Study of the Posterior Spiracles of Drosophila as a Model to Understand the Genetic and Cellular Mechanisms Controlling Morphogenesis

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We have studied the posterior spiracles of Drosophila as a model to link patterning genes and morphogenesis. A genetic cascade of transcription factors downstream of the Hox gene Abdominal-B subdivides the primordia of the posterior spiracles into two cell populations that develop using two different morphogenetic mechanisms. The inner cells that give rise to the spiracular chamber invaginate by elongating into "bottle-shaped" cells. The surrounding cells give rise to a protruding stigmatophore by changing their relative positions in a process similar to convergent extension. The genetic cascades regulating spiracular chamber, stigmatophore, and trachea morphogenesis are different but coordinated to form a functional tracheal system. In the posterior spiracle, this coordination involves the control of the initiation of cell invagination that starts in the cells closer to the trachea primordium and spreads posteriorly. As a result, the opening of the tracheal system shifts back from the spiracular branch of the trachea into the posterior spiracle cells. We analyze the contribution of the ems gene to this coordination. In ems mutants, invagination of the spiracle cells adjacent to the trachea does not occur, but more posterior cells of the spiracle invaginate normally. This results in a spiracle without a lumen and with the tracheal opening located outside it. © 1999 Academic Press

Key Words: tracheal system; morphogenesis; Abd-B; bottle cells; convergent extension.

INTRODUCTION

Many organisms are subdivided into segments, each of them having specific structures at defined positions. Segment-specific structures are formed because the activation of patterning genes by a system of dorsoventral and anteroposterior information results in the localized activation of specific morphogenetic programs. The best known genetic patterning system is the one laid down by the HOX transcription factors. The information encoded by the Hox genes has to be translated into the construction of morphological structures by the control of downstream targets (Botas, 1993; Graba et al., 1997). This means that Hox genes select the development of a structure by locally controlling, through their targets, the morphogenetic behavior of naïve cells. The final morphology of a structure will therefore depend on the combination of morphogenetic mechanisms that are activated during development in the cells that form it. The different morphogenetic mechanisms that can be activated include, among others, the control of cell migration, shape, division, death, and adhesion. The regulation of morphogenesis has an added element of complexity due to the fact that the formation of a structure cannot be controlled in isolation because functionality depends on its proper connection with other elements of the system.

To understand the morphogenesis of a structure at least three factors have to be taken into account. First, which are the genes specifying the cells that compose the organ? Second, what are the morphogenetic mechanisms that the cells forming the organ use to acquire the final shape? Third, how does the organ connect with other elements of the system to form a functional unit? Due to the complexity of understanding morphogenesis at all three levels, we have decided to study the formation of a relatively simple three-dimensional organ: the posterior spiracle of the Drosophila larva. The spiracles and the trachea connect to make a functional tracheal system (Manning and Krasnow, 1993) and they can be considered as a model to understand how genes are involved in the generation of integrated organs. Spiracles are the openings of the insect tracheal system. Externally they connect to the epidermis and internally to the tracheal trunk. The spiracles and the
trachea form from separate populations of cells. The trachea forms from 10 tracheal pits, 1 per hemisegment. The tracheal pits are ectodermal invaginations that branch and fuse together forming the tracheal network. The trachea is attached to the epidermis by the spiracular branch. The attachment of the spiracular branch and the epidermis occurs at the place where the tracheal pit originally invaginated. The spiracular branch lumen, however, collapses in segments where no spiracle forms and does not participate in respiration. The only function of these collapsed spiracular branches is to shed the tracheal cuticle after each molt (Manning and Krasnow, 1993). The Drosophila larva has only two pairs of functional spiracles, the anterior in the first thoracic segment (T1) and the posterior in the eighth abdominal segment (A8). The spiracles are formed from ectodermal cells adjacent to the first and last tracheal pits. During development the spiracles connect to the T1 and A8 spiracular branches, forming a continuous lumen. The anterior spiracle is not functional until late larval stages; therefore, all the gas exchange in the young larva occurs through the posterior spiracle (Manning and Krasnow, 1993).

In this paper we study the morphogenesis of the posterior spiracle. We show that the formation of the posterior spiracle is achieved by two main morphogenetic mechanisms: The internal cells change their shape, whereas the external cells rearrange by intercalation. These cellular mechanisms are controlled by a cascade of transcription factors downstream of the Abdominal-B (Abd-B) gene that pattern the posterior spiracle when it is still a two-dimensional structure. We also show that the mechanism connecting the posterior spiracle to the trachea differs from that used to connect the segmental portions of the trachea into a single network.

