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Genetic analysis of the *Drosophila single-minded* gene reveals a central nervous system influence on muscle development

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Abstract

The *Drosophila single-minded* gene is expressed in the embryonic central nervous system midline cells and plays a critical role in central nervous system development. Additional expression of *single-minded* is found in a subset of ventral muscle precursor cells. Null mutations of *single-minded* result in an alteration of the ventral oblique muscles, such that muscle fibers form inside the embryo above the central nervous system. This defect is due to the mislocalization of a subset of mesodermal precursor cells. The muscle defect observed in *single-minded* null mutations is not due to the absence of *single-minded* expression in muscle precursor cells and likely results from an influence of the central nervous system on ventral muscle development.

Keywords: CNS; Development; *Drosophila*; Muscle; Myogenesis; *Single-minded*

1. Introduction

The somatic musculature of the *Drosophila* larva consists of a segmentally repeated pattern of 30 muscles per hemisegment. Development of these muscles begins with the formation of specific mononucleate precursor cells in the embryonic mesoderm (Bate, 1990). These cells fuse with surrounding mesodermal cells to form syncitial muscle fibers. Initial morphological studies reveal that the pattern of most precursor cells prefigures the pattern of resulting muscle fibers. The lateral and dorsal muscles derive from precursor cells that arise along the body wall. In contrast, precursor cells of the ventral muscles arise internally above the developing central nervous system (CNS) and then migrate to the body wall. These precursor cells not only act as foci for the formation of syncitial muscle fibers but also impart some aspects of identity on the developing multinucleated muscles (Bate, 1990; Dohrmann et al., 1990; Nose et al., 1992).

Both the specification and the differentiation of muscle precursor cells are subject to influences from other tissues. During early somitogenesis in vertebrates, muscle development appears to require the presence of a diffusible substance from the neural tube or notochord (Vivarelli and Cossu, 1986; Buckingham, 1992). In culture, the transition from myoblast proliferation to differentiation is also triggered by extracellular signals (for review, see Olson, 1992). Furthermore, in both vertebrates and invertebrates, proper myoblast migration requires either a target derived signal or guidance cues deposited on the basal lamina (Christ et al., 1986; Hedgecock, et al., 1990; Stern and Horvitz, 1991). Non-autonomous interactions between muscle precursor cells and the ectoderm have been proposed in *Drosophila* but are not well characterized (Lawrence and Brower, 1982; Bate, 1990; Lawrence, 1992).

An increasing number of genes have been identified either in *Drosophila* or vertebrates that influence muscle

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development. The most notable group is the myogenic bHLH gene family that includes the vertebrate *myoD*, *myogenin*, *mrf4* and *myf5* genes, the C. elegans *hlh-1* gene and the *Drosophila nautilus* (*nau*) gene (Krause et al., 1990; Michelson et al., 1990; Patterson et al., 1991; Olson, 1992; Emerson, 1993; Sasson, 1993). Numerous studies have shown that the vertebrate genes are DNA-binding transcription factors that have the ability to drive cultured cells into the myogenic lineage. Recent genetic experiments have shown that *myogenin* muta-

