Development of the *Drosophila* tracheal system occurs by a series of morphologically distinct but genetically coupled branching events

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SUMMARY

The tracheal (respiratory) system of *Drosophila melanogaster* is a branched network of epithelial tubes that ramifies throughout the body and transports oxygen to the tissues. It forms by a series of sequential branching events in each hemisegment from T2 to A8. Here we present a cellular and initial genetic analysis of the branching process. We show that although branching is sequential it is not iterative. The three levels of branching that we distinguish involve different cellular mechanisms of tube formation. Primary branches are multicellular tubes that arise by cell migration and intercalation; secondary branches are unicellular tubes formed by individual tracheal cells; terminal branches are subcellular tubes formed within long cytoplasmic extensions. Each level of branching is accompanied by expression of a different set of enhancer trap markers. These sets of markers are sequentially activated in progressively restricted domains and ultimately individual tracheal cells that are actively forming new branches. A clonal analysis demonstrates that branching fates are not assigned to tracheal cells until after cell division ceases and branching begins. We further show that the breathless FGF receptor, a tracheal gene required for primary branching, is also required to activate expression of markers involved in secondary branching and that the pointed ETS-domain transcription factor is required for secondary branching and also to activate expression of terminal branch markers. The combined morphological, marker expression and genetic data support a model in which successive branching events are mechanistically and genetically distinct but coupled through the action of a tracheal gene regulatory hierarchy.

Key words: branching morphogenesis, tubulogenesis, cell migration, *Drosophila*, trachea

INTRODUCTION

Branched tubular epithelial structures are found in most animals and function to transport gases and liquids in the body. Formation of such structures poses a number of fundamental cellular and molecular questions. How do cells organize themselves into tubes? How are branch points selected and how do new branches arise? What controls the sequence of branching events and the size and direction of branches? Are the same cellular and molecular mechanisms used for all branches? The formation of branched structures is also of interest because of the tremendous amount of human disease associated with such organs, such as coronary artery disease and emphysema, and congenital anomalies like pulmonary atresia.

Most progress in understanding branching morphogenesis has come from studies of explants of mammalian tissues, such as salivary and mammary glands, and cell lines that can be induced to form rudimentary branched structures in vitro (Bard, 1990). Such studies have suggested cellular mechanisms for some types of branching, and they have led to the identification of a number of inducers of branching and other molecules involved in the processes. Branching has been more difficult to characterize in vivo in ways that afford insight into cellular mechanisms and molecules. There are also only limited prospects for in depth genetic analysis of branching processes in vertebrates, with the possible exception of zebrafish. In *Drosophila melanogaster*, a combined cellular and genetic approach is possible and has begun to elucidate the cellular mechanisms and molecules that govern formation of several organs (Bate and Martinez-Arias, 1993). Formation of the *Drosophila* tracheal (respiratory) system, a branched tubular epithelium, is similarly amenable to cellular and genetic analysis (Manning and Krasnow, 1993). With its small number of cells, ~80 per hemisegment, and the availability of a large battery of molecular cell markers, the larval tracheal system provides an excellent system to define the mechanisms of branching morphogenesis in vivo.

The tracheal system delivers oxygen directly to the tissues. Oxygen enters this network of hollow epithelial tubes through the spiracles and diffuses along the major branches until reaching the fine terminal branches (tracheoles) that end blindly on the surfaces of all tissues (Wigglesworth, 1972).
Oxygen passes across the tracheal epithelium into the surrounding tissues. The tracheal epithelium is a monolayer without any surrounding support tissue but with stiff extracellular cuticle that lines its apical (lumenal) surface and prevents the tubes from collapsing (Fig. 1A) (Noirot and Noirot-Thimotée, 1982).

The pattern of tracheal branches is bilaterally symmetric and shows a segmentally repeated structure (Fig. 1H). The pattern within each hemisegment, however, is conspicuously asymmetric: branches are of different lengths and diameters and there is no obvious repeat unit. Except for the most terminal branches, the pattern of branches in each hemisegement is stereotyped and hence must be guided by a fixed developmental program. Little is known about this program and the cellular and molecular mechanisms that govern formation of the tracheal system, although a number of genes have been implicated recently, including the breathless FGF receptor (Glazer and Shilo, 1991; Klämbt, 1993; Affolter et al., 1994; Anderson et al., 1995).

Here we present a cellular and initial genetic analysis of the sequential branching process that gives rise to the larval tracheal system. We describe the cellular structure and cell dynamics of three levels of tracheal branching. We also describe a large and diverse collection of tracheal markers and show that a different set of markers is activated at each step of branching. The cell dynamics and marker expression patterns, along with available genetic data, indicate that different cellular and genetic processes are used at each level of branching. The results further suggest that the different levels of branching are coupled by a genetic regulatory hierarchy that is operative during the branching process.

MATERIALS AND METHODS

Tracheal antibodies and immunocytochemistry

Embryos were fixed for 20 minutes and stained as described (Patel, 1994) unless otherwise noted. A rabbit polyclonal antiserum (TL-1), raised against Punch protein and provided by J. O’Donnell, was used 1:5 and stains the lumen from stage 14 on. Rabbit IgM mAb2A12 (Giniger et al., 1993) was used at 1:10 and stains the lumen from stage 11 on in embryogenesis. The mouse IgM mAb68G5D3 (Giniger et al., 1993) unless otherwise noted. A rabbit polyclonal antiserum (TL-1), raised against Punch protein and provided by J. O’Donnell, was used 1:5 and stains the lumen from stage 14 on. Rabbit IgM mAb2A12 (Giniger et al., 1993) was used at 1:10 and stains the lumen from stage 11 on in embryogenesis. The mouse IgM mAb68G5D3 (Giniger et al., 1993) was used at 1:5 and stains the lumen from stage 14 on. Rabbit antiserum against β-galactosidase (Cappell) was used at 1:1500. Biotinylated secondary antibodies were used at 1:300, followed by avidin-horseradish peroxidase (HRP) histochemistry. For optimal double staining for TL-1 antigen and β-galactosidase, embryos were first stained for β-galactosidase (through the HRP histochemistry) and then for TL-1. Embryo staging was as described (Campos-Ortega and Hartenstein, 1985).

