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Molecular Genetics of the *single-minded* Locus: A Gene Involved in the Development of the Drosophila Nervous System

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Summary

The embryonic neuroepithelium of Drosophila gives rise to the central nervous system. We have studied the mutant phenotype and expression of a gene, *single-minded* (*sim*), which is involved in generating a specific region of this neuroepithelium. In *sim* mutant embryos, a subset of neuronal and nonneuronal precursor cells lying along the midline fail to emerge with the rest of the neuroepithelium. We have identified the *sim* transcription unit and have shown by in situ hybridization to embryos that the *sim* gene is expressed specifically in the midline neuroepithelium. Both the mutant phenotype and the temporal and spatial expression of transcripts suggest that the *sim* gene plays a key role in the emergence of this subset of cells along the midline of the developing central nervous system.

Introduction

The precise cellular patterns generated during neuronal development depend upon the production of specific neurons and specialized nonneuronal cells in characteristic locations, and upon the ability of these cells to interact with one another as neurons find and eventually synapse with their appropriate targets. In Drosophila, many of the events underlying neuronal development have been described at the level of single cells (e.g., Poulson, 1950; Hartenstein and Campos-Ortega, 1984; Thomas et al., 1984; Bastiani et al., 1985). The process of neurogenesis begins early in embryogenesis with the emergence of the neuronal precursors. Just after gastrulation, certain ectodermal cells delaminate from the ectodermal epithelial layer at specific locations to become the precursors of the central nervous system (CNS). Individual precursors acquire unique fates based upon their location within the developing neuroepithelium (Doe and Goodman, 1985), eventually giving rise to stereotyped sets of uniquely identifiable neuronal progeny. The neuronal progeny then differentiate, elongating axons along stereotyped routes to find and eventually synapse with their appropriate targets.

Although these events have been described in some detail at the cellular level, only a few of the molecular mechanisms underlying them are beginning to be elucidated. One of the best-understood events of neuronal development is the first step in neurogenesis: the decision of the ectodermal cells within the developing neuroepithelium to become either neuronal or epidermal progenitors. By identifying mutations causing an overproduction of neuronal precursors at the expense of the epidermis, a set of genes, called the neurogenic loci, has been identified that controls this decision (Poulson, 1937; Lehmann et al., 1983). The recent molecular cloning and sequencing of *Notch* (Wharton et al., 1985; Kidd et al., 1986) and other neurogenic loci (Knust et al., 1987) suggest that at least some of the neurogenic genes are involved in cell–cell interactions underlying the neuroblast/epidermal decision.

We have searched for mutations that disrupt specific features of embryonic neuronal development in order to identify additional genes and eventually gene products involved in the development of the nervous system. In particular, we wished to find mutations that altered the stereotyped pattern of neuronal precursor cells; our expectation is that such mutations might represent genes controlling steps in the acquisition of unique fates by subsets of neuroblasts (NBs). In our initial search for such mutations, we have screened embryos homozygous for previously defined chromosomal deletions. Here we report on the characterization and molecular analysis of a gene, the *single-minded* (*sim*) locus, uncovered by one of the deletions in the screen and involved in neurogenesis. The *sim* locus was originally identified as a lethal complementation group and named *l(3)S8* (Hilliker et al., 1980); we have renamed the locus based on its nervous system phenotype. Analysis of the mutant phenotype and the gene's temporal and spatial pattern of expression indicate that the *sim* gene product is required for the emergence of a specific subset of neuronal and nonneuronal precursor cells lying along the midline of the developing neuroepithelium. In a companion paper (Crews et al., 1987) we present the sequence of the *sim* gene and show that it encodes a protein localized to the nuclei of the midline cells.

Results

Neurogenesis in Wild-Type Embryos

The bulk of the embryonic CNS in Drosophila is produced by a segmentally repeated set of neuronal precursor cells derived from the ventral region of the ectoderm called the neurogenic region (Hartenstein and Campos-Ortega, 1984; Doe et al., submitted). At the cellular blastoderm stage, the presumptive neurogenic region lies ventrolaterally on each side of the embryo, separated by the presumptive mesoderm near the ventral midline. During gastrulation (~3 hr postfertilization) the presumptive mesoderm invaginates along the ventral midline into the interior of the embryo, forming an anterior-posterior groove called the ventral furrow. As a result, the neurogenic region of the ec-

