate that a CNS midline enhancer is contained within a 750 bp region in the 1.4 RR fragment (between −2.1 kb and −1.4 kb relative to *Toll* exon 1) and is partially distinct from the enhancer(s) controlling gut and salivary gland expression. The data further suggest that the CNS midline enhancer consists of multiple elements. One su β-region lies between the 3′ borders of 950 Rd and 770 Rd (approx. 180 bp), and the other between the 5′ border of 1.2 dR and the 3′ border of 320 Rd (approx. 120 bp). Additional elements may lie inbetween. CNS mid-

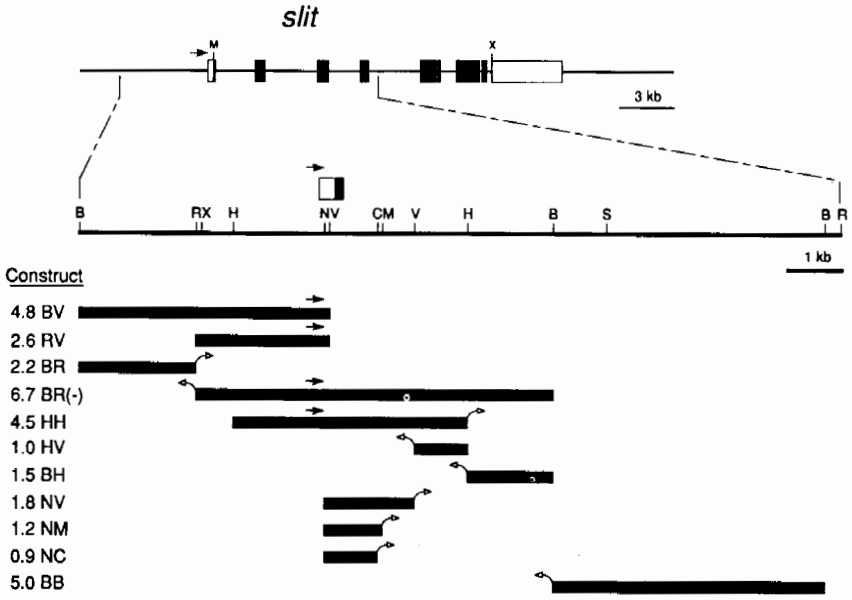


Fig. 5. Genomic structure of the *slit* gene and primary *slit-lacZ* constructs. The *slit* gene is comprised of 9 exons which span 20 kb. The upper map depicts the genomic structure of the *slit* gene (Rothberg et al., 1990). Construct nomenclature and format is as described in Fig. 2. Constructs were made from the region spanning from 4.5 kb upstream to 9.3 kb downstream of exon 1, shown expanded below the primary map. Constructs 4.8 BV and 2.6 RV use the endogenous *slit* promoter, while the remainder of constructs use a weak heterologous promoter to drive *lacZ* expression. Construct 5.0 BB gave no tissue-specific expression. Restriction sites: B = *Bam*HI; C = *Cla*I; H = *Hin*dIII; M = *Sma*I; N = *Nru*I; R = *Eco*RI; S = *Sal*I; V = *Eco*RV; X = *Xba*I.

TABLE 2

slit-lacZ expression summary

Stage	Tissue/Structure	Construct								
		4.8 BV	2.6 RV	2.2 BR	6.7 BR-	1.0 HV 4.5 HH	1.5 BH	1.8 NV	1.2 NM	0.9 NC
10	epidermal stripes		+	+						
	posterior gut	+	+							
11	midline glia	+a		+a		+b				
	lateral CNS cells							+d	+	+e
	cardioblasts	+	+	+f	+					
12–17	ventral epidermis		+		+					
	pharynx-ventral midline of frontal sac		+		+	+				
	brain	+	+		+g			+h	+h	+h
	AMC	+		+						
	midgut	+	+	+					+	+
	malpighian tubules		+		+					
	oenocytes					+	+			

Key: +, initiates β-galactosidase staining in tissue or structure at this stage; a, stains midline glia from stage 11; restricted to MGA and MGM by stage 13; b, stains midline glia, and possibly other dorsally placed MP from stage 11; restricted to MGA and MGM by stage 14; d, only stains cells in thoracic and posterior abdominal segments; e, only stains cells in thoracic segments; progressively weaker in abdominal segments; f, segmentally repeated stripes; g, brain glia; h, commissural brain glia; AMC, antenno-maxillary complex.

line expression of the 1.4 RR fragment is absent in *sim* null mutations (data not shown), a result also obtained for the *Toll* gene (Nambu et al., 1990).

