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Guidance of Peripheral Pioneer Neurons in the Grasshopper: Adhesive Hierarchy of Epithelial and Neuronal Surfaces

Abstract. *An important question in developmental neurobiology is how a neuron finds its way over long distances to its correct target during embryogenesis. Peripheral pioneer neurons in insect embryos have been used for study because of the relative simplicity of the early embryonic appendages, and the accessibility of the identified neurons whose growth cones traverse this terrain. The data presented suggest an adhesive hierarchy of both epithelial and neuronal surfaces that guides the first growth cones from the appendages of the grasshopper embryo.*

Bate first described pioneer neurons in the peripheral (*1*) and central nervous system (CNS) (*2*) of the grasshopper embryo. Since that time, pioneer neurons have emerged as particularly attractive cells with which to study the guidance of neuronal growth cones because of their large size, and the simplicity of the environment through which they navigate. Pathfinding by early differentiating neurons has been extensively studied in the antennae (*1, 3–5*), limb buds (*1, 3, 6–9*), cerci (*10*), and CNS (*2, 7, 11, 12*) of the grasshopper embryo; in the cerci of the cricket embryo (*13*); and in the developing wings of *Drosophila* (*14*) and the moth (*15*). These simple systems may reveal guidance mechanisms common to less accessible growth cones that navigate through more complex environments in these and other organisms.

How do the first growth cones in the peripheral appendages of the grasshopper embryo find their way to the CNS? Bate (*1*) noticed that the first pairs of axons in the periphery became surrounded at particular intervals by other cells, and suggested that if these cells were present early enough, they might serve as "stepping stones" for the first growth cones on their indirect journey to the CNS (*16*). Later, Goodman and co-workers (*11*) noticed that central pioneer neurons turned and grew toward specific neuronal cell bodies in the CNS which they called "landmark cells"; Ho, Goodman, and co-workers (*3, 7*) noticed similar nerve cells, including those described by Bate, in the peripheral appendages appearing to serve a similar landmark guidance role. The idea of the stepping stone–landmark cell was simplified by Bentley and Keshishian (*8*) to the "guidepost cell hypothesis," in which specially placed neurons are the sole source of guidance information. To the exclusion of other sources of guidance, they proposed that the "growth cones from the first pioneers navigate along a chain of cells to the CNS" (*17*).

Another model has been suggested to explain the guidance of peripheral neurons in the developing appendages of insects. Nardi and Kafatos (*18*) previously proposed a proximo-distal gradient of epidermal cell adhesiveness in the developing wing of the moth. Nardi (*15*) then used this model to explain the asymmetric response of growing sensory neurons when confronted with epithelial grafts that changed the proximal-distal axis. Nardi suggested that such an adhesive gradient might also direct the initial polarized growth of the pioneer neurons.

Several important questions are raised by these studies. (i) How do the pioneer neurons initiate their growth cones with a particular polarity, that is, proximally toward the CNS? (ii) What guides pioneer growth cones proximally down the

