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Original Article

Pioneer growth cone migration in register with orthogonal epithelial domains in the grasshopper limb bud

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ABSTRACT At the onset of neural development, pioneer growth cones can migrate over epithelia or neuroepithelia along stereotyped routes that establish the pattern of initial neural tracts. These migration routes may reflect the arrangement of distinct epithelial or neuroepithelial domains. In grasshopper limb buds, a pair of afferent pioneer neurons arise in the tibia and their growth cones migrate on a stereotyped path through the limb to the CNS. In the limb buds, circumferentially-oriented epithelial domains expressing semaphorin-I, annulin, or alkaline-phosphatase, and a longitudinal domain, expressing engrailed, have been described. Using multiple-labeling techniques, we describe the relationships of these domains to each other and to the pioneer neuron pathway. Taken together, these domains establish an orthogonal pattern of regionally specific epithelial molecular markers. During much of their migration across the limb epithelium, the pioneer growth cones are in register with the axes of circumferential or longitudinal epithelial domains.

KEY WORDS: *epithelium, alkaline phosphatase, semaphorin, annulin, pathfinding*

Introduction

Early in embryogenesis the first nerve cells to extend processes often do so across epithelium or neuroepithelium. The paths taken by these "pioneer" growth cones establish the routes of peripheral nerves and the commissures and longitudinal tracts of the central nervous system (Chitnis and Kuwada, 1990; Fraser *et al.*, 1990; Cornel and Holt, 1992; Easter *et al.*, 1993; Wilson *et al.*, 1993; Macdonald *et al.*, 1994). Epithelia may provide directional information to migrating growth cones. Some epithelia are organized into discrete molecule expression domains. Features of these domains, and the borders between them, may provide important information for growth cone guidance.

As characterized in *Drosophila*, insect ectodermal epithelium is organized into epithelial molecular expression domains (Bryant, 1993; Campbell *et al.*, 1993; Couso *et al.*, 1993; Basler and Struhl, 1994; Williams *et al.*, 1994). Molecular interaction between cells in these domains drives morphogenesis. In *Drosophila* limbs, both circumferential and proximo-distal epithelial differentiation may be driven by interactions between a small number of cellular domains. Interactions initially involving a small number of signaling molecules and their receptors appear likely to activate more elaborate and local aspects of limb differentiation as embryogenesis proceeds.

In embryonic limb buds of grasshoppers and cockroaches, one proximo-distal and several circumferential epithelial molecular expression domains have been characterized. As in other insects and arthropods, engrailed is expressed along the proximal-distal axis in the posterior limb compartment in grasshoppers (Bentley and Toroian-Raymond, 1989; Patel *et al.*, 1989). Circumferentially, annulin (Bastiani *et al.*, 1992; Singer *et al.*, 1992), a grasshopper intracellular transglutaminase, and DSS-8 (Norbeck and Denburg, 1991), a cockroach glycoprotein, are expressed distally in limb segments. Alkaline-phosphatase, a highly conserved ecto-enzyme, is expressed in predominantly intra-segmental circumferential bands (Chang *et al.*, 1993). Semaphorin-I (= fasciclin-IV), a member of the semaphorin family of cell surface glycoproteins that effect neural outgrowth (Kolodkin *et al.*, 1993), is expressed in a third series of circumferential bands (Kolodkin *et al.*, 1992).

Afferent pioneer neurons in grasshopper limbs have been a useful model of some features of neural outgrowth, including guidance (Bate, 1976; Caudy and Bentley, 1986; Lefcort and Bentley, 1987), growth cone steering and motility (O'Connor *et al.*, 1990), and disposition of the neural cytoskeleton (Sabry *et al.*, 1991; O'Connor and Bentley, 1993). Isolation of limbs and removal of mesodermal cells and basal lamina has shown that the surfaces of epithelial cells can provide the information necessary for growth cone guidance along the normal route

Abbreviations used in this paper: CNS, central nervous system; T3, third thoracic limb.

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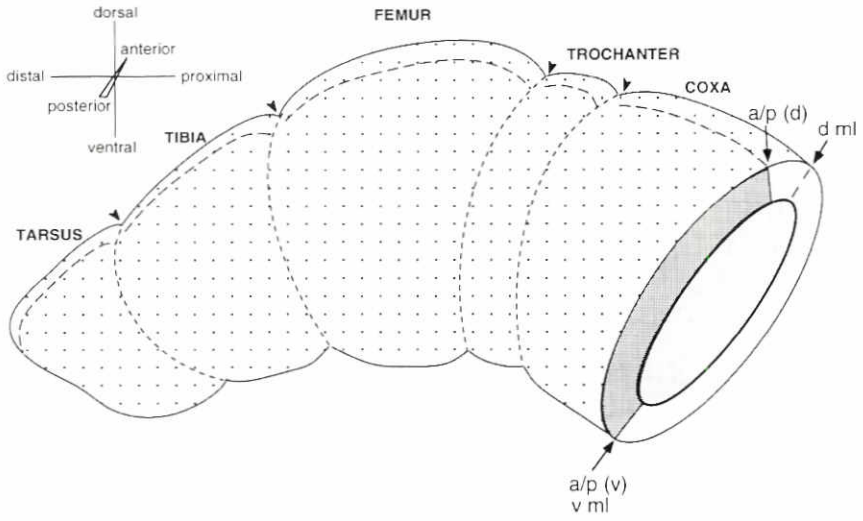

Fig. 1. Schematic diagram of limb morphology. *At the 33.5% stage, the five limb segments (tarsus, tibia, femur, trochanter, coxa) are demarked by circumferential constrictions in the limb epithelium (small-dash line). Longitudinally, the limb comprises a posterior compartment (dark stipple) and an anterior compartment. The ventral boundary between the anterior and posterior compartments (a/p v) is at the ventral midline of the limb (v ml), whereas the dorsal boundary between the compartments (a/p d; large-dash line) is slightly posterior to the dorsal midline (d ml) of the limb.*

(Lefcort and Bentley, 1987; Condic and Bentley, 1989). Growth cones of these neurons migrate across epithelial domains that express annulin, alkaline-phosphatase, and semaphorin-I. The goal of the work reported here was to determine the relationship between the annulin, semaphorin-I, alkaline-phosphatase and engrailed expression domains, and the pathway taken by pioneer neurons with respect to borders between those domains.

