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The *single-minded* Gene of Drosophila Is Required for the Expression of Genes Important for the Development of CNS Midline Cells

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Summary

The *single-minded* (*sim*) gene of Drosophila encodes a nuclear protein that plays a critical role in the development of the neurons, glia, and other nonneuronal cells that lie along the midline of the embryonic CNS. Using distinct cell fate markers, we observe that in *sim* mutant embryos the midline cells fail to differentiate properly into their mature CNS cell types and do not take their appropriate positions within the developing CNS. We further present evidence that *sim* is required for midline expression of a group of genes including *slit*, *Toll*, *rhomboid*, *engrailed*, and a gene at 91F; that the *sim* mutant CNS defect may be largely due to loss of midline *slit* expression; and that the *snail* gene is required to repress *sim* and other midline genes in the presumptive mesoderm.

Introduction

The Drosophila central nervous system (CNS) consists of a diverse set of neuronal and glial cell types that arise from an undifferentiated group of ectodermal cells. Recent progress has been achieved in understanding the initial decision governing whether an ectodermal cell will enter the neural or epidermal lineages (reviewed in Knust and Campos-Ortega, 1989). In addition, several genes have been identified that appear to play a later role in the determination of neuroblast, ganglion mother cell, and neuron identities (reviewed in Thomas and Crews, 1990). Nevertheless, relatively little is understood about the molecular processes that regulate how nerve cells differentiate and attain their unique cellular identities. The study of these problems in Drosophila melanogaster is facilitated by the relative simplicity of its CNS and the ability to use powerful genetic and molecular techniques to identify and analyze developmentally important genes.

We have focused our attention on the molecular and cellular mechanisms that underlie the development of a distinct group of cells that lie along the midline of the Drosophila embryonic CNS. The small number of these specific neurons and glia (about 30 cells per ganglion) as well as the ability to identify individual cells makes this an attractive system in which to study CNS formation. The development of these cells is portrayed schematically in Figure 1A. The cells that eventually occupy the midline, also referred to as "mesectodermal cells," arise from a one cell–wide strip on each side of the blastoderm embryo, which lies at the boundary of cells that will give rise to the mesoderm and those of the neurogenic region (Figure 1, stage 5; Crews et al., 1988). (Cells of the neurogenic region give rise to the lateral cells of the CNS and ventral and lateral epidermis.) During gastrulation, the midline progenitor cells come together at the ventral midline of the ectoderm (Figure 1, stage 6). The nuclei of the midline cells then migrate just inside the epidermis but retain a short cytoplasmic process that extends to the surface of the embryo (Figure 1, stage 10). Later, these midline progenitor cells divide and differentiate into a set of midline neurons, glia, and other nonneuronal cells that lie between the two larger lateral portions of the CNS (Figure 1, stage 15). (In this paper, we will refer to the mature neuronal, glial, and nonneuronal cell types found along the midline as the "CNS midline cells," and the midline cells that have not yet undergone differentiation into those cell types as "midline progenitors.")

The identities of many of the Drosophila CNS midline cells have been determined and are schematized in Figure 1B. There are six midline glia that ensheathe the commissural axon bundles and may play a role in guidance of the axonal growth cones that pioneer those bundles (Crews et al., 1988; Jacobs and Goodman, 1989a). There is one neuroblast, the median neuroblast, that gives rise to a group of neuronal progeny. Other midline neurons include the two midline precursor 1 cells (Thomas et al., 1984) and the ventral unpaired median neurons (VUMs) (Jacobs and Goodman, 1989b), which are thought to derive not from a neuroblast but from the single division of a small midline precursor cell (Bate and Grunewald, 1981). Additionally, there are a small number of cells found along the midline that are neither neuronal nor glial (Jacobs and Goodman, 1989a). Thus, the midline cells comprise a small group of neurons, glia, and nonneuronal cells that have a distinct developmental origin and unique morphology and position within the CNS.

The *single-minded* (*sim*) gene of Drosophila has been shown to play an important role in the development of the midline cells and the normal elaboration of the CNS (Thomas et al., 1988). Mutations in *sim* result in a recessive embryonic lethal phenotype characterized by a collapse of the ventral nerve cord due to loss or misplacement of the midline cells (Figures 2A and 2B; Thomas et al., 1988). The gene has been identified, and antibody staining of embryos using an antiserum raised against a *sim/lacZ* fusion polypeptide (Crews et al., 1988) indicated that *sim* is a nuclear protein. The *sim* protein shares sequence similarity with the *period* (*per*) locus gene product (Crews et al., 1988), a protein that controls the periodicity of biological rhythms in Drosophila and is found in the nuclei of some cell types (Siwicki et al., 1988). Determination of the embryonic expression pattern of *sim* revealed that transcripts are first observed in the midline progenitors of the blastoderm embryo at cellularization (Figure 1, stage 5), while protein is detected within nuclei following gastrulation along the ventral midline of the ectoderm at stages 6–7 (Thomas et al., 1988; Crews et al., 1988). *sim*

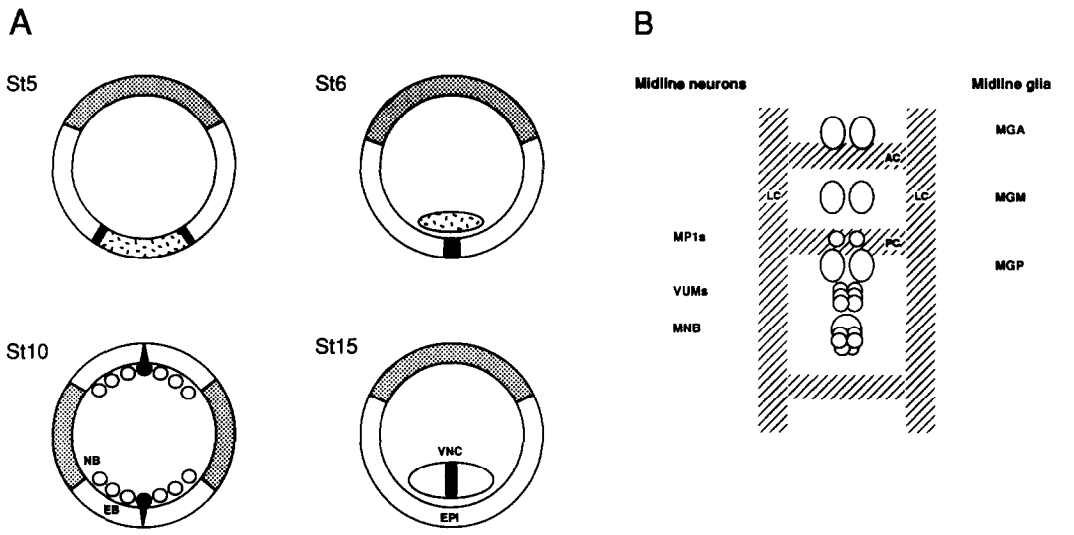

Figure 1. Summary of CNS Midline Cells and Their Development

(A) Four schematic cross-sections of a Drosophila embryo illustrate the development of the midline cells of the CNS. The four stages shown each represent a time period in development as defined by Campos-Ortega and Hartenstein (1985). All four stages are dorsal side up. (St5) This view represents the cellular blastoderm. The dashed cells will give rise to the mesoderm, the two black regions to the midline cells of the CNS, the white cells to the lateral cells of the CNS and the ventral epidermis, and the stippled cells to the dorsal epidermis and amnioserosa. In situ hybridization experiments with a *sim* probe to Drosophila embryos have shown that the regions that give rise to the midline of the CNS are each one cell wide and extend the length of the embryo (Thomas et al., 1988). (St6) Gastrulation results in the invagination of the mesoderm and the migration around the embryo of the other cellular regions. The two single cell–wide layers that give rise to the CNS midline join together at the ventral surface of the ectoderm. (St10) The embryo has undergone germband extension and neuroblast segregation from the ventral ectoderm. The neuroblasts (NB) form their own cell layer internal to the epidermoblasts (EB). The midline progenitors have their nuclei internal and extend a cytoplasmic process to the surface of the embryo. (St15) The neuroblasts and midline progenitors have divided and differentiated into the neurons, glia, and other non-neuronal cells that form the mature embryonic ventral nerve cord (VNC). The VNC lies just above the ventral epidermis (EPI). The midline CNS cells are flanked by the lateral cells of the CNS. St = stage.

(B) The identified CNS midline cells are illustrated in a horizontal view showing a single ganglion of the embryonic CNS. The anterior direction of the embryo is pointed to the top of the page. The hatched regions represent axon bundles: LC, longitudinal axon bundles; AC, anterior commissural bundles; PC, posterior commissural bundles. There are three pairs of midline glia (MG): MGA, anterior; MGM, median; and MGP, posterior. There are three recognizable sets of midline neurons: the two midline precursor 1 cells, the six VUM cells, and the median neuroblast and its progeny.

continues to be expressed in all of the midline progenitors until they differentiate into neurons, glia, and other non-neuronal cells. At this point, detectable expression becomes restricted to the midline glia. Based on these results, it was proposed that *sim* might regulate the expression of a battery of genes that act in the development of the CNS midline cells.