**MATERIALS AND METHODS**

Antibodies and enhancer trap lines used as markers. We have used the following primary antibodies: rabbit anti-spalt (Kühnlein et al., 1994), anti-cut (Blochinger et al., 1990), anti-klumpfuss (Yang et al., 1997), anti-pdf1 (pdf1 is a synonym of nubbin) (Yeo et al., 1995), anti-β-galactosidase (Cappel); mouse anti-engrailed (Patel et al., 1989), anti-crumbb (Tepass et al., 1990), anti-Abd-B (Celünkner et al., 1989), and anti-β-galactosidase (Promega). The following enhancer trap lines were used: trh1, P837 (A1-3-10), P927 (B7-2-22), P1028 (A495.1M2), P1120 (A189.2F3), and btl-lacZ26.81 (Bellen et al., 1989; Bier et al., 1989; Hartenstein and Jan, 1992; Isaac and Andrew, 1996; Klambt et al., 1992). We have also used as markers constructs in which the expression of β-galactosidase was driven by specific enhancers, these are grh-D4 (a gift from Sarah Bray), ems-1.2 (Jones and McGinnis, 1993); and ct-D2.3 and ct-A4.2 (Jack and DeLotto, 1995).

**GAL4 and UAS stocks.** GAL4 and UAS stocks included: klu-GAL4 (Klein and Campos-Ortega, 1997), Arm-GAL4 (Sanson et al., 1996), UAS-dpp (Staehling-Hampton and Hoffmann, 1994), UAS-lacZ (cytoplasmic) (Brand and Perrimon, 1993), and UAS-Nod-lacZ (minus end microtubule motor) (Clark et al., 1997).

**Cuticle preparations and antibody stainings.** For cuticle preparations, 24-h-old embryos were dechorionated in bleach. The vitelline membrane was removed by treatment with heparite and methanol. After washing the embryos in 0.1% Tween they were mounted in Hoyer’s mountant and kept on a hot plate at 60°C for 2 days.

For antibody stainings all primary antibodies were incubated overnight in BBT buffer (Leiss et al., 1988), adding BSA and Goat serum, washed in PBS, and developed using the Vectastain Elite ABC kit. For double stainings nickel chloride salts were added when developing the first primary antibody, but not when developing the second primary, to allow us to distinguish cells positive for each antibody.

Nuclei counts. Embryos were stained with anti-sal antibodies and the number of nuclei in three places scored. The scoring was only performed in the spiracle that was well oriented toward the viewer. Ten or more spiracles where scored for stages 12, 13, and 14 and 7 spiracles for stage 15. The numbers recorded for each spiracle are the circle of sal-expressing cells further away from the spiracular chamber, which is represented as a blue line in Figs. 7B and 7C (the basal circumference of the stigmaphore); the circle of cells in contact with the spiracular chamber, which is represented as a red line in Figs. 7B and 7C (top of the stigmaphore); and the width of the circle of sal-expressing cells, which is represented as a green line in Figs. 7B and 7C (will become the height of the stigmaphore). The latter two counts are more accurate than the first one due to the fact that after st14, the base of the left and right stigmaphores fuse and the decision as to where each stigmaphore ends is a subjective matter. The segmental groove allows us to distinguish unambiguously the sal expressing cells in A8 from those in A9.

Clones. The Tau-Gal slamy clones were induced by Marcos González-Gaitán, who allowed us to use them for this study. The clones were induced using an FRT flip-out cassette made by A. Martí-Sibrana and R. Holmgren (unpublished).

**Mutant fly strains.** The following mutant alleles were used: Abd-B11 (Casanova et al., 1996), ct-Gal (Blochinger et al., 1988), ems93E, ems96G (Dalton et al., 1989), trh9 (Isaac and Andrew, 1996), sal953 (Kühnlein et al., 1994), Df(2L)5 deficient for sal and sal-r (de Celis et al., 1996), btlLG19 (Klambt et al., 1992), Df(2)G4R (deficient for pdm-1 (=nub) and the structurally related gene pdm-2) (Ye et al., 1995), Df(3L) kluX10 (Klein and Campos-Ortega, 1997), grhX10 (Bray and Kafatos, 1991), Df(2L) enE (deficient for en and invected) (Tabata et al., 1995), and Df(3L)H99 (deficient for the three cell death genes reaper, grim, and head involution defective) (Chen et al., 1996; Grether et al., 1995; White et al., 1994).

**RESULTS**

The posterior spiracles are formed in the dorsal part of the eighth abdominal segment (Fig. 1). At 6 h of development (st 11) the morphology of the cells that will form the spiracles in A8 is indistinguishable from that of cells at homologous positions in more anterior abdominal segments (Figs. 1E-1H). At 13 h (st16), the posterior spiracle has all the features present in the mature spiracle. Therefore, the basic morphogenesis of the posterior spiracles is completed in about 7 h.

The posterior spiracle is an ectodermal structure composed of two parts: the spiracular chamber and the stig-
Posterior Spiracle Morphogenesis in Drosophila

To understand the genetic mechanisms involved in posterior spiracle morphogenesis we have started by finding out the regulatory network required for its specification. There are a number of known genes that when mutant affect the posterior spiracle. The formation of the posterior spiracles in A8 requires the HOX transcription factor ABD-B (Sánchez-Herrero et al., 1985); however, ABD-B expression is not restricted to the posterior spiracles but is expressed in the whole of A8 and in other segments (Celnieker et al., 1989). The earliest differentiation of the spiracle cells can be detected at the extended germ band stage (6 h of development) when a small number of transcription factors are activated in the dorsal region of A8 (Figs. 1E and 1H). These are later followed by other genes activated in subsets of cells of the posterior spiracles.

