INTRODUCTION

The ability to develop organs with specific functions has been a fundamental requirement for the evolution of multicellular animals. Many organs, including the lungs, kidneys and circulatory system, are composed of intricate networks of branched tubules. The formation of these branching networks has been intensively studied, mainly by explanting organ or tissue fragments and following or perturbing the morphogenesis in vitro (see Bard, 1990). This work has revealed complex inductive interactions between the branching epithelial tubes and the surrounding mesenchyme and has identified some of the signaling pathways that are used. Recently, a series of elegant experiments in *Drosophila* has defined the elaborate branching morphogenesis of the tracheal system and identified a series of genes required for specific steps in the morphogenesis (Samakovlis et al., 1996a; Guillemin et al., 1996; Lee et al., 1996; Sutherland et al., 1996). In all these examples, the final network is formed by repeated branching of large tubes to form finer and even finer tubes.

There is an opposite form of morphogenesis in which small tubes join together to form larger and less numerous tubes, much as creeks and streams combine to form larger and larger rivers as they move toward the sea. This joining, rather than branching, morphogenesis has been little studied though it is not unusual. For example, during kidney development several initially separate nephrons join to produce a nephron arcade with a single collecting tubule leading to the ureter (Osathanondh and Potter, 1963). Another example is the fusion of the efferent tubules with the nephric duct during testis formation (Saxén, 1987). Joining of tubes is also important during the formation of the vertebrate circulatory system, as the primary capillary beds are remodeled to form the network connecting arteries to veins (Yancopoulos et al., 1998). During tracheal development, dorsal tracheal branches from each segment fuse with their counterparts from the other side of the embryo to connect the left and right sides of the tracheal system (Samakovlis et al., 1996b). Similarly, segmental branches fuse along the sides of the embryo to form the lateral tracheal trunks which connect the tracheae of different segments.

Formation of the larval salivary glands in *Drosophila* provides a simple example of joining morphogenesis. During salivary invagination, ducts from the two sides of the embryo meet at the ventral midline and fuse so that continued invagination produces a single common duct that connects to...
the oral cavity (Fig. 1B). This paper begins to define the genetic control of this example of joining morphogenesis.

In the formation of *Drosophila* salivary glands, several aspects of organogenesis are simplified. First, salivary development occurs rapidly, beginning at 4.5 hours of development and finishing by 10 hours of development. Second, the initial specification of salivary cells occurs within a two-dimensional sheet of cells with no known induction from underlying layers. This initial specification, which is complete by embryonic stage 10 (about 5.5 hours of development), occurs only within a specific region of the anterioposterior axis: parasegment two. The salivary primordium is bilaterally symmetric and consists of approximately 100 cells on either side of the ventral midline (Campos-Ortega and Hartenstein, 1985). The homeotic gene responsible for patterning parasegment 2, Sex combs reduced (Scr), encodes the primary inducer of salivary glands. Embryos lacking Scr have no salivary glands while ectopic Scr results in ectopic salivary glands (Panzer et al., 1992; Andrew et al., 1994). The salivary primordium is continuous across the ventral midline but is limited dorsally by decapentaplegic (Panzer et al., 1992). Third, because the salivary cells do not divide after the initial patterning, further development is not complicated by cell proliferation.

The major subdivision of the salivary primordium distinguishes pregland from preduct tissue. The most dorsal 80-90 cells on each side of the ventral midline constitute the circular pregland domain, also known as the salivary placode (Fig. 1A; Panzer et al., 1992), while the most ventral 20-30 cells become the precursors of the salivary ducts (Fig. 1A; Kuo et al., 1996). After the salivary primordium has been established, gland and duct primordia can be distinguished at the molecular level since expression of several genes is restricted to only one of these domains. For example, *fork head* (*fkh*; Weigel et al., 1989; Panzer, 1992), *Toll* (Gerttula et al., 1988) and *huckebein* (Panzer, 1994; Brönner et al., 1994) are expressed in pregland cells and are specifically excluded from preduct cells. In contrast, expression of *Serrate* (Ser; Fleming et al., 1990; Thomas et al., 1991), *breathless* (*bt*; Klämbt et al., 1992) and *dead ringer* (*dri*; Gregory et al., 1996) is restricted to the preduct cells.

We have shown that two regulatory activities interact to define the border between pregland and preduct cells (Kuo et al., 1996). The first is the EGF receptor (EGFR) signaling pathway, which has multiple functions in *Drosophila* development including the definition of ventral fates in the embryonic ectoderm (Mayer and Nüsslein-Volhard, 1988; Kim and Crews, 1993), wing vein induction, eye development and oogenesis (for a recent review see Schweitzer and Shilo, 1997). In the ventral ectoderm, Spitz is responsible for activating the EGFR pathway in a graded fashion that limits the ventral extent of *fkh* expression to the pregland cells.