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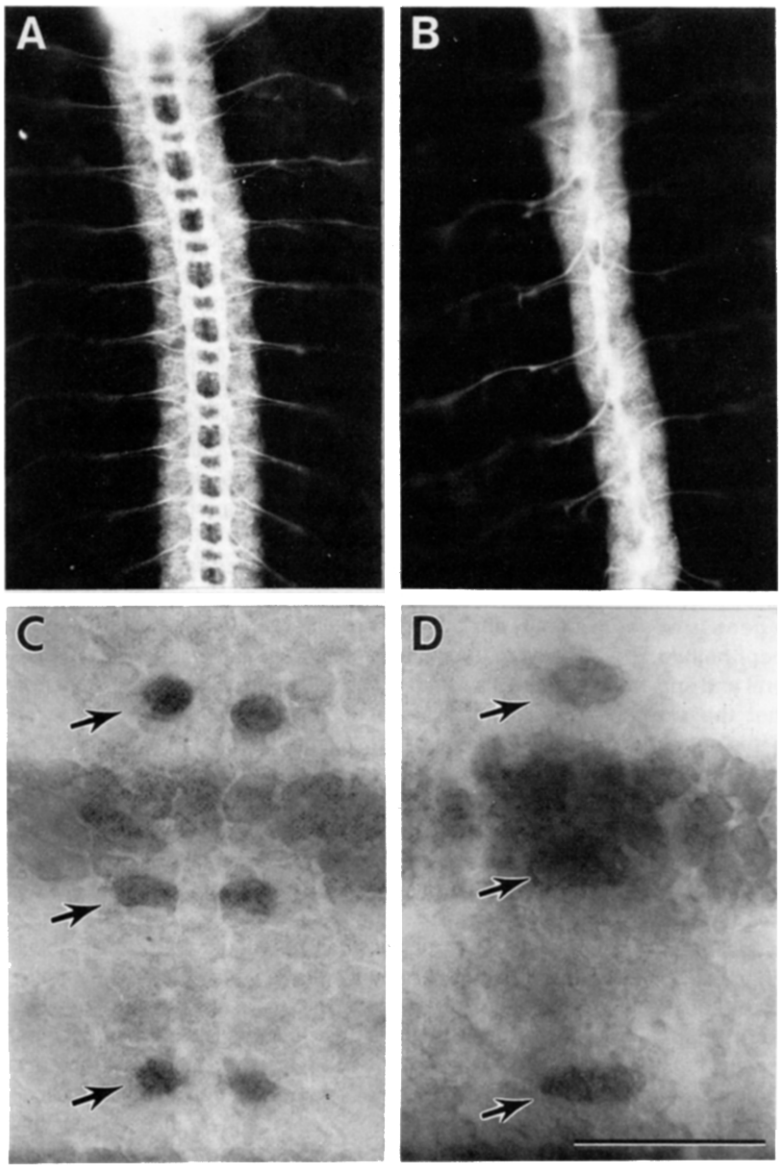

Figure 1. Comparison of Nervous Systems of Wild-Type and *sim* Mutant Embryos

(A, B) Comparison of a dissected wild-type (A) and *sim* mutant (B) embryo stained with fluorescein isothiocyanate–conjugated antibodies against HRP, which stain the cell surfaces of all neurons (Jan and Jan, 1982). Anterior is up; the extent of the ventral nerve cord is shown. In *sim* mutants, cells lying along the midline between the two longitudinal connectives, including the MP1s, the ventral unpaired median neurons, and the MECs, are missing. The two commissural tracts in each segment are missing, and the connectives are fused at the midline.

(C, D) Comparison of the position of MP2 in whole mounts of 7 hr wild-type and *sim* mutant embryos, respectively. Embryos carried p[ry, ftz/lacC], which expresses β-galactosidase from the *ftz* promoter (Hiromi et al., 1985), and were stained with antibodies to β-galactosidase. Anterior is up. Three segments are shown, each containing a pair of MP2 precursors (arrows). In the wild-type embryo each of the bilaterally paired MP2 precursors is located in the first column of NBs, separated by cells lying at the midline. In the mutant the midline cells are absent, and the two MP2 precursors in each segment lie directly adjacent at the midline. The single stripe in each micrograph is the "pair rule" staining of *ftz* expression in the epidermal cells above the plane of focus. (C) is courtesy of C. Doe. Scale bar: (A, B) 80 μm; (C, D) 40 μm.

todermal epithelium is brought to the ventral surface of the embryo.

Just after gastrulation certain ectodermal cells within the neurogenic region begin to enlarge and delaminate dorsally from the ventral surface to become NBs. This process occurs over a period of approximately 4 hr. The NBs act as stem cells, producing families of neurons. As is the case in other insects, individual NBs can be uniquely identified by virtue of their position within the neuroepithelium and the characteristic families of unique neurons they produce. In each segment of the Drosophila ventral nerve cord there are two bilaterally symmetric plates of 25 NBs each plus a single unpaired NB, the median NB (MNB), at the midline (Hartenstein and Campos-Ortega, 1984; Thomas et al., 1984; Doe et al., submitted). In addition to the NBs, there is a second class of neuronal precursors, called the midline precursors (MPs) (Bate and Grunewald, 1981). The MPs lie near the midline and divide only once, producing two daughter neurons (called MP progeny). In Drosophila there is a single MP1 cell and two MP2s per segment. Like the MNB, MP1 is unpaired at the midline, dividing to produce two MP1 progeny neurons; in contrast, the MP2s are bilaterally paired, dividing to give rise to a pair of MP2 progeny on each side of the segment.

Situated between the two bilaterally symmetric NB plates is a midline strip one to two cells wide; in contrast to the lateral neuroepithelium, all of these cells delaminate along with the lateral NBs. These smaller, morphologically distinct cells have been described previously by Poulson (1950) and termed mesectodermal cells because of their location at the dorsal surface of the developing CNS, near the mesoderm. However, since these cells are clearly ectodermal in origin and give rise to both neurons and specialized nonneuronal cells of the CNS, we will refer to them simply as the midline cells of the neuroepithelium. The MNB and the MP1 arise from these midline cells. In contrast, the bilaterally paired MP2s arise more laterally, at the medial edge of the lateral neuroepithelial

plates (Doe et al., submitted). In addition to the MNB and MP1, the midline cells also give rise to a set of six specialized midline ectodermal cells (MECs) in each segment of the CNS. The MECs are nonneuronal cells that lie at the dorsal surface of the CNS and have cell processes extending ventrally around the two axon commissures; the MECs may be involved in the formation of the two axon commissures in each segment. Since there are approximately 12 midline cells in each segment, it is likely that they give rise to other cells within the CNS in addition to the six MECs, the MNB, and the MP1.

By hour 10 the first growth cones are extended from a characteristic set of early-differentiating neurons derived from certain NBs and from MP1 and MP2. The growing axons from these and other neurons can be visualized with antibodies to horseradish peroxidase (HRP), which stain the surface of all neurons (Jan and Jan, 1982). By hour 11, the differentiating neurons have established a framework of axon bundles as shown in Figure 1A. This framework consists of two bilaterally symmetrical longitudinal connectives running the length of the CNS and, in each segment, two commissures connecting the two sides of each ganglion. These axon bundles form the basic scaffold upon which the later-developing neurons will grow.