FIG. 1. Posterior spiracle structure at early and late stages of development. Three-dimensional structure of the posterior spiracles after 15 h of development (left) and two-dimensional organization of the precursor cells of the posterior spiracles at 6 h of development (right). Posterior spiracle of a newly eclosed larva (A) and schematic section of a spiracle (D). In all figures dorsal is up and anterior to the left. The trachea (tr.) connects to the spiracular chamber (sp. ch.) where the filzkörper will form. The filzkörper (Fz.) is a refractile filter formed by cuticle extensions of the cells of the spiracular chamber. The spiracular chamber opens to the outside through the stigma around which there are four branched spiracular hairs (h.). Single cell clones marked with Tau-β-gal (B) showing the elongated "bottle" cell shape of the invaginated spiracular chamber cells. In (C) two of the support cells giving rise to a spiracular hair are marked (black arrowhead), as well as a single internal bottle shape cell (white arrowhead). Close up of segments A6–A8 at stage 11 stained with anti ct (E–G) or anti-nub (H). Both antibodies mark in A8 the cells that will give rise to the spiracular chamber. The embryo in H has also been stained with anti-en and the embryo in F with anti-β-gal to detect the expression of an insert in the breathless gene that labels the tracheal pits. Note that at st 11 (6 h) the cells of the spiracular chamber can only be identified by the expression of the antibodies, as they are morphologically indistinguishable from cells in more anterior segments. The ct-expressing cells (black staining in E–G) are ectodermal cells located on the surface of the embryo, arranged as a two-dimensional sheet posterior to the opening of the tracheal pit. Double staining with anti-en (G) reveals that all ct-expressing cells (black) are in the anterior compartment of the A8 segment. The embryo in (H) has been double stained with anti-Ubx (brown), which is not expressed in the dorsal epidermis of A8. Scale bars, 10 μm.

matophore (Fig. 1D). The spiracular chamber is the internal tube connecting the trachea to the exterior. In the larva this tube forms a very refractile filter, the filzkörper (Fig. 1A). The opening of the spiracular chamber, the stigma, is surrounded by four sensory organs: the spiracular hairs. Clones labeling the spiracular hairs show that each one is formed by four cells related by lineage, two neural and two support cells, the typical structure of a type I external sensory organ (data not shown; Jan and Jan, 1993). The stigmatophore is an external protrusion in which the spiracular chamber is located. When the larva is buried in the semiliquid medium where it feeds, the stigmatophore periscopes out of the medium, allowing the larva to continue breathing.

Although functionally related, the stigmatophore, the spiracular chamber, and the trachea develop in different ways. This is reflected in the timing of their development and by the genes required during development.

Genes Expressed and Required for Posterior Spiracle Development

The activation of cut (ct), empty spiracles (ems), nubbin (nub), klumpfuss (klu), and spalt (sal) genes (Blochinger et al., 1990; Dalton et al., 1989; Klein and Campos-Ortega, 1997; Kühnlein et al., 1994; Ng et al., 1995; Walldorf and Gehring, 1992) does not require the expression of any of the other genes that we have studied, suggesting that they are at the top of the cascade under ABD-B regulation. The ct, ems, nub, and klu genes are expressed in the spiracular chamber in overlapping patterns. The sal gene is not expressed in the spiracular chamber but in the cells that surround it and will form the
stigmatophore. The exclusion of sal from the spiracular chamber is partly due to repression by ct, as in ct mutants sal is now expressed at low levels in the internal part of the spiracle.

Downstream of these putative Abd-B targets other genes are activated. These include the transcription factors grainy head (grh), tracheless (trh), and engrailed (en) (Bray and Kafatos, 1991; Isaac and Andrew, 1996; Patel et al., 1989) and other genes defined by enhancer trap insertions (Bellen et al., 1989; Hartenstein and Jan, 1992). At this level gene regulation depends on more complex inputs. For example, while the P1120 enhancer trap only depends on ct expression, grh and trh expression require both ems and ct function.

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Note. +, normal expression; –, no expression; +/–, expression reduced; ++, ectopic expression; NS, not studied; <sup>a</sup>, antibody, <sup>r</sup>, reporter gene; <sup>p</sup>, P element enhancer trap.

