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Expression of a surface epitope on cells that link branches in the tracheal network of *Manduca sexta*

JAMES B. NARDI

Department of Entomology, University of Illinois, 320 Morrill Hall, 505 S. Goodwin Avenue, Urbana, IL 61801, USA

Summary

A monoclonal antibody (MAb 2F5) to a cell surface epitope labels a small subpopulation of tracheal epithelial cells in each thoracic and abdominal segment of *Manduca*. These cells (nodes) represent the sites within the tracheal network at which invaginating tracheal tubes join during embryonic establishment of the tracheal network. Tracheal nodes are also the sites at which tracheal cuticle fractures during each molt. Since tracheal cuticle is shed through each spiracle, a tracheal node lies between each pair of contralateral spiracles within a segment (commissural node) and between each pair of adjacent, ipsilateral spiracles (lateral longitudinal node). MAb 2F5 first labels presumptive nodal cells of tracheal epithelium immediately prior to the linking of epithelial tubes from successive and opposite spiracles. One cell at the tip of each invaginating tracheal branch labels with MAb 2F5. The highly localized expression of the cell surface epitope recognized by MAb 2F5 may be instrumental in the orderly coupling of tracheal branches during embryonic development. On the basis of immunolabeling of Western blots and tissues, MAb 2F5 is believed to recognize *Manduca* fasciclin II, a member of a class of molecules involved in cell adhesion/recognition.

Key words: cell recognition, cell surface, insect tracheae, nodes, immunolabels, insect evolution.

Introduction

During embryonic development of the tracheal systems in many insects, initially noncontiguous populations of cells join to form a contiguous tubular network extending from the head to the last abdominal segment. The tracheal system is initiated as a series of ectodermal invaginations along the longitudinal axis of the insect embryo (Snodgrass, 1935). As the two invaginating tracheal trunks of a given body segment extend internally, they branch and fuse with each other not only at the ventral midline but also with tracheae from adjacent segments. The complexity of branching patterns for tracheal systems varies considerably among the insects; but in *Manduca sexta* embryos, each tracheal invagination forms three main branches: (1) one branch fuses at the midline with the contralateral tracheal branch from the same segment; (2) one branch fuses anteriorly with a posterior branch from the next anterior segment; and (3) another branch fuses posteriorly with an anterior branch from the next posterior segment.

The points of tracheal fusion during embryogenesis also correspond to the points at which tracheal cuticle breaks at the time of each postembryonic molt (Locke, 1958, 1967). Breakage at each of these key points (nodes) makes possible the periodic shedding of cuticle in all but the finest tracheal branches. The tracheal epithelium at these nodes is unique in that a monoclonal antibody (MAb 2F5) that recognizes a cell surface epitope labels cells of tracheal nodes but none of the remaining tracheal cells. The distribution of this epitope among cells of the tracheal epithelium is examined throughout embryonic and postembryonic development of the *Manduca* tracheal system, and its possible role as a cell recognition epitope is considered.

Materials and methods

Experimental animals

Larvae of *Manduca sexta* were maintained on an artificial diet under controlled lighting (18L:6D) and temperature (26°C) conditions.

Newly deposited eggs collected from the *Manduca* breeding cage were maintained at 26°C. At this temperature embryonic development lasts about 96 h. The stages of insect embryogenesis are conventionally referred to as percentages of development from oviposition to hatching. Timetables for *Manduca* embryonic development published by Dorn *et al.* (1987) as well as by Copenhaver and Taghert (1989) provided additional landmarks for staging of embryos.

Sources of monoclonal antibodies

A supply of MAb 2F5 (also referred to as MAb TN-1) was generously supplied to the author by Drs Paul Taghert and Philip Copenhaver. This antibody was obtained from a

hybridoma screening in which the adult transverse nerve of *Manduca* had been used as a immunogen (Carr and Taghert, 1988).

MAb 2C10 was derived from a mouse that had been immunized with the upper monolayers of epithelia from last instar wing discs of *Manduca*. This antibody stains the surfaces of epidermal epithelial cells as well as tracheal epithelial cells.

The antigens recognized by MAb 2F5 and MAb 2C10 will hereafter be referred to as 2F5 and 2C10 respectively.

Immunolabeling of embryonic and postembryonic tracheal nodes

Staged embryos of *Manduca* were removed from their chorions and embryonic membranes with fine watchmaker's forceps. Dissections were carried out in Grace's insect tissue culture medium (GIBCO). Each embryo was opened dorsally with fine tungsten needles to facilitate penetration of antibodies. Embryos were transferred to phosphate-buffered saline (PBS) containing 4% paraformaldehyde for fixation. The subsequent immunolabeling procedure was identical to that described below for labeling of postembryonic tissues. All immunolabeled embryos were mounted in 70% glycerin in 0.1 M Tris (pH 9.0). The following number of embryos were stained with MAb 2F5: 24% (10), 30–35% (27), 36–40% (11), 41–45% (10), 46–50% (24), 51–65% (7), 80–85% (4). Fewer embryos were stained with MAb 2C10: 50–55% (5), 60–70% (11), 80–85% (5).

