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Identification of Genes Controlling Malpighian Tubule and Other Epithelial Morphogenesis in *Drosophila melanogaster*

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ABSTRACT

The Drosophila Malpighian tubule is a model system for studying genetic mechanisms that control epithelial morphogenesis. From a screen of 1800 second chromosome lethal lines, by observing uric acid deposits in unfixed inviable embryos, we identified five previously described genes (*barr*, *fas*, *flb*, *raw*, and *thr*) and one novel gene, *walrus* (*wal*), that affect Malpighian tubule morphogenesis. Phenotypic analysis of these mutant embryos allows us to place these genes, along with other previously described genes, into a genetic pathway that controls Malpighian tubule development. Specifically, *wal* affects evagination of the Malpighian tubule buds, *fas* and *thr* affect bud extension, and *barr*, *flb*, *raw*, and *thr* affect tubule elongation. In addition, these genes were found to have different effects on development of other epithelial structures, such as foregut and hindgut morphogenesis. Finally, from the same screen, we identified a second novel gene, *drumstick*, that affects only foregut and hindgut morphogenesis.

WHILE the importance of epithelial morphogenesis in development is widely recognized, relatively little is known about its molecular basis. A few molecules, for example, members of the Rho GTPase family, have been identified that modulate the cytoskeleton and control cell shape change in culture (reviewed by Hall 1998). Function of these molecules *in vivo* has been demonstrated in Drosophila embryogenesis: inactivation of Rho disrupts gastrulation (Häcker and Perrimon 1998), while inactivation of Rac disrupts dorsal closure (reviewed by Noselli 1998). In addition to these molecules, additional components essential for the epithelial morphogenetic processes of gastrulation, dorsal closure, and tracheal tube fusion and branching have been identified in genetic screens and characterized molecularly in Drosophila (reviewed by Leptin 1995; Skaer 1997; Hacohen *et al.* 1998; Noselli 1998). It seems likely, however, that more molecules required to establish epithelia and to control the proliferation, shape, and rearrangement of the cells within epithelia remain to be identified.

The Malpighian tubules of the Drosophila embryo provide a useful system for investigating the genetics of epithelial morphogenesis. First, the genetic hierarchy required to establish the tubule primordia and to control their early morphogenesis has been partially elucidated. Second, the tubules constitute one of the simplest possible epithelial organs. As reviewed by Skaer (1993), the tubules develop as outbuddings from a single layered epithelium (the hindgut primordium, or proctodeum), do not become invested with a mesenchyme, and cease cell division relatively early in their development. The fully developed organ consists of only two morphologically distinct cell types and comprises a single layered epithelial tube with proximal and distal portions.

As outlined in Figure 5, the Malpighian tubules arise from the proctodeum. Overlapping expression of the *tailless* (*tll*), *huckebein* (*hkb*), *fork head* (*fkh*), and *wingless* (*wg*) genes at the posterior of the embryo is required to establish and maintain the portion of the proctodeum from which the tubules arise (Weigel *et al.* 1989; Gaul and Weigel 1990; Skaer 1993; Harbecke and Lengyel 1995; Wu and Lengyel 1998). Expression of *Krüppel* (*Kr*) in the tubule anlage is required for the tubules to bud out from the proctodeum (Harbecke and Janning 1989; Skaer 1993). Expression of *cut* in a ring of cells at the future anteriormost region of the proctodeum is required for extension of the buds. All of the aforementioned genes required for early development of the tubules, with the exception of *wg*, encode transcription factors.

In addition to the transcriptional regulation that defines the tubule primordia and controls early events of tubule development, cell signaling plays an important role in later steps of tubule development. Hedgehog is required for the completion of bud evagination (Hoch and Pankratz 1996), while Wingless is required for cell division and morphogenesis in the tubules (Skaer and Martinez Arias 1992; Harbecke and Lengyel 1995). Signaling via the Notch receptor is required to define the single tip cell at the end of each tubule (Hoch *et*

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al. 1994), which leads out the elongation of the tubule (Skaer 1989). Finally, signaling from the tip cell via an EGF-like ligand is required for the proliferation of the distal cells of the tubule (Baumann and Skaer 1993; Kerber *et al.* 1998).