Results

During development of the Ti1 pioneer neuron pathway (31%-35% of embryogenesis), several regions of limb epithelium are traversed by the pioneer growth cones (see Fig. 1 for segment and compartment boundaries). The entire projection is confined to the anterior compartment of the limb, where the pathway typically is established within three limb segments, the femur, trochanter and coxa. Axon extension in the femur and coxa is along the proximo-distal axis of the limb, while in the trochanter it is circumferential. Molecules expressed in specific patterns in these limb regions are candidates for influencing the extension of the pioneer growth cones. We examined the arrangement of epithelial domains, defined by molecular expression patterns, in multiply-labeled 33.5% third thoracic (T3) limb buds using three preparations: intact limbs (Fig. 2), flat epithelial monolayers (fillets) made by unrolling the limb on an adhesive substrate (Fig. 3), and camera lucida drawings showing the labeling intensity over each epithelial nucleus in the anterior limb compartment (Fig. 4).

Labeling of intact limbs with single labels (Fig. 2A,C,E) confirmed previous descriptions (Bastiani *et al.*, 1992; Kolodkin *et al.*, 1992; Singer *et al.*, 1992; Chang *et al.*, 1993). Annulin is expressed in two circumferential bands along the pioneer pathway (Figs. 2A, 4A), first as a narrow band at the distal border of the coxa and second as a narrow band at the distal border of the femur. While the annulin band in the coxa extends circumferentially from the dorsal midline to the ventral midline (Fig. 2A,F), expression is attenuated ventrally in the femur band (Fig. 4A). Semaphorin-I labels three prominent bands at the 33.5% stage. The most proximal band fills the trochanter (Figs. 2C, 4B). Within the femur there is a broad proximal band which is not continuous circumferentially, leaving a patch of unlabeled cells in the middle of the femur (Fig. 2C). A third band found in the proximal tibia (Fig. 2C) is circumferentially continuous from the dorsal to ventral midline. To varying degrees, alkaline-phosphatase labels most cells from the femur-trochanter border to the tarsus-tibia border (Figs. 2E, 4C). Within this region, there is a narrow band of heavy labeling at the femur-trochanter border and a broad heavily-labeled band covering the distal femur and proximal tibia. All alkaline phosphatase bands span the ventral to the dorsal extent of the anterior compartment (Fig. 3A).

Double-labeling of the two annulin and three semaphorin-I bands present at the 33.5% stage shows that these bands occupy interleaved positions along the longitudinal axis of the limb (Fig. 2B). Unlabeled regions separate the bands, except at the trochanter-coxa border. At this border no unlabeled cells are seen between the annulin label in the coxa and the semaphorin-I label in the trochanter. As each of these two domains extends up to the constriction that marks the coxa-trochanter segment border, it appears that the annulin and semaphorin-I domains are contiguous. As the limb develops further, annulin begins expression in the ventral trochanter (Fig. 4A) and semaphorin-I begins expression dorsally in the distal femur (Fig. 5E), resulting in regions of overlap for these two markers.

In the pioneer pathway region, the semaphorin-I and alkaline-phosphatase domains (Fig. 2D) comprise alternating bands that are close to being completely complementary (although neither marker is expressed in the coxa). From proximal to distal along the longitudinal axis of the limb, these complementary domains comprise semaphorin-I in the trochanter, alkaline-phosphatase in the proximal femur, semaphorin-I (non-continuous) in the mid-femur, alkaline-phosphatase in the distal femur, semaphorin-I in the proximal tibia and alkaline-phosphatase in the distal tibia. Between these bands there are no unlabeled cells. Thus along the pioneer pathway the semaphorin-I domains and alkaline-phosphatase domains either abut or overlap. In the distal region of the trochanter where the pioneer growth cones turn ventrally, the domains appear to overlap by one or two tiers of cells, as judged by two criteria. First, alkaline-phosphatase labeling extends proximally past the epithelial constriction that marks the