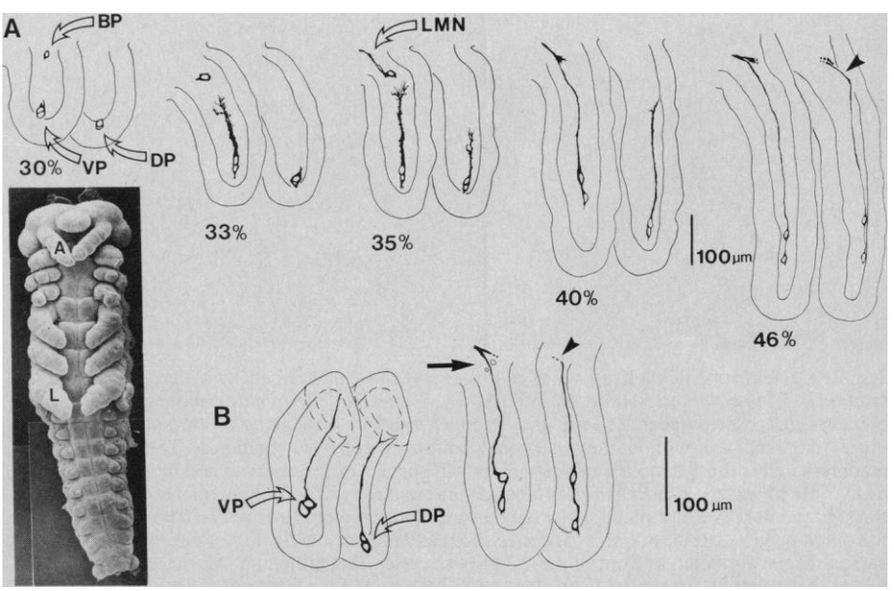

Fig. 1. Camera lucida drawings of antennae stained with the I-5 monoclonal antibody and a biotin-avidin horseradish peroxidase system. (A) Drawings of the antenna spanning the period of 30 to 46 percent of embryonic development reveal the spatio-temporal pattern of neuronal differentiation. In all split-plane drawings, the ventral plane is shown on the left and the dorsal plane on the right. Arrowhead points to where DP axons curve around lateral surfaces to ventral surface of epithelium as they fasciculate with and turn medially along the LMN axons; *VP*, ventral pioneers; *DP*, dorsal pioneers; *BP*, base pioneer; *LMN*, lateral motoneuron growth cone. (B) The left antenna was removed from a 33 percent embryo, and the right antenna was left intact; both were then placed in culture for 43 hours at 29°C and 5 percent CO_2 in a culture medium containing 20.8 nmole β-ecdysterone and 1.7 n*M* juvenile hormone I. In the control antenna (right), the VP growth cones reached the CNS, the BP neuron (left arrow) died as normal, and the DP growth cones made their characteristic ventral and medial turns along the LMN axon and entered the CNS (right arrowhead). In the experimental antenna (left), both pairs of pioneer growth cones have grown proximally along their stereotyped circumferential position to the base of the antenna; the DP growth cones did not turn ventrally and medially. (Inset) The inset on the left is a scanning electron micrograph of a 35 percent grasshopper embryo showing the antenna (*A*) and the metathoracic limb bud (*L*) used in these experiments. [Photograph by Robert Ho and Michael Bastiani]

appendage toward the CNS? (iii) What guides pioneer growth cones to turn away from this simple axial polarity en route to the CNS? (iv) Once they reach the CNS, what guides the pioneer growth cones along their stereotyped pathways? Bentley and Caudy (*9*) answered the third question by selectively killing the landmark cells at the distinctive turn in the limb bud and observing the absence of the normal turn by the pioneer growth cones.

The simplest system in the grasshopper embryo in which to study pathfinding by pioneer growth cones is the antenna in which two pairs of cells, the ventral (VP) and dorsal (DP) pioneer neurons, extend growth cones toward the CNS (*1, 3–5*). Here we present results based on immunocytochemistry, transmission electron microscopy, and tissue culture manipulations of the developing antenna; similar experiments confirm these findings for the developing limb bud as well. Our results suggest that all four questions above can be answered by an adhesive hierarchy of epithelial and neuronal surfaces whereby the pioneer growth cones are (i) initially directed by a polarized epithelium, (ii) guided proximally by an epithelial adhesive gradient, (iii) guided away from this axial polarity by neuronal landmark cells (specifically located neuronal cell bodies and processes), and (iv) guided along their characteristic routes in the CNS by labeled axonal pathways.