Tracheal cell junctions were visualized by confocal microscopy after staining with a guinea pig antiserum to Coracle protein (Fehon et al., 1994), used at 1:5000, followed by a Cy3-conjugated secondary antibody (Jackson). Tracheal lumen was counterstained with mAb2A12 followed by Cy5-conjugated secondary antibody.

Larvae were dissected in phosphate-buffered saline and fixed in 1% glutaraldehyde for 20 minutes. Fixed specimens were incubated with 10 μg/ml RNase A for one hour, stained with 1 μg/ml propidium iodide for 5 minutes, mounted in glycerol and examined by confocal microscopy.

Tracheal enhancer trap lines and mutants

The ~1000 homozygous lethal P[lacZ] enhancer trap lines generated in the laboratory of Alan Spradling were analyzed along with preselected lines showing lacZ expression in or near the tracheal system from large collections from the laboratories of Y. N. Jan (Bier et al., 1989; Hartenstein and Jan, 1992), Matthew Scott, Walter Gehring (Bellen et al., 1989) and Norbert Perrimon (Perrimon et al., 1991). Embryos from each strain were collected overnight and stained with mAB2A12 and anti-β-galactosidase antisera in 36-well polyethylene racks. Cytochemical positions of P elements were determined by hybridization of a biotinylated probe from CaSpeR-β-gal (Thummel et al., 1988) to polytene chromosomes. breathlessLG1 and breathlessLG9 are strong loss-of-function alleles (Klämbt et al., 1992). pointedAA8 is an amorphic allele (Scholz et al., 1993). When expression of enhancer trap markers was analyzed in mutants, the marker gene was heterozygous.

Analysis of tracheal cell division and death

Cell counts were done on heterozygous embryos carrying markers Tracheal-1 or Tracheal-2 double-stained for tracheal lumen and β-galactosidase. BrdU labeling was performed essentially as described (Bodmer et al., 1989). In situ nick translation of DNA ends used biotinylated dUTP and avidin-HRP histochemistry (Hay et al., 1994). Embryos were then stained with mAb2A12 to detect labeling of tracheal cells.

Live imaging of tracheal development

Manually dechorionated embryos were attached to cover slips coated with adhesive and then covered with halocarbon oil. Fluorescein-conjugated dextran (10 mg/ml, ~20×103 M; Sigma) was microinjected into the perivitelline space prior to stage 11. Dye entered the tracheal pits and remained in the tracheal lumen until the end of embryogenesis. Some dye accumulated in intercellular spaces, weakly outlining tracheal cells. Embryos were viewed by confocal microscopy.

Clonal analysis

Clones of β-galactosidase-expressing cells were generated by FLP-mediated recombination (Buenzow and Holmgren, 1995). Embryos 4.5-5.5 hours old carrying both the P[hsFLP] and the P[actin5ClacZ] constructs were raised at room temperature and heat shocked at 32-34°C for 30 minutes. This produced only enough FLP to induce recombination and lacZ expression in ~1% of tracheal hemisegments. Heat-shocked embryos were transferred to 18°C and allowed to develop overnight, then stained with mAb2A12 and anti-β-galactosidase antisera to identify tracheal clones.

RESULTS

The pattern of tracheal branching

The tracheal system forms from 10 clusters of ectodermal cells (Tr1-Tr10) on each side of the embryo, one in each hemisegment from T2 through A8 (Fig. 1C). Each cluster gives rise to a hemisegement of the tracheal system by a series of sequential branching events. In this section, we review the general sequence and pattern of branching and delineate three levels of branching that we call primary, secondary and terminal branching. In the next sections, we describe the cell dynamics of each level of branching. The branching pattern is similar but not identical in different hemisegments (Manning and Krasnow, 1993). Our description focuses on a representative hemisegment, Tr5.

The Tr5 cluster of tracheal cells invaginates and forms an elongate sac of cells that lies just under the surface but still connected to the surface by a thin stalk (the spiracular branch) (Fig. 1, stage 11). Six major buds form along the sac and grow out in different directions and to characteristic lengths (stage
These are the primary branches. The cells that do not grow out to form primary branches remain to form the transverse connective. Primary branches produce secondary branches at stage 15. The first terminal branches form at stage 16 as continuations of secondary branches (Fig. 11). In the larval period, these ramify into extensive arrays of terminal branches (tracheoles) that cover the target tissues (see Fig. 4C). The pattern of primary and secondary branching is stereotyped, although there is some embryo-to-embryo variability in the exact number and position of secondary branches. In contrast, the pattern of terminal branching is highly variable and believed to be dependent on the oxygen needs of the target tissues (Rühle, 1932).

Several primary and secondary branches in each hemisegment do not complete this typical branching sequence. They cease branching and grow towards tracheal branches from neighboring hemisegments, to which they fuse to interconnect the tracheal network. The dorsal trunk anterior and posterior fuse with their partners at stage 14 to form the dorsal trunk (Fig. 11, arrow). Similarly, three secondary branches in Tr5 fuse with their partners to connect the lateral trunk and to form a dorsal anastomosis that connects the right and left sides of the tracheal network (Fig. 11, arrowheads).