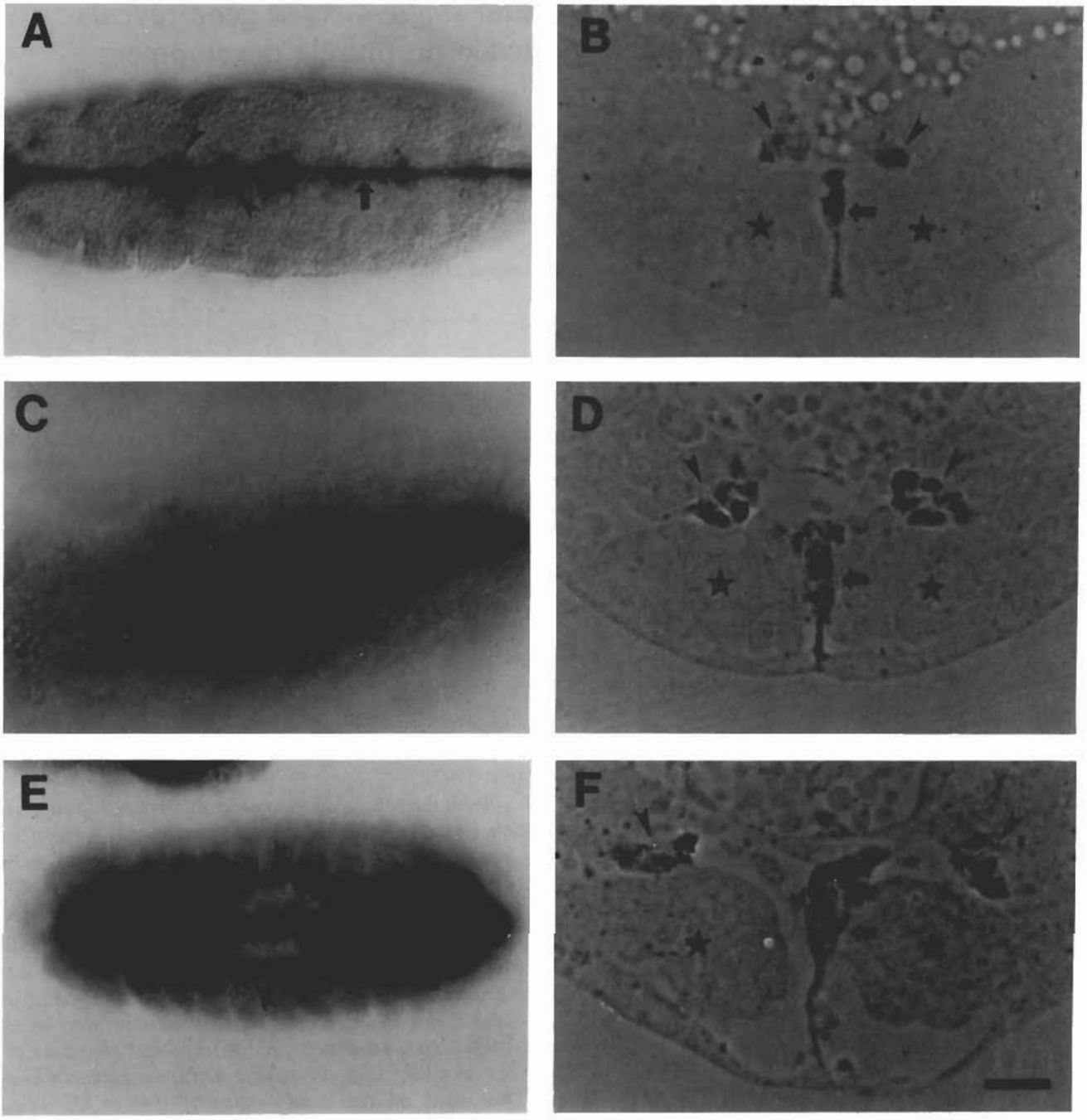


Fig. 1. Expression of *sim* in muscle precursor cells. Expression of *sim* is observed by both in situ hybridization of whole mount embryos using a cDNA probe (A, C and E) and by immunostaining using a SIM polyclonal antiserum followed by preparation of 3 μm thick sections (B, D and F). Whole-mount embryos are ventral views with anterior to the left and sections are transverse. Expression is seen in both the CNS midline cells (arrows) and in a subset of segmentally repeated ventral muscle precursor cells (arrowheads). The lateral CNS is marked by stars. (A and B) Expression of *sim* first appears in the mesoderm at 6–7 h of development (stage 11) in a single cluster of cells per hemisegment. These cells are positioned close to the midline of the developing CNS. (C and D) There is a second, more laterally placed, cluster that appears later in stage 11. (E and F) As germ band retraction commences (stage 12), the muscle precursor cells that express *sim* migrate away from the CNS midline toward the body wall, forming a single row of cells on each side of the embryo. Scale bar indicates 50 μm (A, C and E); 12.8 μm (B, D and F).

tions result in a severe reduction of skeletal muscle, confirming its important developmental role (Hasty et al., 1993; Nabeshima et al., 1993). Similar analyses of individual *myoD*, *myf5* and *hlh-1* loss-of-function mutations have not revealed striking muscle defects (Rudnicki et al., 1992; Braun et al., 1992; Chen et al., 1992). However, a *myoD myf5* double mutant shows an absence of skeletal muscle (Rudnicki, et al., 1993). Thus, the phenotypic results of myogenic gene mutations, the effect of the myogenic genes on muscle formation in culture and the important roles of other bHLH genes in development suggests that bHLH genes play important roles in muscle development.

Here we report that another bHLH protein, the product of the *Drosophila single-minded* (*sim*) gene, is expressed in a subset of ventral muscle precursor cells. Previous work has demonstrated that *sim* is an important regulator of CNS midline transcription and that both the CNS and ventral epidermis are defective in *sim* mutant embryos (Thomas et al., 1988; Mayer and Nüsslein-Volhard, 1988; Nambu et al., 1990; Nambu et al., 1991; Kim and Crews, 1993). The role of *sim* in myogenesis was investigated by genetic analysis. We find that mutations of *sim* do not result in an absence of somatic muscles. However, there is a mislocalization of ventral muscle precursor cells such that muscle fibers form across the inside of the embryo instead of along the body wall. Specific mutations that eliminate *sim* muscle precursor expression but leave intact the CNS expression reveal no abnormalities in muscle formation. Thus, the muscle defect observed in *sim* null mutants results from a non-autonomous influence of the CNS on myogenesis.

2. Results

2.1. Expression of sim in a subset of muscle precursor cells

Previous work has demonstrated that the *sim* gene is expressed prominently in the embryonic CNS midline cells and a subset of the hindgut, anterior midgut and posterior midgut cells (Crews et al., 1988; Nambu et al., 1990). Further analysis of *sim* embryonic expression using an antisera raised against SIM protein or by in situ hybridization indicates that *sim* is also expressed in a subset of ventral muscle precursor cells. Examination of whole mount and sectioned material reveals that *sim* is first detected in the mesoderm in a cluster of 3–5 cells per hemisegment that lie just adjacent to the midline of the developing CNS (Fig. 1A and B). This first cluster appears at 6–7 h of development (stage 11), before germ band retraction commences. Later in stage 11, a second more laterally placed cluster is observed, resulting in two groups of cells per hemisegment (Fig. 1C). Expression extends throughout the labial, thoracic and abdominal segments. Shortly after their appearance, the *sim*-expressing cells migrate laterally away from the midline towards the body wall. This can be seen by comparing Fig. 1A, C and E to each other and Fig. 1B, D

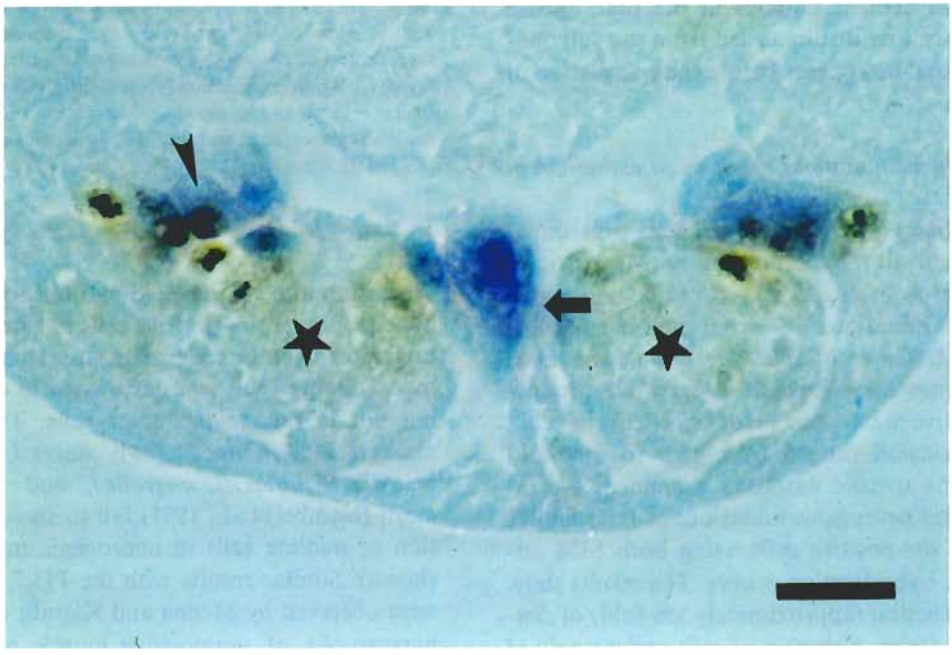


Fig. 2. Overlap of *sim* gene product and enhancer trap-gene E2-3-27 β-galactosidase muscle precursor-cell staining. Embryo carrying the E2-3-27 enhancer trap gene was double stained with a SIM antiserum (blue) and an antibody to β-galactosidase (brown). The E2-3-27 gene was previously shown to be expressed in ventral muscle precursor cells (Hartenstein and Jan, 1992). Examination of a sectioned stage 12–13 embryo reveals that the *sim*-positive cells overlap with the E2-3-27 *lacZ*-positive cells in the mesoderm (arrowhead), indicating that the *sim*-positive cells are muscle precursor cells. The CNS midline cells are indicated by an arrow and the lateral CNS by stars. Scale bar indicates 12.8 μm.