To identify additional genes involved in the formation and/or morphogenesis of the Malpighian tubules, particularly genes that might regulate the cellular basis of morphogenesis (*i.e.*, cell communication, cytoskeleton, cell junctions, and polarity), we screened a collection of lethal lines carrying *P*-element insertion(s) on the second chromosome (Török *et al.* 1993). We identified and mapped seven embryonic lethal alleles, falling into six complementation groups (loci), that cause Malpighian tubule defects. Five of these loci correspond to the previously described genes *barren* (*barr*), *faint sausage* (*fas*), *faint little ball* (*flb*), *raw*, and *three rows* (*thr*). One locus corresponds to a newly identified gene, *walrus* (*wal*). Phenotypic analysis of these mutants revealed a number of genetically distinguishable morphogenetic events required for Malpighian tubule development. In addition, these genes were found to be involved in other gut epithelial morphogenesis (*i.e.*, proventriculus formation, hindgut elongation, and midgut constriction), as well as other morphogenetic processes involving epithelia, such as germ band retraction, dorsal closure, head involution, and epidermal differentiation. Finally, we have also identified another novel gene, *drumstick* (*drm*), which, in contrast to these genes with global effects, specifically affects morphogenesis of hindgut and foregut.

MATERIALS AND METHODS

Fly stocks: We screened the collection of lethal lines with *P*-element insertion(s) on the second chromosome described by Török *et al.* (1993). Alleles of flb^{IK35}, raw^{1}, thr^{1}, *l(2)02516*, and the deficiency "kit" for the second chromosome were obtained from the Bloomington Drosophila Stock Center. $Tp(2;3)odd^{5.1}$ was obtained from the Tübingen Drosophila Stock Center, and the fas^{IIA} allele was obtained from Volker Hartenstein.

Screen for mutants affecting Malpighian tubule and midgut morphogenesis: We screened a total of 1819 lethal lines for defects in morphology of the mature Malpighian tubule by looking for abnormalities in formation of the opaque uric acid deposits that are observable in mature tubules (Skaer 1993). We were also able to identify mutants with defects in midgut morphogenesis; these have a roughly spherical mass of undigested yolk (the "yolk plug") in the center of the mature embryo (Eberl and Hilliker 1988). A set of seven lethal lines was placed in an apparatus of tubes similar to that described by Nüsslein-Volhard (1977), and allowed to deposit embryos for 24 hr at room temperature on an apple juice agar plate on which a drop of yeast had been placed to correspond to the center of each tube. Adults were then removed, a ring of yeast was spread around the edge of each plate (to attract hatched larvae), and the egg collection plates were incubated for an additional 48 hr at room temperature. Unhatched (lethal) embryos left in each tube imprint were then covered with halocarbon oil (series 27, Halocarbon Products Corporation) to render the chorion transparent and examined at 80× by transmitted light in a dissecting microscope. Consistent with previous analyses (Nüsslein-Volhard *et al.* 1984), we found that ~30% of the lethal lines were embryonic lethal. Forty-three embryonic lethal lines were identified from this screen with apparent defective Malpighian tubules and/or midgut and examined in more detail by antibody staining (see below).

Antibody staining and phenotypic analysis: Antibody staining was performed using standard protocols (Ashburner 1989). The monoclonal anti-Crb antibody, a generous gift of Elisabeth Knust (Tepaß *et al.* 1990), was used at a dilution of 1:100 to label the apical surface of ectodermally derived epithelia. The monoclonal anti-Cut antibody, kindly provided by Karen Blochlinger (Blochlinger *et al.* 1990), was used at a dilution of 1:20 to label the nuclei of Malpighian tubule cells. The monoclonal anti-Connectin antibody, kindly provided by Robert White (Meadows *et al.* 1994), was used at a dilution of 1:30 to label the visceral mesoderm that ensheathes the hindgut and foregut. Whole-mount embryos after antibody staining were analyzed with a Zeiss (Thornwood, NY) Axiophot microscope equipped with differential interference contrast optics. Of the 43 putative gut defect lines obtained in the screen, 8 were found by staining with anti-Crb and anti-Cut to have defective Malpighian tubules or other gut defects. For mutant lines found by complementation to be alleles of known genes (see below), transheterozygous embryos were made by crossing these lines with balanced lethal lines carrying a null (or the strongest available) allele of the corresponding genes; embryos were then stained with various antibodies for detailed phenotypic analyses. Embryos were staged according to Campos-Ortega and Hartenstein (1997). Images were acquired by digital camera (Sony, Parkridge, NJ; DKC-5000) using Adobe PhotoShop software. For each figure, embryos or portions of embryos are shown at the same magnification.