The branching process takes ~10 hours, from the middle to the end of embryogenesis, although terminal branching continues in the larval period. Throughout most of embryogenesis, the tracheal system is filled with liquid. About two hours before hatching, the liquid is rapidly cleared from the tubes and they become functional for respiration.

Cell number and distribution in the developing tracheal system

To elucidate the cellular basis of tracheal branching, we first determined the number and positions of tracheal cells during the branching process with the aid of enhancer trap strains in which the marker is expressed in all tracheal cell nuclei. There were ~80 cells in Tr5. These were typically distributed among the different branches as shown in Fig. 2. A small and characteristic number of cells formed each primary branch, although the exact number and positions of cells were not rigidly prescribed (see Fig. 2 legend). Each cell has been assigned a name according to its typical position at stage 16 (Fig. 2).

The total number of tracheal cells did not increase as the primary branches grew and the secondary branches formed and extended throughout the embryo (Fig. 2 legend). Consistent with this, no tracheal DNA synthesis was detected by BrdU labeling after stage 11 (see Methods) and no mitotic figures have been observed in the tracheal system during the same period (Poulson, 1950; Campos-Ortega and Hartenstein, 1985). We also did not detect any dying or dead cells in the developing embryonic tracheal system by in situ nick translation of DNA ends (see Methods). Thus, the dramatic growth of the tracheal network does not involve cell proliferation, death or the egression or ingressio of cells. Rather, new branches form by migration and rearrangement of tracheal cells and by cell elongation.

Multicellular primary branches form by cell migration and intercalation

Primary branches arise by migration of small groups of tracheal cells that organize themselves into tubes as they migrate. In the first stage of this process, tracheal cells migrate out and branches grow as other tracheal cells follow them. In the second stage, no additional cells enter the branches, and branches grow by cell elongation and intercalation.

A close-up view of development of the dorsal branch is shown in Fig. 3. Several tracheal cells migrate dorsally, forming a small bud at stage 12. These continue to move dorsally with other tracheal cells following, generally in pairs, forming a branch of ~6 cells and of relatively constant (2-cell) diameter by the beginning of stage 13. This completes the first stage of dorsal branch formation. Up to this point, the cells in the new branch all appear quite similar and are roughly cuboidal. No cellular processes protruding from the migrating cells are detectable by DIC optics and the cells have lengthened little in the direction of outgrowth. Thus, there does not appear to be any net deformative force on individual tracheal cells as they form a dorsal branch.

The other primary branches begin to form at the same time and in a similar manner as the dorsal branch. Cells migrate out and form new branches of relatively constant, 2-cell diameter, although each primary branch grows to a distinct length and contains a proportionately different number of cells (see Fig. 2, stage 12). This indicates that the length of a primary branch is controlled independently of its diameter.

The second stage of primary branch formation occurs over the next 2-3 hours, from stage 13 to 15. The branches continue to extend (Fig. 3C-E) but no additional cells are recruited into the branches. Instead, the cells in the branch elongate and intercalate from a side-by-side to an end-to-end arrangement. The branches become very narrow as they grow, as if they are being stretched. One or two cells at the base of the dorsal branch appear to recede into the dorsal trunk during this time. Only the primary branches that form the dorsal trunk do not undergo this period of intercalative growth. The cells in these branches remain clustered and the branches stay short and fat as they fuse with their partners.

During the cell migrations and rearrangements described above that give rise to the primary branches, the migrating cells organize themselves into tubular structures. Antibody stains show the presence of a lumen in all primary branches from stage 11 onward (Fig. 1). The same result was also obtained when the tracheal lumen was visualized in living embryos after introduction of fluorescent dyes by microinjection (see Methods). In these latter experiments, there was no leakage of dye from the lumen during outgrowth, demonstrating that the tubes are closed and the integrity of the tracheal epithelium is maintained throughout the branching process.

Secondary branches are formed by individual tracheal cells

A striking finding of the cellular analysis was that each secondary branch is formed by a single tracheal cell. Formation of the two secondary branches of the Tr5 dorsal branch is shown in Fig. 3D-F. At stage 13, the two cells at the end of the dorsal branch, DB1 and DB2, are paired like the other cells in the branch and indistinguishable from them morphologically. However, as the other cells begin to elongate and intercalate, DB1 and DB2 initially remain paired (Fig. 3D), and they develop broad cytoplasmic extensions. Subsequently the two cells begin to separate and grow in different directions (Fig. 3E), each forming a distinct secondary branch.
The other primary branches, except those that form the dorsal trunk, also give rise to secondary branches. Twelve secondary branches typically arise from the Tr5 visceral branch. As in the dorsal branch, when the proximal cells of the visceral branch begin to intercalate and elongate, the more distal cells remain clustered (Fig. 4A). Subsequently, the clustered cells begin to separate and each forms a discrete branch on the gut (Fig. 4B). Although most secondary branches form at the ends of primary branches, several arise at internal positions (Fig. 4D, arrows).

Terminal branches are subcellular tubes formed within long cytoplasmic processes
Most secondary branch cells sprout many terminal branches beginning late in embryogenesis and continuing throughout the larval period. A single cell like the ones shown in Fig. 4A and B forms an extensive array of branches that densely cover its target (Fig. 4C). The subset of secondary branch cells that send out terminal branches we call terminal cells.