and F to each other. By the beginning of germ band retraction (stage 12), the two cell clusters merge and form a single row of *sim*-positive cells on each side of the ventral midline (Fig. 1E). These *sim*-positive cells begin to fuse and acquire the elongated morphology of newly forming muscle syncitia. While expression of *sim* in these cells begins to fade shortly after muscle cell fusion, weak immunoreactivity can be detected in sectioned embryos in a subset of ventral oblique muscles (data not shown).

The evidence presented above suggests that *sim* is expressed in a subset of muscle precursor cells. The identity of these *sim*-positive mesodermal cells is further confirmed by carrying out double label experiments which show both SIM reactivity and expression of a known muscle precursor cell marker. The *lacZ* enhancer trap line E2-3-27 expresses β-galactosidase in a subset of muscle precursor cells, with additional staining in the nervous system (Fig. 2; Hartenstein and Jan, 1992). Expression is continuous throughout development in the muscle precursor cells and the mature ventral oblique muscles which derive from these precursors. Sectioned E2-3-27 embryos double-stained with antibodies against β-galactosidase and SIM show that a subset of the mesodermal cells express both proteins (Fig. 2), indicating that the *sim*-positive cells coincide with identified muscle precursor cells.

In summary, the sim gene is expressed in two ventral clusters of mesodermal cells which likely represent precursor cells to a subset of ventral larval muscles. The expression is transient: it initiates at the time muscle precursor cells are first distinguished from the surrounding mesoderm and disappears before the completion of muscle cell fusion.

2.2. *Neurogenic gene mutations result in an expansion of sim-positive muscle precursor cells*

The *nautilus* (*nau*) gene encodes a bHLH protein expressed in most or all muscle precursors (Michelson et al., 1990; Patterson et al., 1991). It was demonstrated previously that mutations in seven neurogenic loci (*Notch*, *Delta*, *Enhancer of split*, *big brain*, *almondex*, *neuralized* and *mastermind*) result in excessive production of *nau*-positive muscle precursor cells (Corbin et al., 1991). The expansion ranged from two to nine-fold depending on the specific mutation examined. We investigated whether neurogenic mutations cause a similar hypertrophy of *sim*-positive cells using both SIM antisera and in situ hybridization probes. The results show a large overproduction (approximately ten-fold) of *sim*-positive cells in *Delta*, *Enhancer of split* and *neuralized* mutant embryos (Fig. 3A and B). These cells span the embryonic midline as one supercluster. A less severe overproduction (two to four-fold) is observed in *big brain*, *mastermind* and *Notch* mutants (data not shown). Although the SIM antibody and cDNA probes detect