The tracheal networks of larvae were exposed by submerging anesthesized larvae in Grace's culture medium (GIBCO) and making an incision along the entire length of each animal's dorsal midline. The free edges of larval integument were pinned down on a Sylgard surface and the entire gut was removed. Specimens were fixed for 30–60 min with 4% paraformaldehyde in PBS. After at least three rinses in PBS, tissues were placed in PBS containing 3% normal horse serum and 0.1% Triton X-100 (hereafter referred to as PHT) to which was added MAb 2F5 ascites fluid (1:10000) or MAb 2C10 hybridoma supernatant (1:1). Tissues were then incubated for 24 h at 4°C in primary antibody solutions. Before transferring to PHT containing the secondary antibody (biotinylated horse anti-mouse, Vector Laboratories) at a dilution of 1:200, tissues were thoroughly rinsed in PHT. After a 24 h exposure to secondary antibody, tissues were rinsed with PBS+0.05% Tween-20 and then incubated with avidin–HRP (Vector Laboratories) in PBS+0.05% Tween-20 for 2 h at room temperature. After several rinses in PBS, the localization of antibodies was visualized by addition of diaminobenzidine and H_2O_2. Tissues were once again rinsed in PBS and mounted in either (1) Permount (Fisher) after dehydration and clearing or (2) 70% glycerin in 0.1 M Tris (pH 9.0). At least three and as many as six larvae from each of the five larval instars were immunostained with each antibody.

Electrophoresis and Western blotting

Developing adult wings of *Manduca sexta* (4–7 days postpupation) are a rich source of 2F5 and 2C10 cell surface antigens. Wings, rather than tracheae, were used as a source of these antigens since the former tissues can be more easily obtained.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins from wing epithelia (Laemmli, 1970). Wing lysates were applied to 8% gels. After electrophoresis, proteins were electrophoretically transferred from the polyacrylamide gel to a nitrocellulose filter (Towbin *et al.* 1979). The nitrocellulose containing the transferred proteins was preincubated for 8 h at 37°C in PBS containing 5% nonfat dry milk (blot buffer) to reduce nonspecific binding of antibodies. After washing in PBS+0.05% Tween-20 the filter was incubated overnight at 4°C with primary antibody in blot buffer+3% normal horse serum (NHS). MAb 2F5 was diluted 1:10000 and MAb 2C10 was diluted 1:1. As controls, filters with proteins from wing lysates were incubated with blot buffer alone at this stage. Before addition of biotinylated horse anti-mouse secondary antibody (Vector), nitrocellulose filters were washed with PBS+0.05% Tween-20. Filters were left in blot buffer containing secondary antibody (1:200) and 3% NHS overnight at 4°C. Incubation with secondary antibody was followed by several rinses in PBS+Tween-20. The filter was added to avidin–HRP (Vector) in blot buffer and 3% NHS to complete the antibody complex. Following additional rinses in PBS+Tween-20, immunolabeled bands on filters were visualized by addition of diaminobenzidine (1 mg ml^{-1}) and an equal volume of 0.02% H_2O_2 in PBS.

Preparation of tissues for plastic sectioning

Tissues that had been immunolabeled and rinsed in PBS as described above were immediately postfixed with 2% OsO_4 in 0.1 M cacodylate buffer (pH 7.3) for 3 h at 4°C. Following dehydration in graded concentrations of ethanol (10%–100%), tissues were treated with propylene oxide before being embedded in Epon 812. 1.0 μm sections were cut on a Reichert OMU2 ultramicrotome and serially arranged on glass slides. These sections were stained with toluidine blue (1%) in 1% borax. Ultrathin sections were cut on the same microtome and mounted on 200 mesh copper grids prior to staining with uranyl acetate and lead citrate. Sections on grids were examined with a JEOL 100CX transmission electron microscope.

Tissues that had not been immunolabeled were fixed and processed as described previously (Nardi *et al.* 1985), except that ruthenium red was not added during postfixation. Dehydration, embedding and sectioning followed the protocol presented in the preceding paragraph.

Results

Location of tracheal nodes

Tracheal nodes are the sites within the tracheal network (Fig. 1) where tracheal cuticle breaks at the time of each molt (Locke, 1967, 1974). Tracheal cuticle of an insect is continuous with the external cuticle and is pulled through each spiracular opening during a molt. Accomplishing this tracheal molt presents a topological conundrum that is solved by breaking tracheal cuticle in each tracheal branch that lies between two adjacent contralateral or two adjacent ipsilateral spiracles. A node occupies each of these break points (Figs 1, 4).