*fkh* itself is the second regulator of the positioning of the pregland/preduct border. In *fkh*-mutant embryos, expression of the duct marker *Ser* extends dorsally into the gland primordium (Kuo et al., 1996). *fkh* is also responsible for excluding expression of *tracheless* (*trh*) from the gland primordium (Isaac and Andrew, 1996). Thus, *fkh* is critical for the establishment of the dorsal limit of duct fate. Together, the opposing activities of Fkh and the EGFR pathway precisely determine the border between the gland and duct primordia.

The subdivision of the salivary primordium into pregland and preduct defines the expression domains of two regulators that are required for the subsequent development of these tissues. In addition to its function in the establishment of the gland/duct border, *fkh* has another salivary role: *fkh* is necessary for the activation of all tested genes expressed in the gland primordium after its initial establishment (Y. M. K. and S. K. B., unpublished observation; Mach et al., 1996; Lehmann and Korge, 1996). In the duct primordium, *trh* functions to activate duct fate (Kuo et al., 1996) in a way analogous to the role of *fkh* in pregland cells: all tested genes that are expressed in the duct primordium after its initial establishment require *trh* for expression. It is also interesting to note that both *fkh* and *trh* encode autoregulatory gene products (B. Zhou and S. K. B., unpublished data; Isaac and Andrew, 1996), suggesting the possibility that continued expression of these genes is important for maintenance of gland and duct fate, respectively.

Once the initial specification and the primary patterning events are complete, the cells begin characteristic morphogenetic movements that result in mature salivary glands and ducts. The morphogenesis of salivary tissues can be separated into three successive events: formation first of the salivary glands, then the individual ducts and finally the common duct. At the end of stage 11 (about 7 hours of development), the most posterodorsal pregland cells begin to invaginate. The site of invagination progresses anteriorly until all of the pregland cells have been internalized, forming a tubular salivary gland with a single layer of secretory cells surrounding a tubular lumen (Panzer, 1992). *fkh* is required for this first type of invagination, during which the pregland cells leave the ventral surface of the embryo (Weigel et al., 1989; Panzer et al., 1992). As the gland cells invaginate, the preduct cells rearrange to form two parallel rows of cells extending across the ventral midline (Kuo et al., 1996). These cells will form the two types of duct tissue: as we show here, the posterior row becomes the individual ducts and the anterior row becomes mature salivary glands (sg), individual ducts (id) and common duct (cd) can be seen.

Fig. 1. Embryonic glands and ducts arise from the ventral epidermis of parasegment 2. Views are ventral and anterior is to the left. (A) β-galactosidase staining of a stage 11 embryo carrying a reporter construct (*fkh* 1-1000::lacZ; B. Zhou and S. K. B., unpublished data) that labels the salivary gland primordium (sg). Salivary ducts arise from the unstained cells ventral to the circular gland primordia. The duct primordium comprises two subdomains that give rise to the two types of duct tissue. The anteriormost duct cells form the common duct primordium (cd), while the posteriormost duct cells comprise the individual duct primordium (id). These domains have been outlined in black. (B) In a stage 14 embryo carrying a reporter construct that labels all salivary tissues (*fkh*Δ360-505::lacZ; B. Zhou and S. K. B., unpublished data), mature salivary glands (sg), individual ducts (id) and common duct (cd) can be seen.
the common duct. The lateral ends of the individual ducts remain in contact with the gland cells and when they invaginate they continue the tube started during gland invagination. The diameter of the tube, however, is much smaller in the ducts. The individual duct invagination continues to the ventral midline where the left and right sites of invagination fuse (Kuo et al., 1996; Fig. 1B). Finally, the common duct is formed as the anterior row of duct cells move anteriorly and begin to invaginate. The resulting structure connects the lumen of the individual ducts to the pharynx (Fig. 1B). trh plays a critical role in both duct invaginations as neither of the preduct tissues invaginate from the ventral surface in trh-mutant embryos (Kuo et al., 1996; Isaac and Andrew, 1996).

Here we examine the role of the Pax gene eye gone (eyg) in salivary organogenesis. eyg encodes a transcriptional regulator that is most closely related to Pax-B but lacks the N-terminal end of the PAIRED domain shared by most Pax family proteins (Jun and Desplan, 1996). We show that eyg has specific functions in the development of the individual ducts. It is required to distinguish individual from common duct domains and is necessary for the morphogenesis of the cells of the individual ducts. In addition, the precursors of the adult salivary gland, the imaginal ring, require eyg. We also show that known positive and negative regulators of salivary fate regulate eyg and that eyg itself regulates at least one downstream duct marker.