Terminal branches arise as cytoplasmic extensions of terminal cells. A close up view of formation of the first

Fig. 1. General structure and pattern of tracheal branching in embryogenesis. (A) Cutaway view of a major tracheal tube and branch point. (B) Cross-sections of a multicellular primary branch with two intercellular junctions, a unicellular secondary branch with an autocellular junction and a subcellular terminal branch with no junctions. (C-H) Development of the left side of the tracheal system from stage 11 to 16 visualized by staining the lumen with antiserum TL-1 (C-F) and mAb2A12 (G, H). Anterior is left and dorsal is up in all figures. Square bracket, fifth tracheal hemisegment (Tr5).
(I) Development of Tr5. The six primary branches (dorsal branch, DB; dorsal trunk anterior, DTa; dorsal trunk posterior, DTp; visceral branch, VB; lateral trunk anterior, LTa; lateral trunk posterior/ganglionic branch, LTp/GB) are highlighted at stage 12. Secondary branches are highlighted at stage 15. At stage 16, single terminal branches (highlighted) begin to sprout from the end of each secondary branch (except the three fusion branches which are indicated by arrowheads at stage 15). In the late embryo and larva, many more terminal branches form at these positions. The points of transition from secondary to terminal branches are approximate. Solid lines, lumen of Tr5; dashed lines, adjacent tracheal hemisegments; arrow, Tr5-Tr6 dorsal trunk fusion point; TC, transverse connective; SB, spiracular branch. Bar (C-H), 25 μm.
Tracheal branching morphogenesis

The terminal cell DB1 extends a long thin cytoplasmic projection along the surface of the target (Fig. 3F). Initially no lumen is visible by DIC optics or staining with mAb2A12. Subsequently a lumen forms within the cytoplasmic projection (Fig. 3G); the lumen is continuous with the lumen of the secondary branch from which it arises. The cell sends out additional projections, at the level of the cell nucleus or more distally, and the process repeats itself until the cell forms an extensively branched structure as in Fig. 4C (see also Keister, 1948).

There are two topologically distinct ways in which a single cell can form a tube. The cell could curl up until its edges meet and form an autocellular junction like the intercellular junctions of the large multicellular branches (Fig. 1B). Alternatively, a lumen could form in the cytoplasm by fusion of cytoplasmic vesicles along the length of the cell. This would form a ‘seamless’ tube without junctional structures; the cell would look like a doughnut in cross section (Fig. 1B, right). Both types of tracheal tubes have been observed in ultrastructural studies (Noirot and Noirot-Thimotée, 1982; Tepass and Hartenstein, 1994), where the seamless tubes have been termed tracheoles. To determine the distribution of the two types of unicellular tubes in the tracheal system, embryos were stained with antiserum to Coracle protein, which labels the pleated septate junctions at the apicolateral boundaries of ectodermal tissues (Fehon et al., 1994). The major tracheal branches

Fig. 2. Distribution of cells in a tracheal hemisegment. Typical positions of tracheal cell nuclei (filled circles) in Tr5 are shown. There were 78±4 cells (n=6) at stage 12 and 80±0.4 cells (n=4) at stages 16 and 17. The range of cells in each branch at stages 16 and 17 were: DB (5-7), DT (19-21), VB (18-20), LTa (7-10), LTP (4), GB (6-8), SB (6-8), TC (8-10). Cell counts at early stages are less accurate because nuclei are closely packed; nuclei are drawn much smaller than their relative size at stage 12. Cell names are shown at stage 16.

Fig. 3. Cell dynamics of morphogenesis of the dorsal branch. (Top panels) Micrographs of dorsal branch at stages 12-17. Embryos carrying the Tracheal-1 marker (expressed intensely in the nucleus and less intensely in the cytoplasm) were double stained for tracheal lumen (mAb2A12) and β-galactosidase, except the stage 15 specimen which shows only β-galactosidase. mAb2A12 staining begins at stage 14. (Bottom panels) Tracings of the top panels with nuclei in dark grey and cytoplasm in lighter grey. Dashed lines show the approximate positions of the lumen based on double stains for β-galactosidase and three lumenal antigens. The DB1 cell extends many more cytoplasmic processes during the larval period and forms an extensively branched structure. The DB2 cell grows over the dorsal vessel and fuses to the contralateral branch (not shown). Bar, 5 μm.
showed strong Coracle staining at the inter- and autocellular junctions of tracheal cells (Fig. 4E,G,J). In contrast, the terminal branches displayed only weak cytoplasmic and cell surface staining that was also seen in the major branches (Fig. 4G,J). Strong Coracle staining ended abruptly near the position of the nucleus of the terminal cells. Coracle staining thus identifies a transition within a single cell, from tracheal tubes with seams into seamless terminal branches, a transition that is not apparent with mAb2A12 and most other tracheal antibodies which show continuous staining to the end of the terminal branch (Fig. 4F).

The only secondary branches in Tr5 that do not form terminal branches are the three (formed by DB2, LTa4 and LTP/GB8 cells) that grow towards their partners in neighboring hemisegments and fuse to establish tracheal connections. Branch fusion is a complex cellular process which is described elsewhere (Samakovlis et al., unpublished data).

**A different set of markers is activated at each stage of branching**

To begin a systematic genetic analysis of tracheal branching, we examined P[lacZ] enhancer trap lines to identify tracheal cell markers and genes required for tracheal morphogenesis. We analyzed strains from several large collections that together represent several thousand independent P[lacZ] transposon insertions. lacZ expression typically reflects the expression pattern of the endogenous gene at the insertion site (O’Kane and Gehring, 1987). Embryos from each strain were double stained for β-galactosidase and the mAb2A12 lumenal antigen to define tracheal lacZ expression patterns and identify tracheal phenotypes. Over 50 tracheal markers were found (Table 1), many of which identify genes required for tracheal morphogenesis.

Most of the markers showed restricted and, in some cases, dynamic expression patterns in the developing tracheal system, revealing an unexpected molecular diversity among tracheal cells. Many markers were expressed in one of the four general patterns elaborated below. These define four major programs of gene expression that are activated in a defined sequence and in increasingly specific spatial domains in the developing tracheal system, which anticipate or coincide with the three major morphological branching processes described in the previous sections or with branch fusion. Most of the markers were also selectively expressed outside the tracheal system.