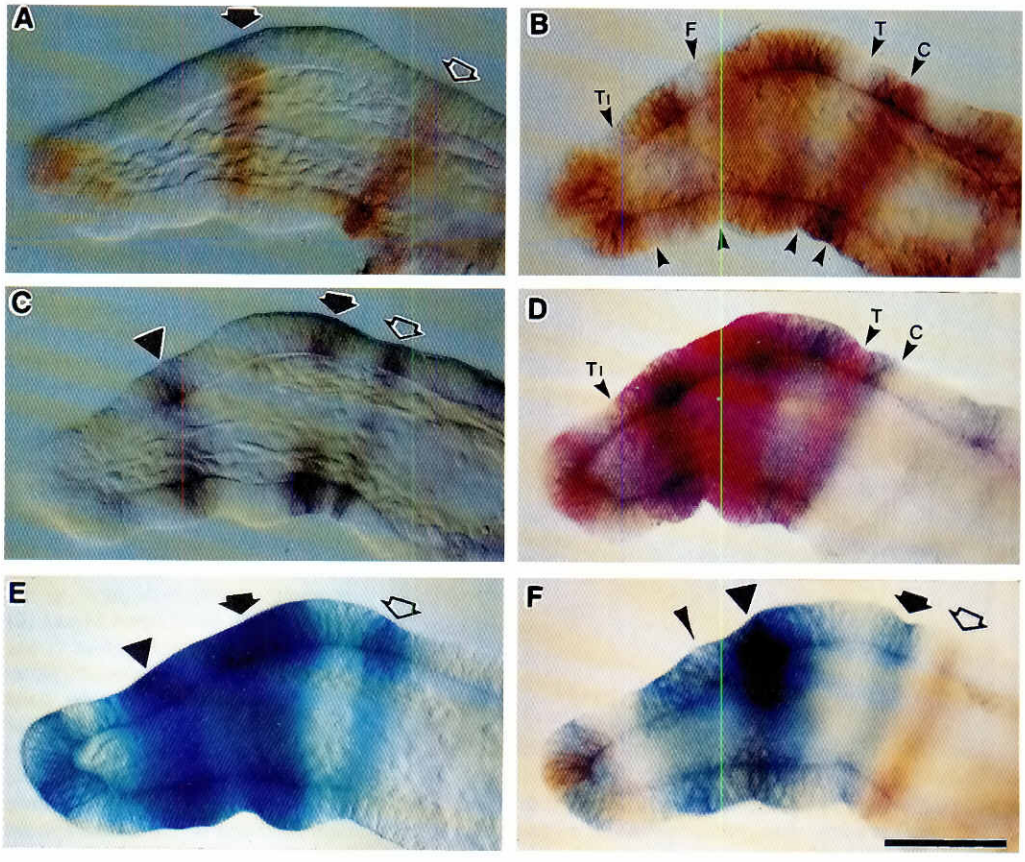

Fig. 2. Circumferential epithelial domains in the T3 limb at the 33.5% stage. **(A)** *Annulin is expressed at the distal border of the coxa (open arrow), the distal border of the femur (solid arrow), and the limb tip (developing tarsus).* **(B)** *Double-labeling indicates that annulin (brown) and semaphorin-I (black) domains abut at the coxa-trochanter border (C). Elsewhere in the limb, regions of unlabeled epithelium separate these bands. T, distal border of trochanter; F, distal border of femur; Ti, distal border of tibia.* **(C)** *Semaphorin-I is expressed throughout the trochanter (open arrow), in a proximal band (solid arrow) in the femur which does not reach the trochanter border, and in a proximal band (triangle) in the tibia.* **(D)** *Double-labeling demonstrates complementary expression of semaphorin-I (black) and alkaline phosphatase (red) from the coxa-trochanter border (C) to the distal border of the tibia (Ti). T, distal border of trochanter.* **(E)** *Alkaline-phosphatase is expressed in a band spanning the trochanter-femur border (open arrow), a dark band (solid arrow) in the distal femur, and a dark band (triangle) in the distal tibia. Lighter labeling between the femur and tibia bands will disappear by the 35% stage (Chang, et al., 1993).* **(F)** *Double-labeling of alkaline-phosphatase (blue) and annulin (brown) confirms the presence of unlabeled cells (semaphorin-I expressing) between the annulin band (open arrow) in the coxa and the alkaline-phosphatase band (solid arrow) spanning the femur/trochanter border, and also the overlap (triangle) in labeling in the distal femur. In the proximal region of the tibia (arrowhead), alkaline-phosphatase expression is beginning to decrease. Dorsal, up; distal, left. Scale bar: A-F, 100 μm (on F).*

trochanter-femur border (Fig. 2E,F), while semaphorin-I labeling extends to the distal border of the trochanter (Fig. 2C). Secondly, in limbs double-labeled for alkaline-phosphatase and annulin, the unlabeled cell band within the trochanter appears to be only one or two cells in width (Figs. 3A,C,D, 5C,D), whereas the semaphorin-I band in the trochanter is 3-5 cells in width (Figs. 4B, 5E; see also Kolodkin *et al.*, 1992). Within the femur, alkaline-phosphatase and semaphorin-I bands appear to complement one another with little or no overlap. In contrast, annulin labeling within the femur (Fig. 5B) lies entirely within the alkaline-phosphatase domain (Fig. 5C,D). Lastly, double-labeling for annulin and alkaline-phosphatase (Fig. 2F) shows that the annulin band in the coxa is well separated from the most proximal alkaline-phosphatase labeling at the coxa-trochanter border (Fig. 2F).

The circumferential staining pattern of annulin, alkaline-phosphatase and semaphorin-I is orthogonal to the expression pattern of engrailed. As previously described (Bentley and Toroian-Raymond, 1989; Patel *et al.*, 1989), engrailed labels the posterior limb compartment of the grasshopper embryo. This compartment runs longitudinally through the limb, with a ventral border at the ventral midline of the limb (Fig. 3B,D) and a dorsal border just posterior to the dorsal midline of the limb (Figs. 1, 5B). Double-labeling shows that the stripe of engrailed expression is roughly perpendicular to the circumferential bands of annulin, semaphorin-I and alkaline-phosphatase expression (Figs. 3B,D, 5B,F).

We evaluated several features of the cellular composition of these domains, including the widths of the bands in number of epithelial cells, the percentage of cells within a domain that express the marker, and the abruptness of transitions between domains, by examining labeled epithelial monolayers (Fig. 3) and by making camera lucida drawings of labeling over every epithelial nucleus in the anterior quadrant (see Materials and Methods) of intact limbs (Fig. 4). All cells within a domain appeared to express the specific marker, although there was significant variation in intensity of labeling. Transitions across domain borders from high to low (undetectable) expression usually were quite sharp, changing abruptly over one or two cell tiers (e.g. annulin, Figs. 3A, 5B and C). The smallest widths of the bands, in terms of numbers of cells, were about 3-5 cells. In the region of the trochanter, three different domains occur within a region about 15 epithelial cells in width (along the longitudinal axis of the limb).