We have addressed the nature of *sim* function by following the developmental fate of the midline cells in *sim* mutants by cytological marking of these cells and by identifying genes whose expression requires *sim* gene function. These experiments indicate that in *sim* mutants the midline cells fail to differentiate properly into the midline neurons, glia, and nonneuronal cells. We further show that *sim* is required for the CNS midline expression of a set of genes including *slit* (*sli*), *Toll* (*Tl*), *rhomboid* (*rho*), *engrailed* (*en*), and a gene at 91F, all of which may play a role in midline cell development. Additional experiments indicate that *sli* is required for the proper development of the midline cells and that the *sim* collapsed CNS phenotype may be largely due to loss of *sli* midline expression. Finally, it is shown that *snail* (*sna*) gene function is required to repress *sim* and other midline genes in the presumptive mesoderm.

Results

Midline Cell Fate Markers

To examine the fate of the midline cells in *sim* or *sli* mutant strains, three approaches for marking midline cells were used: first, β-galactosidase staining of strains containing a P element transposon with *sim* regulatory sequences fused to the β-galactosidase gene, (P[*sim/lacZ*]); second, staining with a *sim* antiserum; and third, β-galactosidase staining of strains containing a P element enhancer trap insertion into the *rho* locus. The P[*sim/lacZ*] insertion is generally the most useful marker, because of its versatility and specificity for the midline cells. The P[*sim/lacZ*] construct was generated from a segment of DNA that includes a set of regulatory elements contained internally within the *sim* gene. The 7.8 kb fragment of Drosophila DNA used to construct the P[*sim/lacZ*] vector is shown in Figure 3. It contains 68% of the *sim* coding sequence and was fused in frame to the β-galactosidase gene residing on the cosP*white*β-gal P element vector.

The suitability of P[*sim/lacZ*] as a midline marker derives from its early expression of β-galactosidase in the midline cells, similar to *sim*. The initial expression of β-galactosidase in the P[*sim/lacZ*] strain is observed in a stage

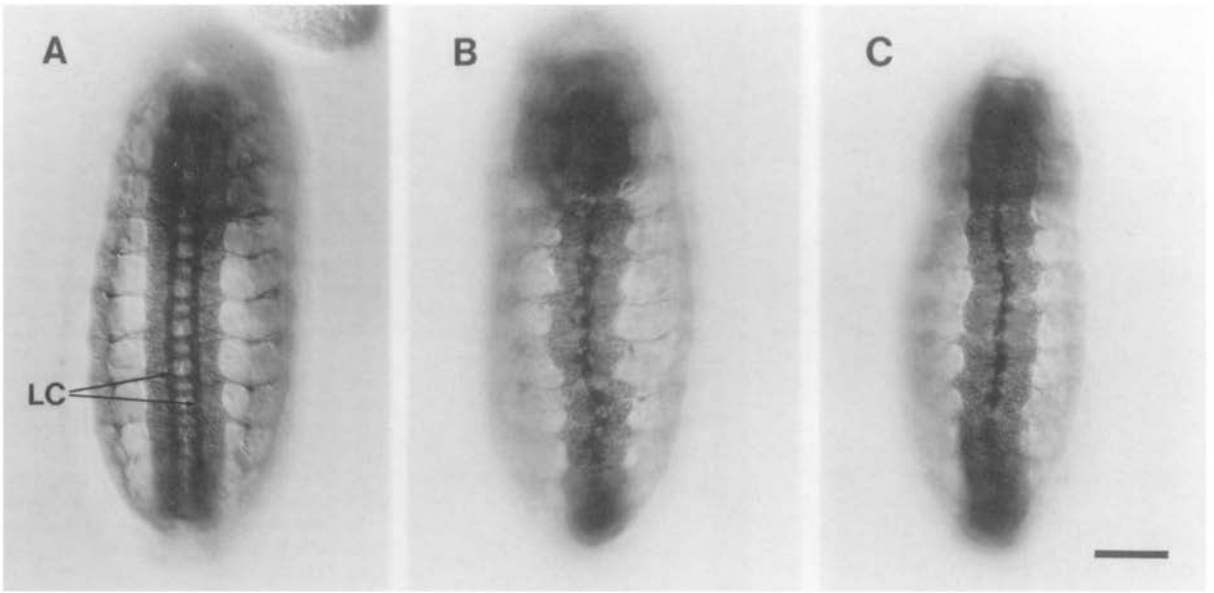

Figure 2. CNS Phenotypes of *sim* and *sli* Mutant Embryos

Embryonic CNS of (A) wild-type, (B) *sim* mutant (*Df[3R]l26d*), and (C) *sli* mutant (sli^{lG107}). Whole-mount stage 15 embryos were stained with anti-HRP antiserum, which reacts with all nerve cells and their axons. Ventral views are shown with anterior pointing to the top of the page. Cells along the midline of the CNS are misplaced in *sim* (Thomas et al., 1988) and *sli* (Rothberg et al., 1988) mutant embryos resulting in a collapse of the CNS and a fusion of the longitudinal connectives (LC). Scale bar: 50 μm.

8 embryo around 3.5 hr postfertilization. Figure 4A shows a ventral view of a stage 10 embryo of the P[*sim/lacZ*] strain stained with an antibody against β-galactosidase. Staining is observed along the midline of the embryo and is localized to cell nuclei. The segment of *sim* coding sequence fused to β-galactosidase thus contains a nuclear localization sequence. A sagittal view of a stage 10 embryo (Figure 4C) shows the nuclei of the midline cells migrating into the neuroblast layer between the mesodermal and epidermal precursors and also shows staining in the stomodeal opening, posterior midgut, and proctodeum. The gut staining is also observed when embryos are stained with a *sim* antiserum. Later, at stage 13 (Figure 4E), the midline cells begin to differentiate into the CNS midline cells and migrate to their appropriate locations in the developing CNS. At stage 15, when nerve cell formation and axonogenesis are essentially complete, β-galactosidase is observed in the midline glia along the dorsal surface of the CNS and in some of the midline neurons and other nonneuronal cells found medial and ventral in the CNS (data not shown). At this stage, endogenous *sim* protein is normally observed only in the midline glia. This difference probably reflects increased stability of β-galactosidase mRNA and/or protein. However, it cannot be excluded that the P[*sim/lacZ*] gene has a slightly different regulation than the *sim* gene. In summary, the pattern of β-galactosidase expression in P[*sim/lacZ*] strains closely resembles the pattern of endogenous *sim* expression.

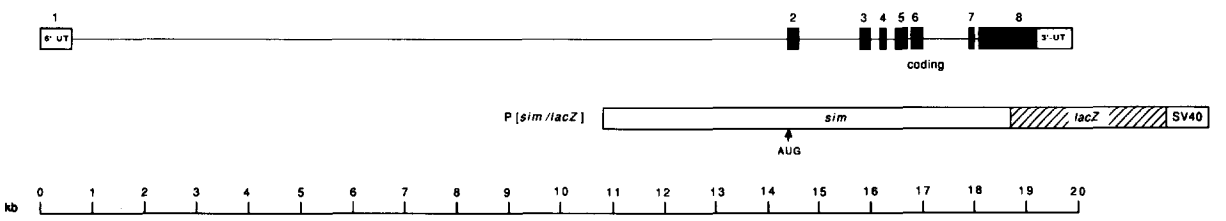

Figure 3. Structure of the *sim* Gene and the P[*sim/lacZ*] Construct

The structure of the *sim* gene (S. T. Crews, J. Thomas, G. de Feo, Y. Kasai, M. G. Muralidhar, and J. Nambu, unpublished data) as determined by DNA sequence analysis is shown at the top. The gene consists of eight exons and they are numbered at the top of the figure. Clear boxes indicate untranslated sequences and the darkened boxes indicate coding sequences. Below is the segment of DNA used to construct the P[*sim/lacZ*] transposon. The relevant sequences of the P[*sim/lacZ*] vector are shown below the gene structure. A 7.8 kb *sim* genomic fragment was fused in frame to the sequences encoding the fifth amino acid of the β-galactosidase gene. The probable initiator AUG is supplied by sequences from the second *sim* exon and is indicated with an arrow, and an SV40 polyadenylation site is located 3′ to the β-galactosidase gene. At the bottom is a scale marked in kilobases (kb).