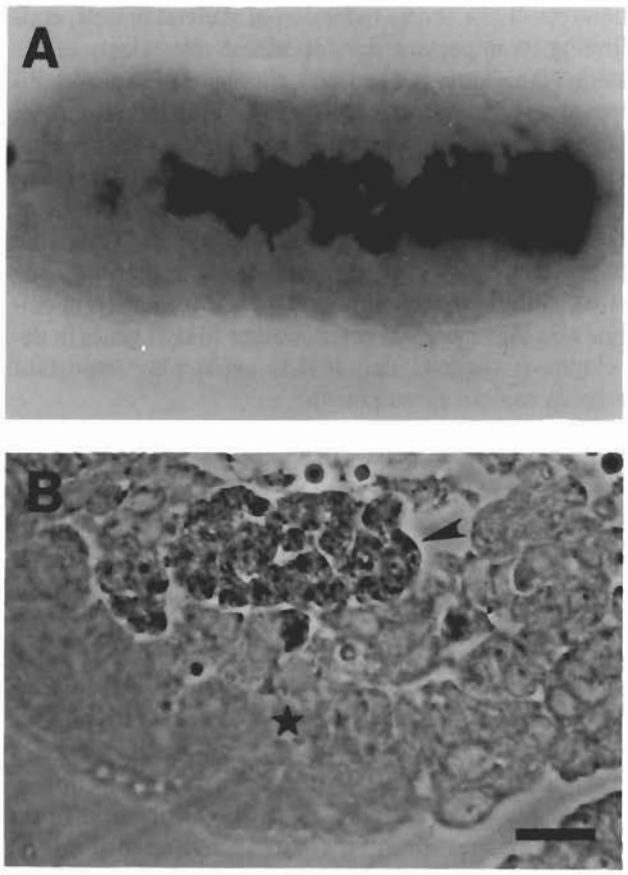


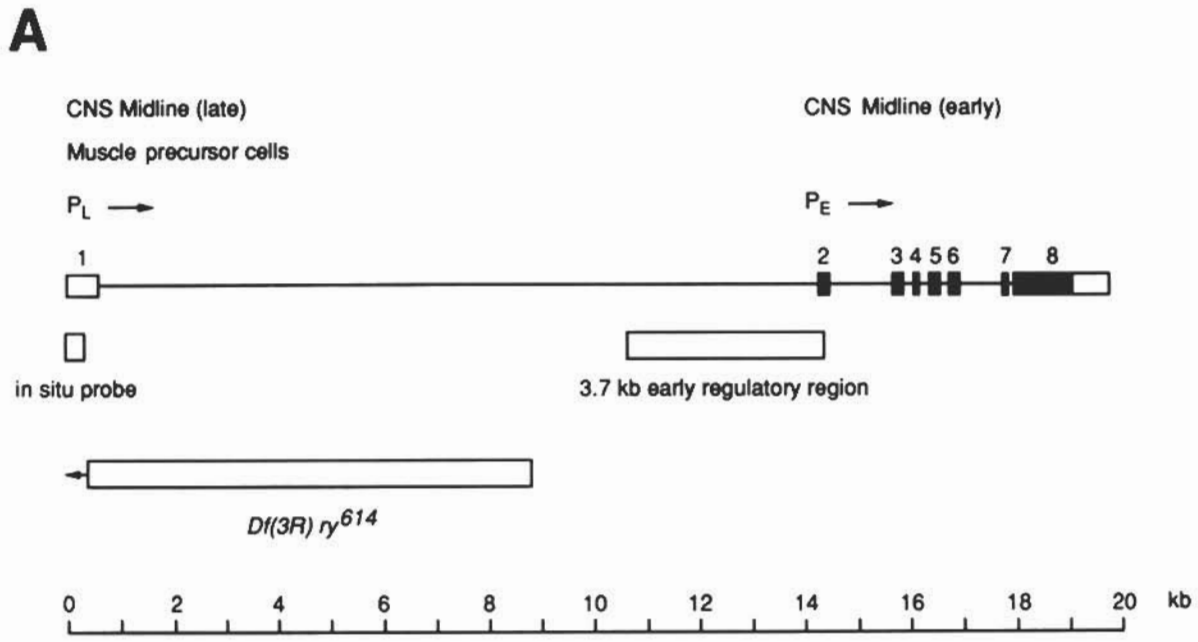
Fig. 3. Neurogenic mutations cause a hypertrophy of muscle precursor cells. The over-production of *sim*-positive muscle precursor cells in a *neu* mutant embryo is revealed by staining with a SIM antiserum. (A) Ventral view of a stage-11 embryo showing the formation of superclusters of *sim*-positive muscle precursor cells in *neu* mutant embryos. (B) Transverse section (ventral surface is at bottom) through a similar embryo as (A) showing the location of the supercluster (arrowhead) above the neuroblast layer (star). Scale bar indicates 40 μm (A) and 12.8 μm (B).

expression in both the CNS midline and mesoderm, the hypertrophy of *sim*-positive cells in a neurogenic mutant originates from mesodermal cells. This was determined by examining four genes expressed in the CNS midline but not in muscle precursor cells. The CNS midline markers P[3.7*sim/lacZ*] (only stains CNS midline; see below), P[*slit/lacZ*], *engrailed*, and P[*center divider/lacZ*] (Nambu et al., 1991) fail to show an overproduction of midline cells in neurogenic mutants (data not shown). Similar results with the P[3.7*sim/lacZ*] marker were observed by Menne and Klämbt (1994). Thus, the hypertrophy of *sim*-positive muscle precursor cells in neurogenic mutants is due to mesodermal expression of *sim*. Furthermore, the degree of overexpression in the mesoderm parallels that seen with a *nau* probe, a known marker for muscle precursor cells. Although it is unclear whether *sim* and *nau* are expressed in the same cells,

these results indicate that the *sim*-positive cells behave similarly to other muscle precursor cells.

2.3. Promoter utilization of sim muscle precursor cell expression

Embryonic transcription of the *sim* gene utilizes two separate promoters (Fig. 4A). The P_E promoter element, which lies just upstream of exon 2, is required for initial *sim* expression in the CNS midline cells and the gut (Nambu et al., 1990). Later expression in CNS midline precursor cells and midline glia is driven off the P_L promoter element upstream of exon 1 (Nambu et al., 1991; Muralidhar et al., 1993). Several experiments indicate that *sim* muscle precursor cell transcription re-



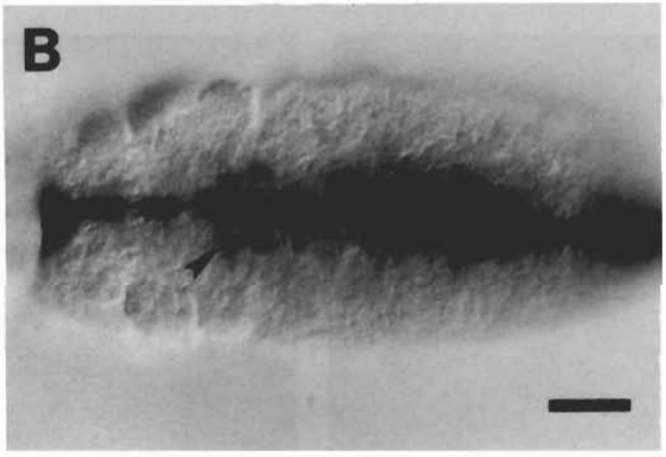


Fig. 4. Promoter utilization of the *sim* gene for muscle precursor cell transcription. (A) The *sim* gene has eight known exons (boxes) and two promoters, an early promoter, P_E, and a late promoter, P_L (arrows indicate direction of transcription). Coding sequences are filled and non-coding sequences open. The early promoter, P_E, drives initial transcription of *sim* in the CNS midline cells. The 3.7-kb fragment shown contains P_E and all sequences required for CNS midline transcription from that promoter. Later transcription in the CNS midline cells as well as expression in muscle precursor cells is dependent on transcription from P_L. The in situ hybridization probe derived from genomic sequences including exon 1 is specific for P_L transcripts. The chromosomal deficiency *Df(3R)ry*^{614} removes P_L but leaves P_E intact. The breakpoint of *Df(3R)ry*^{614} lies within the boxed region and the deleted DNA extends leftward. Scale bar is shown at the bottom in kilobases (kb). (B) Ventral view of a stage 12 whole mount embryo hybridized in situ to the P_L specific probe shown in (A). Transcripts are detected in muscle precursor cells (arrowhead) and in CNS midline cells (arrow). Scale bar indicates 40 μm.

sults exclusively from use of P_L. We have performed in situ hybridization experiments using a DNA probe derived from exon 1 which is specific for P_L transcripts (Fig. 4A). These detect later transcripts in CNS midline precursor cells and in muscle precursor cells (Fig. 4B). In contrast, a transgene containing 3.7 kb of P_E sequences fused to *lacZ* shows only CNS midline and gut expression, lacking expression in muscle precursor cells (Fig. 4A; Kasai et al., 1992). Furthermore, embryos homozygous for *Df(3R)ry*^{614} lack P_L but retain P_E (Fig. 4A). Correspondingly, when stained with a SIM antibody, these embryos show CNS midline but not muscle precursor cell expression.