**FIG. 2.** Hierarchical interactions between genes expressed in the posterior spiracles. The data from Table 1 have been organized in a diagram representing the inferred genetic interactions. The expression of all markers tested depends on Abd-B. The activation of the early targets depends on Abd-B but on no other gene tested. The activation of secondary targets seems to be controlled by a combination of early targets. This scheme does not imply direct regulation which has only been proven for the ABD-B/ems interaction. Possible inputs of Abd-B on the secondary targets have not been tested.
We have analyzed the spiracle phenotypes in mutants for the early Abd-B downstream genes (Fig. 3). In sal mutants the stigmatophore does not form (Fig. 3B), resulting in embryos with a normal spiracular chamber that does not protrude. Conversely, mutations in ems and ct affect the spiracular chamber but not the stigmatophore. Mutations for ems result in a spiracular chamber that lacks a filzkörper and is not connected to the trachea (Fig. 3D). In ct mutants the filzkörper is almost missing, but the trachea is still connected to the spiracular chamber and the spiracular hairs are also missing (Fig. 3C). The abnormal spiracular chamber in ct mutants is not due to the absence of spiracular hairs, as the spiracular chamber is normal in embryos deleted for the achaete-scute complex that also lack spiracular hairs (data not shown).

In tracheal mutants, where the tracheal pits do not form and there is no tracheal network (Isaac and Andrew, 1996), the spiracular chamber cells still invaginate, forming a filzkörper (Fig. 3F). However, this filzkörper is shorter than that of the wild type probably due to a secondary requirement of trh, which is also expressed in the spiracular chamber cells.

These results show that the spiracular chamber, the stigmatophore, and the trachea develop independently of each other. We have not been able to detect any phenotypes in mutants for either klu or nub, indicating that although these genes are expressed in the spiracle, they are either redundant or their function is not required for spiracle morphogenesis.

FIG. 3. Phenotype of mutants affecting the posterior spiracles. Phase-contrast images of the posterior spiracles of wild type (A) and sal (B), ct (C), ems (D), H99 (E), and trh (F) mutants. In (A) the white arrowhead points to the stigmatophore and the black arrowhead points to the spiracular chamber where the filzkörper forms. In sal mutants (B) the filzkörper forms but the stigmatophore does not develop. In ct mutants (C) only fragments of the filzkörper are formed and the spiracular hairs are absent. In ems mutants (D) the spiracular hairs form normally but the filzkörper does not form. In H99 (E) embryos, where there is no apoptotic cell death, a normal posterior spiracle develops. In trh embryos (F), in which the trachea does not form, the stigmatophore is normal and the cells of the spiracular chamber invaginate, forming a filzkörper that is shorter than the normal one.

Fate Map of the Spiracular Chamber at Stage 11

To understand how different mutants affect the behavior of the spiracle cells, we first have to analyze the wild-type development. The morphogenetic movements that give rise to the spiracular chamber start at st 11 after the tracheal pits have invaginated. The ct gene is the best marker for the cells that will make the spiracular chamber as it is expressed in these cells when they are still on the surface and continues being expressed after spiracle invagination. At st 11 ct is expressed in a group of about 70 cells arranged as a two-dimensional sheet. Most of these cells are posterior to the A8 tracheal pit although a few coexpress both tracheal and spiracular markers (Fig. 1F). These cells are located in the dorsal half of the anterior compartment of A8 (Fig. 1G) and at this stage have a shape similar to that of cells at homologous positions in more anterior segments.

To follow the movements of the spiracular chamber cells as they invaginate we have analyzed constructs made with particular enhancers of the ct, ems, and grh genes that drive expression of β-gal in subsets of the cells that express the ct gene at st 11 (Fig. 4). These enhancers are not driving the whole spiracle expression of their genes but are good tools for studying cell specification and the morphogenetic movements of the posterior spiracle cells. The expression of ct in the posterior spiracle is controlled by at least three different enhancers (Jack and DeLotto, 1995), two of which have been used in this study. The ct-A4.2 enhancer (Fig. 6B) marks from st 13 the precursors of the four spiracular hairs (from now on we will refer to this enhancer as ct-four). The ct-D2.3 enhancer is expressed earlier than ct-four and marks three groups of cells from late stage 11 (Fig. 4A). The grh-D4 enhancer of the grany head (grh) gene is expressed in a single group of cells in this area (S. Bray, unpublished; Fig. 4B). The expression of ems in the spiracle is driven at least by one enhancer: ems-1.2 (Jones and McGinnis, 1993). This enhancer marks from st 11 a group of cells abutting the tracheal pit (Fig. 4E).
Double stainings of the ct-D2.3, ems-1.2, and grh-D4 lacZ constructs show that they are expressed in nonoverlapping subsets of cells (Fig. 4, compare C with A and B and H with A and E). As the expression of these constructs is maintained during the morphogenesis of the spiracular chamber, we can follow the cell movements and observe what structures they give rise to at later stages. At st 15, the ems-1.2-expressing cells are located in the deeper areas of the spiracular chamber adjacent to the trachea. The grh-D4 cells are located over the ems-1.2-expressing cells and the ct-D2.3 are located over the grh-D4 expressing cells, closer to the stigma opening (Figs. 4D, 4G, and 4J). The correlation of the expression of these three constructs at st 11 and st 15 allows us to “fate map” the spiracular chamber primordium when it is a two-dimensional sheet of cells (Figs. 4F and 4I). The different spatial expression of these enhancers at st 11 shows that the two-dimensional sheet of cells is already patterned and that the cells invaginate to precise positions during development.