Mapping and expression of zygotic slit regulatory regions

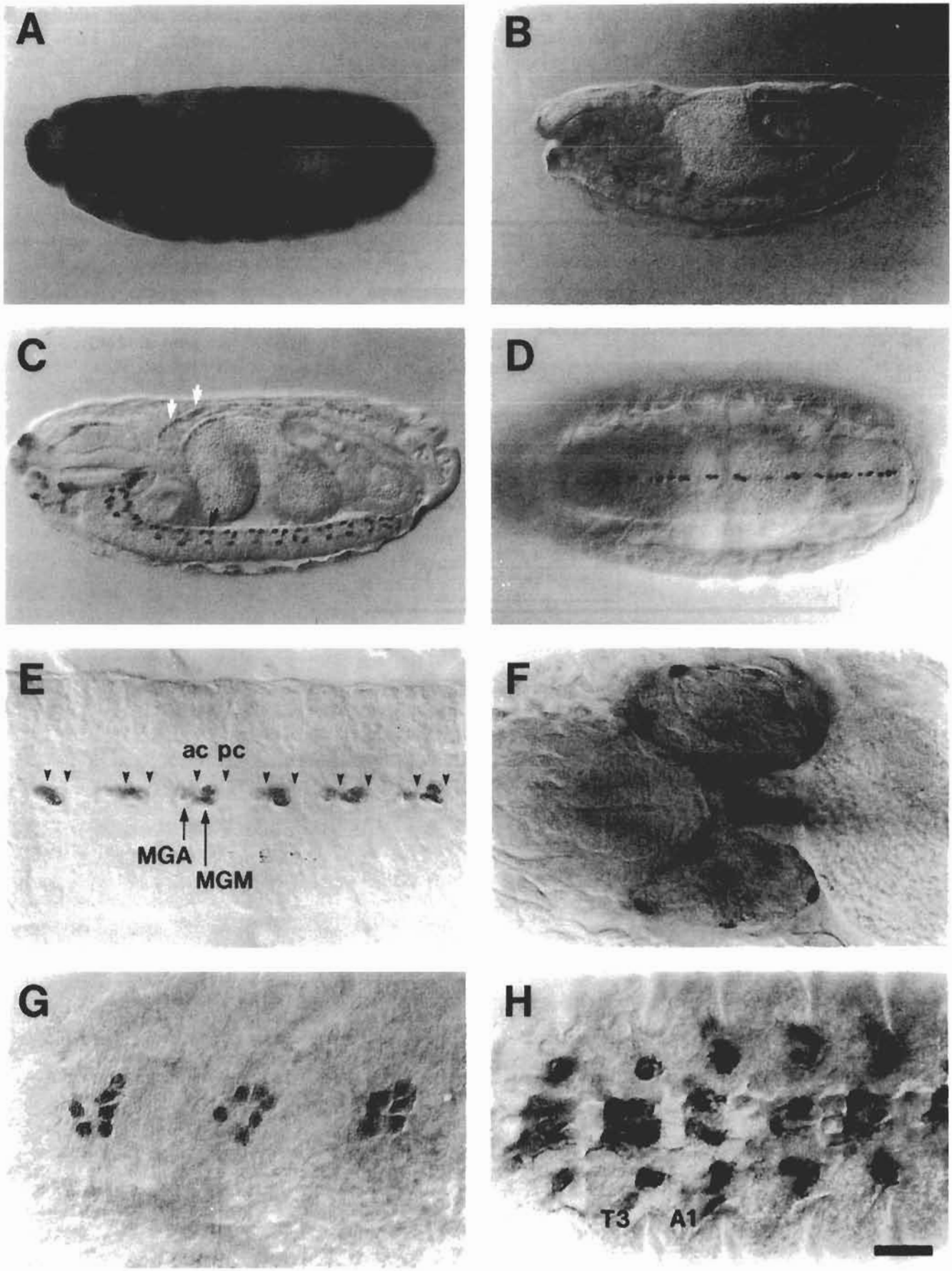
The *slit* gene is expressed prominently in all of the CNS midline precursors early in development, and later becomes restricted to the midline glia (MG) (Fig. 1). Additional domains of *slit* expression, as detected by *in situ* hybridization and antibody staining, include the lateral ectoderm, muscle attachment sites, hindgut, midgut, brain, and dorsal aorta (Rothberg et al., 1988 and 1990). *slit* mutant embryos exhibit a collapsed CNS phenotype, thought to be the result of improper CNS midline cell development (Rothberg et al., 1988, 1990; Nambu et al., 1990).