Neurogenesis in *sim* Mutant Embryos

Our initial screen for mutations that alter embryonic neuronal development, from which the *sim* phenotype was identified, consisted of using an antibody to HRP to examine the nervous systems of embryos homozygous for previously isolated chromosomal deletions. One of these deletions, the third chromosomal deficiency Df(3R)ry^{619}, gives rise to a striking defect in the pattern of neuronal cell bodies. By examining the phenotypes of point mutations and additional deficiencies within the region, we discovered that the phenotype associated with Df(3R)ry^{619} is due to the removal of a single lethal complementation group within the region, the *sim* locus.

Homozygous *sim* mutant embryos are late embryonic-lethal; they develop a nervous system, muscles, and gut. In mutant 11 hr embryos, however, those identified cells derived from the strip of midline cells are missing. These cells include the two MP1 progeny, the ventral unpaired median neurons (VUMs), which are not the progeny of the MNB (Goodman et al., 1984), and the set of specialized ectodermal cells, the MECs. Figure 1B shows the CNS of an 11 hr *sim* mutant embryo stained with the anti-HRP antibody. In the absence of these cells at the midline, the two commissural tracts in each segment are largely missing and the two bilaterally symmetrical longitudinal connectives appear fused into a single connective at the midline.

The nervous system defect caused by mutations in *sim* is not due to the generation and subsequent death of the missing postmitotic cells within the CNS. Instead, the defect is due to a specific alteration in the pattern of precursors that normally give rise to the missing cells. In 6 hr mutant embryos, at the time of emergence of the neuronal precursors, the midline cells that normally delaminate with the NBs to give rise to the MNB, MP1, and the specialized MECs are absent from the delaminating NB sheet, producing a concomitant shift toward the midline of the normally more lateral MP2s and NBs. Thus *sim* mutations appear to delete the midline cells of the NB plate specifically, leaving the bilateral NBs relatively intact.

This shift in the NB pattern can clearly be seen by marking each of the lateral MP2 precursors. The MP2 precursors are the first neuronal precursors to express the *fushi tarazu* (*ftz*) gene product (Doe et al., submitted). Transformed flies carrying the P[ry,ftz/lacC] element, which contains *lacZ* driven by the *ftz* promoter, express β-galactosidase in those cells that express *ftz* (Hiromi et al., 1985). The *ftz*-expressing cells, including the MP2 precursors, can easily be detected with antibodies to β-galactosidase. To analyze *sim* embryos, flies carrying chromosomes bearing a P[ry,ftz/lacC) element were crossed into *sim* mutant lines. In Figures 1C and 1D the position of MP2 in wild-type and *sim* mutant 6 hr embryos carrying this *ftz-lacZ* element is compared. Each MP2 (arrows in Figure 1C) normally arises within the most medial NB column in each segment. In the mutant, however, the midline cells, which are normally situated between the MP2s, are missing, and the MP2s now lie directly adjacent at the midline (arrows in Figure 1D).

Genetics of the *sim* Locus

Mutations at the *sim* locus were originally isolated and identified as a lethal complementation group, called *l(3)S8*, in a detailed genetic study of the 87D,E region surrounding the *rosy* locus (Hilliker et al., 1980). Figure 2 gives a genetic map adapted from their study showing the complementation groups and genetically defined deficiency breakpoints. The *N74* deficiency was generated and characterized by Gausz et al. (1986). We have studied six alleles of the *sim* locus originally isolated by Hilliker et al. (1980): *S8*, *B21-2*, *B30-1*, *B13-4*, *H79*, and *H9*. All alleles are homozygous lethal in combinations with each other, and all show the same nervous system phenotype as deletions of the *sim* locus (ry^{619}, *l26d*, and ry^{81}). Thus each allele behaves as a null mutation, completely removing the wild-type gene function.

The *sim* locus is also defined by deficiency breakpoints flanking the locus (Hilliker et al., 1980). To the right, the proximal breakpoint of Df(3)*l26c* separates *sim* from the neighboring *B16-l* complementation group. To the left, the distal breakpoints of Df(3)ry^{75} and Df(3)ry^{614} separate *sim* from *piccolo* (*pic*). These deficiencies complement the *sim* alleles, and embryos homozygous for each deficiency, although embryonic lethal because of the removal of other essential genes, are sim^{+} since they show a wild-type nervous system phenotype. In addition, individuals carrying either ry^{75} or ry^{614} in *trans* with *l26c* survive to produce fertile adults. Thus the region of the genome between these breakpoints is sufficient to allow wild-type *sim* function and defines the maximum extent of the *sim* locus.

Localization of the *sim* Gene

The DNA in the chromosomal region that contains the *sim* gene has previously been isolated in a chromosomal walk between *ry* and *Ace* (Spierer et al., 1983). Mapping studies