In the thorax of *Manduca* larvae, spiracles are only evident on the prothoracic segment (Fig. 1, PR). Between the spiracle on one side of the prothorax and the first abdominal (A1) spiracle on the same side, one would expect to find only a single tracheal node along the lateral longitudinal trunk of the tracheal system. However, two tracheal nodes are located between these two spiracles on each side of an animal. Closer examination of larvae revealed the presence of a pair of diminutive spiracles near the anterior margin of the metathoracic segment (MT). Each MT spiracle is

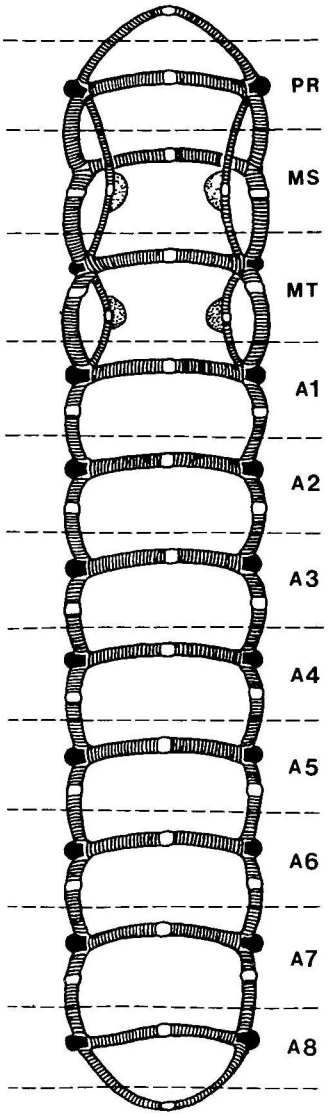

Fig. 1. Schematic diagram of *Manduca* tracheal network. Segment boundaries are indicated with dashed lines. PR, MS, MT=prothorax, mesothorax, metathorax, respectively. A1–A8= abdominal segments 1–8. Spiracles are represented by the solid black sites within the network. All spiracles function during respiration except the diminuitive spiracle of the MT. Tracheal nodes are represented by the clear areas and lie between spiracles. Those nodes that lie on the ventral midline of *Manduca* and join tracheal branches arising in the same segment are the ventral commissural nodes. Those nodes that occupy positions on the two lateral longitudinal trunks of the tracheal network and join tracheal branches that arise in different segments are referred to as lateral longitudinal nodes. Internodal tracheal epithelia are represented by crosshatching of the network. Note that each tracheal branch attached to the wing imaginal discs (stippled, hemispherical objects in MS and MT) has a node where tracheal cuticle breaks at each molt.

located between the above-mentioned two nodes – one within the mesothorax (MS) and the other within the MT. This is a case in which the occurrence of particular morphological features (nodes) predicted the existence of an easily overlooked structure (MT spiracle). The spiracle on the MT has been reported in other caterpillars (Snodgrass, 1935); and although it does not function during respiration of the larva, it does function during molting and serves as the site through which tracheal cuticle from the MS and MT are shed. In the embryo of *Manduca*, tracheal invagination occurs at the site of this future MT spiracle (25% embryogenesis), and the embryonic spiracles of the MT are smaller than other spiracles but still conspicuous. Only during postembryonic development do these spiracles become inconspicuous.

MAb 2F5: A cell surface marker for epithelial cells of tracheal nodes

MAb 2F5 recognizes a cell surface epitope found on a number of cell types in *Manduca* (Carr and Taghert, 1989). Among these cell types is the subset of tracheal cells located at tracheal nodes as well as a subset of neuronal cells in the central nervous system of *Manduca* embryos. It is this immunolabeling pattern of the central nervous system that first suggested the relationship of 2F5 to another characterized cell surface molecule. The immunolabeling pattern of these *Manduca* neuronal cells closely matches that observed for anti-fasciclin II labeling of neural cells in grasshopper embryos (Carr and Taghert, 1988; Snow *et al.* 1988).

The epithelial cells of developing adult wings also are surface labeled with MAb 2F5 and are a rich source of the epitope recognized by MAb 2F5 (not illustrated). Lysates of these wings were fractionated using SDS–PAGE under nonreducing conditions and electrophoretically transferred to nitrocellulose blots. As a control, nitrocellulose blots to which polypeptides of wing lysates had been transferred were exposed to secondary antibody (horse anti-mouse) in the absence of any primary antibody. The secondary antibody from horse consistently recognized a *Manduca* polypeptide with a relative molecular mass of 125×10^3 (Fig. 2). Following immunolabeling of nitrocellulose blots with MAb 2F5, two distinct polypeptide bands having relative molecular masses of 91 000 and 94 000 were visible.