**General tracheal markers**

Thirteen markers were expressed throughout the tracheal system. These include P[lacZ] inserts in breathless (Tracheal-2) (Glazer and Shilo, 1991) and trachealess (Tracheal-1) (Perrimon et al., 1991; Wilk et al., 1996). Most of the general tracheal markers were activated at about stage 11 just before or as the clusters of tracheal precursor cells invaginate (Fig. 5A; Table 1); the earliest tracheal marker known is Tracheal-1 which begins to be expressed in mid or late stage 10. There are some distinctions among the general markers. For example, Tracheal-2 and Tracheal-6 were specifically excluded from the cells of the spiracular branch, cells that become the imaginal tracheoblasts and form the adult tracheal system. Most of the general markers continued to be expressed throughout embryogenesis, although some became more restricted in their tracheal expression (e.g., Tracheal-4 and Tracheal-5).

**Pantip Markers**

Seven markers were expressed in cells at the growing tips of developing primary branches and in the outgrowing secondary branches. Expression of the earliest markers in this class began just after the general tracheal markers, in broad domains that progressively restricted to the cells that form secondary branches.

Expression of the Pantip-2 marker is shown in Fig. 5B. At stage 12, Pantip-2 was expressed in many or all cells of each budding primary branch. As the branches grew, expression of the marker gradually restricted to the cells at the leading ends and eventually to just one or a small number of cells in each primary branch that form secondary branches (~25 cells in Tr5). For example, at stage 14 Pantip-2 expression is strongest in just the two cells (DB1 and DB2) at the tip of the dorsal branch that form secondary branches (Fig. 5B, right panel). This dynamic restriction in marker expression was a common feature of this set of markers. However, Pantip-7 was only expressed late and just in the cells that form secondary branches. We have also found a gene that is expressed in the complementary pattern: in all tracheal cells until turning off at stage 13 or 14 in cells that form secondary branches (N. H., unpublished data).

**Terminal branch markers**

After onset of expression of the general and pantip markers, a set of five markers begins to be expressed in the subset of secondary branch cells that will form terminal branches, the terminal cells. Expression of the Terminal-1 marker is shown in Fig. 5C. There were ~20 expressing cells in Tr5, distributed in small clusters or as isolated cells. Although the expressing cells were widely distributed, each of the markers turned on simultaneously in all cells.

Most but not all secondary branch cells expressed the terminal markers. They were not expressed in the cells that form secondary branches that fuse to other branches, which arise at the same time and in some cases in immediately adjacent positions but express a distinct set of markers.

**Fusion markers**

The earliest markers in this set of four begin to be expressed at the same time or slightly before the earliest terminal markers and also in a highly restricted, but dispersed, set of tracheal cells. Five cells in Tr5 expressed the fusion markers (Fig. 5D). Each of these cells migrates toward and contacts a similar expressing cell in the neighboring tracheal hemisegment and mediates a fusion event. The earliest markers, Fusion-1 and Fusion-2, turned on simultaneously in all fusion cells. We also found three tracheal markers expressed in the complementary pattern (Table 1). The antifusion markers were quite generally expressed in the tracheal system but were off in the fusion cells.

**Branch-specific and regional markers**

In addition to the four major expression patterns described above, we found markers expressed in a variety of other patterns that demonstrate further distinctions among developing tracheal branches. Several demarcated individual primary branches. These include Branch-1, expressed exclusively in the spiracular branch, Branch-2 and another marker (not shown).
which label the visceral branch, and Branch-3 which marks both the spiracular and visceral branches (Fig. 6 and Table 1). There were also regional markers expressed in broader domains encompassing two or more contiguous primary branches, such as Regio-1, Regio-2 and Regio-3 (Fig. 6). Branch-specific and regional markers might be involved in establishing or maintaining the boundaries between branches, or in controlling the special character of each primary branch such as its size, direction of outgrowth and pattern of branching.

A significant number of tracheal markers showed more complex patterns that did not correspond to any obvious aspect of tracheal morphology or function. Two examples are illustrated in Fig. 6 (Complex-1, Complex-2). These complex expression patterns indicate that tracheal cells undergo a substantial degree of diversification during branching, beyond what can currently be explained on morphological grounds.

A clonal analysis demonstrates that branching fates are not assigned to tracheal cells until after their final division

The existence of distinct tracheal cell types and branches, as well as the quite reproducible positions of cells within branches, raises two major questions about the lineal relations of cells in the tracheal system. First, are cells that populate particular branches or regions of the tracheal tree or that share similar branching fates related by lineage? Second, during the substantial migrations of the tracheal epithelium that occur during invagination and branch formation, do sibling cells

\[ \text{Table 1. Examples of the earliest tracheal enhancer trap markers in each class} \]

<table>
<thead>
<tr>
<th>Expression class and marker name</th>
<th>Map position</th>
<th>No. of isolates</th>
<th>Stage ( \beta)-gal begins</th>
<th>Notes</th>
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<td>Tracheal-1</td>
<td>61B3-C1</td>
<td>1</td>
<td>10 (l)</td>
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<td>2</td>
<td>11/12</td>
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<td>11/12</td>
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(a) Some weak, general expression at earlier stages. (b) Not expressed in SB. (c) Later turns off everywhere except terminal cells. (d) Expressed in SB. (e) Expressed in VB. (f) Expressed in GB2 cell. (g) General tracheal expression begins at stage 11; turns off in fusion cells at stage indicated. (h) Expressed in DT fusion cells. (i) Expressed in dorsal region of tracheal system. (j) Expressed in ventral region of tracheal system. (k) See Fig. 6. (l) Wilk et al., 1996. (m) Klambt et al., 1992. (n) Klambt, 1993. (o) Kuhnlein et al., 1994.
remain together or can they separate and populate different parts of the tracheal tree? To address these questions, we analyzed the structures of marked clones of tracheal cells. Random clones of embryonic cells expressing a lacZ transgene were generated by FLP-mediated recombination. Recombination was induced just before and during the last two tracheal cell divisions, generating clones containing two or four cells. Clones were observed in embryos aged to stage 15 or 16, when the final positions and fates of tracheal cells are clear (Table 2, Fig. 7).