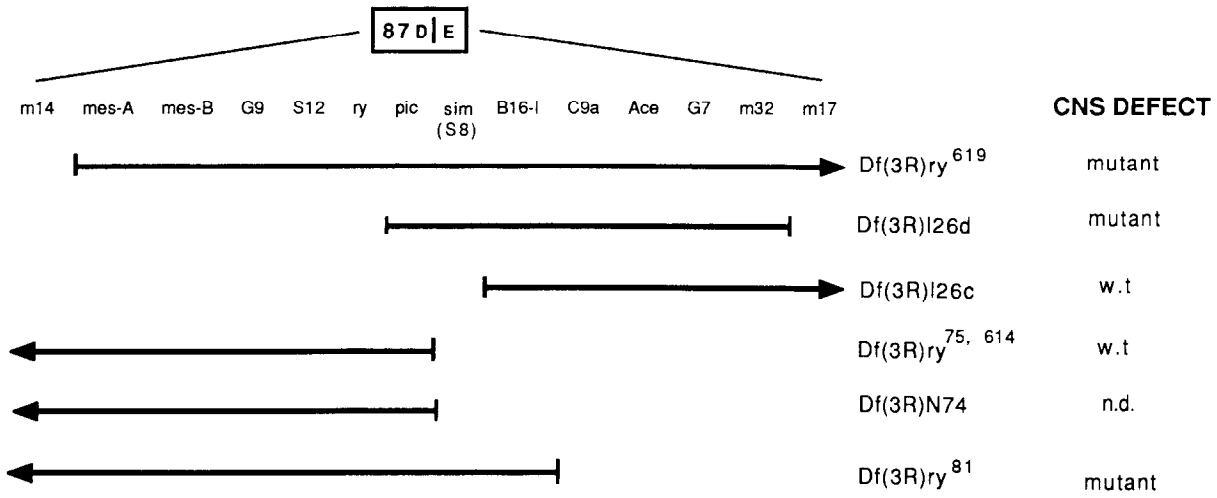

Figure 2. Genetic Map of the Region Surrounding the *sim* Locus

The map is adapted from the work of Hilliker et al. (1980). At the top is the 87D,E cytological interval of the polytene chromosome, which contains the region surrounding the *sim* locus. Below it is the complementation map of the region showing 14 of the complementation groups identified by Hilliker et al. (1980). The extent of deficiencies used in this study and their corresponding neuronal phenotypes (mutant, wild-type, or not determined) are shown. Df(3R)*N74* was isolated and characterized by Gausz et al. (1986). The extent of the *sim* locus is bounded on the right by the breakpoint of Df(3R)*l26c*, on the left by breakpoints of Df(3R)ry^{75}, Df(3R)ry^{614}, and Df(3R)*N74*.

indicated that the *sim* locus should be located within a 60 kb stretch of DNA. We obtained their set of overlapping genomic clones encompassing this region. To localize the *sim* gene more precisely on the DNA map, we mapped the flanking breakpoints defining the *sim* locus of the relevant deficiencies. Polytene chromosomes from larvae heterozygous for a deficiency chromosome were hybridized with biotin-labeled restriction fragments from the region, and assayed for the presence or absence of a signal on the deficiency-bearing chromosome. The breakpoints of the deficiencies are shown as hatched blocks in Figure 3 above the clones and restriction map of the region. The locations of the deficiency breakpoints are derived from a combination of our data and that of Spierer et al. (1983) and Gausz et al. (1986). Since each of the deficiency-bearing chromosomes contains an intact *sim* locus, the gene must lie within the 26 kb interval between the flanking breakpoints shown in Figure 3.

To identify the transcription units within the region, we hybridized Northern blots of embryonic RNA to radiolabeled DNA fragments that together covered the entire 60 kb region of DNA. Hybridizations were done both to total

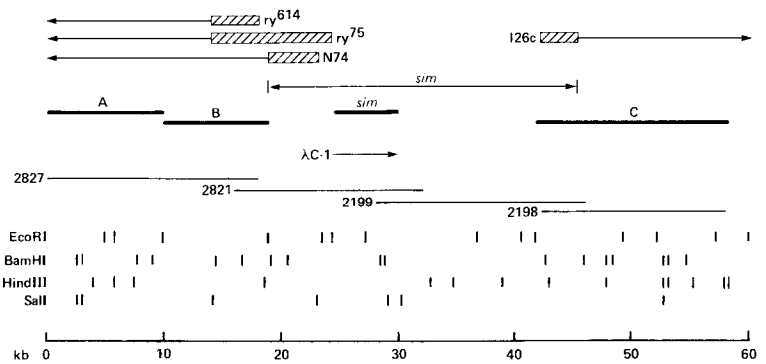

Figure 3. Restriction Map and Transcription Units of the Region Surrounding the *sim* Locus

The 60 kb of DNA surrounding the *sim* locus is indicated at the bottom; the restriction map is above it. This map is identical to that of Bender et al. (1983) except for an additional SalI site at 3 kb and an additional BamHI site at 29 kb. Above the restriction map are the positions of the four genomic clones used in this study, and the location of the λC1 cDNA clone. The locations of the four transcription units (A, B, C, and *sim*) were determined by hybridizing labeled EcoRI fragments to Northern blots. We did not detect hybridization of the EcoRI fragment lying between 52 and 57 kb, but we consider the flanking hybridizing regions to encode the same gene since they both hybridize to a transcript of the same size and developmental distribution. The *sim* transcription unit was more carefully defined by additional genomic and cDNA sequence experiments and by cDNA clone hybridization to Southern blots of restriction-enzyme-digested genomic DNA clones. The breakpoints of the four deficiencies flanking the *sim* locus are shown at the top. The boundaries of each breakpoint are indicated by the hatched boxes. The locations of the ry^{75}, ry^{614}, and *l26c* deficiency breakpoints were derived from a combination of our data and that of Spierer et al. (1983). The breakpoint of *N74* was mapped by Gausz et al. (1986). Shown directly below is the 26 kb stretch of DNA between the *N74* and *l26c* breakpoints, which defines the maximum limits of the *sim* gene.