**Formation of the Spiracular Chamber**

The morphogenesis of the spiracular chamber precedes that of the stigmatophore. As shown above with the lacZ constructs the cells can be traced as they invaginate. The ems-1.2 cells (yellow, Fig. 4F) are the first ones to invaginate and do so after the tracheal pit cells, which are located more anteriorly, have invaginated. After them, grh-D4 cells (green, Fig. 4F) invaginate, followed later by ct-D2.3 cells (red, Fig. 4F). Thus, the invagination is not simultaneous, but proceeds sequentially from anterior to posterior. The ordered invagination results in the anterior cells of A8 being located in more internal positions at stage 15 (Fig. 4I).

The invagination of the spiracular chamber is accompanied by drastic cell shape changes. The cell shapes can be detected either by inducing Tau–β-gal clones (Figs. 1B and 1C) (A. Martí-Subirana and R. Holmgren unpublished) or by using the ct-D2.3 or grh-D4 lacZ lines that express cytoplasmic β-galactosidase in specific cells (Figs. 4D and 4G). Both methods show that the cells of the spiracular chamber have an elongated “bottle” shape, with the nucleus and...
FIG. 5. Polarization of the spiracle chamber cells. Stage 14 embryos in which the klu-GAL4 line has been used to express two different forms of β-gal protein in the posterior spiracles (arrowheads). The first is localized in the cytoplasm and marks the position of the bodies of the cells expressing klu-GAL4 (A). The second form is a fusion between Nod and β-gal and localizes to the apical side of the cells (B), showing that the microtubules are polarized in these cells. Note that the shape of individual cells cannot be observed due to the fact that klu-GAL4 drives expression in most of the cells of the posterior spiracle.

most of the cytoplasm in a basal position and a long thin apical neck extending to the external surface of the embryo. The process of elongation begins when the cell starts invaginating and, in general, the “neck” of the bottle cell is more elongated the further the cell invaginates.

The elongated shape of the cells within the spiracular chamber suggests that their microtubule cytoskeleton might be polarized in the apical–basal axis. To test if this is the case, we have compared the localization of two forms of β-gal protein driven in the spiracular chamber with the klu-GAL4 line (Klein and Campos-Ortega, 1997). One form has a cytoplasmic localization, while the other form is a fusion with the Nod protein and localizes to the minus ends of the microtubules (Clark et al., 1997). Although both proteins are expressed in the same cells, they have a very different distribution. The cytoplasmic form labels homogeneously the cells of the spiracular chamber; in contrast, the Nod-β-gal fusion protein localizes primarily to the lumen of the spiracle, showing that the microtubules are polarized with their minus ends oriented to the apical side of the cell (Fig. 5).

The changes in cell shape could be a passive result of the forces that the cells suffer while they invaginate, or they could be an active process by which the cells contribute to the morphogenetic movements. To distinguish between these two possibilities we have studied the shapes of these cells in embryos in which invagination of the spiracular chamber does not occur. To achieve this we have expressed UAS-dpp driven by Arm-GAL4. In Arm-GAL4 UAS-dpp embryos, although the spiracular chamber stays on the surface, all the structures of the spiracular chamber differentiate (Fig. 6D). In these abnormal embryos the trachea invaginates and connects with the surface of the embryo. Posterior to the A8 tracheal opening, the filzkörper appears as a flat lawn; and posterior to it the four spiracular hairs are aligned in a dorsoventral row (Fig. 6, compare A and B with D and E). Our cell shape markers show that normal cell elongation occurs in these embryos even when the spiracle does not invaginate (Fig. 6F). This shows that cell shape changes are not the passive result of invagination, but an active process at work during spiracle morphogenesis.

Formation of the Stigmatophore

The stigmatophore development is delayed with respect to that of the spiracular chamber. The first gene activated in the stigmatophore is the sal gene (Kühnlein et al., 1994). Sal is initially expressed at st 11 in a subset of cells in the dorsal region of A8. This soon spreads to the posterior compartment and encircles the spiracular chamber cells (Fig. 7A). The expression of sal stabilizes at st 12 with no major gain or loss of sal-expressing cells in the posterior spiracle. Although the number of sal-expressing cells does not change, the circle of sal expression changes shape drastically (Figs. 7A–7D). These changes are not likely due to cell division as, with the exception of the nervous system cells, the last mitoses in the embryo have already occurred.
(Campos-Ortega and Hartenstein, 1997). To analyze why the circle of sal changes shape, we have recorded the number and position of sal-expressing nuclei in the stigmatophore at different stages of development (Fig. 7E). By st 12 sal is expressed in a circle two to three cells wide, with an internal circumference of approximately 25 cells (Fig. 7A). At later stages the width of the sal circle of expression broadens, while simultaneously the inner and outer circumferences become smaller (compare Fig. 7C with Fig. 7B). The decrease of nuclei in the inner and outer circum-