**MATERIALS AND METHODS**

**Drosophila stocks**

A Canton-S stock was used as wild type. trh^{D55} was obtained from V. Hartenstein, fkh^{Y76} from H. Jäckle and Sce{w17} from the Indiana Stock Center. We describe elsewhere Df(3L)eyg^{C1} (69A4-5; 69D4-6), which deletes eyg, and In(3L)eyg^{C53} (69B4-C7), which breaks in or just adjacent to the eyg-coding region (C. Jang, N. A. J. J. Chao, D. Bessarab, Y. M. K., S. Jun, C. Desplan, S. K. B. and Y. H. S., unpublished data). For salivary ducts, In(3L)eyg^{C53} behaves as a hypomorph, as the phenotype of In(3L)eyg^{C53} / Df(3L)eyg^{C1} embryos is more severe than that of In(3L)eyg^{C53} homozygous embryos. A TM6B, Tb balancer chromosome was used to distinguish eyg pupae from their wild-type siblings. In the experiments assaying the ability of individual larvae to survive, one drop of wet yeast was added to each vial containing standard medium per day. The hs-eyg stock used was made by using standard protocols to transform white^{1118} embryos with a construct containing the wild-type eyg-coding region under control of the hsp70 promoter (gift from C. Desplan).

**Immunohistochemistry and in situ hybridization**

Embryos were staged according to Campos-Ortega and Hartenstein (1985). Antibodies mAb Ab22C10 (neuron-specific) and mAb Cq (anti-Crumbs; Tepass et al., 1990) were gifts from C. Goodman and E. Knust, respectively. Polyclonal rabbit anti-beta-galactosidase sera were obtained from 5 Prime-3 Prime Inc., and biotinylated goat anti-rabbit and anti-mouse secondary antibodies were from Jackson ImmunoResearch. Serrate cDNA was graciously provided by R. J. Fleming, and breathless cDNA was a gift from B. Shilo. The eye gone clone was a gift from C. Desplan. All embryo collections, fixations, stainings, in situ hybridizations and color reactions were performed as described previously (Kuo et al., 1996). To examine third instar salivary glands and ducts, we dissected tissue from wandering larvae and fixed it in 4% formaldehyde in PBS for 30 minutes. Salivary tissues were incubated in 1 μg/ml DAPI for 5 minutes, rinsed, and examined.

**Microscopy and photography**

Stained embryos were examined and photographed using a Leica DMRB microscope with Nomarski DIC optics and Kodak 64T film. Slides were scanned using a Nikon Coolscan slide scanner. Images were manipulated using Adobe Photoshop 3.0.

**RESULTS**

**Embryonic eye gone expression includes salivary and sensory primordia and two sets of ectodermal stripes**

Using RNA in situ hybridization, we determined the timing and location of eyg transcription in the Drosophila embryo (Fig. 2A-D). eyg is first detected in the salivary primordium (Fig. 2A) at the beginning of stage 10. Temporal changes in the salivary expression of eyg are described in detail below. By
stage 13 (Fig. 2B), expression can be seen in the dorsal ectoderm in segmentally repeated stripes that arise from the patches of expression seen in A. These stripes span the anterior edge of each thoracic and abdominal segment, with sharp anterior but diffuse posterior borders. There are also ventral stripes of eyg expression in each thoracic segment that extend ventrally across the ventral midline (Fig. 2B).

eyg is also expressed in two pairs of sensory organ primordia. In stage 17 embryos (Fig. 2C,D), eyg is expressed in precursors of the eye-antennal disc in what appear to be the same cells that express eyeless (Quiring et al., 1994). As we will describe elsewhere, eyg is essential for eye development (C. Jang, N. A. J. Chao, D. Bessarab, Y. M. K., S. Jun, C. Desplan, S. K. B. and Y. H. S., unpublished data). The precursors of the larval antennal organ (AO), which is thought to function in olfaction, not only strongly express eyg (Fig. 2A-C) but require it for their development. Although the cells of the AO are present in eyg-mutant embryos, the characteristic finger-like projections known as sensillae are often absent, suggesting a role for eyg in their morphogenesis (Fig. 3). Finally, there are three additional pairs of ectodermal staining areas in the head (Fig. 2D) and three ventral thoracic spots that are either next to or associated with the cells of the leg discs (Fig. 2B).

eye gone expression in salivary tissues becomes refined to the individual ducts and anterior ends of glands

Beginning in stage 10, both the pregland and the posterior preduct cells strongly express eyg (Fig. 4A). At this stage, gland precursors express eyg more strongly than their duct counterparts. As the germ band retracts during stage 12, eyg transcript disappears from the gland precursors, beginning with the posterior regions, leaving a crescent-shaped staining pattern (Fig. 4B). As germ band retraction continues, eyg transcript begins to be restricted to the most posterior of the preduct cells and expression finally completely disappears from the pregland cells (Fig. 4C,D). Later in stage 12, as the individual duct cells are invaginating, eyg transcript completely disappears from the duct cells as well (Fig. 4E). eyg expression, however, reappears and, by stage 14, eyg is expressed in the mature individual ducts and also in the anterior cells of each salivary gland (Fig. 4F). In contrast to trh, eyg expression is never seen in the anteriorly extending common duct. In summary, eyg expression is initially seen throughout the salivary primordium (except the common duct primordium) and then disappears from all cells, mimicking the order of their invagination. The individual duct cells are the last cells to lose expression but eyg expression is reinitiated in them and in anterior gland cells once morphogenesis is complete.