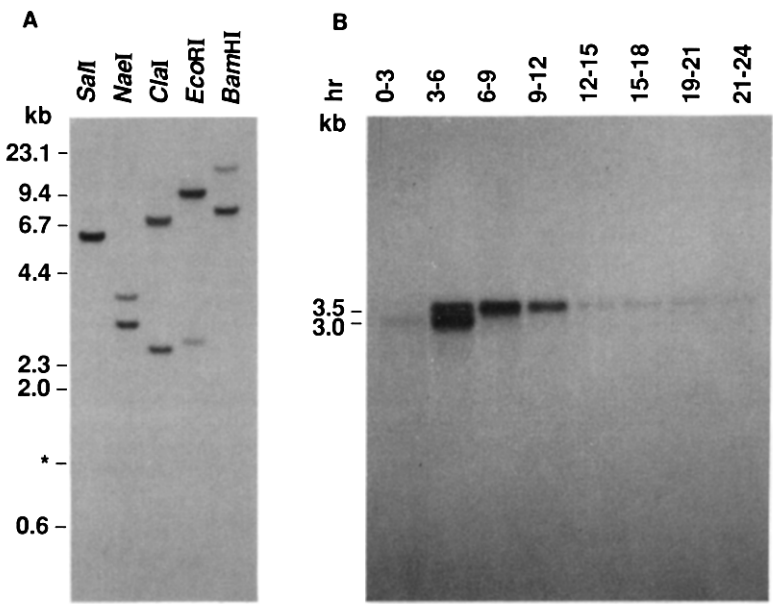

Figure 4. Molecular Analysis of the *sim* Gene
(A) *sim* is present as a single-copy gene. Drosophila Oregon R genomic DNA was digested with five different restriction enzymes, as indicated, and hybridized under moderately stringent conditions to the λC1 cDNA clone. Although two bands in each digest hybridize, each band corresponds to the *sim* locus map. Sizes of the molecular weight markers (in kb) are indicated at left. The asterisk marks the position of a faint 1.1 kb SalI band.
(B) Expression of *sim* transcripts. Poly(A)$^+$ RNA isolated from embryos at successive 3 hr intervals was probed with the λC1 cDNA clone. Lanes are marked at the top with the specific stage; numbers refer to embryonic development in hours postfertilization. Two embryonic transcripts, 3.0 and 3.5 kb in size, are detected.

RNA and to poly(A)$^+$ RNA for greater sensitivity. We estimate that we can detect a transcript as rare as one molecule in 10^6 of the respective RNA samples. Four transcription units, shown in Figure 3, were identified and defined by the occurrence of different-sized transcripts. The locations of the A, B, and C transcription units were determined by Northern blot analysis, and the *sim* transcription unit was further defined by hybridization and sequence analysis of an embryonic cDNA clone, λC1 (see below). Transcription unit A was previously identified by Spierer and his colleagues (Hall et al., 1983; Bossy et al., 1984), but transcription units B, C, and *sim* have not previously been observed in embryonic RNA populations. Transcription units A and B lie completely outside the 26 kb region that defines the *sim* gene, and transcription unit C lies partially outside the region. The only transcription unit that lies completely within the 26 kb interval is the transcript labeled *sim* in Figure 3. In situ hybridization experiments with embryos, as described below, provide strong additional evidence that this transcription unit is indeed the *sim* gene.

For further experiments, we isolated cDNA clones from a 3–12 hr cDNA library constructed by Larry Kauver (Poole et al., 1985). The largest clone isolated, the 2.8 kb λC1 clone shown in Figure 3, was used as a probe in the experiments described below.

To determine whether the *sim* transcription unit is a member of a multigene family, we hybridized, under moderately stringent conditions, the λC1 cDNA clone to restriction-enzyme-cut genomic wild-type DNA. The results of five different digests are shown in Figure 4A. Each of the five digests shows two bands of hybridization, but all of these bands correspond to the *sim* transcription unit. This result indicates that the *sim* gene is a single-copy gene and does not belong to a family of highly related genes.

sim Transcripts

To study the temporal expression of *sim*, Northern blots of poly(A)$^+$ RNA from various stages of development were hybridized with the λC1 cDNA clone. We have examined RNA collected at 3 hr intervals during the entire 22 hr of embryogenesis. As shown in Figure 4B, there are two major transcripts that can be resolved on this gel. A 3.0 kb transcript is expressed at low levels at hours 0–3 of development, and at relatively high levels between hours 3 and 6. No expression of the 3.0 kb transcript is observed after 6 hr. A second transcript, 3.5 kb in length, is first expressed at 3–6 hr, peaks at 6–9 hr, and diminishes at 9–12 hr of development. It is expressed throughout the rest of embryogenesis at low levels. The peak levels of *sim* expression during embryogenesis thus coincide with the period of neurogenesis, and also correlate with the onset of detectable defects in *sim* mutants.

Spatial Expression of *sim* Transcripts

The spatial distribution of *sim* transcripts was investigated by hybridizing a ^{35}S-labeled EcoRI fragment containing most of the *sim* gene (27–32 kb on the map; Figure 3) to whole embryos at specific stages of development, followed by sectioning. Expression is first detected at the cellular blastoderm stage just prior to the commencement of gastrulation, in a two-cell-wide strip of cells running the length of the neurogenic region on each side at the border of the presumptive mesoderm. Figure 5A shows a mid-length cross section of an embryo beginning gastrulation. The ventral furrow is in the process of being formed by the invagination of the presumptive mesoderm, which in cross section consists of approximately eight cells to either side of the ventral midline. In the companion dark-field photograph (Figure 5B), the silver grains are concentrated over a region one to two cells in diameter (arrows) at the border of the neurogenic region and the presumptive mesoderm.