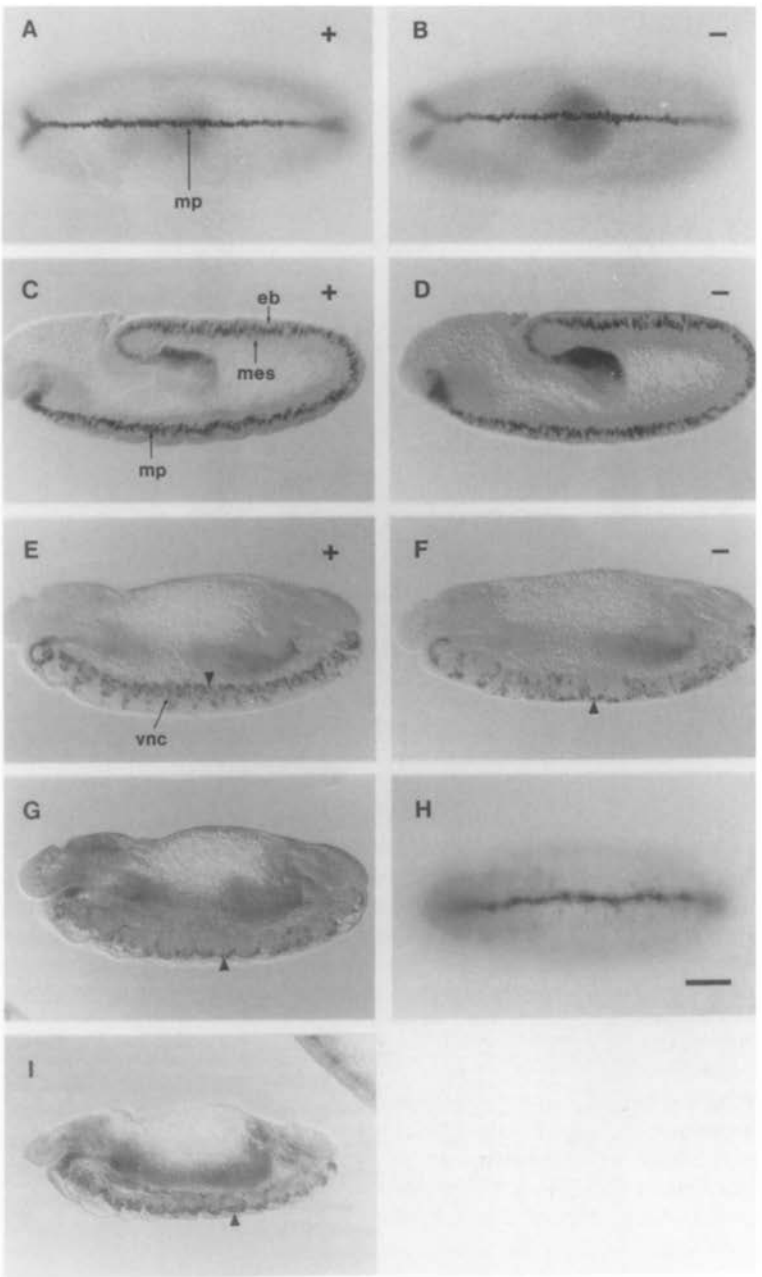

Figure 4. Fate of the Midline Cells in Wild-Type, *sim*, and *sli* Mutant Embryos

Whole-mount embryos containing a P[*sim/lacZ*] chromosome were collected at different stages of development and stained with an antibody against β-galactosidase to visualize the midline cells. (A, C, and E) sim^{+} embryos (noted by the presence of a plus sign in the right-hand corner). (B, D, and F) sim^{-} embryos ($sim^{B21\text{-}2}$ allele, noted by the presence of a minus sign in the right-hand corner). The midline progenitor staining due to the P[*sim/lacZ*] element is similar in both the sim^{+} and sim^{-} stage 10 embryos: (A and B) stage 10 ventral view, (C and D) stage 10 sagittal view. By stage 13, the sim^{+} embryos (E) (sagittal view) show organized CNS midline cells, primarily in the dorsal half of the CNS (arrowhead), whereas the midline cells in the sim^{-} embryos (F) (sagittal view) are disorganized and predominantly found down at the ventral surface of the embryo (arrowhead). Similar results were obtained when midline cell fate was followed in $sim^{B21\text{-}2}$ without P[*sim/lacZ*] using a *sim* antiserum. The midline cells in a *sli* mutant stage 13 embryo (G) (sli^{G107} allele), as identified by P[*sim/lacZ*] expression, are found along the ventral surface of the embryo out of the CNS similar to *sim* mutant embryos (arrowhead), compare to (F). The P[*91F/lacZ*]*242* element is expressed at wild-type levels in the midline CNS cells in *sli* mutant embryos (H) (stage 13, ventral view). The P[*Tl/lacZ*] element is expressed at wild-type levels in *sli* mutant embryos (I) (stage 13, sagittal view). Notice the positively staining cells at the ventral surface of the sli^{-} embryo (arrowhead). All frames have anterior to the left and in the sagittal views, dorsal is up. eb, epidermoblasts; mp, midline progenitors; mes, mesodermal precursors, vnc, ventral nerve cord. Scale bar: 50 μm.

Midline Cell Fate in *sim* Mutants

The fate of the midline cells in *sim* mutants was examined by crossing P[*sim/lacZ*] or P[*rho/lacZ*] into mutant backgrounds, or staining for *sim* protein. These experiments used two ethylmethanesulfonate (EMS)-induced *sim* alleles, $sim^{B21\text{-}2}$ and sim^{H9}, and two deficiencies, $Df(3R)ry^{619}$ and *Df(3R)l26d*. $sim^{B21\text{-}2}$ makes normal levels of a *sim* immunoreactive protein as detected by staining embryos with a *sim* antiserum, whereas the two deficiencies and sim^{H9} fail to make any detectable *sim* protein. The CNS phenotypes of the four *sim* mutations are all severe with each showing a fused connective phenotype when the CNS is stained with anti-HRP. However, $sim^{B21\text{-}2}$ is probably a hypomorphic allele (see below). Stable lines, homozygous for X-linked copies of the P[*sim/lacZ*] chromosome, were generated for each mutant strain. It was important to be able to identify those embryos that were sim^{-} homozygotes, and this was accomplished by maintaining the *sim* mutant chromosome over a balancer that contains a P[*ftz/lacZ*] insertion. When visualized for the presence of β-galactosidase protein, the P[*ftz/lacZ*] insertion displays intense *fushi tarazu* (*ftz*) pair-rule ectodermal stripes and *ftz* CNS expression in both embryos that are homozygous for the balancer chromosome and those that possess a single balancer over the sim^{-} chromosome. Thus, we could unambiguously identify the sim^{-} homozy-

gous mutants, since they do not exhibit P[*ftz/lacZ*] expression. This was particularly important when examining early-stage embryos since the *sim* defects were somewhat subtle. The presence of P[*sim/lacZ*] failed to rescue lethality or the CNS fused connective phenotype of any of the *sim* mutations, although weak rescue of *sim* function remains a possibility.

The fate of the midline cells in the P[*sim/lacZ*]; sim^{B21-2} allele is chronicled in Figure 4 (compare the mutants in [B], [D], and [F] with the wild-type embryos shown in [A], [C], and [E]). Comparison of the mutant embryos with wild-type indicates that β-galactosidase is expressed in the midline cells of the *sim* mutants in normal amounts. The midline progenitor cells appear relatively normal through gastrulation, and the nuclei migrate into the neuroblast layer as in wild-type embryos (Figures 4B and 4D), although they sometimes appear somewhat disorganized compared with wild type.

During stages 11 and 12 the midline cells in wild-type embryos begin to divide and differentiate into midline neurons and glia. The exact lineage and cell movements of midline cell development have not yet been resolved, but characteristic, ordered cell migrations are apparent as the mature cells take their specific positions in the CNS. Generally, it is observed in stage 11 and 12 *sim* mutant embryos that the distribution of midline cells in the CNS is less organized than wild type, and this defect becomes very apparent by stage 13 as the CNS is beginning to take its mature shape (Figure 4F). This disorganization is consistent with the previously published observation indicating that the midline cells were not in their proper position in a stage 11 embryo (Thomas et al., 1988). At stage 13 the midline cells in *sim* mutants are very disordered and tend to be clustered near the ventral surface of the embryo. The same results are observed when the P[*sim/lacZ*] element is introduced in the two EMS-induced *sim* mutants and both deficiency strains. Additional confirmation of these results comes from whole-mount antibody staining experiments with a *sim* antiserum on the sim^{B21-2} allele and in situ hybridization using a *sim* cDNA probe. These reagents both stain sim^{B21-2} homozygous sim^{-} embryos in the midline cells and provide a marker for those cells similar to the P[*sim/lacZ*] chromosome. These experiments showed similar midline cell development as the P[*sim/lacZ*]; sim^{-} strains.

The important point of these experiments is that they indicate that, minimally, *sim* is required for the proper differentiation of the midline cells from their progenitors. These results provide a rationale for the *sim* CNS phenotype. The midline cells fail to differentiate properly and cluster near the ventral surface of the embryo instead of their usual location within the CNS. This leads to the collapse of the two lateral CNS hemiganglia and fusion of the longitudinal connectives. Results described below indicate that alterations in gene expression accompany the CNS disorganization. These experiments do not address whether *sim* plays an earlier function in the formation of the midline progenitors since it is possible that sim^{B21-2}, which makes an immunoreactive *sim* protein, is a hypomorphic allele and that the P[*sim/lacZ*] construct may possess a weak *sim* rescue activity.