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**FIG. 7.** Cell rearrangement of the sal-expressing cells in the stigmatophore. Expression of sal in the stigmatophore at st 12 (A), st 13 (B), st 14 (C), and st 15 (D) (magnification in D is half of that in A–C). The position of A8 is indicated by a bracket. The blue and red lines in B’ and C’ delimit the sal-expressing cells in the stigmatophore. The area inside the incomplete circle of sal expression in A8 (asterisk) invaginates to form the spiracular chamber. Outside the stigmatophore, sal is also expressed in the trachea (arrowhead) and in A9. In the stigmatophore, sal is expressed initially in a two-cell-wide incomplete circle around the cells that will form the spiracular chamber (A). As the cells of the spiracular chamber invaginate, the semicircle closes (B) and the cells start rearranging their positions. As a result, the 2- to 3-cell-wide circle at st 13 (B) becomes 4 to 5 cells wide at stage 14 (C) and about 10 cells wide at stage 15 (green line in B’ and C’ represents the width of the sal circle). Note that due to the rearrangement in cell position, as the width increases, the number of cells on the circumference simultaneously decreases (The circumference is represented in B’ and C’ by the red and blue lines.) (E) Graph illustrating cell rearrangement in the stigmatophore. These data have been obtained by counting the number of nuclei along the three lines represented in B’ and C’. The green line represents the number of nuclei in the width of the sal circle, the red line the circle of cells closer to the spiracular chamber, and the blue line the circle of cells most distal to the spiracular chamber. Cells that initially were along the red and blue lines are displaced to central positions, decreasing the number of nuclei in both circles and increasing the width. Bars in E represent standard error.
ference cannot be accounted for by cell death, as in homozygous embryos for the H99 deletion in which apoptosis does not occur (White et al., 1994) the stigmatophore development is normal (Fig. 3E). These results show that cell rearrangements are responsible for closing the stigmatophore and, indirectly, elongating the stigmatophore.

**Formation of a Continuous Tracheal–Spiracular Lumen**

For the respiratory system to function, the lumen of the spiracular chamber and the trachea must connect. The branch that connects the dorsal trunk of the trachea with the posterior spiracle is the spiracular branch of A8 (Manning and Krasnow, 1993). In segments where the spiracles do not form, the spiracular branch stays attached to the epidermis of the embryo and the lumen collapses.

To study how the spiracular branch of A8 links to the posterior spiracle we have used an antibody against the Crumbs protein (anti-crb) and the markers described above. The Crumbs protein localizes to the apical surface of all epidermal cells and can be used as a marker of the lumen of the trachea and the spiracle (Tepass et al., 1990).

When the trachea primordia first invaginate there are no signs of spiracle morphogenesis. At this point, the stigma (opening of the respiratory system) is formed by the hole in the center of the invaginated tracheal pit of A8 and no spiracular lumen exists. The spiracle cells are still on the surface of the embryo and only later start expressing spiracle specific genes that induce the invagination. The invagination of the spiracular chamber cells occurs from anterior to posterior. The spiracular chamber cells closer to the trachea (those expressing ems-1.2, yellow in Fig. 4F) invaginate attached to the neighbouring tracheal cells (Figs. 8A and 8B). At the same time, the stigma shifts back from the tracheal pit to the ems-1.2-expressing cells. This process spreads posteriorly and as more posterior cells invaginate, the stigma keeps shifting posteriorly first to the grh-D4, and then ct-D2.3 expressing cells (Figs. 8C and 8D) until it reaches its final position. During invagination, the cells of the trachea and the spiracle never lose their connection. The formation of the common tracheal–spiracular lumen can be compared to the movement of a zipper: the stigma shifts back its position between different cells and the invagination process brings into proximity cells that initially were distant in the two-dimensional sheet. The posterior spiracle lumen is formed by the apical surfaces of the cells that invaginate.

**Genes Required for the Connection of the Posterior Spiracle to the Trachea**

We have studied if the genes required for the tracheal and spiracular lumens to connect are different than the genes required to form the continuous tracheal tree. In mutants for the Drosophila FGF and FGF-R homologues branchless...
Formation of the spiracle-trachea lumen in different mutants. Lateral views of stage 13 embryos stained with anti-crb to show the lumen of the spiracle and the trachea. (A) Wild-type embryo with the posterior spiracle forming in the dorsal side of A8 (black arrowhead). At this stage the lumen is well formed in the center of the spiracle (white arrowhead). Note that the expression of crb is stronger in the spiracle lumen than in the tracheal lumen. In Abd-B mutant embryo (B), the posterior spiracle has not formed (the black arrowhead points to the dorsal position where it should be located) there is no spiracle lumen and the trachea opens to the surface directly through the spiracular branch (white arrowhead). Embryos mutant for ems (C–D) stained for crb (C) or carrying the ems-1.2 reporter construct (D). The expression of the construct is much weaker than that in the wild type. Two groups of cells expressing ems-1.2 have not invaginated and are on the surface (white arrowhead points to the anterior group). The posterior spiracles (black arrowhead) have not formed a lumen and the tracheal opening is outside of the spiracle (C, white arrowhead).