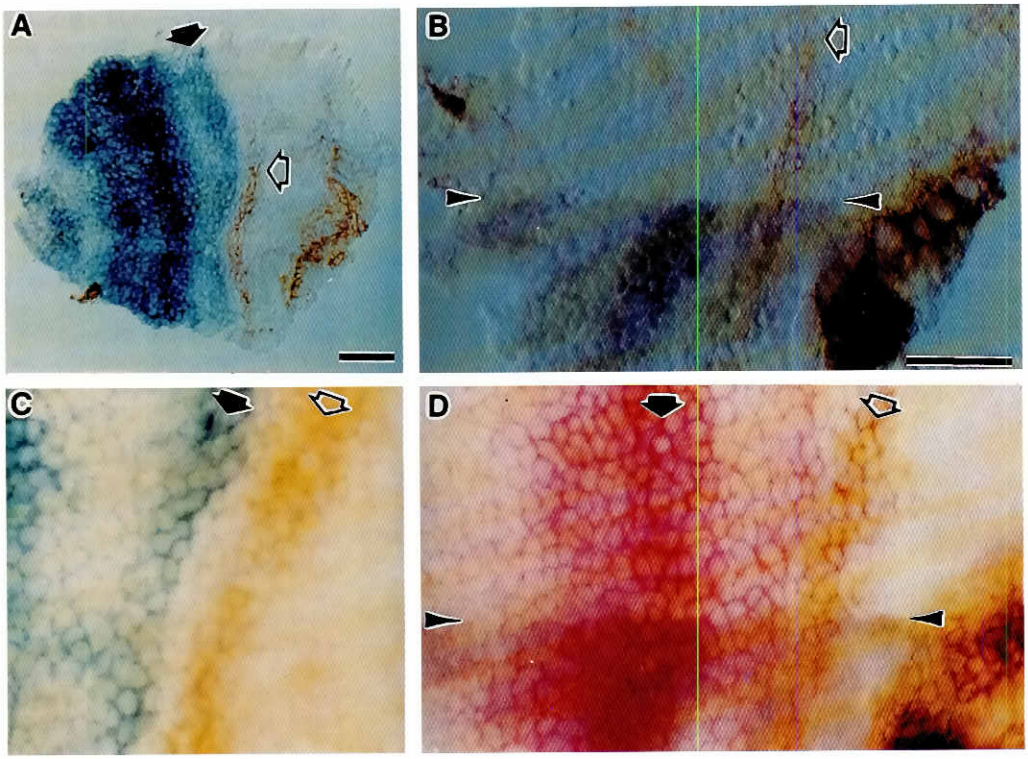

Fig. 3. Epithelial domains in the 33.5% stage T3 limb fillets. (A) *A limb double-labeled for annulin (brown) and alkaline-phosphatase (blue) and prepared as a fillet (epithelium opened posteriorly and unrolled on an adhesive surface, with the mesoderm removed; see Materials and Methods). The circumferential extent of the bands, whose longitudinal positions are shown in Fig. 2F, can be seen. While the alkaline-phosphatase bands, including the femur-trochanter band (solid arrow) extend around the limb circumference, the coxal annulin band (open arrow) ends at the mid-dorsal position.* **(B)** *A limb fillet double-labeled for annulin (brown) and engrailed (black). Engrailed labels the posterior limb compartment from a mid-ventral position (between arrowheads), and is approximately orthogonal to the coxal annulin band (open arrow).* **(C)** *Limb fillet double-labeled as in (A), showing outlines of individual epithelial cells. The coxal annulin band (open arrow) and femur-trochanter alkaline-phosphatase band (solid arrow) each are about three cells in width (the unlabeled cells between these two bands express semaphorin-I).* **(D)** *Epithelial domains of a fillet triple-labeled for alkaline-phosphatase (red), annulin (brown) and engrailed (black). The engrailed border (between arrowheads) is straight and is approximately orthogonal to the coxal annulin band (open arrow) and to the dark alkaline-phosphatase band (solid arrow) in the distal femur. Dorsal, up; distal, left. Scale bars: A and B, 100 μm; C and D, 50 μm (on B).*

From the results described above it is apparent that much of the limb epithelium traversed by the pioneer pathway expresses alkaline-phosphatase, annulin and semaphorin-I. To describe the relationship of the pioneer neuron pathway to the epithelial domains in the anterior limb compartment, we examined multiply-labeled intact limbs (Fig. 4) and fillet preparations (Fig. 5). Typically, the neuron cell bodies are found in the tibia, at the distal border of the annulin patch that lies at the femur border (Fig. 5C,D). The route of the pioneer axons crosses the annulin band (although annulin is not expressed when the growth cones are crossing this patch; Singer *et al.*, 1993), then the alkaline-phosphatase domain in the distal femur, then a domain that does not express any known marker (Figs. 5E, 6) and then the alkaline-phosphatase band at the femur-trochanter border. Immediately after exiting this domain, the axons turn within the trochanter and migrate ventrally along semaphorin-I expressing cells (Fig. 5E). Near the ventral midline (Fig. 5E,F), they make a sharp proximal turn after contacting the Cx1 guidepost cells (Caudy and Bentley, 1986; O'Connor *et al.*, 1990). From the region of the Cx1 cells they migrate parallel to the engrailed domain (Fig. 5F) until they reach the central nervous system. Thus the growth cones cross at least four types of epithelial domains (no label, annulin, semaphorin-I, alkaline-phosphatase), as well as some cells that express more than one marker. During most of their migration the growth cones are migrating in register with the axes of the epithelial domains, that is, either circumferentially (along the semaphorin-I band), or longitudinally (parallel to the engrailed stripe).

Discussion

The Ti1 afferent neurons are the first neurons to undergo axonogenesis in the grasshopper limb bud (Bate, 1976). Their growth cones pioneer a pathway to the central nervous system that is required for establishment of one of the major limb nerves (Klose and Bentley, 1989). Preparation of limb fillets, which involves retaining only the epithelium and cells (immature neurons) derived from it, shows that the epithelium can provide the information required for normal growth cone migration (Lefcort and Bentley, 1987; O'Connor *et al.*, 1990). Normal pioneer pathfinding after enzymatic removal of the basal lamina, suggests that much of this information is available at the plasma membrane of epithelial and neural cells (Condic and Bentley, 1989). These results focus attention on the molecular differentiation of the epithelium.