Salivary eye gone expression is regulated positively by Sex combs reduced and tracheless but is regulated negatively by fork head

Scr, the homeotic gene responsible for patterning parasegment 2, is responsible for the activation of every salivary gene that has been tested. As expected, eyg is not expressed in the salivary primordium of Scr-mutant embryos (Fig. 5C).

We and others showed previously that the trh gene product is necessary for invagination of all salivary duct cells and that it is required for expression of downstream duct markers (Kuo et al., 1996, Isaac and Andrew, 1996). Because eyg is also expressed in part of the salivary duct primordium, we tested the relationship between trh and eyg in the pathway for duct determination. In wild-type embryos, both trh and eyg expression in the salivary primordium begin early during stage 10 (Wilk et al., 1996; Isaac and Andrew, 1996, N. A. J. and S. K. B., unpublished observations). At this stage, eyg expression in trh-mutant embryos is indistinguishable from expression in wild-type embryos (Fig. 5A,B). Therefore, initiation of eyg expression in the salivary primordium is independent of trh. In early stage 12, however, eyg expression becomes dependent on trh. Although eyg is expressed strongly in the posterior preduct cells of wild-type embryos, this expression is completely absent in trh-mutant embryos (compare Fig. 5D,E). It is eyg maintenance, then, and not its initiation, that depends on trh. We also tested whether trh expression depends on eyg and...
eye gone is required for salivary ducts

Fig. 4. eyg exhibits a dynamic expression pattern in embryonic salivary tissues as visualized by in situ hybridization. A-F are ventral views of embryos in successive stages of salivary development. (A) Salivary placode and posterior duct cells in stage 10 embryos express eyg strongly. As germ band retraction begins at the beginning of stage 12, eyg transcripts disappear from the cells of the placode in an order mimicking their invagination, resulting in crescent-shaped patches of eyg-expressing cells (B). As germ band retraction continues, eyg transcription becomes restricted to the posterior duct cells as they change their shape to form a transverse line of cells (C,D). Immediately afterwards, the individual ducts begin to invaginate at the lateral ends of this line of cells. Surprisingly, as stage 12 is ending, eyg transcripts completely disappear from salivary tissues (E), but reappear in individual ducts and the anterior ends of the glands in stage 13 embryos (F). Note that eyg is not expressed at any stage of development in cells that will form the common duct (compare F to Fig. 1B).

found that trh expression is unaffected in eyg null-mutant embryos (data not shown).

Recall that fkh plays an important role in establishing the pregland/preduct border by dorsally limiting duct-specific gene expression. trh, like eyg, is also initially expressed throughout the gland primordium. Isaac and Andrew (1996) demonstrated that, in fkh-mutant embryos, trh transcript never disappears from the pregland cells. Does fkh play a similar negative regulatory role in eyg transcription? When the wild-type eyg expression pattern is compared to that of fkh-mutant embryos, it becomes clear that fkh indeed negatively regulates eyg. eyg expression persists in gland precursors in fkh-mutant embryos (Fig. 5F,G). Thus, fkh represses expression of trh and eyg, both of whose expression disappears from the pregland cells at

Fig. 5. Positive and negative transcriptional regulators of salivary development also control eyg expression. eyg expression is visualized by in situ hybridization. A, D, F are wild-type embryos that can be used as same-stage references for mutants in the same rows. In stage 11 embryos, removal of Scr (C) results in lack of eyg expression in parasegment 2. In contrast, trh mutations do not affect eyg expression at this early stage (B). By mid-stage 12, however, eyg transcription has become dependent on trh (E). (G) In embryos that lack fkh, expression of eyg remains in the presumptive gland cells. Embryos lacking both trh and fkh (H) display a similar pattern: persistent eyg expression in the presumptive gland cells.
approximately the same time. eyg plays no role in regulation of fkh expression (data not shown).

Armed with the knowledge that (1) fkh is responsible for the exclusion of both trh and eyg from the pregland cells and (2) trh is necessary for maintenance of eyg expression in the duct cells, it is possible to ask whether fkh represses eyg in the pregland cells simply by repressing trh or if fkh downregulates trh and eyg independently. To address this question, we constructed embryos that were doubly mutant for trh and fkh. If the reason for eyg disappearance from the pregland cells in wild-type embryos is disappearance of trh, then we would predict that eyg expression would not persist in trh fkh-mutant embryos. eyg expression, however, does persist in pregland cells in trh fkh-mutant embryos, suggesting that trh plays no role in eyg repression by fkh (Fig. 5H).