Polypeptides transferred to nitrocellulose blots from reducing gels failed to react with MAb 2F5, suggesting that disulfide bonds exist in the antigens recognized by MAb 2F5 and that antigenicity is destroyed by disruption of these bonds.

The characterization of antigens recognized by MAb 2F5 on Western blots as well as the immunolabeling patterns of tissues are both consistent with the 2F5 antigens being fasciclin II of *Manduca*. First, the molecular size of the antigens is very similar to that for fasciclin II of the grasshopper (Snow *et al.* 1988; Harrelson and Goodman, 1988). Second, several disulfide bonds are found in fasciclin II of grasshopper, and the presence of disulfide bonds in the 2F5 antigens of *Manduca* is inferred from Western blot analysis.

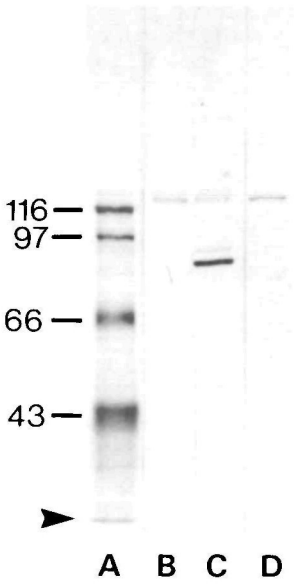

Fig. 2. Immunoblotting of *Manduca* wing lysates (day 4) with MAb 2F5 (B,C,D). Proteins were fractionated with SDS–PAGE on 8% gels and transferred to nitrocellulose. Positions of dye front (arrowhead) and biotinylated protein standards (Bio-Rad) are indicated ($M_r\times10^{-3}$). (A) Molecular weight standards; (B) control, nonreducing gel labeled with secondary antibody in absence of MAb 2F5; (C) nonreducing gel labeled with MAb 2F5; (D) reducing gel labeled with MAb 2F5.

As expected for a surface molecule involved in homophilic binding, the 2F5 antigens are localized primarily to those cell surfaces that adjoin other cells expressing these antigens. 2F5 antigens are distributed over both the basal and lateral surfaces of nodal cells from postembryonic tracheae (Fig. 3). The interface between nodal cells and internodal cells does not label with MAb 2F5 (Figs 3B,4D). Each nodal cell at this interface therefore shows a nonisotropic distribution of the 2F5 eptiope. Individual cells are somehow able to fine-tune their distribution of these surface molecules.

Whereas the pattern of immunostaining with MAb 2F5 changes dramatically within the nervous system, mesoderm and epidermis of *Manduca* during development (Carr and Taghert, 1988; 1989), the distribution of the 2F5 epitope within the tracheal epithelium remains unchanged throughout the second half of embryonic development and all of postembryonic development.

MAb 2C10: A cell surface marker for all epithelial cells of tracheae

This immunolabel delineates all cells of the tracheal epithelium and has helped to clarify the relationship between cells at nodes and cells of internodal epithelium (Fig. 4B). Surfaces of tracheal cells do not label with MAb 2C10 until the last quarter of embryogenesis. However, all tracheal cells label with MAb 2C10 throughout postembryonic development.

Embryonic development of the tracheal network

Major tracheal tubes begin invaginating at prospective spiracular sites at about 25% embryogenesis. These tracheal tubes extend and follow stereotypic pathways until they fuse at sites corresponding to future tracheal nodes. Between the inception of tracheal invagination and the fusion of tracheal epithelium at lateral longitudinal nodes, approximately 20% of embryogenesis transpires. (Fusion of tracheal branches to form the ventral commissural trunks is obscured by the presence of the overlying nerve cord. See Figs 1 and 4C.) During this portion of embryonic development, the anterior invaginating trachea of each abdominal segment grows parallel to the embryonic midline until it reaches the segmental boundary. At this point the anterior branch curves along the posterior side of the boundary in a dorsal direction for about 50 μm (double arrowheads in Fig. 5C, D) before it reverses its direction and moves both ventrally as well as anteriorly after crossing the segmental boundary (Fig. 5A–E). The posterior tracheal branch from segment *n* grows about 25 μm in a dorsoposterior direction until it contacts the anterior tracheal branch from segment *n+1* (Fig. 5A–D). During the growth of tracheal branches, only a single cell labels with MAb 2F5 at the tip of each major branch. Tip cells frequently extend processes during this period (Fig. 5B). Immunolabeling of these tip cells is not evident until shortly before fusion of anterior and posterior branches – i.e. not until they are separated by a distance of about 25 μm (Fig. 5A); and only cells at the tips of tracheal branches that are destined to form nodes label with MAb 2F5. This antibody labels these