Genetic analysis: Information on the localization of the *P* element(s) in the identified lines was obtained from the Berkeley Drosophila Genome Center. The deficiency "kit" for the second chromosome from the Bloomington Drosophila Stock Center was used to localize a number of the lethal mutations from the screen. In addition, two multiply marked second chromosomes, *S Sp Tft N-2G Pu* and *al dp b pr c px sp*, were used for meiotic mapping of alleles of *wal* and *drm*, respectively. Revertant chromosomes were generated from line *k14036* (*wal*) by *P*-element excision as described by Török *et al.* (1993).

RESULTS

Identification of seven loci affecting epithelial morphogenesis: From a screen of over 1800 lethal lines (see materials and methods), 8 lines were identified that produced lethal embryos with abnormal Malpighian tubules and various additional defects in epithelial maintenance and morphogenesis. On the basis of complementation tests among these lines and with other previously known genes and deficiencies, as well as *P*-element mapping and reversion studies, these 8 lines are concluded to correspond to seven loci, as described below.

Although each line contains one or two *P*-element insertion(s) on the second chromosome, in only three out of eight lines did the *P*-element map position correspond to the map position of the mutant locus giving the phenotype (see materials and methods; Table 1).

TABLE 1

Second chromosome lethal mutations involved in epithelial morphogenesis and maintenance

Gene	Line	Cytology/genetic location	*P*-element insertion(s)[a]	Fails to complement	Complements	Malpighian tubule phenotype	Hindgut/foregut phenotype	Other epithelial phenotypes
drumstick (drm)	*k11011*	23E-24A	43E4-5; 58C1-2	*Tp(2;3)odd*$^{5.1}$	*Df(2R)pk78s* *Df(2R)X58-8*		Failure in HG elongation and PV formation	
raw	*k0373*	2-19	46F1-2	*raw*^{1}	*Df(2R)X1*	Not fully elongated tubules	LI collapsed	Incomplete GMR and DC
barren (barr)	*k14014*	38B2	38B1-2	*Df(2L)TW84*		Not fully elongated tubules	Incomplete PV morphogenesis and PG invagination	No MG constructions Incomplete HI Abnormal tracheae Holes in epidermis
walrus (wal)	*k14036*	48B6-7[b]	48B6-7	*l(2)02516*	*Df(2R)en30*	Early defects in bud evagination Later convoluted tubules	Incomplete PV morphogenesis and PH invagination Wide, corrugated ES	No MG constrictions Incomplete HI Abnormal tracheae
faint sausage (fas)	*k04221*	50B7-9	57E6-7; 91F10-11	*fas*^{IIA}	*Df(2R)PuD17*	Globular buds Defective bud extension	Small, misshapen SI LI cells lack polarity Short FG without PV	No MG constrictions Defective DC and HI Holes in epidermis No SG or PS invagination
three rows (thr)	*k01302* *k07336*	55A1	25D1-2 46E1-2	*thr*^{1}	*Df(2L)cl-h3* *Df(2L)tkv*^{2} *Df(2L)sc19-8* *Df(2R)X1*	Short tubules with fewer but larger cells	Short HG Short FG	No MG constrictions Incomplete HI Abnormal tracheae Holes in epidermis
faint little ball (flb)	*k05115*	57F1-2	57F1-2	*Df(2R)Pu-D17 flb*^{IK35}		Very reduced tubules		No MG constrictions Failure in GMR and DC Short SG No head epithelium

DC, dorsal closure; ES, esophagus; FG, foregut; GMR, germ band retraction; HI, head involution; HG, hindgut; LI, large intestine; MG, midgut; PH, pharynx; PS, posterior spiracle; PV, proventriculus; SG, salivary gland; SI, small intestine.