Analysis of the 101 identified clones revealed two results. First, there were no consistent relationships in the final positions or ultimate fates of sibling cells, beyond the fact that they tended to populate the same general region of the tracheal hemisegment. For example, in the seven clones that included the DB1 terminal cell (Fig. 7A-G), its siblings populated different primary branches and none formed terminal branches, although all of the cells resided in dorsal positions in the tracheal tree. Second, while tracheal cells tended to remain near their siblings, many sibling cells separated from one.

Fig. 4. Unicellular secondary branches and subcellular terminal branches. (A) Terminal cells of a visceral branch in early stage 15. Terminal cells are clustered (bracket) but secondary branches have not formed. Terminal cell nuclei, brown (Terminal-1 marker). Lumen, black (mAb2A12). (B) Visceral branch terminal cells several hours later after the cells have separated and each has formed a separate secondary branch (arrow). (C) A single visceral branch terminal cell several days later (third instar larva) after the cell has formed an extensive network of terminal branches on the gut. The image is a combined series of confocal optical sections, with tracheal lumen visualized under phase contrast, and tracheal and gut nuclei visualized by staining with propidium iodide (pseudocolored blue). The terminal cell nucleus (arrow) is located at the point where the secondary branch ramifies into over fifty terminal branches. (D) Two hemisegments of the lateral trunk at stage 16, double-stained to show lumen (black, mAb2A12) and tracheal cell nuclei and cytoplasm (brown, Tracheal-1 marker). Most unicellular secondary branches arise at growing ends of primary branches like the branches from LTa1, 2 and 3 cells (arrowheads), but others like those from LTp/GB10 cells arise internally (arrows). TC, transverse connective. GB, ganglionic branch. (E) Section of the dorsal trunk at stage 16 stained with Coracle antiserum, showing the characteristic strong labeling of cell junctions. Anterior is down in this panel. (F-H) Ganglionic branch at stage 16 double stained to show lumen (mAb2A12) and cell junctions (Coracle). A distinct seam of Coracle staining indicating an autocellular junction extends down to the proximal edge of the terminal cell nucleus (arrow). Lumenal staining continues past the nucleus into the terminal branch. Asterisks, GB1 (bottom) and GB2 (top) nuclei. Arrowhead, edge of nerve cord. (I-K) A late stage 16 dorsal branch of an embryo carrying the Tracheal-1 marker and double stained for β-galactosidase to show tracheal cells and Coracle to show cell junctions. The DB3, 4 and 5 cells have intercalated; Coracle staining marks the autocellular junction along the length of each cell and the junctions between cells. An autocellular junction is also seen in DB1 but ends at the nucleus (arrows), where terminal branching begins. Bars: A-D, 10 μm; E-K, 5 μm.
another and some ended up widely dispersed in the tracheal tree (Fig. 7P-T). This demonstrates a substantial capacity for cells to move with respect to one another within the tracheal epithelium during development.

The results show that at the time of clone marking, the movements of tracheal cells with respect to one another, the primary branches that they will occupy and their ultimate fates with respect to secondary branching, terminal branching and branch fusion have not yet been specified. These must be assigned after the last tracheal cell division, which occurs just before primary branching begins and coincides with the earliest restricted expression of tracheal markers.

**Genes required for early branching events are also required to activate expression of later markers**

How are tracheal cells assigned their different branching fates during the branching process? For the four major classes of tracheal markers, later markers were always activated within the expression domains of the earlier markers. This suggested that the markers might be organized in a genetic regulatory hierarchy, with early marker genes required for proper expression of later genes. This idea was tested by examining expression of later markers in mutants of earlier marker genes. The Tracheal-2 marker is an insertion in *breathless* (*btl*), which encodes a receptor tyrosine kinase. In *btl* loss-of-function mutants, tracheal cells invaginate to form the elongate sacs, but primary tracheal branches fail to develop (Klämbt et al., 1992). We found that, in contrast to several general tracheal antigens (antisera 84, mAb2A12, TL-1, mAb68G5D3) which are expressed normally in *btl* mutants (Klämbt et al., 1992 and our unpublished observations), the mutants failed to express or expressed only weakly or sporadically the Pantip-1, Fusion-1 and Terminal-1 markers (Fig. 8B and data not shown). Thus *btl* is required not only for formation of the primary branches, but also for activation of the gene expression programs that underlie formation of the secondary and terminal branches.

The function of the Pantip-1 gene was investigated in a similar manner. We first demonstrated that Pantip-1 is an allele of *pointed* (*pnt*), an ETS domain transcription factor that has been extensively characterized in CNS and eye development and has also been implicated in tracheal development (Klämbt, 1993). The Pantip-1 P*[lacZ]* insert mapped to 94F, the same cytological position as *pnt* (Scholz et al., 1993 and below); and the Pantip-1 mutation failed to complement the lethality and tracheal phenotype of a *pnt* null allele, *pnt*<sup>D88</sup>. We analyzed the tracheal defects in *pnt*<sup>D88</sup> mutants and found that the early steps in tracheal development occurred normally through the beginning of primary branch formation. Most primary branches continued to grow and some reached their normal destinations, although occasionally primary branches appeared to collapse and retract. Strikingly, no secondary branches ever formed, even among those primary branches that reached their normal destinations (Fig. 8H). Expression of late tracheal markers was also disrupted. The Terminal-1 and Terminal-3 markers were not expressed (Fig. 8D and data not shown). In contrast, the Fusion-1 and Fusion-2 markers were expressed in broader domains. There were typically several clustered cells expressing Fusion-1 and Fusion-2 at the end of the dorsal branch and other positions where a single expressing cell is seen in wild type (Fig. 8F). Thus, *pnt* is required for secondary branch formation as well as for activating and repressing expression of marker genes that underlie terminal branching and branch fusion, respectively.