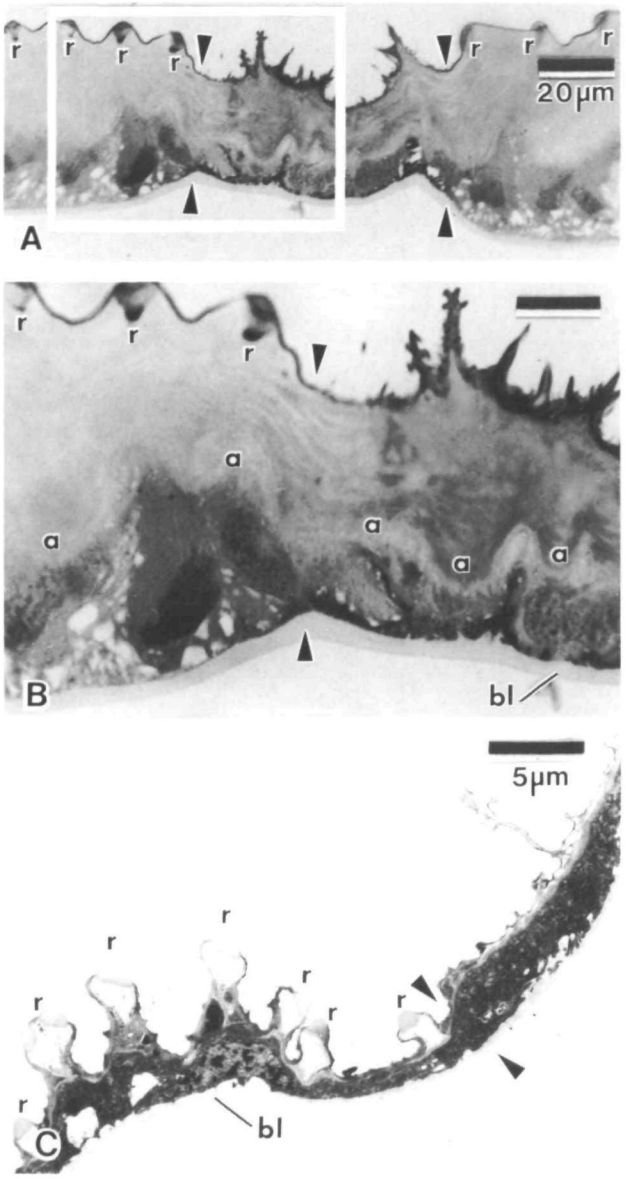

Fig. 3. Sections of nodes from lateral longitudinal trunk of fourth larval instar *Manduca*. Each node has been sectioned parallel to the long axis of each tracheal trunk, and only one side of each tubular cross section is shown. Apical cuticle of trachea is at top; basal lamina (bl) of trachea is at bottom. Taenidial ridges (r) of cuticle overlie internodal cells of trachea, and spiny cuticle covers the apical surface of nodal cells. The interface between nodal cells and internodal cells is marked by arrowheads. Tissue in A and B was taken from a late fourth instar larva, and tissue in C was removed from an early fourth instar. More cuticle has been produced by the cells of the late fourth instar tracheal epithelium (A,B). (A) Cells of the tracheal node are flanked on both sides by internodal cells. This tissue has been immunolabeled with MAb 2F5 and then lightly stained with toluidine blue. The lateral and basal surfaces of nodal cells stain darkly with immunolabel. Bar=20 μm. (B) An enlarged view of the enclosed area in 4A. The convoluted interface between cuticle and the apical surfaces of tracheal cells is marked by the letters *a*. Bar=10 μm. (C) Section of early fourth instar trachea viewed with transmission electron microscopy. This section shows the interface between nodal (on right) and internodal cells (on left). Cuticle at interface changes abruptly from distinctive ridges of taenidia (r) to spiny cuticle over nodal cells.

embryonic cells in both the presence and absence of detergents that permeabilize cells, supporting the claim that the 2F5 epitope is a cell surface epitope in embryos.