[a] From Berkeley Drosophila Genome Project.

[b] Revertants were obtained after *P*-element excision.

For lines *k05115* and *k14014*, construction of transheterozygotes with deficiencies uncovering the site of the *P* insertion gave embryos with the same phenotype as homozygotes. Complementation analysis revealed that *k05115* is a *P*-element insertion in *faint little ball* (*flb*; Nüsslein-Volhard *et al.* 1984), which encodes the Drosophila epidermal growth factor (EGF) receptor (Schejter and Shilo 1989), and *k14014* is an insertion in *barren* (*barr*), which encodes a protein required for sister-chromatid separation (Bhat *et al.* 1996). Finally, in line *k14036*, the position of the *P*-element insertion defines a newly described locus, *walrus* (see below).

For the remaining lines, deficiencies uncovering the *P* insertion(s) complemented the *P* insert chromosomes in lethality and failed to yield embryos with defects characteristic of the homozygous line (Table 1). In a number of cases, additional complementation tests between these lines and the deficiency "kit" for the second chromosome, as well as characterization of specific defects in embryogenesis, suggested candidate genes mapping to sites different from that of the *P*-element insertion. In this way, four lines, *k04221*, *k01302*/*k07336*, and *k03703*, were shown to carry alleles of three previously identified genes (Table 1). *k04221* is an allele of *faint sausage* (*fas*; Nüsslein-Volhard *et al.* 1984), which encodes an extracellular protein involved in neuron delamination (Lekven *et al.* 1998); *k01302* and *k07336* are strong and weak alleles, respectively, of *three rows* (*thr*; Nüsslein-Volhard *et al.* 1984), which, like *barr*, encodes a protein required for sister-chromatid separation (D'Andrea *et al.* 1993; Philp *et al.* 1993); and *k03703* is an allele of *raw* (Nüsslein-Volhard *et al.* 1984), which has recently been cloned and encodes a novel protein involved in dorsal closure (A. Letsou, personal communication).

Two previously undescribed loci were identified. One of these has been named *walrus* (*wal*), for the head defects seen in cuticle preparations of the mutant larvae. We conclude that *wal* maps at the site of the *P*-element insertion in line *k14036* on the basis of the following. First, the *wal* phenotype is reverted by *P*-element excision. Second, *k14036* does not complement another lethal line, *l(2)02516*, which contains a *P* element inserted at the same cytological position (48B6-7, Table 1). Third, meiotic recombination (see materials and methods) maps *wal* to 2-59, which corresponds roughly to the cytological position of the *P* element in line *k14036*. The fact that this line complements the only known deficiency, *Df(2R)en30*, reported to uncover the *P*-insertion site is probably due to an error in mapping of the deficiency, since *l(2)02516* also complements this deficiency.

The novel locus *drumstick* (*drm*) was identified on the basis of the fact that the Malpighian tubules, though normal in morphology, are located more posteriorly in mutant than in wild-type embryos. Unlike the other mutants described here, which all affect the morphogenesis of multiple epithelial structures, the *drm* mutation affects only hindgut elongation and folding of the proventriculus. Although the *drm* mutation is lethal, it is not embryonic lethal; recombination was used to separate the *drm* mutation in the *k11011* line from an embryonic lethal locus. Meiotic mapping placed *drm* between *al* and *dp*, while complementation tests with different deficiencies and translocations in this region further refined the location of *drm* to cytological region 23E-24A. However, as there is no available deficiency uncovering this region, we cannot determine whether the *drm* mutation in *k11011* is a null allele. Below we describe the Malpighian tubule phenotype of the mutants identified in the screen. In addition, many of the mutants also affect development of other epithelial structures (hindgut, foregut, midgut, tracheae, etc.). These phenotypes are described in subsequent sections.