Embryos deficient for eye gone are defective in the convergence and extension required to form the individual ducts

We compared the morphogenesis of eyg-mutant duct primordia to that of wild type to determine the role that eyg plays in duct development. In wild-type embryos, the salivary ducts arise as a result of two successive convergence and extension events (Fig. 6A-C). Convergence and extension is a common developmental process during which cells intercalate to narrow the tissue while at the same time lengthening it in a perpendicular axis (Irvine and Wieschaus, 1994). The Serrate (Ser) gene is expressed in the entire duct primordium throughout its development and is a useful marker in these cells (Kuo et al., 1996). As the germ band is retracting in wild-type embryos, the duct primordium narrows from about 6-8 cells in the anterioposterior axis to 2 cells. At the same time, it extends laterally across the ventral midline (Fig. 6A,B). The result of this first convergence and extension is two parallel rows of cells separated by a cleft (Kuo et al., 1996). The posterior row of cells expresses eyg (Fig. 4D) and will form the individual ducts. The anterior row of non-eyg-expressing cells, however, converges towards the ventral midline and extends anteriorly to form the common duct (Fig. 6B,C). In embryos homozygous for the eygC1 deficiency or the hypomorphic eygC1/eygC53 combination, the duct primordium never completes the first convergence and extension. Instead of forming the two parallel rows of cells extended across the ventral midline, the preduct cells remain as compact clumps, often slightly separated by the ventral midline (Fig. 6E,F).

Because eyg normally functions in convergence and extension of the duct primordium, we examined the resulting terminal phenotype of eyg-mutant embryos. In wild-type embryos, each of the two salivary glands is attached at the anterior end to individual ducts, which fuse ventrally into the common duct which empties into the pharynx (Fig. 7A). We determined that approximately three quarters of eygC1 or eygC1/eygC53 embryos have no individual ducts at all (Fig. 7C). The remaining eyg-mutant embryos have only one individual duct linking a gland to the common duct (Fig. 7B). In these embryos, the lone individual duct is almost always malformed. The individual duct precursors, instead of invaginating, remain in a group and move anteriorly as head involution occurs. In these mutant embryos, the anterior end of the salivary glands is sealed so that the gland resembles a closed sac with no connection to the mouth. There are no other visible defects in the salivary glands.

Even when no individual ducts form in eyg-mutant embryos, the common duct can still form, although it does not connect to anything at its posterior end (Fig. 7E). In some cases, the common duct in eyg-mutant embryos was visibly much broader than in wild-type embryos, suggesting that the presumptive individual duct cells had been converted to common duct. To test this possibility, we took advantage of an enhancer trap in the dead ringer (dri) locus (Gregory et al., 1996) that labels the nuclei of all duct cells as well as a few cells in the pharynx. We counted 56±4 stained nuclei per wild-type embryo, 27±2 of them associated with the individual ducts and the remaining 29±3 associated with the common duct or pharynx (n=10 embryos). In eyg-mutant embryos (eygC1/eygC1), all of the labeled cells were clustered together in the common duct region, often appearing as a pouch open to the pharynx anteriorly but closed at its posterior end. This cluster contained 42±4 nuclei (n=10), suggesting that, in eyg-mutant embryos, many of the presumptive individual duct cells contribute to the common duct structure.

We also determined when during the development of these mutant embryos the salivary glands lose contact with the duct tissue. In wild-type embryos, all gland cells have invaginated by the end of germ band retraction (stage 13). In eyg-mutant embryos, however, it is common to find gland cells
immediately adjacent to presumptive duct cells and still on the ventral surface of the embryo after germ band retraction is complete. In many cases, then, the completion of gland invagination was delayed in eyg-mutant embryos.

In summary, in eyg-mutant embryos, the duct primordia fail to converge and extend across the midline resulting in the absence of individual ducts. Invagination of the salivary gland cells begins normally but becomes temporarily stalled at the gland/duct boundary until the gland cells finally break loose from the duct cells. In these mutant embryos, many of the presumptive individual duct cells join with the presumptive common duct cells to form an unusually large common duct that does not connect to the glands.

The salivary duct phenotype of eye gone-mutant embryos can be rescued with heat-shock-driven eye gone

Because the eygC1 deficiency removes 20 chromosomal bands, another salivary duct gene might lie in this interval and account for the ductless phenotype. Mutant embryos carrying two copies of a hs-eyg transgene were heat shocked for an hour at 4.5±1.5 hours AEL. After 12 hours of additional development at 25°C, the heat-shocked embryos were collected, stained and compared with non-heat-shocked controls. Without heat shock, 76% had no individual ducts and 24% had one individual duct (Table 1). With heat shock, however, 44% of embryos were rescued to the wild-type number of individual ducts (two) and only 18% had no individual ducts. The remainder had one individual duct. Although many of the rescued ducts were somewhat malformed – abnormal in diameter or length – a small number of them were indistinguishable from wild type.

We can therefore achieve partial rescue of the salivary duct phenotype by a single pulse of heat-shock-induced eyg. We conclude that the gene removed by eygC1 whose absence results in the individual duct phenotype is eyg.