As in appendages of other insects (Bryant, 1993; Campbell *et al.*, 1993; Basler and Struhl, 1994), the grasshopper limb bud epithelium is organized into longitudinal and circumferential epithelial domains. Four domains, expressing engrailed, semaphorin-I, annulin, and alkaline-phosphatase have been described separately (Bentley and Toroian-Raymond, 1989; Patel *et al.*, 1989; Bastiani *et al.*, 1992; Kolodkin *et al.*, 1992; Singer *et al.*, 1992; Chang *et al.*, 1993). Here, using multiple-labeling, we have examined the arrangement and juxtaposition of these domains during pioneer growth cone migration (Fig. 6).

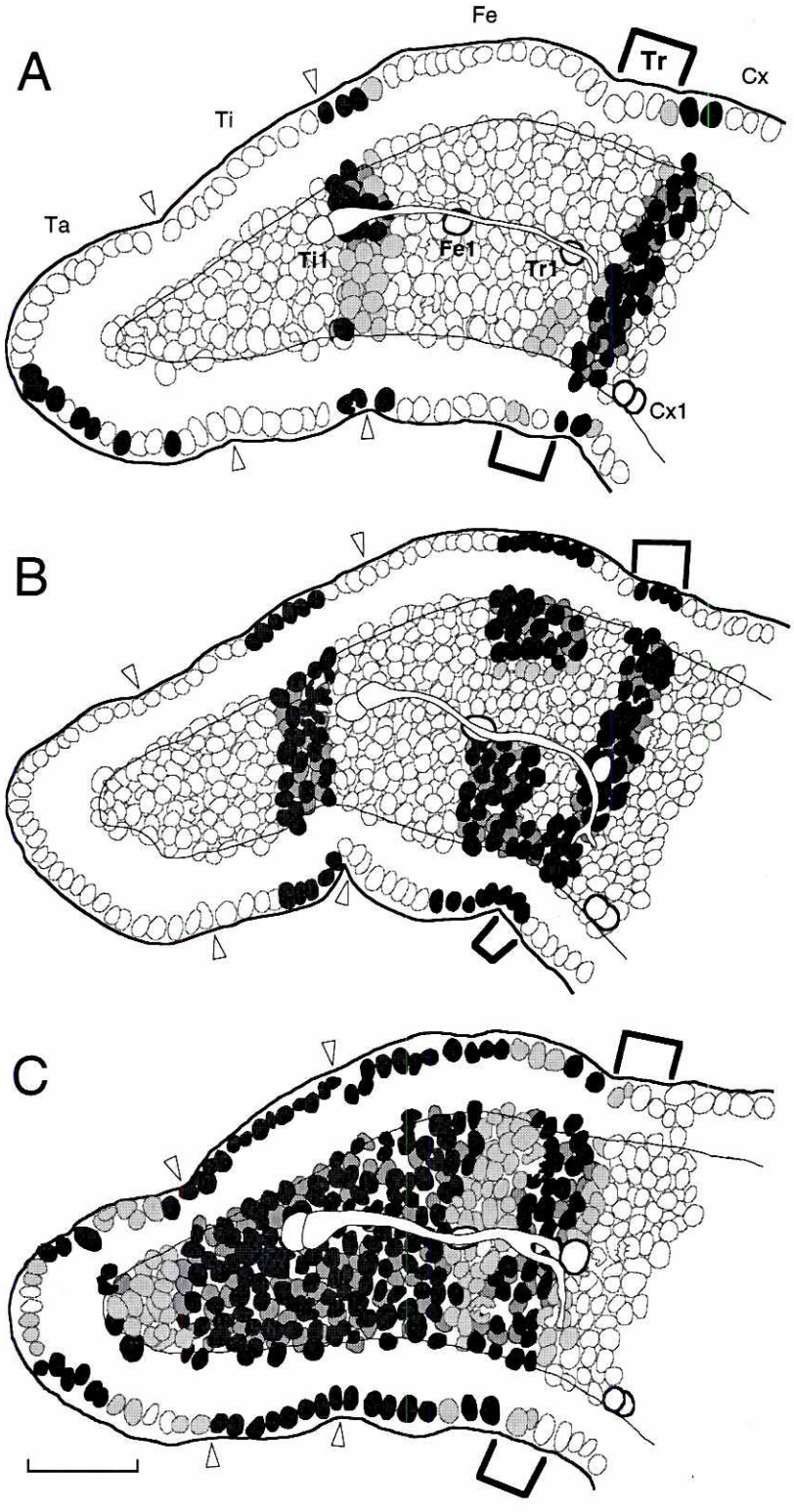

Fig. 4. Cell composition of epithelial domains in 33.5% stage T3 limbs. *Camera lucida drawings of three limbs labeled for pioneer neurons (anti-HRP antibody), one epithelial domain (annulin, or semaphorin-I, or alkaline-phosphatase), and all epithelial nuclei (Hoechst 33258). All nuclei in the anterior quadrant of the limb epithelium, and all nuclei along the dorsal and ventral midline are shown (see Materials and Methods). Dark and medium stipples indicate superficial and deep nuclei, respectively, of cells that strongly express the epithelial marker; the light stipple indicates all nuclei of cells that weakly express the marker.* **(A)** *Annulin is expressed in a band about three cells wide in the distal coxa, and a small patch in the distal femur. Weak labeling is developing ventrally in the femur band, and in the ventral trochanter.* **(B)** *Semaphorin-I is expressed throughout the trochanter, in a circumferentially incomplete band in the proximal femur, and in a complete band in the proximal tibia.* **(C)** *Alkaline-phosphatase is expressed in a band about three cells wide at the femur-trochanter border, and a broad band from mid-femur to the distal end of the tibia. This broad band is in the process of separating into distinct femur and tibia bands (Chang et al, 1993). The pioneer growth cones turn in the trochanter after crossing the alkaline-phosphatase band (C), and before the annulin band (A), and extend ventrally on the semaphorin band (B). Arrowheads mark segment boundaries; trochanter is bracketed. Ta, tarsus; Ti, tibia; Fe, femur; Tr, trochanter; Cx, coxa; Ti1, pioneer neurons; Fe1, Tr1, Cx1, guidepost neurons. Dorsal, up; distal, left. Scale bar, 50 μm.*