Midline Cell Development in *sli* Mutants

sli is a gene originally identified as having a mutant larval cuticular defect (Nüsslein-Volhard et al., 1984) and later shown to have sequence similarity to the *Notch* gene product (Rothberg et al., 1988). The *Notch* and *sli* proteins both display a series of repeats similar to those found in epidermal growth factor and both are likely to be involved in cell–cell interactions. *sli* mutants have an embryonic CNS phenotype similar to that of *sim* (Rothberg et al., 1988; see Figures 2B and 2C); in both *sim* and *sli* mutants the longitudinal connectives of the CNS are fused together. Given the similarity of their CNS phenotypes, we decided to investigate further the fate of the midline cells in *sli* mutant embryos by using the P[*sim/lacZ*] chromosome as a midline cell marker. When the fate of the midline cells are compared in sli^{-} embryos and sim^{B21-2} embryos, it is apparent that the midline cells behave in a similar fashion (Figure 4G shows a P[*sim/lacZ*]; sli^{-} embryo at stage 13; compare to Figure 4F). The midline progenitors appear relatively normal, but the cells fail to properly differentiate and take their normal positions within the CNS. Ultimately, the cells end up along the ventral surface of the embryo. Since the *sli* gene is expressed in the midline cells subsequent to *sim*, *sli* expression may require *sim* function, and the effect on CNS differentiation observed in *sim* mutant embryos may be largely due to loss of *sli* expression. This possibility was tested directly as described below.

Requirement of *sim* for Midline Gene Expression

The nuclear localization of the *sim* protein, its restricted expression in the midline cells, and the dramatic effect of *sim* mutations on midline cell development suggest that *sim* is required for the expression of a set of genes that play a crucial role in the normal development of these cells. Several genes have been identified that are expressed in midline cells and are potential candidates for requiring *sim* function for their expression. We tested whether the expression of these genes requires *sim* by examining their expression in a *sim* mutant background. Lack of expression of a putative downstream gene in the midline cells in stage 9–11 sim^{-} embryos, when the cells still reside along the midline, can be interpreted as requiring *sim* gene function. Below, we describe the effects of *sim* mutations on midline gene expression of five different genes: *sli*, *Tl*, *en*, *rho*, and a gene at 91F associated with an enhancer trap insertion.

sli

The *sli* gene is expressed in most cells of the embryo; however, it exhibits particularly high levels of expression in the midline progenitor cells and later in the midline glia (Rothberg et al., 1988). We have examined *sli* expression using a P[*lacZ*] insertion into the *sli* locus provided by A. Kolodkin and C. Goodman. The insertion, *E-158*, is lethal, but does not show the *sli* mutant CNS defect at 25°C. β-galactosidase expression along the midline due to the

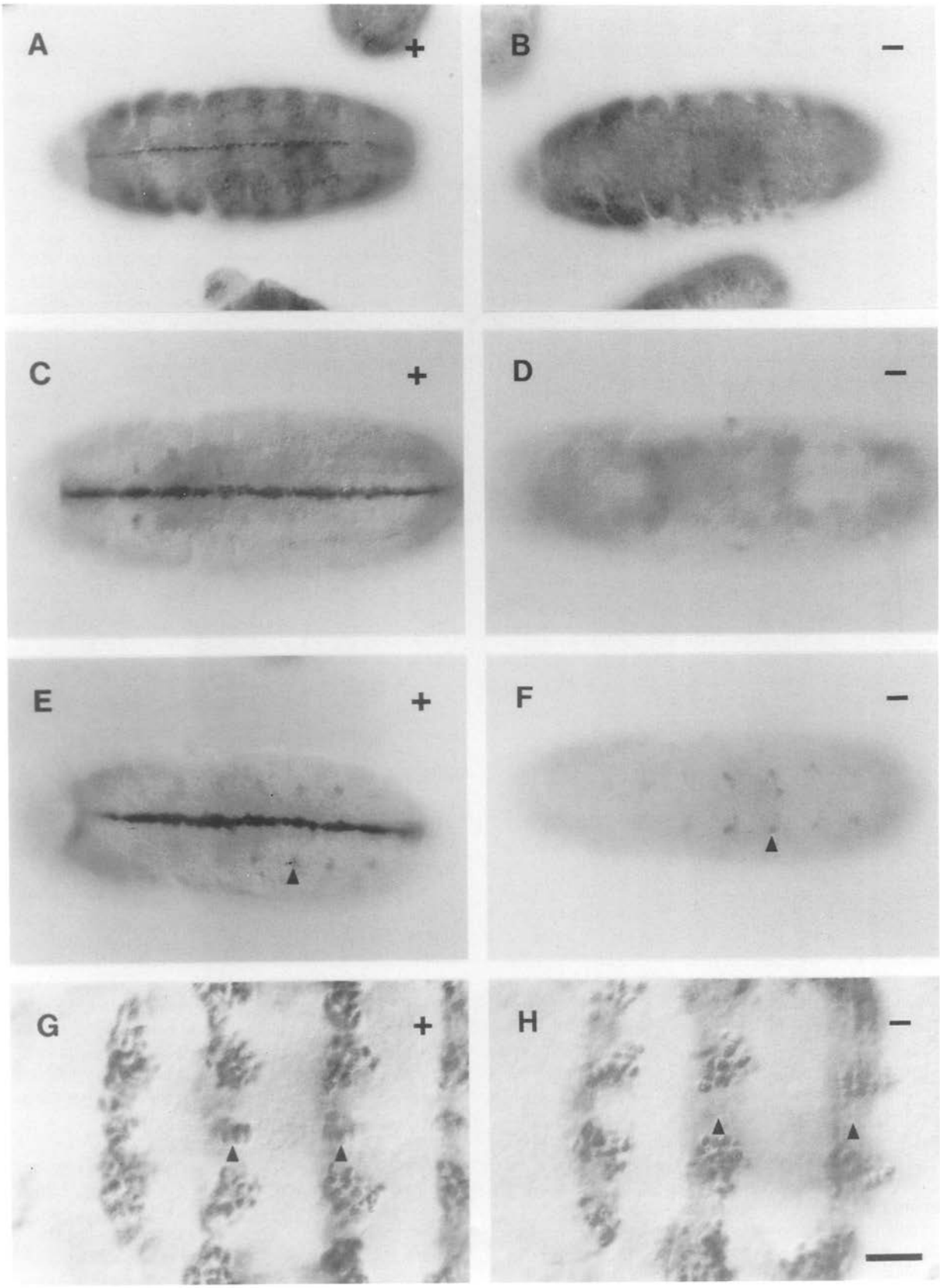

Figure 5. Requirement of *sim* for Midline Gene Expression

Absence of midline staining of a group of midline genes is observed in mutant embryos homozygous for *sim* deficiencies, and hypomorphic alleles of *sim* also generally show reduced or absent midline expression.

P[*sli/lacZ*]*E-158* insertion is first observed above the uniformly staining background in stage 10 embryos about 40–60 min after *sim* protein is detected in cell nuclei. Because of the occurrence of *sli* expression throughout the embryo, we focused on stage 11 embryos, when *sli* midline staining is high relative to other cells.

The results indicate that *sli* midline expression is absent or diminished in *sim* mutants (Figures 5A and 5B). No expression of P[*sli/lacZ*]*E-158* is observed along the midline in the *sim* deficiency *Df(3R)l26d*, although the nonmidline expression is similar to wild type. Reduced expression in a small number of midline cells is usually observed in the $sim^{B21\text{-}2}$ EMS-induced allele (data not shown), suggesting that this is a hypomorphic allele. Staining of $sim^{B21\text{-}2}$ mutant embryos with a *sim* antiserum indicated that the midline progenitors were present in approximately the same number as wild type in stages 9–11 when these observations were generally made. Thus, normal *sli* midline expression requires *sim* function. These results suggest not only that *sli* expression requires *sim* function, but also that in *sim* mutants the collapsed CNS phenotype may be largely due to the absence of *sli* expression in the midline cells.

Tl

The *Tl* gene is expressed both maternally and zygotically and probably plays multiple roles during embryogenesis (Gerttula et al., 1988). The sequence of the *Tl* protein suggests that it may act as a transmembrane receptor and signal transduction molecule (Hashimoto et al., 1988). Maternally, it is required for establishing the dorsal–ventral axis of the embryo (Anderson et al., 1985). However, *Tl* also exhibits a complex zygotic expression pattern, and this expression appears to be required for larval viability. The pattern of *Tl* zygotic expression includes cells of the anterior and posterior midguts, trachael and salivary gland placodes, and the midline progenitors (Gerttula et al., 1988). *Tl* is first expressed in the midline progenitors of wild-type embryos around stage 9 about 30–60 min after *sim* nuclear protein is detected and persists in these cells and their progeny throughout embryogenesis.