The entire *slit* coding region is contained within the 37 kb smart 19D cosmid clone provided by J. Rothberg and S. Artavanis-Tsakonas. This clone has approximately 4.5 kb of sequence upstream of *slit* exon 1. Because *slit* has 4 large introns, both upstream and intronic sequences were tested for enhancer activity.

A summary of the primary *slit-lacZ* constructs made is shown in Fig. 5. The 4.8 BV and 2.6 RV constructs use the *slit* promoter fused to *AUG-lacZ*; the remaining constructs use enhancer-tester vectors previously mentioned. A summary of expression patterns of these constructs and the stage at which staining initiates is shown in Table 2.

We have identified regions of *slit* DNA which mimic most of the wild type *slit* expression pattern. This includes late midline glial expression; early CNS midline precursor staining was not observed with one ex-

Fig. 6. *slit-lacZ* expression patterns during embryogenesis. Shown are β-galactosidase staining patterns of *slit-lacZ* constructs (A, B) 4.8BV, (C, E) 2.2BR, (D) 4.5HH, (F) 6.7BR(−), (G) 580Vd, (H) 1.2NM. All panels show whole mount preparations. Anterior is to the left in all embryos, and dorsal is facing the top of the page in (A,B,C,G). (A,B) Construct 4.8 BV. (A) Saggital view of a stage 11 embryo with epidermal stripes and posterior midgut and hindgut expression. (B) Saggital view of a stage 14 embryo staining in the dorsal aspect of the ventral nerve cord (arrow). These staining cells are midline glia (MG), whose processes ensheath the paired commissural axon bundles. The anterior commissure is visualized as a hole in each cell cluster. (C) Construct 2.2 BR. This is a saggital view of a stage 16 embryo showing β-galactosidase staining in the MG nuclei (black arrow) and a segmentally repeated subset of cardioblasts (white arrows) and other tissues (see Table 2). (D) Construct 4.5 HH, ventral view of a stage 16 embryo shows MG expression. (E) Construct 2.2 BR. High power ventral view of 6 ganglia in a stage 16 CNS that demonstrates late expression in MG subset. Positions of the anterior and posterior commissural axon bundles (ac and pc) at a position medial to the longitudinal connectives are designated by labelled arrowheads. Note staining in two cells in the MGM position and one cell in the MGA position. Constructs 4.5 HH and 1.0 HV stains the same subset of MG shown here for construct 2.2 BR (data not shown). (F) Construct 6.7 BR(−). High power dorsal view of the supra-esophageal ganglia of the brain of a stage 17 embryo. Stained glial cells are observed at the periphery of the ganglia. (G) Construct 580 Vd (See Fig 7A). High power lateral view of a stage 17 embryo demonstrates oenocyte expression (Rizki, 1978). Segments A1, A2, and A3 are shown. (H) Construct 1.2 NM. High power ventral view of 5 ganglia of a stage 14 CNS. The thoracic segments (T3 is marked) show staining of 4 para-midline cells/ganglion, while abdominal segments stain 2 cells in the same position. Two more laterally placed cell clusters also stain on each side of the midline. Scale bar: (A, B) 64 mm; (C, D) 50 mm; (E,F) 16 mm; (G,H) 20 mm.