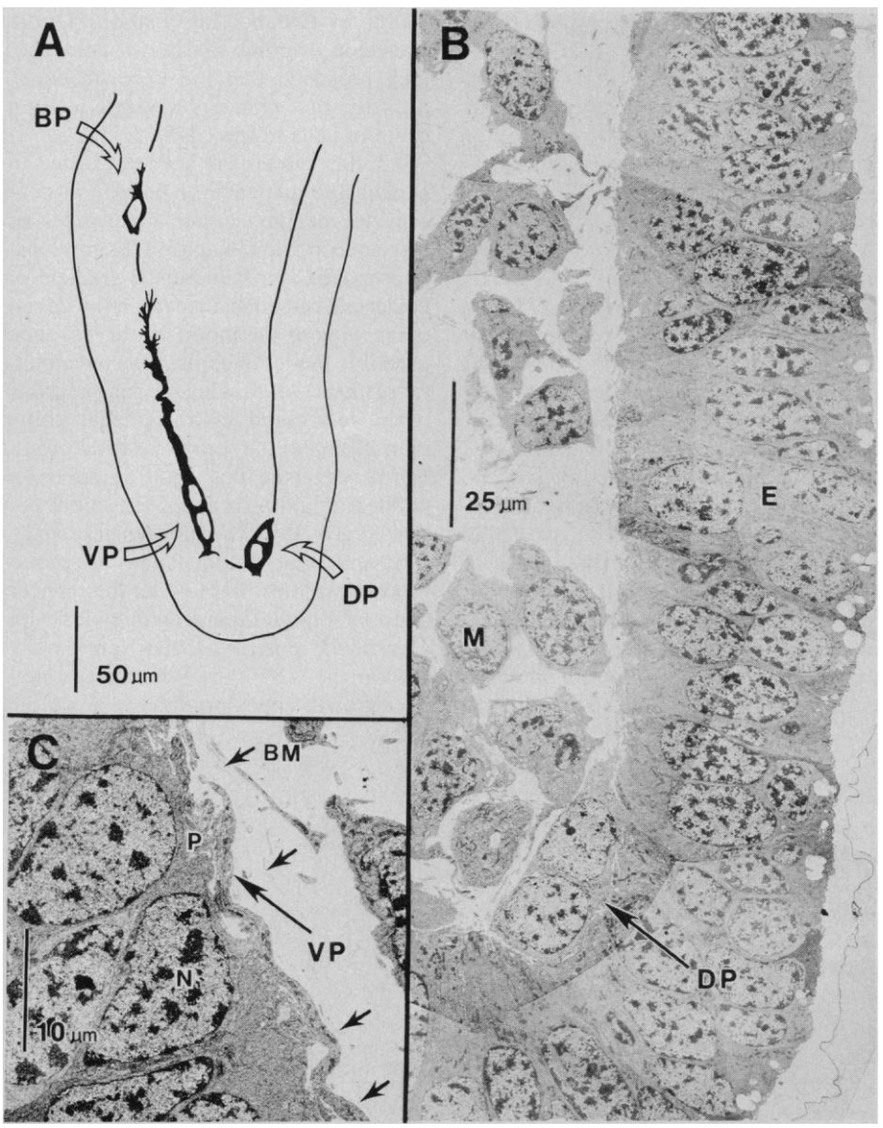

Fig. 2. (A) Camera lucida drawing of a 33 percent antenna stained with the I-5 monoclonal antibody; side view with ventral on left and dorsal on right; *VP*, ventral pioneers; *DP*, dorsal pioneers; *BP*, base pioneer. The DP growth cones must navigate along the dorsal epithelium for more than 200 μm without any neuronal landmark cells for guidance. The nearest such landmark cell is the BP, on the opposite (ventral) surface of the antenna and more than 200 μm away. (B) Montage of transmission electron micrographs of a 33 percent antenna sectioned through the dorsoventral plane, showing the dorsal surface of the antenna. The DP's at the tip have just begun to send out growth cones. Special landmark cells do not appear on the inside surface of the epithelial in front of the DP growth cones. *E*, epithelium; *M*, mesodermal cells in lumen of antenna. (C) Transmission electron micrograph of the VP growth cone in a 33 percent embryo, showing the growth cone under the basement membrane (*BM*) and in direct contact with the surface of the epithelial cells. The epithelial cells show an intrinsic polarity in that they overlap the next most proximal cell on its luminal surface; *N*, nucleus of epithelial cell; *P*, process of same cell over its proximal neighbor.

The pioneer neurons and all other neurons in the early grasshopper embryo can be visualized with the I-5 monoclonal antibody (*3, 19*) or with the serum antibody to peroxidase (*8, 20*). Two pairs of pioneer neurons appear in a precise temporal sequence at the distal tip of the antenna and extend growth cones toward the CNS early in development (Fig. 1A). First the pair of VP's appears at about 29 percent, and then the pair of DP's appears at about 30 percent (*1, 3, 4*). They pioneer the two axonal pathways (one ventral and the other dorsal) within the antenna. At approximately the same time, a single base pioneer cell (BP) appears on the ventral surface at the antennal base and extends its growth cone toward the CNS, contacting and fasciculating with the lateral motoneuron (LMN) growth cone extending outward from the CNS (*3, 4*). The VP neurons are about 100 μm from the BP neuron and on the same (ventral) surface of the epithelium when they initiate their growth cones (Fig. 1A). The DP neurons, in contrast, are on the opposite (dorsal) surface and well over 200 μm [and thus beyond the filopodial grasp which rarely exceeds 50 μm (*21*)] from the BP when they initiate their growth cones (Figs. 1A and 2A). Only when the DP growth cones are within about 30 to 50 μm of the LMN growth cone do they change their axial pathway as they fasciculate with the LMN axon and follow it ventrally and medially into the CNS (Fig. 1A). By this time the BP is dead.