We have used a P[*lacZ*] insertion into the *Tl* gene (*F336*; provided by Yasushi Hiromi and Corey Goodman) to examine its midline expression in *sim* mutant and wild-type embryos. The P[*Tl/lacZ*]*F336* element was recombined onto the *sim* deficiency $Df(3R)ry^{619}$ and balanced over TM3 P[*ftz/lacZ*] to enable the identification of the homozygous *Tl* mutant embryos. Analysis of P[*Tl/lacZ*]*F336* expression in *sim* mutant embryos indicates that *Tl* expression is absent along the midline in the *sim* deficiency strain (Figures 5C and 5D). Expression is also absent along those sections of the gut in which both *sim* and *Tl* are expressed.

en

Expression studies have shown that the *en* segment polarity gene product is localized to a set of segmentally repeated ectodermal stripes early in embryonic development (DiNardo et al., 1985). These cells give rise to neuronal precursor cells in the posterior region of each CNS ganglion, and *en* continues to be expressed in the progeny of these precursors. These cells include subsets of the median neuroblast progeny and VUM cells (Patel et al., 1989b). Similar to the effects of several other segmentation genes on CNS development (Doe et al., 1988; Patel et al., 1989b), *en* may contribute to the specification of nerve cell identity.

Since there exists a sustained level of *en* expression in the posterior CNS midline cells from the blastoderm to the mature CNS, it is relatively easy to see if there is an effect of *sim* on *en* expression. When *sim* point mutants or deficiencies are stained with a monoclonal antibody reactive with *en*, staining along the midline is normal until approximately stage 9–10. At this time and later, expression in the midline progenitors is absent. This can be seen in Figures 5G and 5H, showing that there are no cells along the midline that express *en* in *sim* mutant embryos.

91F Gene

Enhancer trap screens have provided a number of interesting candidate genes that may require *sim* function for their expression. One of them is a gene that maps to the region 91F, on the third chromosome, and was first identified by two insertions, *87* and *242*, found by Yasushi Hiromi and Corey Goodman. We have also identified another insertion into this region, *BA01*, that has midline expression (S. Hu, B. Matthews, and S. T. Crews, unpublished data). These lines all show staining in the midline progenitors (Figure 5E) and are expressed in all or most of the CNS midline cells. Initial expression is observed at the end of stage 9, about 30–60 min after the first observation of *sim* nuclear expression. All three inserts display the same midline staining pattern, but differ with respect to other cells in the embryo that do or do not stain. The DNA at this region has been cloned and a gene has been identified with similar midline expression to the enhancer trap lines as determined by whole-mount in situ hybridization

(A and B) Staining of stage 11 whole-mount embryos containing a P[*sli/lacZ*]*E-158* chromosome with anti-β-galactosidase. Ventral views of sim^{+} (A) and sim^{-} (*Df[3R]l26d*) (B) embryos are in similar focal planes and show the lack of midline staining in *sim* mutant embryos compared with sim^{+} embryos.

(C and D) Staining of stage 11 sim^{+} (C) and sim^{-} ($Df[3R]ry^{619}$) (D) embryos containing a P[*Tl/lacZ*]F336 chromosome with anti-β-galactosidase. Staining along the midline is absent in *sim* mutant embryos.

(E and F) Staining of stage 11 embryos containing the P[*91F/lacZ*]*242* chromosome. The ventral views of sim^{+} (E) and sim^{-} embryos ($Df[3R]ry^{619}$) (F) show absence of staining along the midline in *sim* mutant embryos. Staining is observed in both sim^{+} and sim^{-} embryos in longitudinal glioblasts (arrowheads), which are cells not derived from the midline progenitor cells (Jacobs et al., 1989).

(G and H) Staining of stage 11 embryos with an anti-*en* monoclonal antibody. Ventral views of sim^{+} (G) and sim^{-} (*Df[3R]l26d*) (H) embryos are both in the same focal plane. Notice the absence of staining along the midline in *sim* mutant embryos in contrast to the staining observed in the sim^{+} embryos (arrowheads).

Scale bars: (A–F) 50 μm, (G–H) 20 μm.

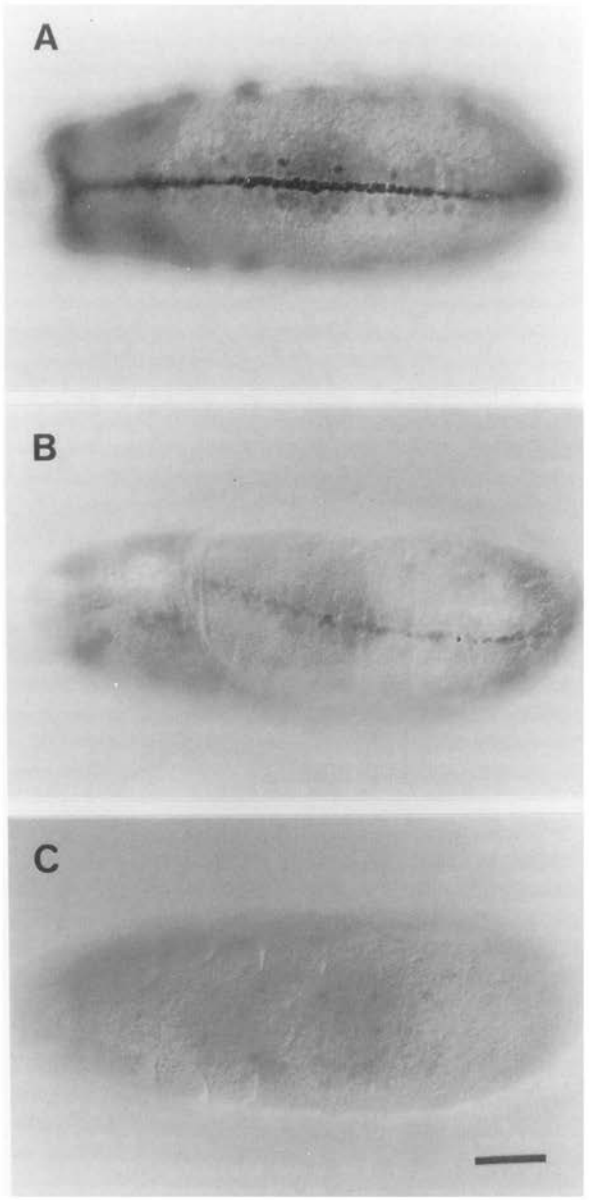

Figure 6. Midline Expression of P[*rho*/*lacZ*] Requires *sim*

(A) Staining of a stage 11 wild-type embryo containing two copies of P[*rho*/*lacZ*] with anti-β-galactosidase shows strong midline staining.

(B) Anti-β-galactosidase staining of a stage 11 embryo containing a single copy of P[*rho*/*lacZ*] that is a *sim* transheterozygote with the genotype $Df(3R)ry^{619}/sim^{B21\text{-}2}$. Notice the presence of the midline cells at this stage and the low level of midline staining.

(C) Anti-β-galactosidase midline staining of P[*91F*/*lacZ*]*242* is absent in $Df(3R)ry^{619}/sim^{B21\text{-}2}$ stage 11 embryos, even though the midline cells are present at this time; see (B). The few staining cells lateral to the midline are longitudinal glioblasts (see Figures 5E and 5F). All views are ventral except (B). Scale bar: 50 μm.

(B. Matthews, Y. Hiromi, C. Goodman, and S. T. Crews, unpublished data).

The P[*91F*/*lacZ*]*242* insertion was recombined onto a *sim* deficiency, $Df(3R)ry^{619}$, and balanced over TM3 P[*ftz*/*lacZ*]. Examination of these embryos for β-galactosidase expression reveals that 91F expression is completely abolished along the midline (Figures 5E and 5F). The only staining present is found in a set of longitudinal glial precursors whose expression is due to the *ftz* promoter used in this enhancer trap line (Jacobs et al., 1989). Transheterozygotes of *sim* deficiency and *sim* EMS-induced alleles ($Df[3R]ry^{619}/sim^{B21\text{-}2}$) also show no midline expression of P[*91F*/*lacZ*]*242* (Figure 6C). Similar results were obtained for another insertion in the 91F locus, P[*91F*/*lacZ*]*87*, that was recombined onto $Df(3R)ry^{619}$; the midline staining was absent in the *sim* homozygous mutant embryos, and the nonmidline staining was unaffected.

rho

rho mutants display a number of alterations in the larval cuticle, peripheral nervous system, and CNS (Mayer and Nüsslein-Volhard, 1988). The CNS defect has not been carefully analyzed, but shows a collapsed commissure defect similar to *sim* but less severe. Whether this defect is due to alterations in midline cell development is unknown. The gene has been cloned and is expressed prominently in the CNS midline progenitors and their progeny (Bier et al., 1990). The earliest expression of *rho* closely parallels *sim* and precedes the expression of *sli*, *Tl*, and the 91F gene. We have used an enhancer trap insertion in the *rho* locus, which closely resembles *rho* gene expression (Figure 6A), to assess whether *sim* is required for *rho* expression. Midline expression of P[*rho*/*lacZ*] in a $sim^{B21\text{-}2}/Df(3R)ry619$ transheterozygote is present but greatly reduced (Figure 6B) compared with wild type (Figure 6A). The residual P[*rho*/*lacZ*] midline staining indicates that the midline cells are present in the transheterozygote at stages 10–11. Expression of P[*rho*/*lacZ*] was detectable, but even further reduced, in homozygous deficiency backgrounds. Thus, *sim* is required for high levels of *rho* midline expression through embryogenesis.