Effects on steps of Malpighian tubule morphogenesis: Normal development of the tubules has been reviewed by Skaer (1993). Below we discuss the sequential steps in tubule development that are affected in six of the mutants identified in our screen (we do not discuss *drm* because it affects only hindgut elongation and proventriculus folding).

Bud evagination (and recruitment): In the early gastrula, the Malpighian tubules share the same anlage with the hindgut. At the end of germ band extension (stage 10), two pairs of buds evaginate from the proctodeum, at the junction of the hindgut and posterior midgut primordia. As shown in Figure 1A, cells continue to be recruited from the proctodeum into the Malpighian tubule primordia during germ band shortening (stage 12). This cell recruitment, which we define as part of bud evagination, is defective in *wal* mutant embryos: instead of being incorporated into the everting buds, many cells of the primordia remain in the proctodeum as a ring (Figure 1E). Even at later stages (14 and 15), a few Cut staining cells are observable in the ring (not shown); the cells that have been recruited into the buds, however, manage to undergo subsequent cell rearrangements. The resultant *wal* tubules are fairly elongated but convoluted (not shown).

Bud extension: As the tubule cells continue to be recruited into the buds during stage 12, cells already in the buds participate in another morphogenetic process, bud extension, which is completed by the end of stage 13. In this process, the cylindrical buds extend and become narrower both proximally and distally, resulting in a crescent-shaped morphology (Figure 1B). In *fas* mutant embryos, the tubule primordia cells appear to evert into buds, proliferate, and complete recruitment successfully; however, by the end of stage 13, the tubules have not extended, but rather remain arrested as four globules of densely packed, Cut-stained cells (Figure 1F). Thus, *fas* is required for the cylinder-to-crescent transition, presumably for the movement of cells toward the tubule tips, and/or for the rearrangement of cells leading to reduction in tubule diameter near the tips.