A
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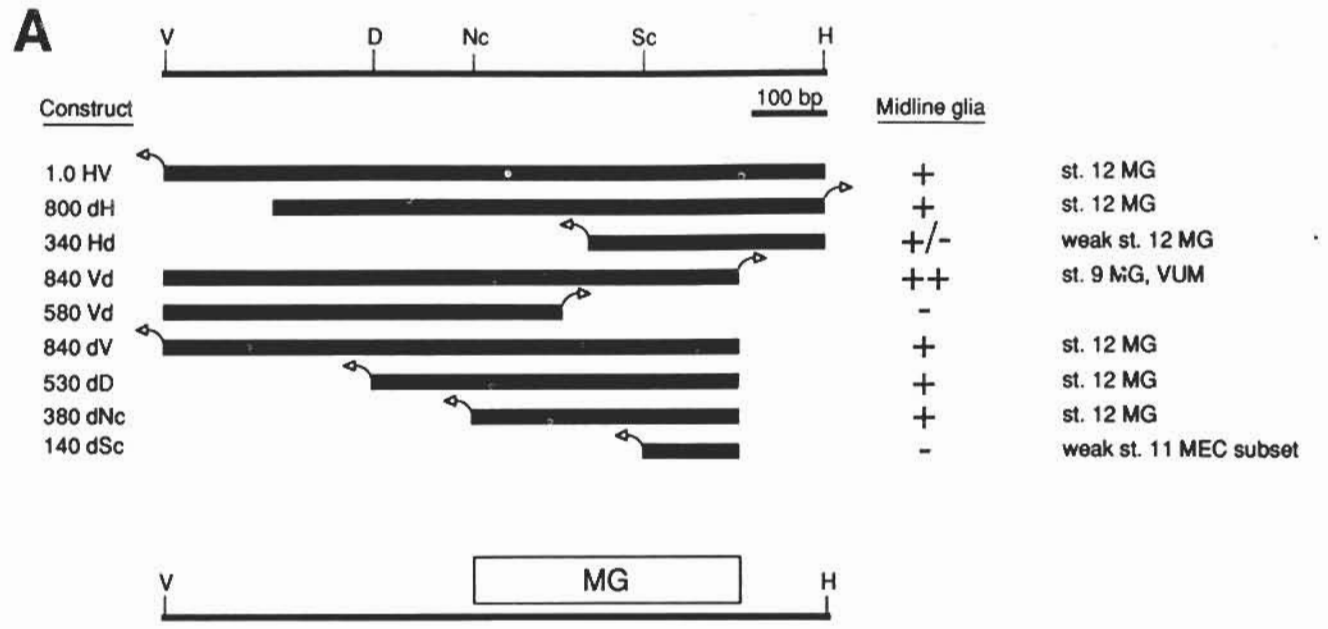
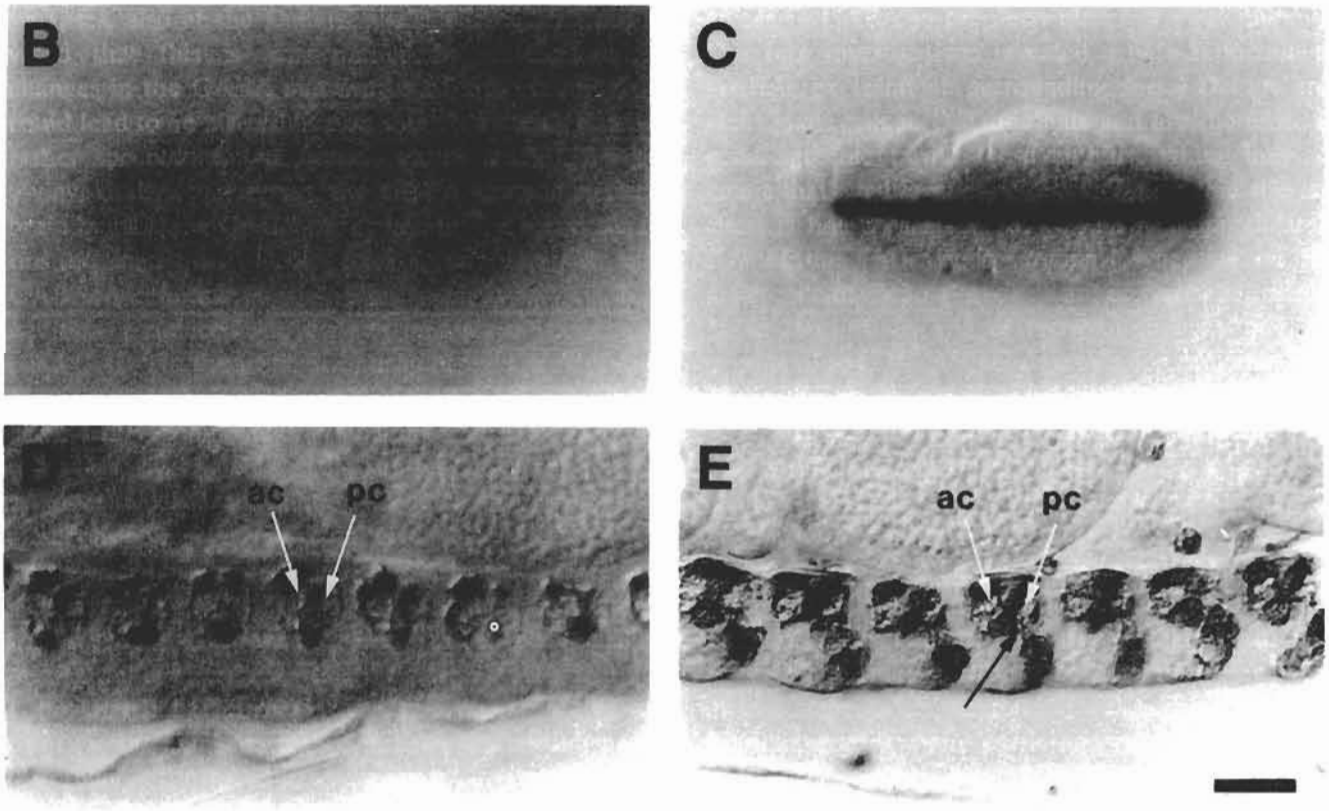
ception noted below (construct 840 Vd). Most of the *slit* expression pattern is mimicked by the β-galactosidase pattern of construct 4.8 BV. During germ band extension, expression of this construct is prominent in epidermal stripes and the posterior midgut and hindgut (Fig. 6A). After germ band retraction, expression is present in midline glia, cardioblasts, midgut, malpighian tubules, antenno-maxillary complex, and brain (Fig. 6B). Construct 4.8 BV was subdivided into two non-overlapping fragments 2.6 RV and 2.2 BR. Most of the cell types that expressed *lacZ* in 4.8 BV also expressed *lacZ* in either 2.6 RV or 2.2 BR. Construct 2.2 BR