When the antenna is removed from the embryo and grown alone in tissue culture (*22*), the DP growth cones extend proximally in a normal fashion toward the antennal base ($N = 12$) (Fig. 1B). Interestingly, they do not randomly wander around the circumference, but rather stay along their correct circumferential position, even when the antenna is removed distal to the BP cell and the antennal base ($N = 6$). These results eliminate the notion of a chemotactic gradient diffusing from the CNS or the antennal base and thus guiding the pioneer growth cones down the antenna toward the CNS (*23*).

The DP growth cones extend toward

the base of the antenna along the inside surface of the epithelium with no special neuronal landmark cells protruding from this columnar epithelium within filopodial grasp (Fig. 2, A and B). For the DP neurons, the only source of initial guidance is the epithelium itself. Immediately after the cell division of their precursor cell, the pioneer neurons are already polarized; the two cell bodies are aligned along the proximo-distal axis at the tip of the antenna (Figs. 1A and 2, A and B). The initial proximal extension is made when the cell bodies of the DP neurons are nestled in and surrounded by the epithelium at the tip (Fig. 2, A and B).

Transmission electron microscopy reveals that the DP growth cones extend under the developing epithelial basement membrane (Fig. 2C) and that their filopodia are in direct contact with the epithelial surface. These results suggest that the DP filopodia are responding to information along the epithelial surface. This information is likely to be in the form of an adhesive gradient of surface molecules (*24*) similar to that proposed by Nardi (*15*). In addition, the epithelial cells express an intrinsic polarity in their shape. The more distal cells overlap the luminal surface of the more proximal cells; this is particularly prominent near the antennal tip where the neurons make their initial polarity decision (Fig. 2B).

If the DP growth cones, however, were only guided by the epithelial polarity, it is likely they would never find their correct entrance to the CNS, but rather would continue to grow anteriorly along the dorsal surface of the epithelium. Normally, however, the DP growth cones make a characteristic ventral and medial turn at the base of the antenna once they are within filopodial grasp (within 30 to 50 μm) of the LMN growth cone (the BP cell is dead by this time). It appears as if filopodial adhesion to this landmark, the LMN growth cone, guides the DP growth cones toward the CNS once they reach the base of the antenna. For example, when the distal portion of the antenna is removed from the embryo and grown without the CNS (and thus without the LMN growth cone) in tissue culture, the DP growth cones extend proximally and remain on the dorsal surface of the epithelium ($N = 8$) (Fig. 1B).

Our results suggest two sources of guidance for the pioneer growth cones in the antenna: polarity information in the form of an adhesive gradient along the epithelium, and further pathway information in the form of specific early-differentiating neurons called "landmark cells." As for whether these mechanisms apply to the developing limb bud, we find that the pioneer neurons in the limb bud initiate and extend their growth cones proximally without contacting any distinctive landmark-guidepost cells. They make a similar proximal extension when the distal portion of limb buds are grown alone in culture, here too without the aid of the landmark-guidepost cells, and thus also eliminate a gradient diffusing from the CNS or base of the limb bud ($N = 6$). We conclude that axial information along the epithelium itself initially guides the pioneer growth cones in the limb bud toward the CNS, just as in the antenna.

Our results are in contrast to the guidepost cell hypothesis (*8, 9*) which states that cell F1 (*8*) [called cell 2B in (*3*)] in the limb bud is within filopodial grasp of the pioneer neurons before the pioneer neurons initiate their growth cones, and thus that filopodial contact with the F1 cell initially guides the pioneer growth cones proximally toward the CNS. Furthermore, cell F1 is supposed to display a distinctive cell surface antigen (*20*), and become dye-coupled via the pioneer filopodia, before being directly contacted by the pioneer growth cones (*17*).

However, we have used the same antibody against peroxidase (*20*), as well as extensive intracellular dye injections with Lucifer yellow (*7*) and Nomarski optics observations ($N > 20$), and have been unable to repeat these observations on the F1 cell. Rather, the F1 cell does not bind tagged antibody, nor does it appear to have differentiated from the epithelium or become dye-coupled to the pioneer growth cones until just after the growth cones have extended past the presumptive F1 location and reached the F2 cell.