Proliferation of nodal cells during the Manduca *life cycle*

At the time that tracheal branches fuse during

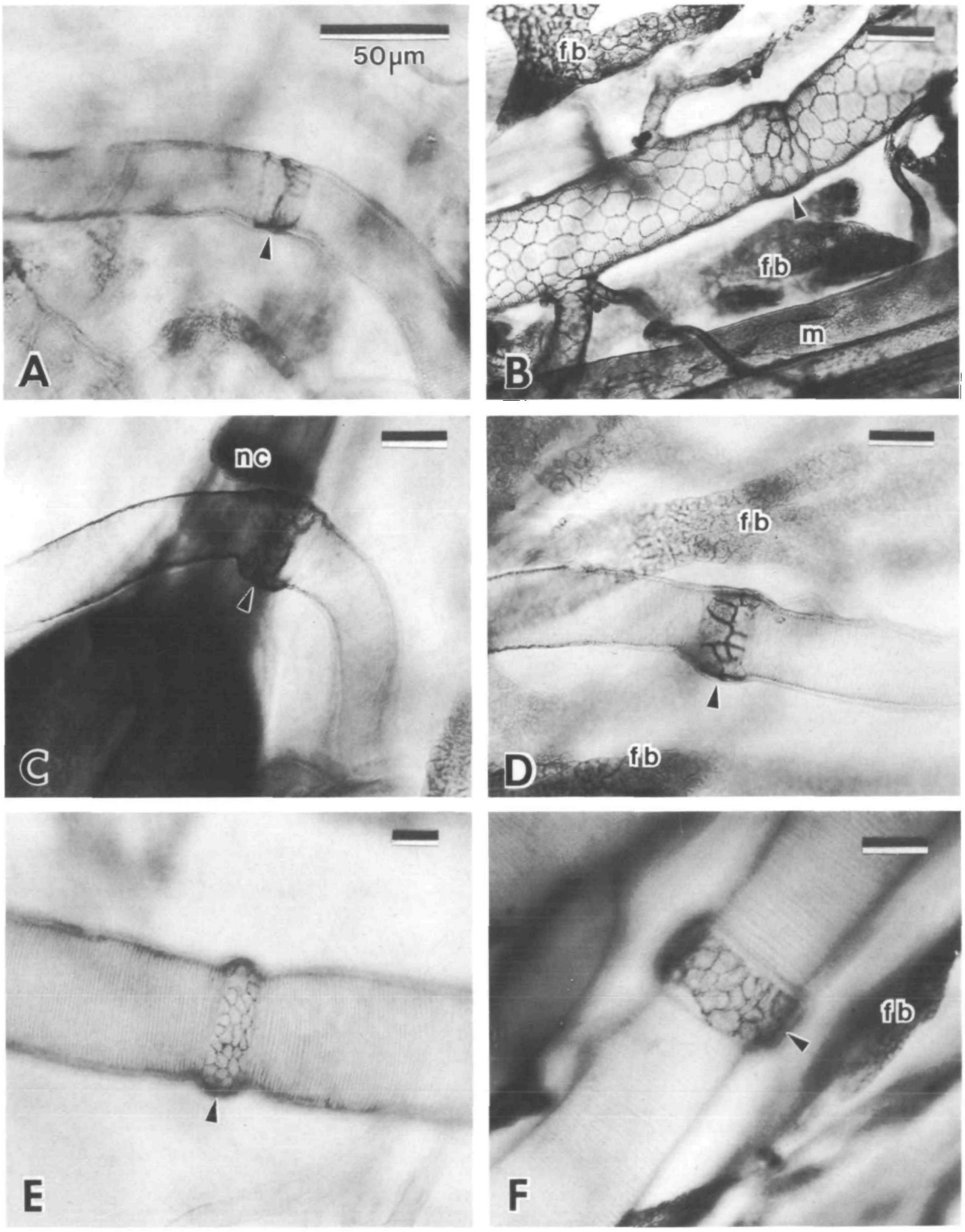

Fig. 4. Whole mounts of postembryonic tracheal nodes immunolabeled with either MAb 2F5 (A,C–F) or MAb 2C10 (B). Tracheae were photographed in abdominal segments 1–4. Arrowheads point to nodes. Fat body=fb. Muscle=m. Each bar represents 50 μm. (A) Lateral longitudinal trachea of first instar larva labeled with MAb 2F5. (B) Lateral longitudinal trachea of third instar larva labeled with MAb 2C10. (C) Ventral commissural trachea of third instar larva labeled with MAb 2F5. This trachea lies ventral to the nerve cord (nc). (D) Lateral longitudinal trachea of third instar larva labeled with MAb 2F5. (E) Lateral longitudinal trachea of fourth instar larva labeled with MAb 2F5. (F) Lateral longitudinal trachea of fifth instar larva labeled with MAb 2F5.