Table 1. Heat-shock eyg can rescue eyg salivary duct phenotype

<table>
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<tr>
<th>Genotype</th>
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<th>1</th>
<th>2</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>–</td>
<td>0</td>
<td>0</td>
<td>100 (31)</td>
<td>100 (31)</td>
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<tr>
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<td>23.8 (15)</td>
<td>0</td>
<td>100 (63)</td>
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<tr>
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<td>+</td>
<td>17.6 (6)</td>
<td>38.2 (13)</td>
<td>44.1 (15)</td>
<td>100 (34)</td>
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</table>

In salivary ducts, breathless but not Serrate is activated by eye gone

btl codes for a Drosophila FGF receptor homolog that is critical to tracheal and midline glial cell development (Klämbt et al., 1992), while Ser encodes a Notch ligand containing a single EGF repeat (Fleming et al., 1990). Although neither is required for duct morphogenesis (Klämbt et al., 1992; Y. M. K., unpublished observation), they are expressed during most of duct development and therefore serve as useful duct markers (Fig. 8A,C). As part of the evidence that trh is required for all aspects of duct development, we showed previously that Ser and btl expression depend on trh (Kuo et al., 1996). Since trh also regulates eyg, we tested whether eyg might act as an intermediate in the regulation of btl and Ser by trh. We found that btl expression in duct cells is strongly reduced in embryos lacking eyg (Fig. 8B). In contrast, expression of Ser is not downregulated in the duct cells of eyg-mutant embryos even though the duct primordia are not fused as in wild-type embryos (Fig. 8D).

Fig. 7. Embryos lacking eyg lack individual salivary ducts. A-C are ventral views while D and E are lateral views. Salivary glands (sg), individual (id) and common ducts (cd) in A-C are shown by β-galactosidase staining of a reporter construct that labels all salivary tissues (fkh Δ360-505: lacZ; B, Zhou and S. K. B., unpublished data). When this construct was crossed into eygC1/eygC35 animals, we found that approximately 25% of mutant embryos had only one intact individual duct (B) while the remaining 75% had no individual ducts at all (C), although presumptive duct tissue still stains with this reporter. When an eygC1 embryo is stained with mAb Cq, which labels epithelial lumens, an intact common duct is sometimes seen (between arrows, E). This common duct is often of wider diameter than in wild-type embryos (D).
**eyg is required for imaginal ring development**

In *eyg*-mutant animals, there is another salivary tissue affected in addition to the individual duct: the imaginal ring. The precursors of many adult *Drosophila* tissues are set aside during embryonic development. These cells multiply during larval development and do not differentiate until metamorphosis. For salivary glands, the adult precursor is a ring of cells at the junction of the salivary gland and the individual duct. During larval growth, the imaginal ring cells remain diploid while both the salivary gland and duct cells become highly polyploid. Thus, during third instar, the imaginal ring nuclei can be distinguished as characteristically smaller than those of the neighboring gland and duct cells (Fig. 9A). Note in Fig. 4F that expression of *eyg* in late embryos is not restricted to the individual ducts alone, but also extends to a few cells at the anterior end of the glands. Because this domain of expression appears to overlap the region that will become the imaginal ring, we tested whether in *eyg*-mutant larvae the imaginal ring is affected. In *eyg*-mutant third instar larvae lacking individual ducts, no small, imaginal ring nuclei are present at the ends of glands, suggesting that *eyg* is required for imaginal ring development (Fig. 9B). This result also suggests that the imaginal ring is formed from the most anterior salivary gland cells, those that are adjacent to individual duct cells. These are the only cells that express both *eyg*, which is required for formation of the individual ducts, and *fkh* (Panzer et al., 1992), which is required for formation of the glands.

**Salivary glands and ducts are dispensable for larvae**

Although homozygous *eyg* animals die as embryos, *eyg*/*eyg* animals hatch and pupariate and some of them even survive to adulthood. We took advantage of the fact that these animals are viable as larvae to determine whether or not salivary glands and ducts have any functional role in larval growth. Even though 70% of *eyg*/*eyg* larvae have no individual ducts and the salivary glands are closed sacs, almost all of them survive until pupae. This result suggests that larvae do not need salivary ducts and glands in order to grow and survive. An alternate possibility is that wild-type siblings of these ductless larvae might expectorate digestive enzymes from their salivary glands into the food and digest the food outside of the animal. Gregg et al. (1990) termed this phenomenon social digestion since several larvae typically cluster around a pit in the food that is being consumed. This external digestion might explain why *eyg*/*eyg* larvae survive. Mutant larvae without ducts might be able to consume predigested food courtesy of their siblings. If so, we might not expect individually raised ductless larvae to survive. To determine whether wild-type siblings enabled ductless larvae to survive, we grew larvae in isolation, one per vial, and determined the genotypes of recovered pupae (Table 2). Surprisingly, ductless larvae survive just as well by themselves as they do when they are with their siblings. Thus, under these culture conditions, salivary glands and ducts are dispensable.

<table>
<thead>
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<th>Genotype</th>
<th>If <em>eyg</em>/<em>eyg</em> is embryonic or larval lethal, expect</th>
<th>Larvae reared many per vial</th>
<th>Larvae reared one per vial</th>
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<td>100</td>
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**DISCUSSION**

**eye gone functions in salivary development**

In this study, the tissue that we have examined in the most detail is the salivary duct. The ducts form as a result of two successive convergence and extension events and have proven useful as a model system for studying joining morphogenesis. By examining *Ser* expression in presumptive duct cells of *eyg*-mutant embryos, we have discovered that the duct primordia fail to converge and extend across the ventral midline, resulting in the absence of individual ducts. The invaginating glands (which in wild-type embryos are in continuous contact with the individual ducts) maintain contact with the duct primordia only until germ band retraction is complete and then break apart from the duct primordia and finish their invagination. These deviations from wild type result in embryos that lack individual ducts and whose glands are not connected to the oral cavity.