Our adhesive hierarchy hypothesis for the guidance of pioneer growth cones is reminiscent of Steinberg's differential adhesion hypothesis for the morphogenetic control of embryonic tissues (*25*). Our results suggest that the filopodia of the pioneer growth cones in the antenna and limb buds of the grasshopper embryo express an adhesive hierarchy, whereby the surfaces of neurons are preferred over the surfaces of the epithelial cells. Given only the epithelium, the growth cones extend proximally along its surface, appearing to follow an epithelial adhesive gradient. Given a choice in the periphery, however, of neurons versus epithelium, the filopodia preferentially adhere to the neuronal surfaces and thus guide the growth cones onto these neuronal cell bodies and axons. Given a choice in the CNS of different axon bundles, certain neuronal surfaces appear to rank higher in the adhesive hierarchy than others; they invariably choose a particular axon bundle on which to extend (*26*), similar to the observation of selective fasciculation by central neurons that led to the labeled pathways hypothesis (*27*).

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16. The pioneer axons of the limb bud follow an indirect route to the CNS. After extending proximally, the growth cones make a posterior turn; near the posterior margin of the limb, they make a second medial turn and extend to the CNS (*1, 6*).
17. Bentley and Keshishian (*8*) reported that (i) the pioneer growth cones (the Til cells) extend toward and contact each of these guidepost cells, called F1, F2, and CT1, (ii) they become dye-coupled to each of these cells at a distance via their filopodia, and (iii) these guidepost cells are distinctive in that "each cell in this group displays on its surface at least one type of molecule which distinguishes it from surrounding cells and does so before arrival of the pioneer axons."
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21. Filopodia have a diameter of 0.1 to 0.2 μm and a length that can exceed 50 μm. According to D. Bray [in *Cell Behavior*, R. Bellairs, A. Curtis, and G. Dunn, Eds. (Cambridge Univ. Press, New York, 1982), p. 299] filopodia are randomly extended from the growth cone and retracted in a contractile cycle. If a filopodium makes contact and its adhesion is weak, then it is retracted; if adhesion is strong, however, tension is then increased in that direction during the contractile cycle and the growth cone advances toward the point of attachment [differential adhesion of filopodia, P. Letourneau, *Dev. Biol.* **66**, 183 (1975)].
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26. Once they enter the CNS, the pioneer growth cones are confronted with a scaffold of axonal pathways, and yet invariably choose a particular pathway. For example, in the limb bud, they extend anteriorly along an ipsilateral medial axonal pathway.
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Inheritance of Functional Foreign Genes in Plants

Abstract. *Morphologically normal plants were regenerated from* Nicotiana plumbaginifolia *cells transformed with an* Agrobacterium tumefaciens *strain containing a tumor-inducing plasmid with a chimeric gene for kanamycin resistance. The presence of the chimeric gene in regenerated plants was demonstrated by Southern hybridization analysis, and its expression in plant tissues was confirmed by the ability of leaf segments to form callus on media containing kanamycin at concentrations that were normally inhibitory. Progeny derived from several transformed plants inherited the foreign gene in a Mendelian manner.*

Agrobacterium tumefaciens, the causative agent of crown gall disease, is capable of transferring a DNA segment (designated T-DNA), located between specific border sequences, from its tumor-inducing plasmid (Ti plasmid) into the nuclear DNA of infected plant cells (*1*). Expression of T-DNA-encoded tumor genes in the transformed cell provides a selectable trait for recognition of those cells in culture; namely, the ability to grow on medium without added phytohormones. Unfortunately, this trait interferes with regeneration of normal fertile transformed plants (*2*).

Recently, we (*3*) and others (*4*) have constructed chimeric genes that function as dominant selectable markers in plant cells, thus making the tumor genes unnecessary for identification of transformants. Our chimeric gene contains the coding sequence of the bacterial gene for neomycin phosphotransferase II (NPTII) joined to the 5′ and 3′ regulatory regions of the nopaline synthase (NOS) gene, which is expressed constitutively in higher plant cells (*5*). We have shown that petunia and tobacco cells transformed with this chimeric NOS/NPTII/NOS gene are readily selected and are highly resistant to kanamycin (*3*).