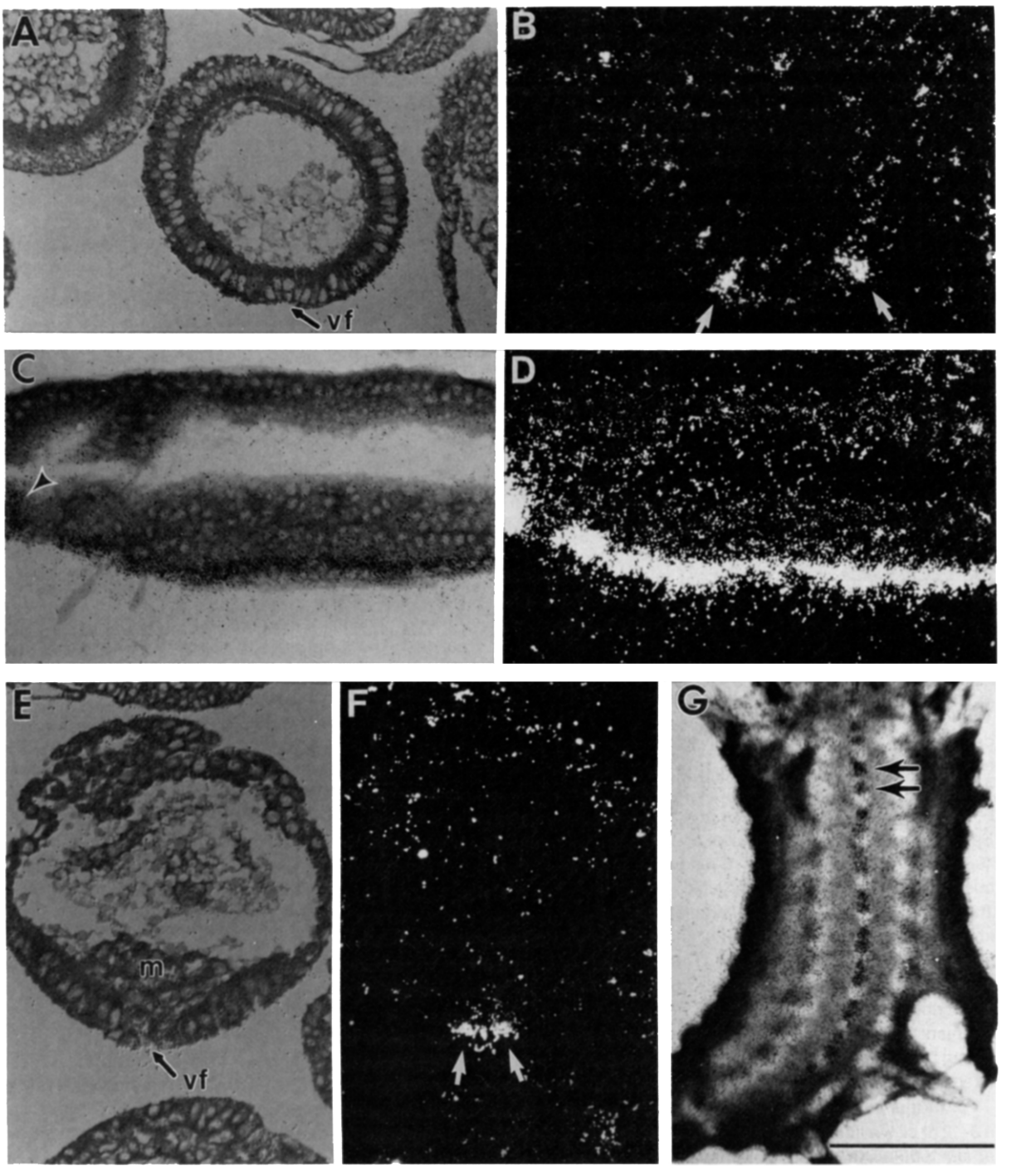

Figure 5. Spatial Expression of *sim* Transcripts in Embryos

A ^{35}S-labeled probe containing the 5′ EcoRi genomic fragment of the *sim* transcription unit was hybridized to whole embryos that were then sectioned for autoradiography (A–F), or hybridized to whole-mount dissections (G) as described in Experimental Procedures. For the sections, bright-field and dark-field pairs of micrographs are shown.

(A, B) A cross section of a late cellular-blastoderm stage embryo that has just begun gastrulation. Expression is first seen at this stage in a strip one to two cells wide (arrows in dark field) running the length of the neurogenic region at the border of the presumptive mesoderm, which is starting to invaginate along the ventral furrow (vf).

(C, D) A longitudinal section through the ventral furrow of a slightly older embryo at the close of gastrulation, showing the distribution of *sim* transcripts as a continuous strip of cells running the length of the ventral furrow. There is also hybridization anterior to the ventral furrow (arrowhead), encircling the presumptive anterior midgut invagination.

(E, F) A cross section of an embryo that has just completed gastrulation, which results in internalization of the presumptive mesoderm (m) along the ventral furrow (VF). Cells expressing *sim* have shifted to the ventral midline (arrows in dark field).

(G) Hybridization of *sim* sequences to a dissected 11 hr embryo, showing the two regions of hybridization in each segment of the CNS. Arrows point to the two regions of hybridization in a single segment. Scale bar: (A, B, G) 100 μm; (C, D) 130 μm; (E, F) 90 μm.

A cross section of a slightly older embryo, which has finished gastrulation and is beginning to show germ-band elongation, is shown in Figure 5E. Here, the presumptive mesoderm has invaginated to the interior of the embryo, and the labeled cells (more clearly shown in dark field; Figure 5F) are now situated at the ventral midline. Figure 5C is a longitudinal section cut obliquely through the ventral midline, showing hybridization to a continuous strip of cells running the length of the ventral furrow. Interestingly, there is also hybridization to cells surrounding the stomodeal invagination anterior to the ventral furrow (arrows). Although the participation of these cells in the development of the CNS has not been studied in detail because of the complexity of the anterior segments giving rise to the embryonic brain, neuronal precursors giving rise to neurons of the brain are derived from this region (Campos-Ortega and Hartenstein, 1985).

By hour 6 hybridization is seen over the entire strip of midline cells, which have delaminated from the ectodermal epithelium along with the rest of the neuronal precursors. At hour 11 hybridization is seen along the midline of the developing nervous system. Figure 5G shows the pattern of hybridization to a whole-mount dissection of an 11 hr embryo. In each segment there are two distinct regions of hybridization at the midline (arrows). Although the resolution of the in situ hybridizations does not permit visualization of expression by single cells, these regions correspond to the positions of those cells missing in the mutants.