DISCUSSION

The posterior spiracles present several advantages for the study of morphogenesis: they are conspicuous and easy to observe; they develop fast; and there are many molecular markers that can be used to identify the cells of the spiracle before they are different from their neighbors, allowing their movements to be followed during the morphogenetic process. As the posterior spiracles are formed at 15 h of development, we can study most mutations in genes involved in the control of morphogenesis as they allow the embryo to develop beyond this stage.

Morphogenetic Mechanisms Required for the Formation of the Posterior Spiracles

Cell proliferation and cell death are two major morphogenetic mechanisms (Conlon and Raff, 1999; Vaux and Korsmeyer, 1999). Neither appears to play a major role in posterior spiracle formation. This is shown by the fact that mutations that abolish apoptosis make a normal spiracle and that the morphogenesis of the posterior spiracle starts at late st 11, after the last mitoses in the embryonic epidermis have finished (Campos-Ortega and Hartenstein, 1997). On the other hand, control of cell shape and cell rearrangements play an important role in the formation of the spiracular chamber and the stigmatophore.

We have shown that cell elongation occurs simultaneously to the invagination of the spiracular chamber cells. The cell shape changes are not due to mechanical forces imposed on the invaginating cells, as in embryos in which invagination does not occur, the cells change shape at the appropriate time of development. The elongation does not occur simultaneously in all cells, but starts in the more anterior ones (Figs. 10A–10D) and, in general, the invaginating cells keep contact with the external surface of the embryo. This results in the cells that have invaginated earlier being deeper in the spiracular chamber and longer (compare Figs. 4D and 4G). The elongated cells have polar-
ized microtubules with their minus ends toward the apical surface. We cannot at this point say if the polarization of the microtubules is the cause of the elongation of these cells or if it is required for the stabilization of the shape. Other cytoskeletal components are probably also important to achieve the cell shape, as stainings with phalloidin–rhodamine show that high levels of actin accumulate in the narrow processes of the bottle cells (data not shown).

The invagination of the cells starting in the anterior part of the spiracular chamber primordium and spreading posteriorly suggests the involvement of a relay signaling mechanism. However, several experiments indicate that the well-timed invagination is controlled cell autonomously by transcription factors activated in the two-dimensional primordium. First, in mutants for the transcription factor ems the cells neighboring the tracheal pit do not invaginate. This stops the progression of the lumen but does not affect the invagination of more posterior cells. Second, genetic markers specific for different cells of the spiracular chamber are activated in the two-dimensional primordium before the onset of the morphogenetic movements. Therefore, in the posterior spiracle, signaling might be important for the spatial activation of the expression of transcription factors in the two-dimensional primordium, and these result in the control of cell elongation autonomously. However, we cannot discard the alternative possibility that a signaling wave spreads through the primordium, informing the cells when to activate the invagination mechanisms. In this hypothesis ems mutant cells would have the invagination mechanism blocked, but they would be capable of relaying the signal to more posterior cells.

The formation of elongated bottle cells is common during the invagination of tissues in several organisms. In Drosophila, bottle-shaped cells form transiently during the invagination of the tracheal pits and in the formation of the ventral furrow during mesoderm invagination (Costa et al., 1994). The major difference of these cells and those we describe here is that, in the posterior spiracle the bottle cell shape becomes stabilized. Bottle-shaped cells have been described in the dorsal lip of the blastopore in amphibians and in the vegetal plate of the sea urchin where these cells are critical for the initiation of invagination (Keller, 1981; Kimberly and Hardin, 1998).

The morphogenetic mechanism used for the formation of
The stigmatophore is completely different. The cells do not change shape very markedly but rearrange their positions in the epithelium in a mechanism which is similar to the convergent extension movements described during the gas-
trulation of organisms ranging from vertebrates such as Xenopus or zebra fish, to Caenorhabditis (Warga and Kim-
mel, 1990; Williams-Masson et al., 1998; Wilson and Keller,
1991). During convergent extension, cells that are side by side intercalate, changing their positions so that one be-
comes anterior to the other (Figs. 10F–10I). This rearrange-
ment results in the elongation of the epithelium that will
form the stigmatophore. At the same time, convergent ex-
tension in the stigmatophore solves the problem that the
invagination of the spiracular chamber causes to the integ-
riety of the epithelium: cell rearrangement is the solution
the stigmatophore cells use to make a smaller contact
surface with the shrinking apical surfaces of the spiracular
chamber cells.