2.4. Mutations of *sim* affect ventral muscle development

To determine the role of *sim* in muscle development, the organization of the embryonic musculature was analyzed in *sim* mutant strains. The muscle fibers were visualized using the muscle-specific monoclonal antibody 3E2 (Fujita et al., 1982) or a P[*myosin heavy chain/lacZ*] transgene (Hess et al., 1989) which was detected by staining for β-galactosidase. Embryos homozygous for *sim* null mutations (*sim*^{H9} or *sim*$^{B13\text{-}4}$) show a dramatic alteration in the embryonic muscle pattern. Several of the muscle fibers (including 15, 16 and 17) cross above the CNS, interior to the embryo, and attach to the body wall on the opposite side of the embryo (Fig. 5B and D). The remaining ventral muscles are shifted towards the ventral midline of the epidermis. This ventral shifting of muscles may result from ventral epidermal defects present in these embryos (Kim and Crews, 1993). Based on tracings of video projections, the number of ventral muscles in *sim*-mutant embryos appears to be the same as wild type (data not shown).

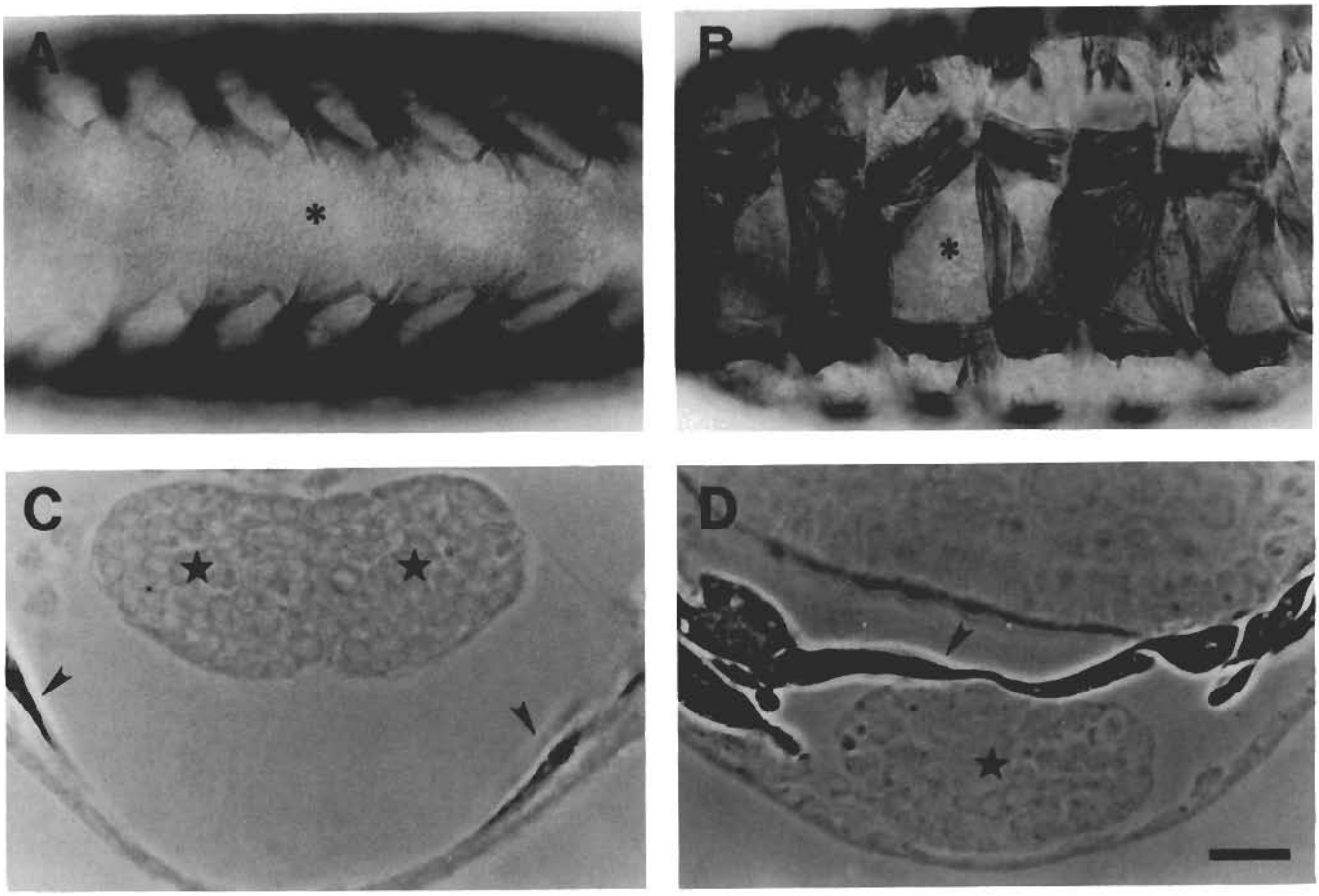


Fig. 5. Null mutations of *sim* affect ventral muscle development. All embryos are stages 15–17. (A) Ventral view of a wild type embryo stained with the muscle specific antibody MAB3E2 showing the normal arrangement of the ventral musculature. The ventral midline is designated by asterisks. Anterior is to the left. (B) Ventral view of a *sim*$^{B13\text{-}4}$ mutant embryo showing that muscles are shifted ventrally and some span the midline (asterisk). (C) Transverse section of a wild type embryo carrying a muscle-expressing P[*myosin/lacZ*] transgene, stained with an anti-β-galactosidase antibody. The ventral oblique muscles (arrowhead) run along the body wall and attach to the epidermis ventrolateral to the CNS. In this section, the CNS (stars) has pulled away from the body wall. (D) Transverse section of a *sim*$^{B13\text{-}4}$ embryo with P[*myosin/lacZ*] stained for β-galactosidase. The ventral oblique muscles (arrowhead) cross above the CNS (star) and attach onto the opposite side of the embryo. Scale bar indicates 25 μm (A and B) and 12.8 μm (C and D).

However, because of the abnormal layering of muscles in these mutants, the presence of specific ventral oblique muscles cannot be identified. The dorsolateral embryonic muscles appear normal.