Discussion

The first evidence of neurogenesis in Drosophila is the segregation of the neuronal precursors from the ventral neuroepithelium. By hour 5, certain of these ectodermal cells have enlarged and delaminated to become uniquely specified neuronal precursors. In grasshopper embryos, which share with Drosophila an almost identical pattern of neurogenesis, ablation experiments have shown that individual neuroblasts acquire unique fates based upon their position within the neuroectoderm, and that interactions between cells occur to produce the precise pattern (Doe and Goodman, 1985). The nature of the steps involved in the acquisition of unique fates by individual precursors is still unknown. By identifying mutations that alter the patterns of precursor cells during neurogenesis, one might define genes directly involved in this process. From both the mutant analysis and the spatiotemporal expression of *sim* transcripts, the *sim* locus appears to be such a gene.

During normal development those ectodermal cells lying at the ventral midline after gastrulation all delaminate with the NBs, forming a strip one to two cells wide of morphologically distinct smaller cells between the two bilaterally symmetric plates of NBs. These cells normally give rise to neurons and to specialized ectodermal cells lying at the midline of the CNS, including MP1, the MNB, the VUMs, the MECs, and most likely some additional, as yet unidentified cells.

In *sim* mutant embryos these midline cells are missing from the NB layer. The fate of the missing midline cells in *sim* mutants is at present unknown. One possibility is that these cells simply fail to delaminate along with their neighbors and are left behind in the epithelium. Alternatively, these cells may end up in ectopic locations within the mesoderm during gastrulation. In either case, we have never seen evidence for the differentiation of the MNB and MP1 progeny or any neurons in ectopic locations, as indicated by the lack of expression of neuronal markers such as the anti-HRP (Jan and Jan, 1982) and SOX-II (Goodman et al., 1984) antigens by any cells within the mesoderm or ventral ectoderm of mutant embryos. A third possibility, which we have not ruled out, is that the midline cells die in *sim* mutants; however, we have not seen any overt signs of cell death in the mutants.

The first evidence of the expression of *sim* transcripts is at the cellular blastoderm stage. In situ hybridization experiments show that *sim* is expressed in a remarkably precise anterior-posterior stripe, one to two cells wide, on both sides of the embryo. Each stripe of *sim*-expressing cells lies within the presumptive neurogenic region at the border of the presumptive mesoderm. These cells will become the midline cells of the neuroepithelium after gastrulation. Although morphologically indistinguishable from neighboring cells at this time in development, these cells are clearly different by virtue of their expression of the *sim* gene.

This situation is analogous to that of the segmentation genes, which are expressed in dorsal-ventral stripes (e.g., Hafen et al., 1984; Ingham et al., 1985; Harding et al., 1986). In the same sense that expression of segmentation genes is required for normal differentiation of portions of the anterior-posterior segmental pattern, the expression of *sim* may be thought of as being required for differentiation of a portion of the dorsal-ventral pattern—in this case, the midline cells within the neuroepithelium. This conceptual framework was originally proposed by Doyle et al. (1986) for the homeobox-containing gene *zen*, whose expression pattern, like that of *sim*, is an anterior-posterior stripe, but along the dorsal surface, and whose expression is necessary for the proper differentiation of the amnioserosa.

After gastrulation, as the very first NBs begin delaminating from the ectodermal epithelium, all of the midline cells still appear to be expressing *sim*, and form a continuous strip running the length of the germ band. However, by this stage in development the midline cells also share an additional feature distinguishing them from surrounding ectodermal cells of the neuroepithelium. In contrast to the more lateral neuroepithelium, where only a portion of the ectodermal cells delaminate to become NBs, all of the *sim*-expressing midline cells delaminate with the NB layer. It is also at this stage that we first observe the pattern defects in *sim* mutant embryos: the absence of all of these cells from the delaminating precursor population. Taken together, these observations suggest that the *sim* gene product plays a key role in the emergence and differentiation of the midline cells of the neuroepithelium, perhaps by controlling the expression of genes involved in their proper delamination. In the companion paper (Crews et

al., 1987) we lend support to this hypothesis by showing that the *sim* gene encodes a nuclear protein.

In 11 hr embryos it appears that cells within the CNS arising from the pool of midline cells are also expressing *sim* transcripts. Although these cells include MP1 progeny, the MNB, the VUMs, and the MECs, the resolution of the in situ hybridization experiments is not sufficient to discern the expression of *sim* in individual identified cells. Therefore it is possible that not all, but only a subset of cells derived from the midline cells express *sim* at this later time. Whatever the case, the continued expression of *sim* by postmitotic cells within the CNS suggests a further role for *sim* in neuronal development. The potential role of *sim* at these later stages is unknown, since in embryos homozygous for each of the available alleles, the *sim*-expressing cells are absent. The generation and study of temperature-sensitive conditional alleles of *sim* might provide us with insight into the possible functions of *sim* later in neuronal development.

Experimental Procedures

Strains

Descriptions of strains used can be found in Lindsley and Grell (1967) and Hilliker et al. (1980). The wild-type strain Oregon R was used for both histochemistry and Southern blots. The deficiencies and point alleles of *sim* were maintained as balanced lines with either MKRS or TM6B. Flies were raised on standard cornmeal–agar medium at 25°C. Staged collections were made on standard grape–agar plates from the balanced lines or from crosses between them. For the MP2 marking experiments, a strain of the genotype P[ry, ftz/lacC]; ry^{506} was used (Hiromi et al., 1985) for the example of normal development; for the mutant analysis, the genotype of the strain used was P[ry,ftz/lacC]; Df(3R)ry^{619}/TM6B.