The Patchwork Formation of the Posterior Spiracle
Could Be a Result of a Step by Step Evolution

Many insects have one pair of spiracles per segment from
T2 to A8, but this number has varied during evolution and
also during development (Keilin, 1944). For example, while
in the larva of Drosophila there are only two pairs, posterior
spiracles in A8 and anterior spiracles in T1, most segments
of the adult have functional spiracles. In the larvae of other
insect species, the number of functional spiracles varies (0,
1, 2, 8, 9, or 10), and in many cases this variation is
considered to be an adaptation to the physiological require-
ments of the larva to the particular environment in which it
develops (Keilin, 1944). In the genus Drosophila the shape
and size of spiracles can vary. Some species having similar
shapes as Drosophila melanogaster and others being longer
or shorter (Wheeler, 1987). This variability suggests that the
shape of the posterior spiracles is under strong selective
pressure and that the morphology they now have is a
relatively recent adaptation.

The trachea and the posterior spiracle develop indepen-
dently, each using a different morphogenetic mechanism
that is regulated by independent genetic pathways. For
example, trachea formation does not require Abd-B but is
dependent on the trachealless gene for the invagination;
while for the formation of the posterior spiracle, trachealless
plays a secondary role and Abd-B is fundamental. The same
independent development applies to the external and inter-
nal parts of the posterior spiracle. The spiracular chamber
requires ems and ct but it does not express sal while the
opposite is true in the stigmatophore. All three genes are
expressed in many other tissues (Bloehinger et al., 1990;
Dalton et al., 1989; Kühnlein et al., 1994; Waldorf and
Gehring, 1992) and have been coopted in the spiracle
downstream of Abd-B. This patchwork formation for a
structure that has to be perfectly connected to be functional
relies on a perfect integration of the three different genetic
pathways and morphogenetic mechanisms. This integra-
tion might have been achieved by a step by step evolution.
Initially the spiracular branch opening could act as a stigma
for air exchange. Later on in evolution, using a different
genetic pathway, the adjacent cells could be recruited to
form filter-like structures as the filzkörper. Finally, a last
group of cells could be recruited to form the periscopic
structure of the stigmatophore. This scenario would explain
the patchwork development of the posterior spiracle we
now see, as at each of these stages a different morphoge-
netic mechanism would have been used with its control
being under the regulation of a new subset of transcription
factors.

The Posterior Spiracles Gene Regulatory Cascade

Abd-B is necessary and sufficient to induce posterior
spiracle development in the dorsal part of the trunk of the
larva. In Abd-B mutants the posterior spiracles do not form
and, conversely, ectopic expression of ABDB protein in
anteri or segments results in ectopic posterior spiracles
(Castelli-Gair et al., 1994; Kuziora, 1993; Lamka et al.,
1992; Sánchez-Herrero et al., 1985). These experiments
indicate that Abd-B regulates all the morphogenetic mecha-
nisms that lead to the formation of the posterior spiracle.
Abd-B is expressed on A8 both in the ventral and dor-
sal region, so there must be some positional information
that modifies the transcriptional outcome of Abd-B expres-
sion. We can only guess that the interaction of the ABDB-
protein with cofactors expressed differentially in a dorso-
ventral pattern or an interaction with signaling molecules
required for dorsoventral patterning such as the TGF-
homolog decapentaplegic (dpp) will give the intrasegmental
differences.

The first genes that we have found to be expressed in a
posterior spiracle restricted pattern are a group of early
responsive transcription factors whose expression does not
depend on each other but requires ABDB. We call these
group early targets and, though only ems has been shown to
be a direct target (Jones and McGinnis, 1993), their timing
of expression suggests that they could be regulated directly
by ABDB at the transcriptional level. The expression of the
early targets ct, ems, klu, nub is restricted to the primor-
dium of the spiracular chamber and with a slight delay the
sal gene becomes expressed in the surrounding cells that
will become the stigmatophore. This is the first indica-
tion of the existence of two distinct populations of cells in
the spiracle suggesting that it is at this stage that the spirac-
ule is patterned into stigmatophore and spiracular chamber.
Secondary targets seem to be either expressed in the stigmato-
phore or in the spiracular chamber, in agreement with the
observation that both cell populations develop using differ-
ent morphogenetic strategies.

With the available data, the genetic network of the
stigmatophore seems to be a simple cascade downstream of
sal. The genetic network of the spiracular chamber shows a
more complicated relationship with several early transcrip-
tion factors acting in parallel. This richness of transcription
factors could provide the positional information required for the precise spatial activation of the cell markers expressed when the spiracular chamber primordium is still two dimensional (Fig. 4F).

In summary, in this paper we have described the development of an essential part of the tracheal system: the posterior spiracles. The spiracles are a good model to study the cellular and molecular mechanisms controlling cell shape and cell rearrangements, two mechanisms which are used during the morphogenesis of a variety of organisms. The relative simplicity and experimental accessibility of the posterior spiracles of Drosophila should help to understand how these basic processes are controlled.

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