*eyg* is necessary but not sufficient to distinguish individual from common duct

If presumptive individual duct cells do not form individual ducts in *eyg*-mutant embryos, what happens to these cells? Three observations suggest that, in these mutants, many of the individual duct cells become associated with the common duct. First, in *eyg*-mutant embryos lacking any individual duct but containing an intact common duct, the common duct often appears much larger in diameter than normal. Second, using the *dri* enhancer trap, we counted duct cells in wild-type and *eyg*-mutant embryos. In wild-type embryos there are about 29 cells in the common duct and 27 in the individual ducts. In *eyg* mutants the expanded common duct has about 42 cells, suggesting that about half the individual duct cells have become associated with the common duct. Third, none of the common duct cells in *eyg* mutants express the individual duct marker *btl*, suggesting that the excess cells that have joined the common duct have lost their individual duct identity. We conclude that the *eyg*-mutant individual duct cells are likely to be transformed into common duct cells, since they can incorporate into the common duct epithelium and no longer express *btl*.

*eyg* may be required for imaginal ring development

The absence of small, diploid nuclei from the anterior end of *eyg*-mutant, third instar salivary glands suggests that *eyg* is required for the formation of the salivary imaginal rings. Another alternative is that the imaginal ring is specified in *eyg*-mutant larvae but that, when the glands pull away from the ducts, the imaginal ring cells remain with the common duct. We have not yet been able to test this directly by recovering the common duct from third instar *eyg*-mutant larvae and looking for diploid cells. However, we think this is an unlikely alternative because when we used the *dri* enhancer trap to label the duct cells but not the gland cells of *eyg*-mutant embryos, we did not see unlabeled cells associated with the enlarged common duct.

The expression of *eyg* in the anterior cells of the invaginated gland/duct junction of wild-type and *eyg*/*eyg* third instar larvae by DAPI staining of dissected salivary glands. (A) In a preparation from a wild-type mid-third instar larva, the small nuclei of the imaginal ring (ir) are seen at the gland/duct junction. (B) Salivary glands of *eyg*/*eyg* larvae lack small, imaginal ring nuclei.

**Fig. 9.** *eyg* is necessary for imaginal ring development.
glands suggests that it is these cells that will become the imaginal ring. The pattern of DNA synthesis in these cells is consistent with this interpretation. In the larval cells of the gland, the first round of endoreplication begins at around 8.5 hours (Smith and Orr-Weaver, 1991), but the imaginal cells remain diploid and do not divide until after hatching. By following BrdU incorporation, one can see, in whole-mount preparations, that most of the cells of the invaginated gland have incorporated the label, but the anterior boundary is sharp and does not include the rounded anterior end of the gland (Smith and Orr-Weaver, 1991; Sigrist and Lehner, 1997; Y. M. K., unpublished observation). Thus it appears that the boundary of eyg expression is closely aligned with the boundary between quiescent and endoreplicating cells.

Model for salivary determination

Salivary cells make at least three fate decisions

The salivary primordium is initially established by the positive action of Scr and the negative action of Dpp (Panzer et al., 1992; Andrew et al., 1994). The first subdivision of the primordium occurs when salivary cells interpret the output of the EGFR signaling pathway and decide to either express fkh and become gland or express trh and become duct (Kuo et al., 1996; Isaac et al., 1996).

eyg can now be added to our model of salivary tissue determination (Fig. 10). eyg is required for the second decision, the distinction between individual and common ducts. It is expressed specifically in individual duct cells and is critical for development of these cells as individual rather than common duct. In eyg-mutant embryos, presumptive individual duct cells are converted to common duct. We therefore conclude that trh-expressing salivary cells express eyg and become individual duct or do not express eyg and become common duct.

eyg also appears to be involved in the third cell fate decision, the choice between gland and imaginal ring. As discussed above, imaginal ring cells appear to arise at the juxtaposition of individual duct and gland and to require eyg for their development.