Histochemistry

Staged embryos were dissected and filleted on glass slides, fixed in 2% paraformaldehyde for 10 min, then washed for 15 min in PBS and for 15 min in PBS containing 0.1% Triton X-100 and 0.2% bovine serum albumin (PBT). Embryos were incubated with a 1:400 dilution of fluorescein isothiocyanate–conjugated goat anti-HRP (Cappel) in PBT, 2% normal goat serum (PBTN) for 2 hr at room temperature, and washed for 30 min in PBS. Whole-mount embryo preparations were modified from procedures described in Mitcheson and Sedat (1983). Embryos were dechorionated in 50% bleach, washed in PBS, and fixed in an 8:2 mixture of heptane and 4% paraformaldehyde for 10 min. Embryos were removed from their vitelline membranes by replacing the paraformaldehyde with methanol and shaking vigorously. The embryos were rehydrated in PBS, washed in PBT for 15 min, and incubated with a 1:500 dilution of rabbit anti-β-galactosidase serum (Cappel) in PBTN overnight at 4°C followed by a 2 hr wash in PBT. Embryos were incubated in a 1:500 dilution of HRP-conjugated goat anti-rabbit antibody (Cappel) in PBTN for 4 hr at room temperature, washed in PBT, and allowed to react with 3,3′-diaminobenzidine (0.5 mg/ml) and hydrogen peroxide (0.003%). For mounting, embryos were dehydrated and cleared in methyl silicilate.

Southern Blots

Five micrograms of Drosophila genomic DNA was digested with a restriction enzyme, electrophoresed, and blotted onto nitrocellulose. The blot was hybridized with 10^6 dpm/ml of the λC1 cDNA clone insert that was ^{32}P-labeled by hexanucleotide priming (Feinberg and Vogelstein, 1983). The hybridization was carried out at 42°C in 50% formamide, 5× SSPE, 1× Denhardt solution, 0.1% SDS. The blot was washed three times for 15 min in 2× SSC, 0.5% SDS at room temperature, then washed twice for 30 min in 0.1× SSC, 0.1% SDS at 65°C. Size markers were prepared by digesting bacteriophage λ DNA with HindIII, end-labeling with ^{32}P, and mixing the DNA with EcoRI-cut Drosophila DNA.

Northern Blots

Five micrograms of poly(A)$^+$ RNA or 15 μg of total RNA extracted from Drosophila embryos was electrophoresed on a formaldehyde–agarose gel and blotted onto nitrocellulose or Nytran nylon membranes (Schleicher & Schuell). The blot was hybridized to the λC1 insert and washed under the same conditions used for Southern blot hybridizations. Size markers were prepared by mixing 0.2 μg of the Bethesda Research Laboratories RNA ladder with Drosophila poly(A)$^+$ RNA. The marker bands were revealed by hybridization to ^{32}P-labeled λ DNA.

Transcriptional Mapping

The transcriptional units around and including the *sim* gene were first identified by individually hybridizing the four ^{32}P-labeled genomic clones shown in Figure 3 to Northern blots of total embryonic RNA and poly(A)$^+$ RNA. Each blot contained eight samples of embryonic RNA collected at successive 3 hr intervals. The transcriptional units were mapped more precisely by hybridizing EcoRI fragments to Northern blots containing 0–3 and 3–6 hr embryonic RNAs (all four transcriptional units identified were expressed at these time periods). The *sim* gene was mapped by hybridization of the λC1 cDNA clone to Southern blots of restriction-enzyme-cut genomic clone DNA, and by sequence analysis of both the cDNA clone (Crews et al., 1987) and the *sim* gene (Crews and Thomas, unpublished data).

Isolation of the λC1 cDNA Clone

cDNA clones corresponding to *sim* embryonic transcripts were isolated from a 3–12 hr cDNA library constructed by Larry Kauver (Poole et al., 1985) that was screened with an EcoRI genomic DNA fragment (27–32 kb on the map; Figure 3) containing part of the *sim* gene. The longest of these cDNA clones, λC1, is 2.8 kb in length.

Polytene Chromosome In Situ Hybridization

Chromosome squashes were performed essentially as described by Pardue and Gall (1975); hybridization was with biotinylated probes as in Langer-Safer et al. (1982). Regions of hybridization were visualized with a streptavidin–HRP (Bethesda Research Laboratories) complex, followed by a standard reaction with 3,3′-diaminobenzidine.

In Situ Hybridization to Embryos

Devitellinized, fixed embryos prepared as described above were processed for hybridization by a modified procedure of Levine et al. (1983). ^{35}S-labeled probes were made by hexanucleotide priming (Feinberg and Vogelstein, 1983) and boiled for 20 min prior to use; 1 mM dithiothreitol was used throughout the hybridizations and initial wash. After four hr washes in 1× SSC at 60°C, embryos were dehydrated in ethanol and embedded in methacrylate as described by Mahoney and Lengyel (1987). Blocks were then sectioned at a thickness of 1 μm and mounted on slides. After removal of the plastic in Xylene, sections were coated with NTB-2 emulsion (Kodak) and exposed for 2 days to 3 weeks. For the hybridizations to dissected 11 hr embryos, embryos were filleted on glass slides as in Thomas et al. (1984), fixed for 20 min in 2% paraformaldehyde, and processed for hybridization as above.

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Notes Added in Proof

The work referred to throughout as Doe et al., submitted, is now in press: Doe, C. Q., Hiromi, Y., Gehring, W. J., and Goodman, C. S. (1988). Expression and function of the segmentation gene *fushi tarazu* during *Drosophila* neurogenesis. Science, in press.

Ultrastructural analysis has indicated that the MECs are specialized glial cells which are likely to be involved in the formation of the two axon commissures in each segment (R. Jacobs and C. S. G., unpublished data).