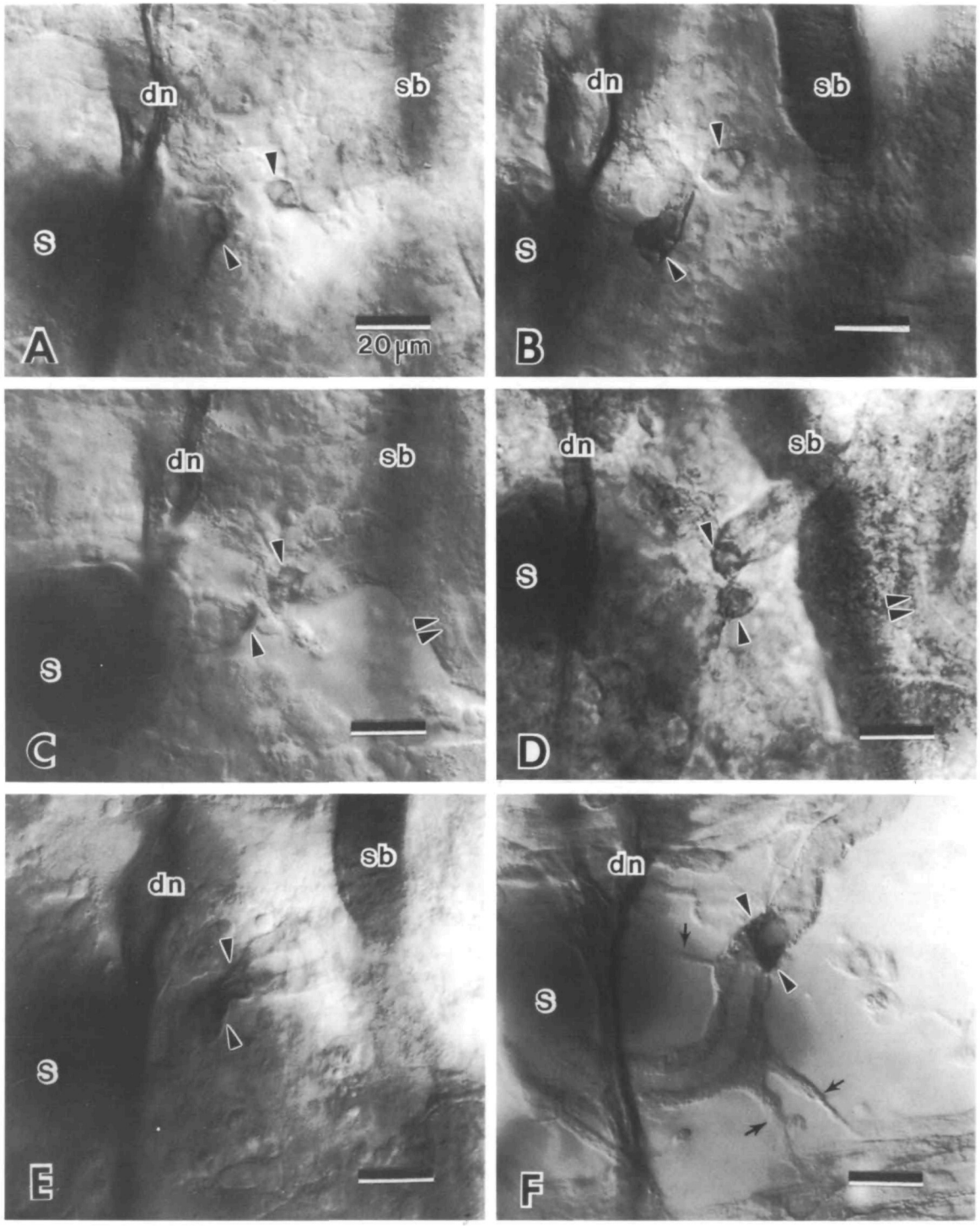

Fig. 5. Embryonic development of lateral longitudinal nodes between abdominal spiracles 1–4. Anterior is to the left; dorsal is at top. Landmarks include spiracles (S), dorsal nerve (dn), and segmental boundary (sb) separating segment *n* on left side of figure from segment *n+1* on right side. A ventrally pointing arrowhead indicates immunolabeled tip cell of anterior tracheal branch. A dorsally pointing arrowhead marks the immunolabelled tip cell of the posterior tracheal branch. Double arrowheads in C and D point to the tracheal branch that lies along the posterior side of the segmental boundary. Each scale bar represents 20 μm. (A) Immunolabeling of tracheal cells appears at around 46% embryonic development. (B) 46% embryogenesis. The tip cell of the posterior tracheal branch is extending processes toward the anterior tracheal branch. (C and D) 46% embryogenesis. The relationship between tip cells of the tracheal branches immediately prior to fusion of tracheal branches. (E) 47% embryogenesis. Fusion of anterior and posterior tracheal branches has just occurred. (F) 63% embryogenesis. The tracheal node is clearly delineated by immunolabel (arrowheads) and incipient tracheal branches are forming (arrows). The segmental boundary has been displaced to the right of the figure.

embryogenesis, the expression of 2F5 is restricted to two cells at each tracheal node. Throughout the first larval stadium, each tracheal node still contains only two cells and MAb 2F5 label is localized to these tracheal cells (Fig. 4A) By the third larval stadium, the number of cell diameters spanning the length of a node is still only two (Fig. 4B,D), although the number of cells occupying the circumference of each node has increased. (The topography of each node makes precise counting of cell number of the circumference difficult after the first stadium.) From the fourth larval stadium through the adult, the length of each node, as measured in terms of cell number, does not increase beyond 3 or 4 cells. The number of cells along a given circumference of a node, however, increases at each molt as tracheal circumference increases (Fig. 4D–F).