**DISCUSSION**

We have carried out a cellular analysis of branching morpho-
genesis of the larval tracheal system, including morphological studies, marker expression studies and a clonal analysis. Although new branches arise sequentially from a morphologically homogenous cluster of cells and form a continuous network of branches, these studies show that tracheal branching is not an iterative process in which the same branching mechanism is used repeatedly at successively finer scales. Rather, each of the three levels of tracheal branching that we define appears to involve different cellular mechanisms and different sets of tracheal markers and genes. The results lead us to propose that the development of the tracheal system occurs by a series of distinctive branching events, and that a gene regulatory hierarchy controls and couples the different steps of branching.

Three levels of tracheal branching
The three levels of tracheal branching are summarized in Fig. 9 and in more detail below. Six primary branches form first, followed by ~20 secondary branches and hundreds or more terminal branches in each hemisegment. In addition, we distinguish a special set of branches that interrupt the usual sequence of branching and instead fuse to other tracheal branches. The tremendous branching of the tracheal network occurs by cell migration and elongation, not proliferation. But the different steps of branching utilize these basic cellular processes in a variety of ways to form the different tracheal branches.

Primary branches
Primary branches form in two stages. The first is a period of cell migration and rearrangement. A few tracheal cells begin to migrate and other tracheal cells follow into the developing branch, with little change in the shapes of the cells except as necessary to form a new tube. This is followed by a period of branch lengthening and narrowing that occurs by intercalation and elongation of the cells. Primary branches express a common set of markers and require a common set of genes for their formation. Mutations in breathless (Tracheal-2) and two new genes identified in our screen arrest development of all primary branches just as they start to bud (Klämbt et al., 1992).

Secondary branches
Secondary branches are formed by individual tracheal cells located mostly at the growing ends of primary branches. Although secondary branches arise at widely dispersed positions in the hemisegment, they all begin to form around the same time, approximately five hours after primary branches start to form. The cellular process by which these unicellular branches form is not clear, although our finding that an autacellular junction spans the length of some secondary branches indicates that the lumen is extracellular with the cell wrapped around it. Expression of pantip markers precedes secondary

**Fig. 6.** Expression patterns of branch-specific, regional and complex tracheal markers. Expression patterns are shown at stage 14. Expression ends abruptly at boundaries between branches for branch-specific and regional markers. Micrographs show close up views of the regions indicated by brackets: visceral branches of Tr4-6 are shown for Branch-2; dorsal branches of Tr4-6 are shown for Regio-3; lateral trunk of two central hemisegments is shown for Complex-1. Bars: left and middle, 10 μm; right, 5 μm.

**Fig. 7.** Structures of twenty marked clones of tracheal cells. Approximate positions of nuclei of the two or four sibling cells in each clone are shown. A-P, clones that contained at least one cell in the dorsal branch. P-T, clones in which sibling cells were widely dispersed.
branch formation and expression restrict to precisely the cells that give rise to secondary branches. Pantip genes regulate secondary branching, as null mutations in pnt (Pantip-1) eliminate all secondary branches and mutations in two other pantip genes increase the number of secondary branches but leave primary branches intact (N. H., unpublished data).

Terminal branches
Terminal branches are subcellular tubules that arise as long cytoplasmic extensions of terminal tracheal cells that have already formed a secondary branch. A narrow lumen forms in each cytoplasmic extension creating seamless tubules that transport oxygen from the secondary branch to the ends of the terminal cell.

Terminal branching is associated with the expression of terminal markers which are activated about 2 hours before terminal branches start to form. These markers regulate terminal branching, as loss-of-function mutations in one of the genes eliminates all terminal branches and mutations in two others also selectively affect terminal branches (Guillemin et al., 1996; K. G., unpublished data). Unlike the earlier branching events, the pattern of terminal branching is highly variable and regulated by target tissue oxygen need (Wigglesworth, 1954). The genetic program controlling terminal branching must therefore be influenced somehow by the physiological needs of its targets.

Fusion branches
Two primary branches and three secondary branches in Tr5 do not follow the usual sequence of branching. They cease branching and grow towards specific branches in neighboring hemisegments. These cells express fusion markers and undergo a distinct morphogenetic program that culminates in branch fusion. Loss-of-function mutations in two fusion markers selectively disrupt branch fusion, demonstrating that this process is also under separate genetic control (C. S., G. M. and M. A. K., unpublished data). Fusion cells remain specialized after branch fusion is complete. At later stages, they are involved in cuticle molting and tracheal growth control and have been referred to as tracheal node cells (Locke, 1958).

Individual tracheal cells can undergo more than one program of branching
The results show that some tracheal cells that form primary branches go on to form secondary branches and many secondary branch cells go on to form terminal branches. Individual tracheal cells containing branches with junctional structures (secondary branches) and others that lack them (terminal branches or tracheoles) were first noted in ultrastructural studies (Tepass and Hartenstein, 1994). Our results show that the different branching processes occur sequentially and not simultaneously in tracheal cells and that a later branching process can be disrupted without perturbing the earlier one (Fig. 8; K. G., unpublished data).