2.5. Lack of an identifiable autonomous role of sim in muscle development

The previous section indicates that *sim* function is required for proper muscle formation. However, in addition to expression in the muscle precursor cells, *sim* is also expressed in the CNS midline cells. Genetic experiments indicate that formation of both the CNS midline and ventral epidermis require *sim* function (Thomas et al., 1988; Mayer and Nüsslein-Volhard, 1988; Nambu et al., 1991; Kim and Crews, 1993). This raises the question of whether the muscle defects observed in *sim*-mutant embryos are autonomous and due to the absence of *sim* muscle precursor cell expression, or non-autonomous and the result of defects in the CNS or ventral ectoderm. This is particularly relevant since some of the ventral muscle precursor cells arise near the midline of the developing CNS, migrate outward along the surface of the CNS and attach to the ventrolateral epidermis. Conceivably, the CNS and/or epidermis could influence formation or migration of ventral muscle precursor cells and their attachment to the epidermis.

This issue was addressed by utilizing a mutant strain, $Df(3R)ry^{614}$, that eliminates the *sim* P_L promoter element which drives muscle precursor transcription, but leaves intact regulatory elements that control early *sim* CNS midline cell expression (Fig. 4A). Embryos homozygous for $Df(3R)ry^{614}$ show normal early CNS midline expression but expression is absent in muscle precursor cells and the midline glia. The CNS and ventral epidermis appear normal in homozygous mutant embryos. Staining of neuronal cell bodies and axons with anti-HRP (Jan and Jan, 1982) show a normal appearing CNS (Thomas et al., 1988) and staining with an enhancer trap line AA41 (Crews et al., 1992) reveal that the midline glia are present (Kim and Crews, unpublished). The ventral epidermis is analyzed with the BP28 *lacZ* transgenic strain (Kim and Crews, 1993) and the cells appear normal (Kim and Crews, unpublished). When the embryonic musculature is examined by P[*myosin/lacZ*] or MAB3E2 staining, no defects in muscle morphology, organization or gene expression were detected (data not shown). This implies that *sim* expression in muscle precursor cells does not play a key role in muscle development.

2.6. Ventral muscle precursor cells are mislocalized in sim mutant embryos

As described earlier, *sim* null mutant embryos show a dramatic muscle defect in which a group of ventral muscles cross above the CNS and attach to the contralateral epidermis. Further insight into the cause of this defect was obtained by following earlier events in myogenesis using a P[*Toll/lacZ*] transformant strain (Wharton and Crews, 1993). This construct is expressed in a subset of ventral muscle precursor cells, the precursors to muscles 6, 7, 15, 16 and 17. Expression of this marker is dynamic, beginning with a single cell per hemisegment at early stage 12. Later in stage 12, expression is seen in four precursor cells per hemisegment with additional cells staining as muscle cell fusion proceeds. P[*Toll/lacZ*] continues to be expressed throughout the differentiation of these cells into mature muscle fibers. Embryos double-stained for both *sim* and P[*Toll/lacZ*] expression indicate that they are primarily expressed in non-overlapping groups of cells, although they may overlap in one or two cells per hemisegment (data not shown). The P[*Toll/lacZ*] gene is expressed in some of the muscles affected in sim^{H9} and $sim^{B13\text{-}4}$ null mutants and serves as a useful marker to follow the development of the precursor cells which give rise to these muscles. Given its absence of expression in most *sim*-expressing muscle precursors cells, it is not used as a marker to follow the fate of those cells.

The number of P[*Toll/lacZ*]-positive precursor cells in *sim* mutant embryos is the same as wild type, consistent with the observation that most or all muscles are present in *sim* null mutants. However, the positioning of these precursor cells in *sim* mutant embryos is altered from the time that the marker is first expressed. In wild type embryos, the P[*Toll/lacZ*]-positive muscle precursor cells form a uniform row several cell diameters away on either side of the ventral midline of the dorsal surface of the CNS (Fig. 6A). In *sim* mutant embryos, some of the P[*Toll/lacZ*]-positive cells are located closer to the midline at varying positions between the midline and their normal lateral position (Fig. 6B and C). Despite the incorrect location of these precursor cells, they are able to fuse with surrounding mesodermal cells to form the muscle fibers which cross internal to the CNS and attach onto the contralateral side of the embryo (Fig. 6C). It thus appears that the muscle defect in *sim* null mutants results from the abnormal localization of ventral muscle precursor cells. Since this defect is not dependent on autonomous expression of *sim*, it must reflect a role of the CNS or, less likely, the ventral epidermis in muscle precursor cell development.

3. Discussion

3.1. Relevance of sim to muscle development

Evidence presented in this paper indicates that the *sim* gene is expressed transiently in a subset of ventral muscle precursor cells. The location of the *sim*-positive cells during embryogenesis is consistent with these cells being muscle precursor cells. This was further confirmed by showing that *sim* expression in the mesoderm overlaps with a known muscle precursor marker, the enhancer