In summary, we have shown that *sim* function is required for the normal expression of five genes. Four of the genes reveal no detectable expression in *sim* deficiency strains, with P[*rho*/*lacZ*] being the exception in showing strongly reduced expression. The data presented in this paper usually compare expression of the midline genes in late germband extended wild-type and *sim* mutant embryos when the difference in expression is most dramatic. However, it is worth mentioning that at no earlier or later time of development in *sim* deficiency strains is there any detectable midline expression of genes such as *sli*, *Tl*, and the 91F gene. Additionally, four of the genes were shown to have low or undetectable expression in hypmorphic genetic backgrounds in which cell fate analysis has revealed the presence of the midline cells at the same time of development. Thus, the effect on expression of these genes observed in *sim* mutants is not due simply to the absence of the midline cells.

Expression of Tl and the 91F Gene in sli Mutants

To understand further the hierarchy of events involved in midline development and gene expression, we looked at the expression of *Tl* and the 91F gene in *sli* mutant embryos to see if *sli* played a role in their expression. The results indicate that P[*91F*/*lacZ*]*242* and P[*Tl*/*lacZ*] are expressed in *sli* mutants throughout development (Figures 4H and 4I, stage 13 is shown). The stained midline cells

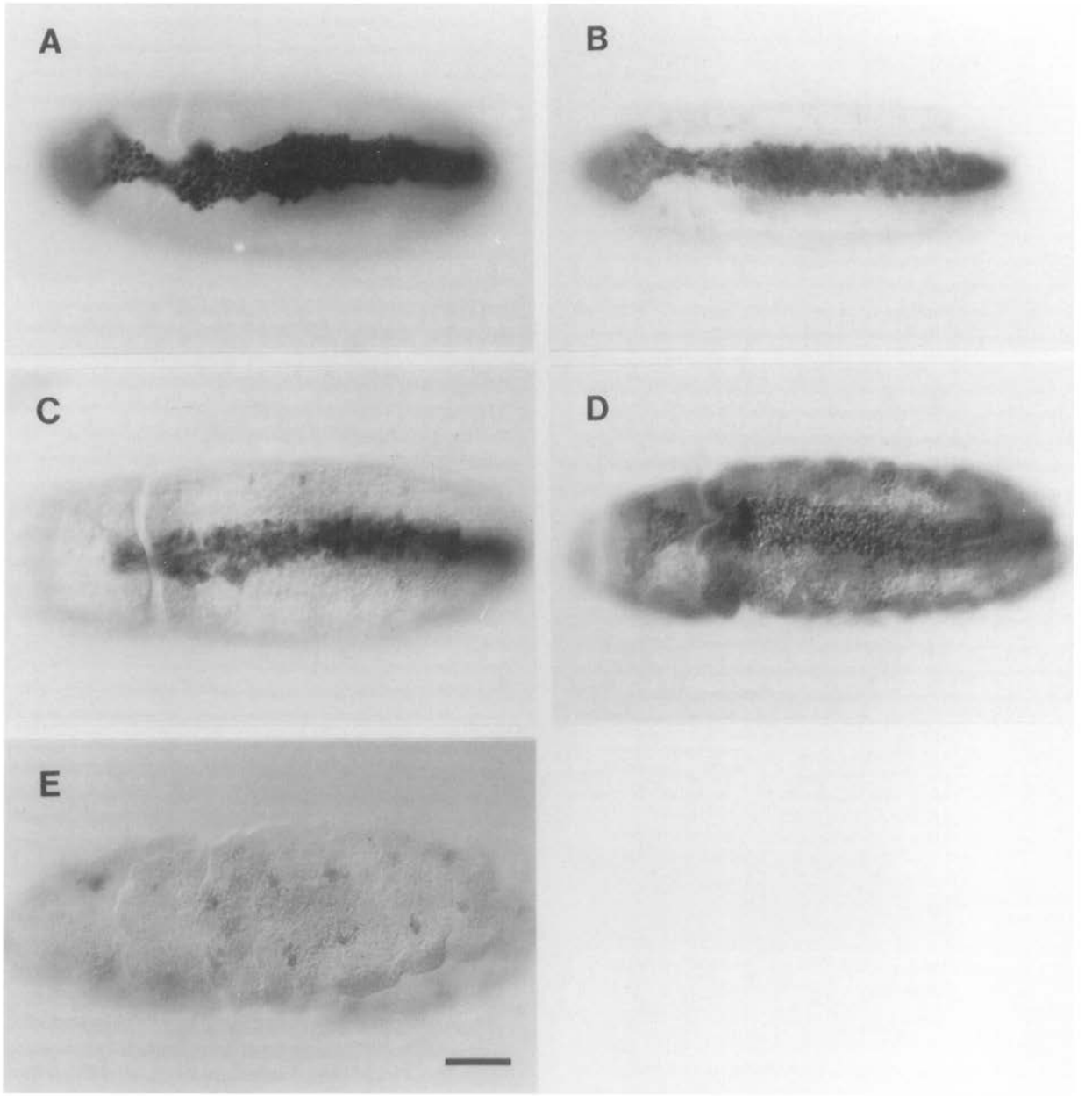

Figure 7. Altered Expression of *sim* in *sna* Mutants Results in a Similar Altered Expression of Other Midline Genes

Whole-mount *sna* mutant embryo (A) (*sna*$^{4.26}$ allele) carrying P[*sim/lacZ*] shows expression of β-galactosidase in an expanded region corresponding to the cells that in wild-type give rise to the mesoderm. *sna*$^{-}$ embryos containing the P[*Tl/lacZ*]*F336* (B), P[*91F/lacZ*]*242* (C), and P[*sli/lacZ*]*E-158* (D) elements showing expanded β-galactosidase expression similar to P[*sim/lacZ*]. No expression of P[*91F/lacZ*]*242* along the ventral surface is seen in a *sna*; *sim* double mutant embryo (E), indicating that the expanded expression of P[*91F/lacZ*]*242* observed in *sna* mutant embryos is dependent on *sim* function. The ventral surfaces of the embryos are shown. Scale bar: 50 μm.

can be observed along the ventral surface of a stage 13 or older *sli*$^{-}$ embryo (Figure 4I) similar to cells stained in a P[*sim/lacZ*]; *sli*$^{-}$ embryo (Figure 4F). These results show that while *sli* activity is required for normal midline cell development, it is not required to initiate or maintain P[*91F/lacZ*]*242* or P[*Tl/lacZ*] expression.

Ectopic Expression of *sim* in *sna* Mutants Results in Ectopic Expression of Downstream Genes

We have examined the expression of *sim* in the background of a number of genes that affect the dorsal–ventral axis. One of the most interesting results was the effect on *sim* by the *sna* gene. Mutations in *sna* result in a lack of ventral furrow formation, and analysis of larval cuticles implies that the gene affects only the formation of the mesodermal cells (Nüsslein-Volhard et al., 1984). Whole-mount in situ hybridization to Drosophila embryos using a *sna* cDNA probe indicates that before gastrulation *sna* is expressed specifically in the cells that give rise to mesoderm (data not shown). The expression of *sna* precedes

that of *sim*, and experiments were initially performed to determine whether *sna* regulates the expression of the *sim* gene. In situ hybridization experiments using a *sim* cDNA probe indicate that *sim* expression occurs over the ventral region of the blastoderm embryo in *sna* mutant embryos. Ectopic expression of β-galactosidase along the ventral surface of *sna* mutant embryos harboring the P[*sim/lacZ*] chromosome is also observed (Figure 7A). Thus, *sna* is normally required to repress *sim* expression in the cells that give rise to mesoderm.

One prediction of a model in which *sim* is required for the expression of downstream midline genes is that ectopic expression of *sim* should result in a parallel ectopic expression of the downstream genes. We have tested this by examining the expression of the *Tl*, *sli*, and 91F genes in *sna* mutants. The results shown in Figure 7B indicate that P[*Tl/lacZ*]*F336* is ectopically expressed as a broad strip of cells in the ventral section of *sna*$^-$ embryos. Examination of P[*91F/lacZ*]*242* in a mutant background revealed that the 91F gene is also expressed as a broad ventral stripe (Figure 7C); similar results have been obtained by examining the expression of an independent P[*lacZ*] insertion into the 91F gene (P[*91F/lacZ*]*87*) in *sna* mutants (Y. Hiromi and C. Goodman, personal communication). Similarly, P[*sli/lacZ*]*E-158* was also expressed at high levels, consistent with midline cell expression, as a broad ventral strip of cells in *sna* mutant embryos (Figure 7D). The requirement of *sim* for the ectopic expression of the downstream genes in *sna* mutants was shown by measuring the expression of P[*91F/lacZ*]*242* in *sna*; *sim* double mutants. The result shown in Figure 7E indicates that P[*91F/lacZ*]*242* expression is absent in the double mutant, confirming that the midline expression of the 91F gene requires *sim* function.