The results show that two of these markers, semaphorin-I and alkaline-phosphatase are expressed in alternating bands of cells that are close to complementary. In the region extending from the proximal border of the trochanter to the distal border of the tibia, some cells at every position along the proximo-distal level axis of the limb express at least one of these markers (at any given axial position, not all cells around the limb circumference express the marker). Proximal to the trochanter, a third marker, annulin, is expressed distally in the coxa. Proximal to the annulin band is a region in which no markers have been identified.

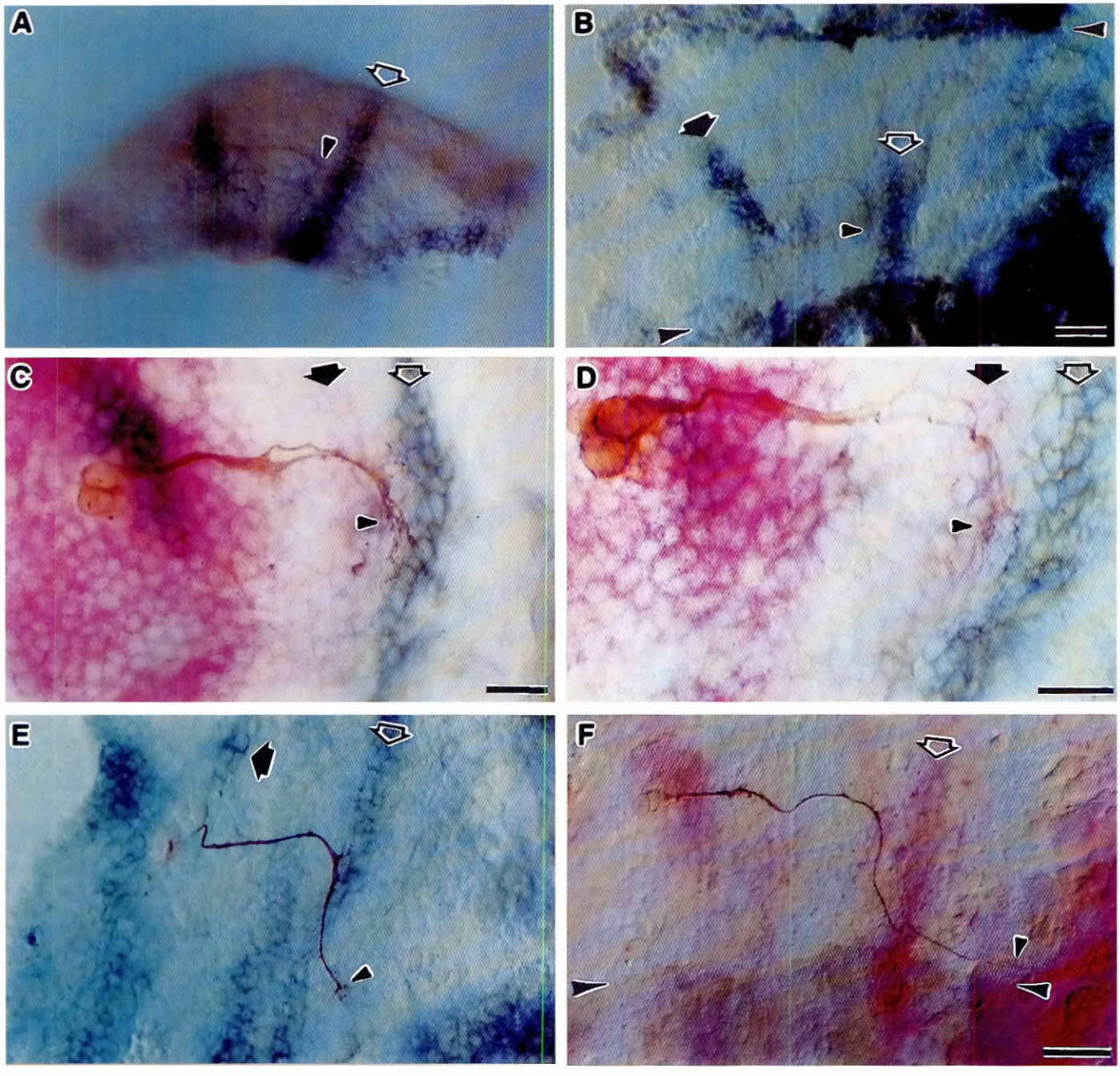

Fig. 5. Pioneer neuron pathway through epithelial domains. (A) *Double-labeling of the Ti1 afferent pioneer neurons (brown) and annulin bands (black) in an intact limb shows the pioneer growth cones (arrowhead) approaching the coxal annulin band (open arrow) at the 33% stage.* **(B)** *A limb fillet labeled as in (A), and with the posterior compartment also labeled for engrailed (black). Engrailed labeling defines the dorsal (left pointing arrowhead) and ventral (right pointing arrowhead) borders between the anterior and posterior limb compartments. The Ti1 path lies within the anterior compartment. The pioneer growth cones (small arrowhead) have crossed the annulin patch (solid arrowhead) in the distal femur, and have extended ventrally just distal to the coxal annulin band (open arrowhead).* **(C,D)** *Two triple-labeled limb fillets at the 33.5% stage with the pioneer neurons labeled (brown), and with the epithelium labeled for annulin (black) and alkaline-phosphatase (red). The neurons arise in the tibia just distal to the femur annulin patch; the growth cones (arrowheads) are turning ventrally in the trochanter after crossing the alkaline-phosphatase band (solid arrows) and before reaching the coxal annulin band (open arrows). Unlabeled semaphorin-I expressing cells lie between these two bands.* **(E)** *Double-labeling of the Ti1 pioneer neurons (brown) and semaphorin-I (black) on a fillet at the 34% stage. The pioneer growth cones (arrowhead) have completed their ventral migration along the trochanter semaphorin-I band (open arrow) and have just turned proximally toward the CNS. In the femur, the distal and dorsal band (solid arrow) of semaphorin-I is beginning to label.* **(F)** *A limb fillet at the 35% stage triple-labeled for Ti1 neurons (brown), annulin (red), and engrailed (black). After turning proximally and crossing the coxal annulin band (open arrow), the pioneer growth cones (small arrowhead) have migrated parallel to, and about one cell diameter anterior to, the ventral compartment border (between large arrowheads). Dorsal, up; distal, left. Scale bars: A and B, 50 μm (on B); C and D, 25 μm; E and F, 50 μm (on F).*

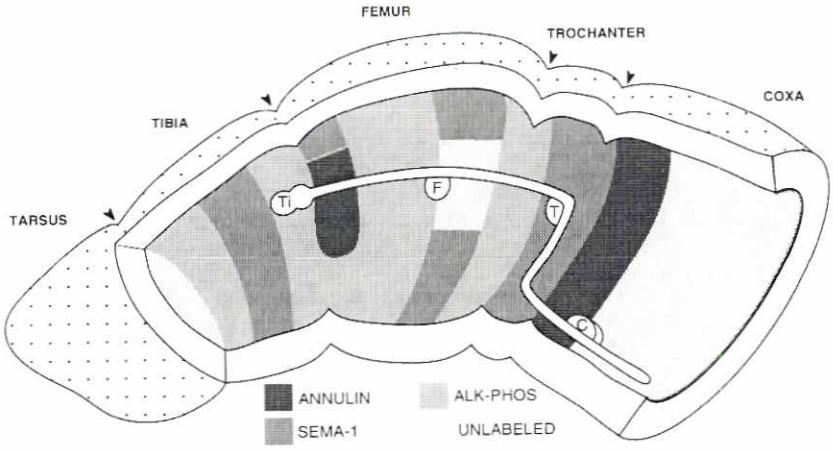