A tracheal gene regulatory hierarchy controls and couples the different levels of branching
There is surprising diversity in the expression patterns of tracheal enhancer trap markers. We can distinguish over twenty different tracheal cell types in the typical hemisegement based on marker expression patterns. How does such diversity arise in the developing epithelium? How do individual branches and individual tracheal cells acquire different identities, as manifest by their specialized programs of gene expression and morphogenesis? Clonal analysis showed that cell lineage does not play an important role in the specification process. Branch positions and branching fates must thus be assigned to tracheal cells after their final division, which occurs just as primary branching begins.

An important clue to the question of how tracheal cell diversity is generated emerged from the analysis of the temporal and spatial expression patterns of the four major classes of tracheal markers. These sets of markers are sequentially activated during development, with general tracheal markers expressed first followed by pantip markers and finally terminal and fusion markers. Tracheal cells gradually acquire their fates during the branching process.

**Fig. 8.** Requirement for breathless (Tracheal-2) and pointed (Pantip-1) in tracheal branching and marker expression. (A, B) Expression of Pantip-1 (pointed) marker in a Tr5 dorsal branch of a stage 13 wild-type embryo (A) and a breathlessG18 homozygote (B). Embryos are double stained for tracheal lumen (TL-1) and β-galactosidase. The nuclei of three tracheal cells (DB1, 2 and 3) expressing Pantip-1 are indicated by a bracket in A. In the mutant, Pantip-1 is not expressed at high levels in the severely truncated dorsal branch (bracket in B) or elsewhere in the tracheal system, although expression is maintained in the epidermis (dotted area) and other tissues. (C-F) Expression of the Terminal-1 marker (C,D) and the Fusion-1 marker (E,F) in the Tr5 dorsal branch of stage 14 wild-type (C,E) and homozygous pointed mutant embryos (D,F). The DB1 terminal cell does not express Terminal-1 (bracket in D) while expression of Fusion-1 expands from the single DB2 cell to three cells (bracket in F). (G,H) The Tr6 and Tr7 visceral branches (mAb2A12) of stage 16 wild-type (G) and homozygous pointed mutant (H) embryos. Secondary branches are seen in wild type (brackets in G) but not the mutant (brackets in H). G is a montage. Bars: A-B, C-D, E-F, G-H, 5 μm.
At each transition, the later markers were always activated in a subset of the cells that expressed the earlier markers and there was close temporal coupling (about 2 hours) between each set of markers. We also found that two early tracheal genes (*breathless* (Tracheal-2), a general marker, is required for primary branch formation and to stimulate expression of pantip markers. *pointed* (Pantip-1) is required for secondary branch formation and activates expression of terminal markers. *Terminal-1* is required for terminal branch formation (Guillemin et al., 1996).

These results lead us to propose that many tracheal genes are organized into a regulatory hierarchy in which the genes expressed at each level of branching serve two functions. The first is to control that level of branching. The second is to regulate expression of genes involved in the next level of branching. This serves to coordinate and couple the different branching events and ensure that the different steps of branching occur in proper sequence.

Implicit in the proposed regulatory scheme are other, as yet unspecified, spatial inputs. All tracheal cells express general tracheal markers implicated in primary branching, yet only six groups of cells migrate out and form primary branches. There must be spatial signals which select these groups of cells. The same is true at the next level of the branching hierarchy. Expression of the pantip genes precedes expression of terminal branch and fusion markers, but not all cells that express pantip markers turn on fusion and terminal markers and, of those that do, some turn on terminal markers and others turn on fusion markers. There must be spatial signals that select which cells within the pantip expression domain turn on terminal and fusion markers.

There are at least two possible sources of such spatial input. One is that genes are expressed in the developing tracheal epithelium in patterns that overlap these genes, such as the regional- and branch-specific markers. These could function combinatorially with the general tracheal genes and pantip genes in the regulation of branching events. For example, visceral branch markers such as Branch-2 and Branch-3 could ensure that all cells expressing pantip markers in the visceral branch turn on terminal markers and undergo terminal branching and that none express fusion markers. The other possible source of regulatory input is tissues neighboring the tracheal system, which could provide local inductive cues that impinge on the proposed tracheal regulatory hierarchy.

The morphological processes and molecular control of tracheal branching are clearly more complex than just the processes described here. We have focused on similarities among branches of a given level, but there are also important differences. For example, each primary branch grows to a different length and most, but not all, undergo a second growth phase involving cell intercalation. Each also grows in a distinct direction across different substrates. These properties may be controlled by branch-specific or regional genes or the genes with more complex expression patterns.

By elucidating some of the key cellular events in tracheal development and by providing a battery of tracheal enhancer trap markers, this work provides a framework for an in depth genetic and molecular analysis of branching. Many of the enhancer trap markers identify genes required for proper tracheal development. The phenotypes of these and other tracheal mutants can now be described with cellular precision, and their relationship to other tracheal genes and markers rapidly established and placed in the proposed regulatory hierarchy. Most importantly, our work has subdivided tracheal branching into distinct morphological and genetic steps, each of which can now be dissected in cellular and molecular detail.

**Implications for morphogenesis of other branched structures**

Throughout this century, biologists and mathematicians have wondered how successive waves of branching can be controlled to generate extensively branched tubes of successively finer scale like the mammalian lung (Thompson, 1917). One appealing idea is that the same mechanism of branching is used iteratively but at successively finer scales, like a fractal process (West and Goldberger, 1987). This might apply to the arborization of terminal tracheal branches. However, our analysis of earlier stages of branching suggests an alternative, mathematically less elegant means of accomplishing the same, namely, by employing fundamentally different cellular and molecular mechanisms of tubulogenesis, each accommodated to a different scale and coupling them genetically to ensure that they occur in the right sequence and generate the required final structure.

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