Discussion

sim Is Required for CNS Midline Cell Differentiation

Previously, it was shown in *sim* mutants that the longitudinal axon bundles of the mature embryonic CNS (stage 15 or later) become juxtaposed and that the CNS midline cells that normally separate them are absent or misplaced (Thomas et al., 1988). This defect could be observed earlier in development in a late stage 11 embryo. However, the exact fate of these cells was unknown. We find that the midline cells clearly fail to differentiate properly and take their proper position within the CNS in *sim* mutants. Even in potential hypomorphs, such as *sim*$^{B21\text{-}2}$ and P[*sim/lacZ*]; *sim*$^-$, the midline neurons and glia fail to form properly from their progenitors. These cells are eliminated from the CNS and reside near the ventral surface of the embryo. Null alleles of *sim* may reveal more severe phenotypes, but this has been difficult to analyze with the cell-marking reagents available.

Around the time when morphological defects appear in *sim* mutants, defects in gene expression occur in a set of genes normally expressed in the midline cells. Loss of expression of genes such as *sli*, *Tl*, *en*, *rho*, and 91F in *sim* mutants is consistent with the idea that these cells have not adopted their usual cell fate. Although the CNS midline cells in *sim* mutants acquire neither their proper fate nor their normal positions within the mature CNS, we do not know whether they adopt an alternative fate. They do not express any known genes that correlate with differentiated midline cells (i.e., *sli*, 91F gene, etc.) with the exception of *sim* and perhaps *rho*. The cells are possibly blocked as a type of midline progenitor or differentiate into another cell type.

sim Is Required for the Transcription of Midline Genes

We have shown that *sim* is required for the expression of five genes expressed in the midline cells: *sli*, *Tl*, *en*, *rho*, and the 91F gene. These experiments do not indicate how *sim* functions; it may act directly to control expression of these genes or it may play an indirect role. It was shown that the expression of *sli*, *rho*, *en*, and the 91F gene is absent in deficiencies of *sim*, and absent or reduced in hypomorphic alleles such as *sim*$^{B21\text{-}2}$ and the transheterozygote *sim*$^{B21\text{-}2}$/*Df(3R)ry*^{619}. Cell fate analysis of *sim*$^{B21\text{-}2}$ using a *sim* antiserum and the transheterozygote using P[*rho/lacZ*] indicates that the midline cells are present during the time (stage 9–11) when gene expression was assayed. Thus, reduced expression in these mutant embryos is due to a loss or reduction of gene expression and not simply due to rapid cell death.

Because *sim* nuclear expression precedes that of *sli*, *Tl*, and the 91F gene by a short time period, about 30–60 min, it is possible that *sim* is acting on the midline expression of these genes in a direct fashion. However, whether it acts directly or indirectly, *sim* does appear to be required for the transcription of these genes. The P[*Tl/lacZ*] insert, whose expression requires *sim* function, has been shown to lie upstream of the normal *Tl* transcriptional start site (H. Clark, K. Anderson, Y. Hiromi, and C. Goodman, personal communication), suggesting that *Tl* transcription requires *sim* activity. Similarly, the P[*sli/lacZ*]*E-158* insertion, whose expression requires *sim* activity, has also been mapped to either the 5′-flanking or 5′ untranslated region of the gene (J. Rothberg and S. Artavanis-Tsakonas, personal communication). The *sim* requirement for *en* and *rho* expression is less clear since these genes are expressed in the midline progenitors at the same time as *sim*. Their expression is then extinguished or reduced only later in the development of these cells in the absence of *sim*. Our results suggest that maintenance of high level *en* and *rho* expression in the midline cells is dependent on *sim*. This mode of *en* regulation is similar to the multi-tiered regulation required for the maintenance of *en* expression in the epidermal stripes (DiNardo et al., 1988).

Although *sim* encodes a nuclear protein, sequence analysis of its protein coding region does not provide strong evidence that it is a transcriptional regulatory factor. It has partial similarity to the *per* gene product of Drosophila, which is found in the nuclei of some cell types (Siwicki et al., 1988). However, since it is unknown how *per* functions within the nucleus, we are still uncertain as to the precise biochemical function of *sim*. There exists a polyglutamine tract at the carboxyl terminus of *sim*. Similar regions in other nuclear proteins have been shown to

be transcription activation domains (Courey and Tjian, 1988), but a similar role in *sim* requires experimental analysis. Our identification of potential target genes for *sim* regulation should ultimately allow us to determine the mode of action of a novel type of nuclear protein.

sna Is Required for the Repression of CNS Midline Genes in the Mesodermal Anlage

Genes such as *sim*, *Tl*, and P[*91F/lacZ*]*242* are normally not expressed at high levels in the ventral mesodermal anlage. However, mutations in the *sna* gene result in the activation of *sim* and genes downstream of *sim*, such as *Tl*, *sli*, and the gene at 91F, in these cells. The observation that the *Tl*, *sli*, and 91F genes are activated ectopically in *sna* mutants is consistent with the idea that *sim* is required for their midline expression. Lack of expression of P[*91F/lacZ*]*242* in *sna*; *sim* double mutants confirms that the ventral expression requires *sim* and is not simply due to loss of a direct regulation by *sna*. These results imply that *sna* acts as a ventral repressor of midline gene expression. *sna* mutants result in a lack of ventral furrow formation, and this may be due to a conversion of mesodermal cells to CNS midline cells. Consistent with this view is the observation that in *sna* mutants the ventral cells express all of the genes examined so far (*sim*, *Tl*, *sli*, and 91F) that are characteristic of midline cells. The predicted protein sequence of *sna* contains a zinc finger motif characteristic of DNA binding proteins (Boulay et al., 1987), and it is reasonable to predict that *sna* may directly repress the transcription of the *sim* gene in the mesodermal anlage.

It will be interesting to see whether genes such as *sim* and *sna* that act early in embryogenesis to control the development of the midline cells in Drosophila will have vertebrate homologs that perform a similar function. Homologous genes would most likely control the development of the floor plate cells, a group of specialized cells that lie at the base of the vertebrate spinal cord. It appears that the floor plate cells may share similar developmental and functional properties with the insect CNS midline cells (Dodd et al., 1988; Jacobs and Goodman, 1989a).

Midline Cell Development and the Function of *sim*

One model of *sim* function is that it acts as a master regulatory gene that directs the transcription of a battery of genes involved in the development of a specific group of CNS cells—those that lie along the midline. *sim* may thus function like the *twist* gene (Thisse et al., 1988), which is a transcription factor thought to act early in development to specify the mesodermal lineage in Drosophila. *twist* is first expressed in those blastoderm cells destined to give rise to mesoderm and continues to be expressed in those cells throughout mesodermal development. Similarly, *sim* is initially expressed in the blastoderm midline progenitors and continues to be expressed throughout the development of this cell lineage.

A model can also be proposed that describes the hierarchy of events involved in the development of the midline cells of the CNS. The early maternal-effect and zygotically expressed genes that set up the dorsal–ventral axis of the embryo result in the expression of *sim* in the blastoderm at its single cell–wide coordinates between the presumptive mesodermal cells and the cells that will give rise to the lateral cells of the CNS. In a similar fashion, genes that set up the termini and anterior–posterior axis of the embryo restrict the formation of the midline CNS cells to a subset of the blastoderm cells. *sim*, and perhaps other genes, is required for the expression of a group of downstream genes, such as *sli*, *Tl*, *rho*, and the 91F gene. These genes continue the developmental program of midline CNS development, which includes neuronal and glial cell formation, axon guidance, and functional differentiation.