shows expression in the midline glia, a subset of cardioblasts, ventral epidermis and ventral pharynx, antenno-maxillary complex, midgut, and malpighian tubules (Fig 6C). Construct 2.6 RV, like 4.8 BV, is expressed during stage 10 in epidermal stripes, but its restriction to the brain and cardioblasts in older embryos is much weaker (data not shown).

Constructs 4.5 HH and 6.7 BR(−) contain the *slit* endogenous promoter, but the *lacZ* gene is fused to its own weak promoter. Despite the fact that they contain overlapping regions of DNA, the two constructs show different staining patterns. Construct 4.5 HH stains the MG (from stages 11–12; Fig. 6D), the midline of the frontal sac, and oenocytes. Another construct which contains the 3′ most 1.0 kb of the 4.5 kb HH fragment (construct 1.0 HV) reveals the same MG staining as 4.5 HH, despite containing a different weak promoter (P-element vs *hsp70*) and orientation. Construct 6.7 BR(−) specifically stains cardioblasts and a subset of brain cells, probably glia based on their placement at the periphery of the CNS, their small elongated nuclei, and their spindle-shaped processes (Fig. 6F). The different expression of constructs 4.5 HH and 6.7 BR(−) are likely due to competition between a strong, endogenous *slit* promoter within these fragments and the weak promoter in the vector, the only elements identified lying immediately adjacent to the weak promoter in the construct.

These *slit-lacZ* constructs show there are separate fragments of the *slit* gene which are capable of driving MG expression, upstream (2.2 BR) and downstream (1.0 HV) of exon 1. Immediately following germband retraction, both construct 2.2 BR and 1.0 HV stain all of the developing MG (6 per segment), but both lines progressively restrict expression to both median midline glial (MGM) cells and one anterior midline glial (MGA) cell in each segment (Fig. 6E).

Constructs 1.8 NV, 1.2 NM, and 0.9 NC were designed to test sequences between fragment 1.0 HV and exon 1 whose *lacZ* expression might have been masked by the competing *slit* promoter in construct 4.5 HH. These constructs stain a small set of commissural brain glia, the midgut, and a segmentally repeated set of cells in the lateral CNS. Constructs 1.8 NV and 0.9 CN show expression in thoracic segments, while 1.2 NM is expressed in all segments (Fig. 6H). It is not clear what contribution these elements make, if any, to *in vivo slit* expression, since transcription of *slit* has not been reported in the lateral CNS.