Fig. 6. Schematic diagram of the pioneer neuron pathway through epithelial domains in a T3 limb. *In this cut-away diagram, the posterior limb compartment has been removed from all segments except the tarsus, allowing the interior surface of the anterior compartment to be viewed. Epithelial domains expressing annulin, semaphorin-I (sema-I), and alkaline phosphatase (alk-phos) are demarked by stippling as indicated (as is unlabeled epithelium). The pioneer axon (Ti1) pathway is shown as well as the locations of immature (guidepost) neurons Fe1 (F), Tr1 (T), and Cx1 (C). The Ti1 pioneer neurons arise within the tibia, just distal to the annulin expressing patch of cells in the femur. Their axons grow proximally through the femur to the trochanter. They turn after crossing the band of alkaline-phosphatase expressing cells at the trochanter border and grow ventrally within the trochanter on semaphorin-I expressing cells. At the ventral end of the semaphorin-I band, they make filopodial contact with the Cx1 guidepost cells, and turn proximally across the annulin band in the distal coxal. They then migrate parallel to the ventral compartment border (see Fig. 1), and about one epithelial cell diameter inside the anterior compartment, until they reach the CNS.*

Most regions of the epithelium express only one of the three circumferential band markers tested here. Some epithelial domains, such as the semaphorin-I and annulin domains at the coxa-trochanter border, are contiguous. In other regions, such as the semaphorin-I and alkaline-phosphatase domains at the femur-trochanter border, there is a short zone of overlap, comprising one or two cell tiers, between domains. In some restricted regions, there is complete overlap of one expression domain by another. For example, all the cells in the annulin patch in the distal femur (Fig. 5B) also express alkaline-phosphatase (Fig. 1E). Thus, although most epithelial cells express only one of these three circumferential markers, they are not mutually exclusive.

What role do these domains play in growth cone guidance? Like other pioneers, the Ti1 cells appear to by guided by three types of cues, local guidepost cells, pathways, and more global gradients. Guidepost cells are specifically positioned cells or cell clusters that provide contact guidance to growth cones within filopodial reach (Palka *et al.*, 1992; Kuhn *et al.*, 1995). In development of the mammalian visual system, for example, special cells at the optic chiasm play this role in guiding retinal ganglion cell axons (Sretevan *et al.*, 1995). For the Ti1 pioneers, three guidepost cells and clusters provide a high affinity cue over part of the pathway (Caudy and Bentley, 1986; O'Connor *et al.*, 1990). More global positioning information can be provided by diffusible gradients (Kennedy *et al.*, 1994) or by gradients of cell surface molecules (Cheng *et al.*, 1995; Drescher *et al.*, 1995). In insect limbs, proximo-distal molecular gradients have been demonstrated in the basal lamina (Norbeck *et al.*, 1992), on which the Ti1 pioneer growth cones normally migrate (Anderson and Tucker, 1988; Condic and Bentley, 1989). A global dorso-ventral gradient that directs ventral growth of the pioneer neurons also may be present. Following treatment with anti-semaphorin-I antibodies, Ti1 growth cones can turn ventrally at many positions along the limb axis (Kolodkin *et al.*, 1992). Involvement of a specific signaling system is suggested by the frequent failure of ventral turning following enzymatic release of GPI-anchored molecules in the limb (Chang *et al.*, 1992).