The cuticular surfaces of nodal and internodal cells

With the light microscope, the taenidia (regularly spaced ridges of cuticle) can be observed in tracheal whole mounts on the apical surfaces of internodal epithelia (Fig. 4D–F). Taenidia are not evident on the apical surfaces of nodal cells viewed under the same conditions. In whole mounts of tracheae, however, taenidia could be obscured by other structures present at tracheal nodes.

Sections of tracheal surfaces clearly show that taenidia are absent from nodes and present only in internodal regions. A spiny cuticle covers the apical surfaces of nodal cells (Fig. 3). The cuticular reinforcement of tracheae provided by taenidial ridges is absent from nodal regions of tracheae that provide the weak cuticular links in the tracheal network.

Discussion

Cellular recognition and the formation of tubular networks

From numerous descriptions of precise tissue and cellular interactions that are involved in animal morphogenesis, it is evident that recognition cues on cell surfaces must be specified with great spatial and temporal fidelity. To generate the complex tracheal network of *Manduca*, links must be established among tracheal branches in adjacent hemisegments – both contralateral and ipsilateral. A specific cell surface epitope is restricted to only those cells of the tracheal epithelium that are involved in the linking process during embryogenesis. The spatial and temporal distribution of this surface epitope suggests that it is found on molecules that mediate cellular recognition and adhesion. These same molecules may also function as important cues for growth cone guidance and neuronal recognition in *Manduca* and other insects (Carr and Taghert, 1988; Harrelson and Goodman, 1988).

In addition to the tubular organization of the tracheal system in insects, the growing tips of embryonic tubules and ducts in vertebrates are known to establish precise contacts during the formation of a number of mesodermal tissues (Balinsky, 1975; Zackson and Steinberg, 1987). The free ends of mesonephric tubules join with the pronephric duct to form the Wolffian duct; the ureter forms as an extension of a bud from the pronephric duct into metanephrogenic tissue. The formation of connections among blood vessels (endothelial tubes) probably also involves the expression of cell recognition molecules at key sites. However, what specific recognition molecules mediate these morphogenetic events is not known.

Tracheal systems in other insects

In many insects the relationship among the number of tracheal invaginations in embryos, the number of functional spiracles in postembryonic stages, and the number of tracheal nodes is not the straightforward relationship that exists for *Manduca*. With the exception of the nonfunctional metathoracic spiracle of *Manduca*, a functional spiracle forms at the site of each tracheal invagination; and a tracheal node lies between each pair of adjacent ipsilateral spiracles and between contralateral spiracles of the same segment (Fig. 1). Insects can have any number of pairs of functional spiracles from zero to ten. In *Drosophila* only certain spiracles function between molts as sites for gas exchange. During the first larval stadium, only the tenth pair of spiracles is functional; while in the second and third instars, both the first and the tenth pair of spiracles are functional. At molting, however, nonfunctional spiracles on other segments act as sites through which tracheal cuticle is shed (Whitten, 1980). Molting of tracheal cuticle through several nonfunctional spiracular openings implies that tracheal nodes lie between these spiracles. A polyclonal antiserum to the molecules recognized by MAb 2F5 should label tracheal nodes of other insect species. The number of labeled nodes found in a given species should correspond to the number of tracheal invaginations during its embryonic development and the number of spiracles that function at each molt.

Implications for insect evolution

Although tracheae are not unique to the insects, tracheal networks that extend between body segments are found only in the insects. Myriapods (centipedes and millipedes) and many of the primitive wingless insects (Apterygota) have tracheal systems that are autonomous not only within a segment but also within a hemisegment (Snodgrass, 1935; Imms, 1967). During embryogenesis in these arthropods, invaginating tracheal tubes not only fail to fuse at the midline but also fail to join between segments. Their tracheal tubes undergo no fusion during embryogenesis to form the integrated tracheal networks found in the winged insects (Pterygota). This coupling of tracheae between segments and hemisegments has contributed to the efficiency of respiration in those insects with wings and may even have been an essential precondition for the evolution of flight in insects. Fusion of tracheal branches during the embryogenesis of winged insects implies that linking molecules are present on the tip of each extending branch. Without such a cell recognition

label, the orderly linking of different tracheal branches would probably not be possible.

The generation of morphological novelty is the basis of macroevolutionary change. A one-to-one correspondence rarely exists between genes and morphology. However, in the case of the gene(s) coding for cell recognition molecules present at growing tips of tracheae, the route from gene(s) to cells to phenotype can be easily traced. Clearly, an understanding of cell–cell interactions during development contributes to an appreciation of the relationship among genes, morphology, and evolution.

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