Dissection of 1.0 HV slit late, midline glia regulatory region

Additional experiments were performed to more precisely dissect the boundaries within fragment 1.0 HV that drive MG expression. Bidirectional deletions of fragment 1.0 HV were generated, cloned into enhancer-detector vectors, and transformed into flies. The results of these transformations are summarized in Fig. 7A.

Two deletions were generated from the 5′ *Eco*RV site. The 800 dH deletion construct gives staining indistinguishable from construct 1.0 HV, despite being in the opposite orientation and employing a different promoter (*hsp70*). A further deletion (340 Hd) maintains staining in the MG but at much reduced levels relative to 1.0 HV and 800 dH.

Deletions from the other end yield surprising results. Construct 840 Vd yields β-galactosidase expression in a segmentally repeated subset of CNS midline precursors beginning during stage 9, compared with its parent construct 1.0 HV, which initiates *lacZ* expression during stages 11–12 (compare Fig. 7B to 7C). Later in development, additional β-galactosidase staining is observed in a set of ventrally placed midline neurons as well as the MG (compare Fig. 7D to 7E). In some segments, processes emanate from the ventral neurons and insert between the anterior and posterior commissures (arrowhead, Fig. 7E), a characteristic of VUM cell axons. Deletion construct 580 Vd lacks midline staining.

Further deletions of fragment 840 Vd were per-

Fig. 7. Deletion of *slit* fragment 1.0 HV and the late, MG restricted regulatory region. (A) Summary of 1.0 HV fragment deletion constructs and MG staining features. Deletion fragments and their transcriptional orientation relative to a weak heterologous promoter-*lacZ* are depicted below an expanded map of fragment 1.0 HV. MG expression which resembles the parent constructs 4.5 HH/1.0 HV is designated with a '+'; increased expression with '++'; decreased expression with '+/−'; and no expression with '−'. '+' constructs generally initiate β-galactosidase expression during stage 11–12, with expression restricted to MGA and MGM later in embryogenesis. 140 dSc weakly stains a subset of mesectodermal cells beginning at stage 10 as well as lateral epidermis. The 'MG' element is defined by construct 380 dNc, and can be divided into two suβ-regions that are both required for normal MG *lacZ* expression based on analysis of the 340 Hd construct. Restriction sites: D = *Dra*I; H = *Hin*dIII; Nc = *Nco*I; Sc = *Sca*I; V = *Eco*RV. (B) Construct 1.0 HV. Ventral view of stage 11 embryo demonstrating initial weak staining in a subset of midline cells. (C) Construct 840 Vd. Ventral view of stage 11 embryo demonstrating high levels of staining in most of the CNS midline cells. This construct initiates *lacZ* expression during stages 9–10 in a subset of CNS midline cells, and is also expressed in parts of the midgut, haemocytes, ventral epidermis, and the midline of the frontal sac. (D) Construct 4.5 HH. Higher power saggital view a of stage 17 CNS showing the MG and their processes which ensheathe the anterior and posterior commissures (ac and pc, white arrows). Seven ganglia are shown (T3–A6). (E) Construct 840 Vd. Saggital view of stage 16 CNS showing high levels of MG staining, as well as additional staining cells in the ventral 50% of the nerve cord. The dark arrow shows axonal processes inserting between the ac and pc, characteristic of VUM cell axons. Scale bar: (B,C) 64 mm; (D,E) 20 mm.

formed in order to define the 5′ boundary of the region responsible for MG expression. Constructs 840 dV, 530 dD, and 380 dNc all stain the MG similarly to construct 1.0 HV, initiating during stages 11–12. Thus, the smallest fragment able to drive wild type (1.0 HV) levels of MG expression is a 380 bp region within the 1.0 HV fragment. Apparently, enhancer orientation and/or promoter choice (P-element vs *hsp70*) plays an important role in eliciting earlier expression from this fragment, because constructs 840 dV and 840 Vd initiate midline staining at different times. Analysis of all of the deletions suggest at least two positive components of the 1.0 HV MG regulatory region: one residing in fragment 340 Hd and another in the 5′ suβ-region of fragment 380 dNc (Fig. 7A). Expression of the *slit-lacZ* MG enhancer (4.5 HH) is absent in *sim* null mutations (data not shown), a result also obtained for the *slit* gene (Nambu et al., 1990).