A concomitant of orientation along a single gradient is that growth cone wandering in the other dimension is not constrained. Epithelial expression domains can serve to constrain such wandering. For example, motoneuron growth cones emerging from the spinal cord appear to be constrained to follow a pathway through the anterior regions of somites by the expression of repulsive molecules within the posterior regions of somites (Davies *et al.*, 1990). In the grasshopper limb, the narrow circumferential band of expression of semaphorin-I constrains the ventral turn of the pioneer growth cones to a specific location (the trochanter) along the longitudinal axis of the limb. If semaphorin-I is blocked by antibodies or fab fragments, growth cones then turn ventrally at many locations along the longitudinal axis (Kolodkin *et al.*, 1992). Growth on the semaphorin-I band does not appear to be caused by repulsion from the adjacent domain (annulin; Fig. 6) because growth cones readily migrate onto the annulin domain when semaphorin-I is blocked. Thus the semaphorin-I domain appears to form a narrow, positive pathway for growth cone migration.

For a portion of their route to the CNS, Ti1 growth cones migrate along the ventral edge of the anterior compartment. In this region, the pathway may be constrained by proximity to a border, rather than confinement within a narrow epithelial domain. Growth cones do not cross into the posterior compartment. This may be because the posterior compartment is not conducive to migration of anterior compartment neurons, although afferent neurons can cross compartment boundaries in *Drosophila* (Palka *et al.*, 1981). Another alternative may be that proximity to diffusible signals emanating from the posterior compartment effects differentiation of the band of anterior compartment cells adjacent to the border, as has been demonstrated in *Drosophila* (Basler and Struhl, 1994; Diaz-Benjumea *et al.*, 1994; Williams *et al.*, 1994). In the grasshopper, the Ti1 growth cones migrate along a chain of cells of undetermined origin that do lie adjacent to the compartment border (Caudy and Bentley, 1986).

Multiple labeling of epithelial domains during pioneer growth cone migration has provided more detailed information on the nature of the substrate traversed by the growth cones. The observation that for much of their route migrating growth cones are in register with either circumferential or longitudinal epithelial domains, and experimental tests of epithelial surface molecules

(Kolodkin *et al.,* 1992; Chang *et al.,* 1993), support the conclusion that epithelial expression domains are an important element of the growth cone guidance system in the limb. In this system, as in a variety of vertebrate systems (Chitnis and Kuwada, 1990; Fraser *et al.,* 1990; Cornel and Holt, 1992; Easter *et al.,* 1993; Wilson *et al.,* 1993; Macdonald *et al.,* 1994), molecular characterization suggests that a high degree of spatial organization of early embryonic epithelium and neuroepithelium contributes significantly to the guidance pioneer growth cones and establishment of the initial peripheral and central pathways of the nervous system.

Materials and Methods

Schistocerca americana eggs were collected from a colony maintained at the University of California at Berkeley. Individual eggs were dissected and staged according to percentage of embryogenesis completed (Bentley *et al.,* 1979; Caudy and Bentley, 1986). To prepare fillets, limbs were opened along the posterior midline and unrolled flat on a poly-l-lysine (5 mg/ml) coated substrate; mesodermal cells were removed with a suction pipette, leaving the ectodermal epithelium and neurons (Lefcort and Bentley, 1987; O'Connor *et al.,* 1990).

For labeling neurons with lipophilic dyes, pioneer neuron cell bodies exposed on a limb fillet were contacted for a few seconds with a crystal of DiI (Molecular Probes) on a micropipette (O'Connor *et al.,* 1990). Following a 15-30 min period for dye diffusion, dye was photoconverted with 1 mg/ml diaminobenzidine and ultraviolet irradiation to form a precipitate (Sandell and Masland, 1988). In intact limbs, neurons were labeled (Jan and Jan, 1982) with anti-horseradish peroxidase antibodies (Cappell Laboratories) using a biotin-avidin-HRP intensification kit (Vector Laboratories).

For labeling of limb epithelium, alkaline-phosphatase expressing cells were labeled with alkaline-phosphatase detection kit I (red-product) or kit III (blue product; Vector Laboratories; Chang *et al.,* 1993). Semaphorin-I, engrailed and annulin were labeled with monoclonal antibodies 6F8, 4D9 (kindly provided by C.S. Goodman) and 7H7 (Singer *et al.,* 1992). Primary antibodies were detected with peroxidase-conjugated secondary antibodies (brown reaction product; Patel, 1994), and intensified in some cases with nickel chloride (black reaction product; Patel, 1994). For multiple labeling, the individual protocols for each label were performed in series, in the following order: neurons (DiI), semaphorin-I (mAb 6F8), endogenous alkaline-phosphatase (Vector kit), engrailed (mAb 4D9), annulin (mAb 7H7). For labeling of epithelial nuclei with Hoechst 33258 (Sigma), fixed embryos were incubated in the dark for 30 min in 0.1 mg/ml Hoechst 33258 in 0.1M Tris-HCl, pH 7.2, rinsed in Tris-HCl, and stored in the dark.

For camera lucida drawings, intact limbs were labeled with anti-HRP antibody (for neurons), Hoechst 33258 (for nuclei), and one epithelial domain marker (annulin, or semaphorin-I, or alkaline-phosphatase). In a plane bisecting the limb dorso-ventrally, an outline of the limb epithelium and all epithelial nuclei within this outline were drawn. In tangential planes moving toward the anterior face of the limb, constrictions marking all segment boundaries, the Ti1 neurons, and all epithelial nuclei in the anterior quadrant of the limb were drawn. For each epithelial marker, labeling intensity in the epithelial nuclei was recorded as strong, weak, or not detected. Fifteen single-labeled limbs (five for each marker), and four limbs double-labeled for annulin and alkaline-phosphatase were drawn.

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