We now report that kanamycin-resistant plant cells obtained with our vector system regenerate to morphologically normal plants. These plants carry a functional kanamycin-resistance gene and produce viable seeds. Analysis of progeny shows that the chimeric kanamycin-resistance gene is inherited and is expressed as a dominant Mendelian trait.

We used the previously described pMON120 intermediate vector to introduce the chimeric NOS/NPTII/NOS gene into the *A. tumefaciens* Ti plasmid. The pMON120 plasmid also contains an intact NOS gene as a scorable transformation marker. This NOS fragment includes the nopaline-type T-DNA right border sequence (*6*). Because this additional border sequence is initially carried on a separate plasmid, we refer to our system as the split end vector (SEV) system. Figure 1 shows how this system is used. The pMON128 plasmid [pMON120 containing the chimeric kanamycin-resistance gene (Fig. 1B)] is introduced by conjugation into *A. tumefaciens* cells, where homologous recombination with a resident octopine-type Ti plasmid [pTiB6S3 (Fig. 1A)] occurs. The resultant cointegrate plasmid pTiB6S3::pMON128 (Fig. 1C) contains a hybrid T-DNA in which the nopaline-type right border sequence is positioned between the kanamycin-resistance gene and the tumor genes of the resident Ti plasmid. Use of the nopaline T-DNA border sequence during infection results in the transfer of a short T-DNA segment (Fig. 1E) which contains the kanamycin-resistance gene and an intact NOS gene but does not contain genes for tumor formation or octopine synthase (*7*). The short-transfer transformants can be regenerated to give intact plants as described below.

Transformation of *Nicotiana plumbaginifolia* cells was carried out with the engineered *A. tumefaciens* train containing the chimeric NOS/NPTII/NOS gene by the method of cocultivation (*3*).

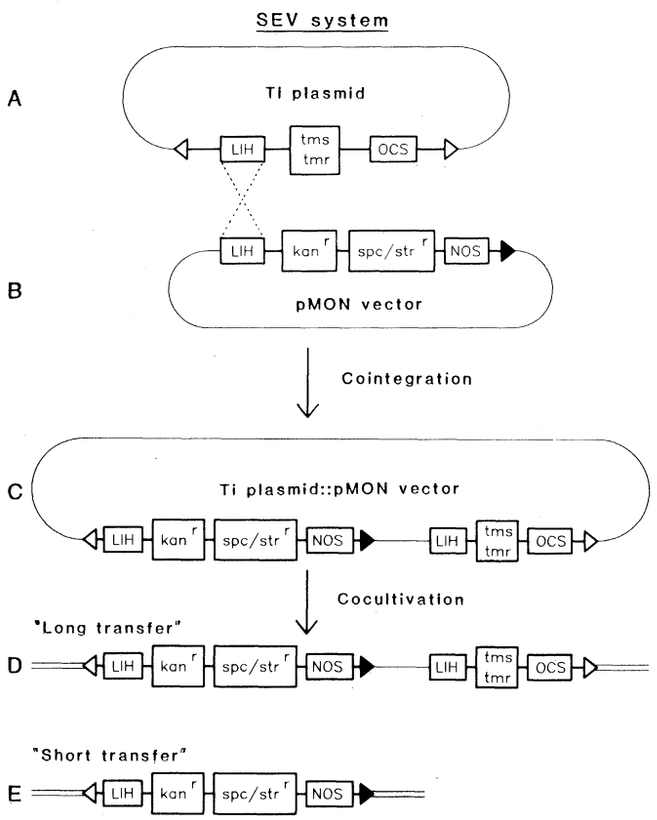

Fig. 1. Steps in the SEV system for plant cell transformation. The arrows represent the T-DNA border sequences. *LIH* is a region of homologous DNA for recombination. The tumor genes are represented by *tms* and *tmr* (*11*); *OCS* and *NOS* are octopine and nopaline synthase genes, respectively. The chimeric kanamycin-resistance gene is designated as *kan*^{r}. The bacterial spectinomcyin - streptomycin resistance determinant for selection of cointegrates is designated *spc/str*^{r}. Reciprocal recombination of (A) a resident Ti plasmid (pTiB6S3) and (B) pMON120 derivative (pMON128) yields (C) the cointegrate, pTiB6S3::pMON128. After cocultivation and selection for kanamycin-resistant plant cells either (D) the entire hybrid T-DNA or (E) a truncated T-DNA without tumor genes is transferred into the plant genome.