Discussion

As a first step in the study of CNS midline transcriptional regulation, we report the isolation of CNS midline enhancers within the Drosophila *slit* and *Toll* genes. An additional result of this analysis is the identification of DNA fragments which can direct *lacZ* transcription in most of the known sites of tissue-specific expression of these genes. These diverse cell types include nurse cells, epidermis, midgut, foregut, hindgut, sensory precursors, muscle precursors, salivary gland, cardia, oenocytes, and the brain (Tables 1 and 2). These may be generally useful as cytological markers, for transgene construction, and for studying gene regulation in these tissues.

In the *Toll* gene, we identified a 750 bp region that directs high levels of *lacZ* expression early in CNS midline cell development, corresponding to endogenous *Toll* expression. This region also contains enhancers which mimic *Toll* expression in the stomodeum, proctodeum, and salivary placode. Within this DNA fragment, finer deletions suggest that there are two suβ-regions that are required for the high levels of early midline expression: deletion of the 5′ suβ-region results in the loss of all midline expression, while absence of the 3′ suβ-region results in low yet detectable levels of midline expression.

The *slit* gene is expressed early in CNS midline precursor cells, and later in the midline glia. No separate fragment was identified in *slit* that clearly drives early midline transcription. However, two separate fragments of the *slit* gene (2.2 BR and 1.0 HV) were identified that direct *lacZ* expression later in the MG: one upstream of exon 1 and the other downstream. These apparently redundant fragments mimic the endogenous late expression of *slit* in the MG. Finer dissection of the *slit* 1.0 HV fragment indicates that it also requires divisible elements for high levels of MG transcription. There is one suβ-region that confers low-level MG expression and another that, by itself, is not sufficient for detectable MG expression. These CNS midline regulatory regions from *slit* and *Toll* both exhibit properties in common with tissue-specific enhancers in many systems, including orientation independence, multiple control elements, and promiscuous interaction with heterologous promoters.

Isolation of fragments within the *slit* and *Toll* genes with CNS midline enhancer activity represents a first step toward understanding how gene regulation in the CNS midline cells is controlled throughout neurogenesis. Additionally, DNA fragments in the *sim*, *veinlet* (*rhomboid*), and *Krüppel* genes have also been reported which possess this activity (Kasai et al., 1992; Ip et al., 1992; Hoch et al., 1990). Expression of these midline regulatory regions requires *sim* function, and future experiments will focus on how Sim and other transcription factors act through these enhancers to control the development of the CNS midline cell lineage.

Materials and Methods

slit and Toll genomic DNA

Phage clone TP-15 is an EMBL4-based clone with a 12 kb insert containing approximately 9 kb upstream and 3 kb downstream of the 3′ end of *Toll* exon 1 (Hudson, 1989).

Genomic cosmid clone smart 19D contains the entire *slit* coding region as reported by Rothberg et al. (1990).

P-element transformation vectors

Casper-AUG-βgal (Thummel et al., 1988) and HZ50PL (Hiromi and Gehring, 1987) were used as described in 'Injection constructs' below. Both vectors result in cytoplasmic localization of *lacZ*.

CPLZ, CPLZN, and C4PLZ are enhancer detector vectors analogous to HZ50PL except that they are *white* based and encode nuclear *lacZ* via a transposase-*lacZ* fusion. All three vectors are Casper-based (Pirrotta, 1988) and were constructed in the following way. A 4.1 kb *Hin*dIII-*Sma*I fragment of P-lacW enhancer trap vector, which contains the P-element promoter (up to −48 relative to the start of P-element transcripton) and N-terminal transposase sequence fused in frame with *lacZ* (Bier et al., 1989) and 3′ eukaryotic polyadenylation sequences, was cloned into *Hin*dIII-*Nru*I cut pHSX (Jones and Rubin, 1990) to form plasmid PLZ. This plasmid was then transformed

in *dam- E. coli* strain GM2163 (New England Biolabs) to demethylate one of the two flanking *Xba*I sites in the polylinker. PLZ was then cut with *Xba*I and the P-*lacZ* fusion was then cloned into *Xba*I cut Casper or Casper 4 (Pirrotta and Thummel, 1991) to form CPLZ or C4PLZ, respectively. CPLZN was made by inserting a *Not*I linker (BRL; cat. no. 5308LC) into *Eco*RI cut and Klenow filled-in CPLZ. As in HZ50PL, the direction of *lacZ* transcription is toward the nearest P-element end, so as to minimize position effects. Unique restriction sites between the divergent P-*lacZ* and mini *white* cassettes are as follows: CPLZ: *Sph*I, *Bam*HI, *Eco*RI; CPLZN: *Sph*I, *Bam*HI, *Not*I; C4PLZ: *Sph*I, *Bam*HI, *Spe*I, *Sfi*I, *Not*I, *Sst*II, *Kpn*I, *Eco*RI. The presence of approximately 500 bp of repeated 5′ P sequence appears not to interfere with transformation efficiency. These vectors are available upon request.