It is interesting that *sli*, *rho*, and *Tl* all have protein sequence motifs indicating that they may be involved in cellular adhesion, communication, or signal transduction. The *sli* protein contains a set of repeats similar to those found in epidermal growth factor, *Notch*, and *lin-12* (Rothberg et al., 1988); *Tl* contains a set of leucine-rich repeats found in several proteins implicated in protein–protein interactions (Hashimoto et al., 1988), and *rho* encodes a putative transmembrane protein (Bier et al., 1990). A prominent role of *sim* in specifying midline cell identities may be its requirement for the activation of a set of specific cell–cell interaction gene products in the midline progenitors. These interactions would participate in the differentiation of the progenitor cells into CNS midline cells. Thus, midline cell development may require cell–cell interactions, just as cell–cell interactions are important for the development of the Drosophila visual system (Cagan and Ready, 1989; Rubin, 1989; Zipursky, 1989) and the decision of an ectodermal cell to become either a neuroblast or an epidermoblast (Knust and Campos-Ortega, 1989). Our results directly demonstrate that *sli* is involved in the formation of the CNS midline cells. Since *sli* has a severe midline phenotype similar to *sim*, it could play a particularly important role in the development of these cells. Presumably, it is predominantly the absence of midline *sli* expression in *sim* mutant embryos that results in the collapsed CNS phenotype. The effects of mutations of *Tl*, *rho*, and the 91F gene on midline CNS development are presently unknown.

en is a homeobox-containing transcription factor (Fjose et al., 1985; Poole et al., 1985); it thus may regulate the transcription of genes involved in midline CNS development. It is restricted to a subset of midline cells and their progenitors that lie in the posterior region of each ganglion. These cells include a subset of the VUM cells and median neuroblast progeny. The effects of *en* mutations on CNS midline development are unknown, but it may be involved in specifying the identity of the midline progenitors that lie in the posterior of the segmental neuroepithelium, analogous to its role in establishing cell identity in the blastoderm. Later in midline cell development, *en* may also play a role in the differentiation of their neuronal progeny.

sim is expressed throughout the embryonic development of the midline cells. Initially, it is expressed in all of the midline progenitors; these cells will give rise to midline neurons, glia, and other nonneuronal cells. This early function appears necessary for the expression of many or all of the genes involved in differentiation of the progenitor

cells. Later, *sim* expression is restricted to the midline glia. This later expression of *sim* may be required for the maintenance of expression of one or more of the genes involved in midline cell differentiation. Alternatively, it may be required for the expression of a set of genes that act specifically in the function of the midline glia. Future progress in understanding the role of *sim* in regulating the expression of midline genes and in directing the development of midline cells will come from detailed biochemical, cell culture, and germline transformation experiments.

Experimental Procedures

Drosophila Strains

The *sim* strains used were obtained from the Chovnick laboratory. The $sim^{B21\text{-}2}$ and sim^{H9} alleles were both EMS induced. The $Df(3R)ry^{619}$ and *Df(3R)l26d* chromosomes are mutant for the *sim* gene and genes flanking *sim* on both sides (Hilliker et al., 1980), and thus are likely to be null alleles. All *sim* strains were kept as balanced stocks over TM3, TM6B, or MKRS balancer chromosomes. The *sna* allele, $sna^{4.26}$, is a strong allele derived from an X-ray mutagenesis (Nüsslein-Volhard et al., 1984). The *sli* allele, sli^{iG107}, was EMS induced (Nüsslein-Volhard et al., 1984) and does not make detectable *sli* protein (Rothberg et al., 1988). Both strains were obtained from the Bowling Green stock center.

The P[*ftz/lacZ*]/TM3 balancer was created by genetically transposing an X chromosome P[*ftz/lacZ*] element (Hiromi et al., 1985) onto TM3 *Sb e pp ry*. The source of P element transposase was the P[$ry^{+}(\Delta 2\text{-}3)$] chromosome (Robertson et al., 1988). β-galactosidase expression from the P[*ftz/lacZ*] TM3 strain can be reliably detected beginning in the syncytial blastoderm through stage 15 of embryogenesis.

Construction of P[*sim/lacZ*] and Germline Transformation

A 7.8 kb BamHI fragment of the *sim* gene was cloned into the BamHI site of cosP*white*β-gal. This vector contains a mini-*white* gene (Pirrotta, 1988), sequences for P element transposition, and the *lacZ* gene with an SV40 polyadenylation site at its 3′ end. Insertion of the *sim* DNA fragment results in a predicted fusion protein of the 482 N′-terminal amino acids of *sim* coupled to β-galactosidase starting at amino acid 7.

P[*sim/lacZ*] DNA was injected (Rubin and Spradling, 1982) into w^{1118} flies with p25.7wc helper P element DNA (Karess and Rubin, 1984), and four independent transformants were obtained. One insertion was on the X chromosome and the other three were autosomal. Three of the four lines are homozygous viable, and they all showed the same spatial, temporal, and quantitative expression pattern when analyzed for β-galactosidase expression by X-Gal histochemistry. Staining is localized to the nuclei of cells presumably due to the presence of a nuclear localization sequence on the *sim* coding sequence fused to β-galactosidase.

Cell Fate Analysis of *sim* Mutant Strains Using P[*sim/lacZ*]

Cell fate analysis of *sim* mutants was carried out by crossing the P[*sim/lacZ*] X chromosome onto *sim* mutant backgrounds. This was done with the EMS-induced *sim* alleles $sim^{B21\text{-}2}$ and sim^{H9}, and the deficiencies $Df(3R)ry^{619}$ and *Df(3R)l26d*. All of the *sim* alleles were balanced over P[*ftz/lacZ*] TM3 except *Df(3R)l26d*, which was kept over MKRS. Each strain was stained with anti-HRP to confirm that about one-fourth of the embryos had a *sim* mutant CNS phenotype.

Enhancer Trap Lines

The 242 and 87 P element insertion lines were isolated and generously donated by Yasushi Hiromi. Both inserts were mapped to 91F. The 242 line was created by an insertion using a P element with a *ftz* promoter fused to *lacZ*. The presence of the *ftz* promoter in this line probably results in the expression of β-galactosidase observed in the lateral glioblasts (Figure 5E; Jacobs et al., 1989). The 87 line was derived from a screen using a P element promoter. Both lines were recombined onto $Df(3R)ry^{619}$ to observe the effect on midline β-galactosidase expression in a *sim* mutant background. This recombinant chromosome was balanced over P[*ftz/lacZ*] TM3. Crosses between $sim^{B21\text{-}2}$/P[*ftz/lacZ*] TM3 and P[91F/*lacZ*] $Df(3R)ry^{619}$/P[*ftz/lacZ*] TM3 to generate a *sim* transheterozygote indicated that the observed loss of midline staining in embryos without the balancer was due solely to the absence of *sim*.

The enhancer trap insertion into the *sli* locus, $sli^{E\text{-}158}$, was generously provided by Alex Kolodkin and Corey Goodman. Homozygotes of $sli^{E\text{-}158}$ are lethal at 25°C, and the insertion chromosome fails to complement sli^{iG107}. However, it does not show a *sli* mutant CNS phenotype at 25°C. The insertion maps within about 500 bp 5′ to the start site of translation in either the *sli* 5′ untranslated or flanking region. The effect of a *sim* mutant background on P[*sli/lacZ*] E-158 expression was determined using the following strains: P[*sli/lacZ*]*E-158*/CyO; $sim^{B21\text{-}2}$/MKRS and P[*sli/lacZ*]*E-158*/CyO; *Df(3R)l26d*/MKRS. The *sim* homozygous mutant embryos could not be directly observed. However, midline staining was observed in all stage 11–13 P[*sli/lacZ*]*E-158*/CyO embryos allowed to develop at 25°C, whereas loss of midline staining was observed in about one-fourth of the embryos resulting from the P[*sli/lacZ*]*E-158*/CyO;*Df(3R)l26d*/MKRS stock and was reduced or absent in about one-fourth of the embryos from the P[*sli/lacZ*]*E-158*/CyO; $sim^{B21\text{-}2}$/MKRS stock.

A P element insertion into the *Tl* locus, F336, was provided by Yasushi Hiromi and was generated using his enhancer trap vector with the *ftz* promoter. This insertion, which resides at 97D, was recombined onto a chromosome with $Df(3R)ry^{619}$ and balanced over P[*ftz/lacZ*] TM3.

A P element enhancer trap insertion likely to reside in the *rho* locus was isolated in our laboratory using a P element promoter vector (S. Hu, B. Matthews, and S. T. Crews). The P element is assumed to reflect *rho* gene expression because the insertion maps to 62A, the location of *rho*, and expresses β-galactosidase in a pattern similar to the *rho* gene and another enhancer trap insertion known to reside in the *rho* locus (Bier et al., 1990). The P[*rho/lacZ*] insertion was recombined onto a $Df(3R)ry^{619}$ chromosome balanced over P[*ftz/lacZ*] TM3, and its expression was assessed in homozygous deficiency and $Df(3R)ry^{619}/sim^{B21\text{-}2}$ transheterozygote backgrounds.

Antibodies and Histochemistry

Embryos containing a P[*lacZ*] element were stained for β-galactosidase expression using a monoclonal antibody against β-galactosidase (1:500 dilution; Promega). *en* expression was assayed using a monoclonal antibody that recognizes *en* (4D9) kindly provided by Nipam Patel, Tom Kornberg, and Corey Goodman (Patel et al., 1989a). The whole-mount antibody staining procedure was essentially that of Patel et al. (1989a), and antibody reactivity was visualized using a peroxidase mouse IgG Vectastain ABC kit (Vector) and diaminobenzidine. Experiments using *sim* antiserum were performed in a similar fashion using a peroxidase rabbit Vectastain ABC kit. Embryos were cleared in methyl salicylate and either mounted in Permount or in methyl salicylate beneath a bridged coverslip. The embryos were examined and photographed using a Zeiss Axiophot microscope and Nomarski optics.

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