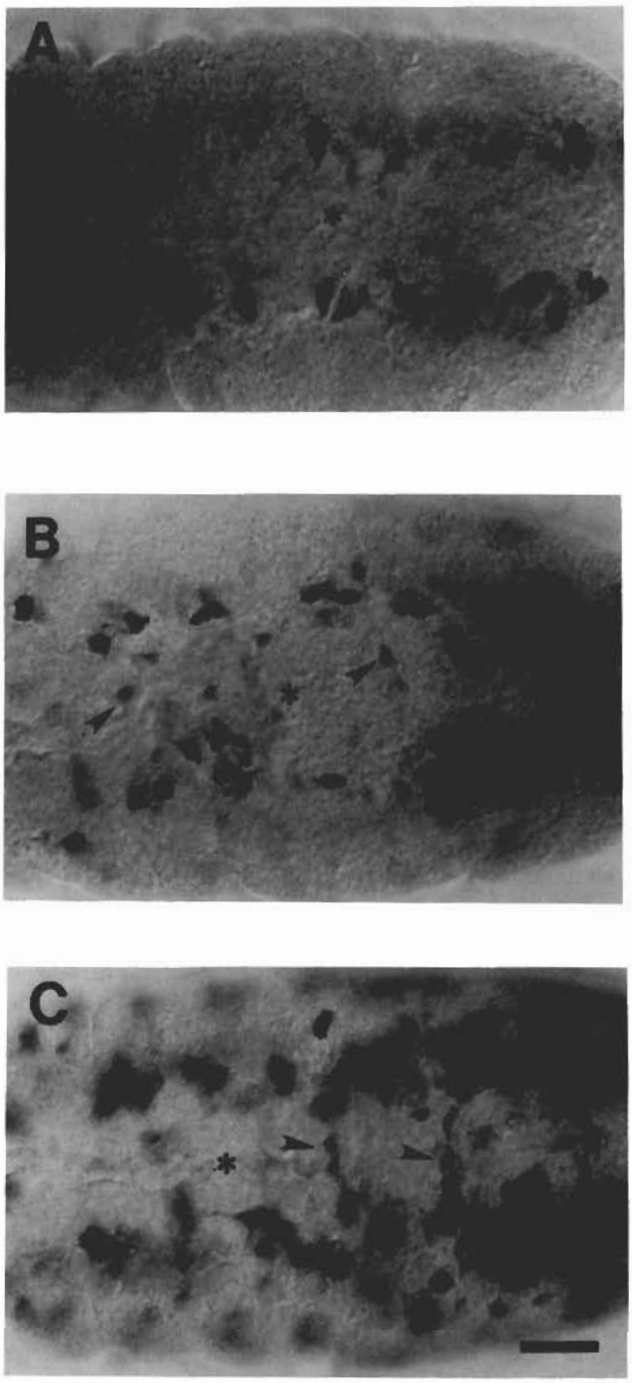


Fig. 6. Mislocalization of ventral muscle precursor cells in *sim* null mutant embryos. Ventral views of whole-mount embryos are shown with the ventral midline marked by an asterisk; anterior is to the left. (A) Ventral view of a stage 12 wild type embryo carrying a P[*Toll/lacZ*] transgene and stained with an antibody against β-galactosidase. Expression of β-galactosidase is observed in a subset of ventral muscle precursor cells (arrowhead). (B) Ventral view of a stage 12 *sim* $^{B13\text{-}4}$ mutant embryo stained with anti-β-galactosidase antibody. Some P[*Toll/lacZ*] expressing cells are mislocalized (arrowheads) compared to wild type and lie closer to the ventral midline. (C) In a slightly older mutant embryo (stage 13), the mislocalized muscle precursor cells undergo fusion (arrowheads) developing into the muscles which span across and above the CNS. Scale bar indicates 25 μm.

trap line E2-3-27. Finally, neurogenic mutations result in an over-production of *sim*-positive cells similar to the expansion of the *nautilus*-positive muscle precursor cells.

The expression pattern of *sim* is consistent with a role in either establishment of the identity of this group of muscle precursor cells or in controlling a function unique to these cells. However, mutations which specifically eliminate *sim* expression in these cells do not have an observable effect on their development. Our analysis should detect the addition or absence of even single muscles and the transformation of muscles that are morphologically distinct. There are several explanations for the lack of an observable *sim* phenotype. The *sim* gene may play a subtle role in muscle development which we are unable to detect, its function may be compensated for by redundancy or its expression in muscle precursor cells may be adventitious and not functionally significant. At this point, we have no basis to argue for or against any of these possibilities.

The issue of gene redundancy is difficult to address because only a few genes with candidate roles in *Drosophila* myogenesis have been identified. Mutations in other vertebrate and invertebrate bHLH genes, however, do not always lead to severe muscle defects. Mutations in the C. elegans *hlh-1* gene do not cause gross muscle defects (Chen et al., 1992). Mutants of the mouse *myoD* and *myf5* genes individually do not have detectable skeletal muscle defects (Rudnicki et al., 1992; Braun et al., 1992). However, *myoD*, *myf5* double mutant embryos completely lack skeletal muscle indicating functional redundancy (Rudnicki et al., 1993). The undetected or absent role of *sim* in muscle precursor cell development contrasts with its important regulatory role in CNS midline and ventral epidermis development. In those two cases, the entire cell lineage fails to develop in the absence of *sim* function. It is interesting that the muscle specific expression of *sim* is due to transcription from a promoter element distinct from that required for CNS midline development. The P_L promoter drives *sim* transcription in muscle precursor cells and in CNS midline precursor cells and midline glia. However, mutations that remove P_L lead to normal muscles and CNS. Thus, *sim* P_L expression appears to be developmentally less important than P_E expression.

3.2. Role of the CNS in muscle development

Analysis of *sim* null mutations reveals a striking muscle defect. Some of the ventral muscles lie inside the embryo, crossing above the CNS and attaching onto the contralateral epidermis. General differentiation of a precursor cell into a muscle fiber does not appear to be affected, since in *sim* mutant embryos these precursor cells still form muscles and express muscle specific genes such as P[*myosin/lacZ*] and the MAB3E2 antigen. Further analysis with a P[*Toll/lacZ*] marker which stains a subset of ventral muscle precursor cells indicates that at

least some of the muscles which develop internally are derived from improperly positioned precursor cells. Since the absence of *sim* expression in muscle precursor cells is not responsible for this defect, the conclusion is that either the CNS or ventral epidermis is responsible; both these tissues are defective in *sim* mutant embryos. The close proximity between the CNS and the ventral muscle precursor cells, however, points to the CNS as more likely to influence early muscle precursor cell development rather than the ventral epidermis.

Genetic analysis of the muscle pattern in other mutant strains reinforces the idea that the CNS plays a specific role in the early development of the ventral muscle precursor cells. Mutations in the *slit* gene affect the formation of CNS midline precursor cells (Nambu et al., 1990) but have a normal ventral epidermis (Nüsslein-Volhard et al., 1984; Kim and Crews, unpublished). These mutations also show ventral muscle defects similar to those of *sim*, with muscle fibers crossing internal to the CNS (Lewis and Crews, data not shown). Mutations in three *spitz* class genes, *pointed*, *rhomboid* and *spitz*, show muscle (Bier et al., 1990; Rutledge et al., 1992; Klämbt, 1993), ventral epidermal (Mayer and Nüsslein-Volhard, 1988; Kim and Crews, 1993) and CNS midline defects (Klämbt et al., 1991). Midline glia development is affected in *spitz* class mutants but they do not show the severe CNS midline defects observed in *sim* and *slit* mutant embryos. The *spitz* class mutants also do not exhibit the striking muscle alteration seen in *sim* mutants in which muscles form inside the embryo and cross over the CNS. The other *sim* muscle defect in which some ventral group muscles fail to attach at the proper sites on the epidermis, projecting more ventrally, is shared by the *spitz* class mutants. Given the similarity of epidermal defects between *sim* and *spitz* class mutants (Mayer and Nüsslein-Volhard, 1988; Kim and Crews, 1993), this alteration in the attachment sites of some ventral muscles likely results from the epidermal defects in these mutant embryos. Consequently, amongst these five mutants, the muscle defect in which muscles form internally correlates with severe defects in the CNS midline cells but not with defects in the epidermis.