Transgene construction

Toll

Construct 1.7 RS was made by cloning a 1.7 kb *Eco*RI-*Ssp*I fragment, which spans from the first *Eco*RI site upstream of the longest cDNA isolated to an *Ssp*I site 65 bp upstream of the 5′ splice donor of exon I, into Casper-AUG-βgal cut with *Bam*HI, filled-in with Klenow, and then cut with *Eco*RI. This fragment contains start sites of maternal transcription (Hudson, 1989). Construct 1.4 RR was made by cloning this 1.4 kb fragment into *Eco*RI cut CPLZ. Constructs 6.5 RL and 6.5 LR were made by cloning the 6.5 kb upstream fragment into *Eco*RI cut pHSX. pHSX contains two *Not*I sites flanking the polylinker which were used to shuttle both orientation inserts into *Not*I cut HZ50PL.

For the 1.4 RR fragment deletion series, the 1.4 kb *Eco*RI fragment was cloned into *Eco*RI cut Bluescript (BS) II KS − (Stratagene) in both orientations. Exonuclease III serial deletions were performed using Erase-a-base kit (Promega), with the BS II KS − /1.4 RR subclones cut with *Eco*RV and *Apa*I as a template. Deletion clones of the appropriate size were cut with *Not*I and *Kpn*I and cloned into *Not*I-*Kpn*I cut C4PLZ.

slit

Constructs 4.8 BV and 2.6 RV were made by first cloning the 4.8 kb *Bam*HI-*Eco*RV and 2.6 kb *Eco*RI-*Eco*RV fragments into *Bam*HI-*Eco*RV or *Eco*RI-*Eco*RV cut BS II KS −, respectively. These subclones were cut with *Bam*HI-*Kpn*I or *Eco*RI-*Kpn*I and cloned into similarly cut Casper-AUG-βgal. The downstream *Eco*RV site is 190 bp from the *slit* presumed initiator methionine, and, because these constructs drive appropriate *slit* expression, are thought to contain the start site of transcription. Constructs 6.7 BR(−), 4.5 HH, 1.5 BH, 1.8 NV, 1.2 NM, and 0.9 NC were made by subcloning these inserts into pHSX, and cutting with *Not*I and cloning into *Not*I cut HZ50PL. Construct 1.0 HV was made by cloning the 1.0 kb *Hin*dIII-*Eco* RV intronic fragment into pHSX, and similarly via *Not*I ends, into *Not*I cut CPLZN. Construct 2.2 BR was made by cloning this fragment into BS II KS −, and using the *Not*I and *Kpn*I sites which flank the BS polylinker, to clone into *Not*I-*Kpn*I cut C4PLZ.

The 1.0 HV *slit* deletion series was generated in a manner analogous to the 1.4 RR *Toll* deletion series. Deletions 800 dH, 840 Vd, and 580 Vd were cloned into *Not*I-*Kpn*I cut HZ50PL, while deletions 340 Hd, 840 dV, 530 dD, 380 dNc, and 140 dSc were cloned into *Not*I-*Kpn*I cut C4PLZ.

P-element transformation

P-element transformation was performed as described in Rubin and Spradling, 1982. Pp25.7wc (Karess and Rubin, 1984) was used as source of transposase.

Drosophila strains

w^{1118} and ry^{506} or ry^{42} embryos were used as hosts for injection of *white* and *rosy* based P-element vectors, respectively.

Immunohistochemistry

Embryos were collected on yeasted grape-juice agar collection plates for allotted times, dechorionated in 50% bleach for five minutes, and processed for staining with a monoclonal antibody against β-galactosidase (Promega) or X-gal, as described in Nambu et al., 1990. At least two independent transformants of each construct were stained (the lone exception being the *slit* 1.8 NV construct). Embryos were mounted in 100% methyl salicylate or an 80/20 mix of canada balsam/methyl salicylate for permanent mountings.

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