Duct gene regulation

\(\text{btl}\) is expressed throughout duct development, strongly in individual duct cells but weakly in the cells of the common duct (Kuo et al., 1996). \(\text{Ser}\) in contrast, is expressed in both individual and common duct at a high level throughout duct development (Kuo et al., 1996). In \(\text{trh}\)-mutant embryos, expression of \(\text{btl}\) and \(\text{Ser}\) is never seen in duct cells, implying that \(\text{trh}\) functions to activate both of these and probably other duct genes. In this study we have shown that \(\text{trh}\) is also responsible for the maintenance of eyg expression in the individual duct cells. By examining expression of these duct markers in eyg-mutant embryos, we tested whether \(\text{trh}\) acts via eyg in the activation of \(\text{Ser}\) and \(\text{btl}\). While the presumptive individual duct cells continue to express \(\text{Ser}\) in the absence of eyg, \(\text{btl}\) expression is strongly reduced, suggesting that \(\text{trh}\) acts via eyg to activate \(\text{btl}\) but not \(\text{Ser}\). While \(\text{trh}\) is necessary for expression of all the individual and common duct genes that we have tested, while eyg is necessary for activation of individual duct-specific genes alone. Figure 11 summarizes the genetic interactions known to regulate duct determination.

eye gone and sensory organ development

In both vertebrates and invertebrates, Pax genes have been shown to be critical for the development of many sensory tissues. Here we have shown that the newly described \textit{Drosophila} Pax gene eyg has similar functions. Although eyg encodes a Pax family protein most closely related to Pax-6 genes (C. Jang, N. A. J. Chao, D. Bessarab, Y. M. K., S. Jun, C. Desplan, S. K. B. and Y. H. S., unpublished data; Jun and Desplan, 1996), it lacks landmarks common to Pax-6 proteins. Eyg, for example, contains only the ‘RED’ (C-terminal) half of the characteristic PAIRED DNA-binding domain (Jun and Desplan, 1996). \(\text{Pax-6}\) homologues in \textit{Drosophila}, mouse and humans have all been shown to be critical for eye development (Quiring et al., 1994; Glaeser et al., 1992; Hill et al., 1991). Similarly, adult flies lacking eyg have eyes that are either reduced in size or completely absent (C. Jang, N. A. J. Chao, D. Bessarab, Y. M. K., S. Jun, C. Desplan, S. K. B. and Y. H. S., unpublished data). In addition, ectopic expression of eyg results in ectopic eyes (C. Jang, N. A. J. Chao, D. Bessarab, Y. M. K., S. Jun, C. Desplan, S. K. B. and Y. H. S., unpublished data).

\(\text{Pax}\) genes have also been shown to be critical to the development of non-eye sensory structures. Missense mutations in human PAX3 cause profound sensorineural deafness (Asher et al., 1996). In mouse, \(\text{Pax-6}\) is expressed in the olfactory bulb and some \textit{Seay} alleles display nasal defects (Stoykova and Gruss, 1994; Walther and Gruss, 1991). \(\text{Pax-6}\) is also expressed in the chemosensory organs of the ribbonworm \textit{Lineus sanguineus} (Loosi et al., 1996) and the squid olfactory organ (Callaerts et al., 1997). Vanario-Alonso and coworkers (1995) have demonstrated a role for \textit{Drosophila paired} in ventral organ (VO) determination. The VO is thought to function in chemosensation and is also in close physical proximity to another chemosensory organ: the antennal organ (AO). eyg is strongly expressed in the AO primordium and its development is affected in eyg-mutant embryos. Most of the AO cells are still present in these mutants, but many of the sensillae fail to differentiate properly. In this report, we have described eyg expression in two sensory organ primordia: that of the eye and that of the AO. It is clear that eyg has functions...
in sensory organ development that are similar to those of its Pax gene family relatives.

**Functional roles of salivary glands and ducts**

In a final set of experiments, we tested whether salivary glands are important for growth and survival of Drosophila larvae. We know that in mammals salivary glands provide several important functions. They provide lubrication for mastication and secrete amylase to aid in the digestion of complex carbohydrates. In addition, mammalian salivary glands produce antibacterial peptides and recent studies have shown that impaired salivary gland function can increase the prevalence of dental caries and cause overgrowth of several pathogenic microorganisms (Enwonwu et al., 1994; Papas et al., 1993). It is unclear whether Drosophila salivary glands have a similar antibacterial function although some molecular components of the Drosophila immune response such as Toll and 18-wheeler are expressed in salivary glands (Gerttula et al., 1988; Eldon et al., 1994). Mammalian salivary glands also produce buffering action and salivary mucins provide a protective coating on oral tissues (Papas et al., 1993; Slomiany et al., 1996).

Although Drosophila salivary glands actively secrete numerous proteins during larval growth and it has been proposed that some of these are digestive enzymes, the only functionally identified products of the salivary glands are the glue proteins, which aid larvae in adhering to substrata for pupariation (Korge, 1975; Beckendorf and Kafatos, 1976; Fraenkel and Brookes, 1953). In this study, we took advantage of the salivary ductless phenotype present in eyg-mutant larvae to test whether glands and ducts are actually required for growth and viability. Ductless larvae can grow and pupariate, even in the absence of their wild-type siblings. Although this result rules out an obligate requirement for salivary glands and ducts, we do not believe this proves that they are completely dispensable. In this experiment, to each single wild-type or mutant larva in each vial, we added a drop of liquid yeast each day to prevent dehydration of the medium. These pampered larvae survived regardless of their duct phenotype. It may be true that, in the wild, this disadvantage would be selected against and these larvae would not fare as well as they do in the shelter of laboratory conditions.

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