This postulated interaction between the CNS and mesoderm could take several forms. The CNS could be involved in specifying the identity of the ventral muscle precursor cells or it could affect precursor cell localization. Currently, there exists only a limited number of reagents available to address this problem so the issue cannot be resolved completely. However, evidence presented here suggests that the CNS affects muscle precursor cell localization. The P[*Toll/lacZ*] gene is expressed in a subset of ventral muscle precursor cells and thus is a cytological marker of muscle precursor cell identity. Since the number of P[*Toll/lacZ*]-positive cells and the timing of their appearance does not appear altered in *sim* mutant embryos, precursor cell identity may be normal. However, the cells are not in their proper position indicating a defect in localization.

This mislocalization of muscle precursor cells in a *sim* mutant could result from interactions between muscle precursor cells and either CNS midline cells or cells of the lateral CNS. Homozygous *sim* mutant embryos completely lack developed CNS midline cells but the spatial organization of the lateral halves of the CNS is also altered (Thomas et al., 1988; Nambu et al., 1991). How could the CNS affect muscle precursor localization? One possibility is that the CNS directs the initial position of the ventral muscle precursor cells that arise in the adjacent mesoderm. In this case, the disruption of the CNS in *sim* mutant embryos would lead to a mispositioning of specific precursor cells. An alternative hypothesis is that the CNS may be required for proper muscle precursor cell migration. Such non-autonomous cues are thought to be important for myoblast migration in both invertebrates and vertebrates (Christ et al., 1986; Hedgecock et al., 1990; Stern and Horvitz, 1991). Some of the *Drosophila* ventral muscle precursor cells migrate along the basement membrane that covers the dorsal surface of the CNS as seen with *sim* staining. It is interesting that a subset of the CNS midline cells, called the mesectodermal strand cells, synthesize laminin, an extracellular matrix protein known to direct cell migration in vertebrates (Montell et al., 1989; Goodman et al., 1989). These cells and their ability to synthesize laminin are disrupted in *sim* mutant embryos (data not shown). However, at present it is unclear whether the mislocalized P[*Toll/lacZ*]-positive cells initially form in the wrong location or fail to migrate properly since the P[*Toll/lacZ*] gene is expressed too late to directly observe migration.

The CNS midline cells play a number of important developmental and function roles (Nambu et al., 1993). In the CNS, these cells are required developmentally for proper formation of the CNS axon scaffold (Thomas et al., 1988; Klämbt et al., 1991) as well as serving as functional neurons and glia in the mature CNS. In addition, they play a role in the development of the ventral epidermis, possibly via a signaling pathway involving the *spitz* group of genes (Mayer and Nüsslein-Volhard, 1988; Kim and Crews, 1993). We have shown in this paper that the CNS influences muscle development and this may also be mediated by the CNS midline cells.

4. Experimental procedures

4.1. Drosophila strains

The sim^{H9}, $sim^{B13\text{-}4}$ and $Df(3R)ry^{614}$ strains were obtained from the A. Chovnick laboratory and were maintained over a TM3 balancer. The mutations sim^{H9} and $sim^{B13\text{-}4}$ are protein nulls (Nambu et al., 1991). Muscle defects were detected in balanced $Df(3R)ry^{614}$ stocks, but these were removed by outcrossing *sim* onto an

Oregon R background. The following neurogenic alleles were used: *neu*^{IIIA86}, *bib*^{1D05}, *E(spl)*^{R1}, *mam*^{m199}, *N*^{55e11} and *Dl*^{X} (Corbin et al., 1991, Lindsley and Zimm, 1992). P[*Toll/lacZ*] (constructs 6.5 RL and 6.5 LR in Wharton and Crews, 1993) contains 6.5 kb of Toll genomic sequences fused to a weak heterologous promoter. This construct is expressed in the precursor cells and mature ventral muscle fibers 6, 7, 15, 16 and 17. The enhancer trap line E2-3-27 originated from the Y. N. Jan laboratory (Hartenstein and Jan, 1992) and was obtained from the Bloomington Stock Center (stock designation P750). The P[*myosin heavy chain/lacZ*] line was obtained from the laboratory of S. Bernstein (Hess et al., 1989).

4.2. Immunostaining and in situ hybridization

Embryos were collected and stained as described previously (Nambu et al., 1990) with a SIM antisera, anti-β-galactosidase (Promega) or the monoclonal antibody 3E2 (Fujita et al., 1982; obtained from laboratory of S. Benzer), followed by horseradish peroxidase histochemical staining (Vectastain Kit from Vector Labs). In the case of double stained embryos, the β-galactosidase antibody was detected with horseradish peroxidase histochemical staining (Vectastain Kit) and the SIM antibody was detected with an alkaline phosphatase conjugated secondary antibody (Promega). The embryos were stained first for SIM and then for β-galactosidase. The SIM antisera was generated in rabbits using a bacterially-synthesized TrpE fusion protein (Kleid et al., 1991) containing the N-terminal two thirds of the SIM protein. The antisera was affinity purified using a bacterially produced full length SIM protein (Studier and Moffatt, 1986) bound to Sepharose (Pharmacia). Stained embryos were mounted in 80% Canada balsam/20% methyl salicylate. Whole-mount in situ hybridizations were carried out using digoxigenin-labeled DNA probes (Boehringer Mannheim) as described by Tautz and Pfeifle (1989) and mounted in 80% glycerol. Whole-mount embryos were viewed and photographed using a Zeiss Axiophot with Nomarski optics.

4.3. Tissue sectioning

Antibody-stained embryos were dehydrated and mounted for sectioning as described previously (Nambu et al., 1991). Dehydrated embryos were placed in a staining dish with a 1:1 mix of acetone and Epon-Araldite where they remained until the acetone evaporated. The embryos were then mounted in fresh blocks of Epon-Araldite and baked at 60°C for 12–15 h. Sections (3μm) were cut, mounted with Permount and photographed using phase-contrast optics.

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