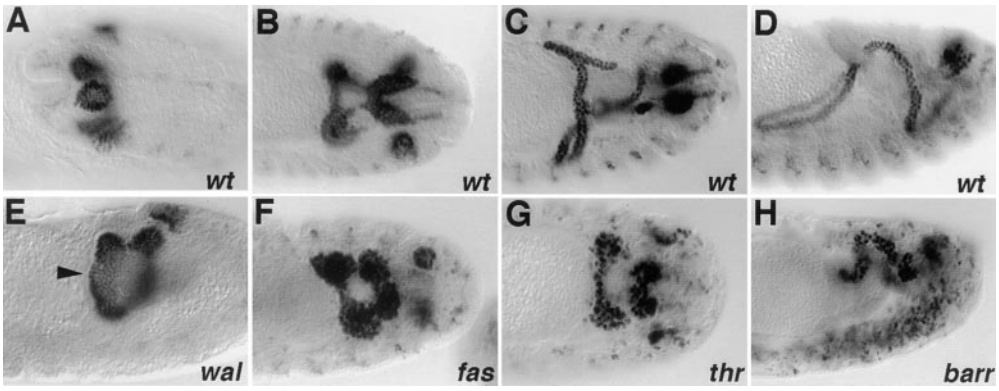

Figure 1.—Mutations affecting Malpighian tubule development. Embryos of wild-type (A–D) and *wal* (E), *fas* (F), *thr* (G), and *barr* (H) mutants stained with anti-Cut. In wild type, the Malpighian tubule precursor cells, as detected by Cut staining, are initially a ring of cells located at the junction between midgut and hindgut at stage 10. These cells then evert into four small buds that are still connected by the ring (A). During stage 12, these precursor cells continue to divide, and cells still in the ring migrate out into the buds (recruitment, a continuation of bud evagination); cells already in the buds rearrange (bud extension). As a result, by early stage 13, most Malpighian tubule cells are now in four extended, crescent-shaped tubules (B). The Malpighian tubule cells complete their final postblastoderm mitosis by the end of stage 13 (Skaer 1989); the circumference of the four primitive tubules at this stage is about eight cells (Janning *et al.* 1986; Skaer and Martinez Arias 1992). The primitive tubules continue to elongate during stages 14 (C) and 15 (D) by a process of convergent extension (intercalation of cells). By the end of stage 16, when this cell rearrangement process (tubule elongation) is completed, the circumference of the fully developed tubules has narrowed to two cells. In *wal*, bud evagination appears defective during stage 12, as indicated by a larger ring of anti-Cut labeled cells (arrowhead in E) than that of wild-type. In contrast, in *fas*, bud evagination appears complete, while bud extension is inhibited, revealed by the presence of four globular-shaped buds instead of wild-type crescent-shaped buds (F). Bud extension is also defective in *thr* (G); note that there are fewer cells with larger nuclei in the partially extended buds due to the failure of postblastoderm mitosis (Philp *et al.* 1993). Tubule elongation is defective in *barr*, revealed by shorter and wider tubules with more cells in their circumference (H) than seen in wild type (D).

A phenotype similar to that of *fas* embryos is seen in *thr* embryos, namely, that extension of the tubular buds is inhibited (Figure 1G). A difference is that while tubule extension is blocked in *fas* embryos, it is only retarded in *thr* embryos, so that the tubules in the latter embryos continue development but become arrested during elongation (described below).

Tubule elongation: After completion of recruitment and bud extension, there is no further cell division. At this point (end of stage 13), the circumference of each of the four primitive tubules comprises about eight cells (Janning *et al.* 1986; Skaer and Martinez Arias 1992). By a process of cell rearrangement (convergent extension) during stages 14 and 15, the tubular circumference is reduced to only two cells, and the tubules are concomitantly extended further (Skaer 1993; Figure 1, C and D). Four loci identified in the screen, *barr*, *flb*, *raw*, and *thr*, affect this process; the tubule phenotype of *flb* and *raw* has been described (Baumann and Skaer 1993; Jack and Myette 1997) and is shown for *barr* in Figure 1H. For embryos mutant for all of these loci, Malpighian tubule development does not proceed beyond the stage where tubules are about four to six cells in circumference (Figure 1H and data not shown). We note that three of these four loci affecting tubule elongation are required for cell division: *flb* is required for reception of a signal from the tip cell that promotes cell proliferation in the tubule (Skaer 1989; Baumann and Skaer 1993; Kerber *et al.* 1998), while *barr* and *thr* both encode proteins required for segregation of sister chromatids throughout the embryo (D'Andrea *et al.* 1993; Bhat *et al.* 1996).

Hindgut morphogenesis: In the blastoderm stage embryo, the hindgut primordium is established as a ring of cells bordering the posterior midgut primordium (Technau and Campos-Ortega 1985; Harbecke and Janning 1989; Diaz *et al.* 1996; Wu and Lengyel 1998). Invagination of the hindgut anlage follows that of the posterior midgut primordium. During germ band extension, the hindgut primordium (proctodeum) extends along the anterior-posterior axis by both cell division (which ceases during stage 11) and cell rearrangement. As the germ band shortens, the hindgut bends back toward the posterior pole and reverses its orientation; it continues lengthening by cell rearrangement until stage 14. The fully developed hindgut, as observed by anti-Crb staining of the stage 16 embryo, can be divided into three subregions: the small intestine, which connects to the posterior midgut; the large intestine; and the rectum, which terminates in the anus (Hoch and Pankratz 1996; Figure 2A). The mature hindgut is surrounded by visceral mesoderm (Figure 2B), which derives from a mass of mesodermal cells on the future ventral side of the hindgut that can be identified at stage 11 and spreads to surround the hindgut during stage 12 (not shown).

Four mutants identified in the screen, in addition to displaying defects in Malpighian tubule development and in other aspects of epithelial morphogenesis (see below), also show hindgut defects. In *raw* embryos, there is an extremely narrow tube, or thread, of Crb staining connecting the small intestine and rectum (Figure 2C). That this constitutes a connection (a very reduced or collapsed large intestine) between small intestine and

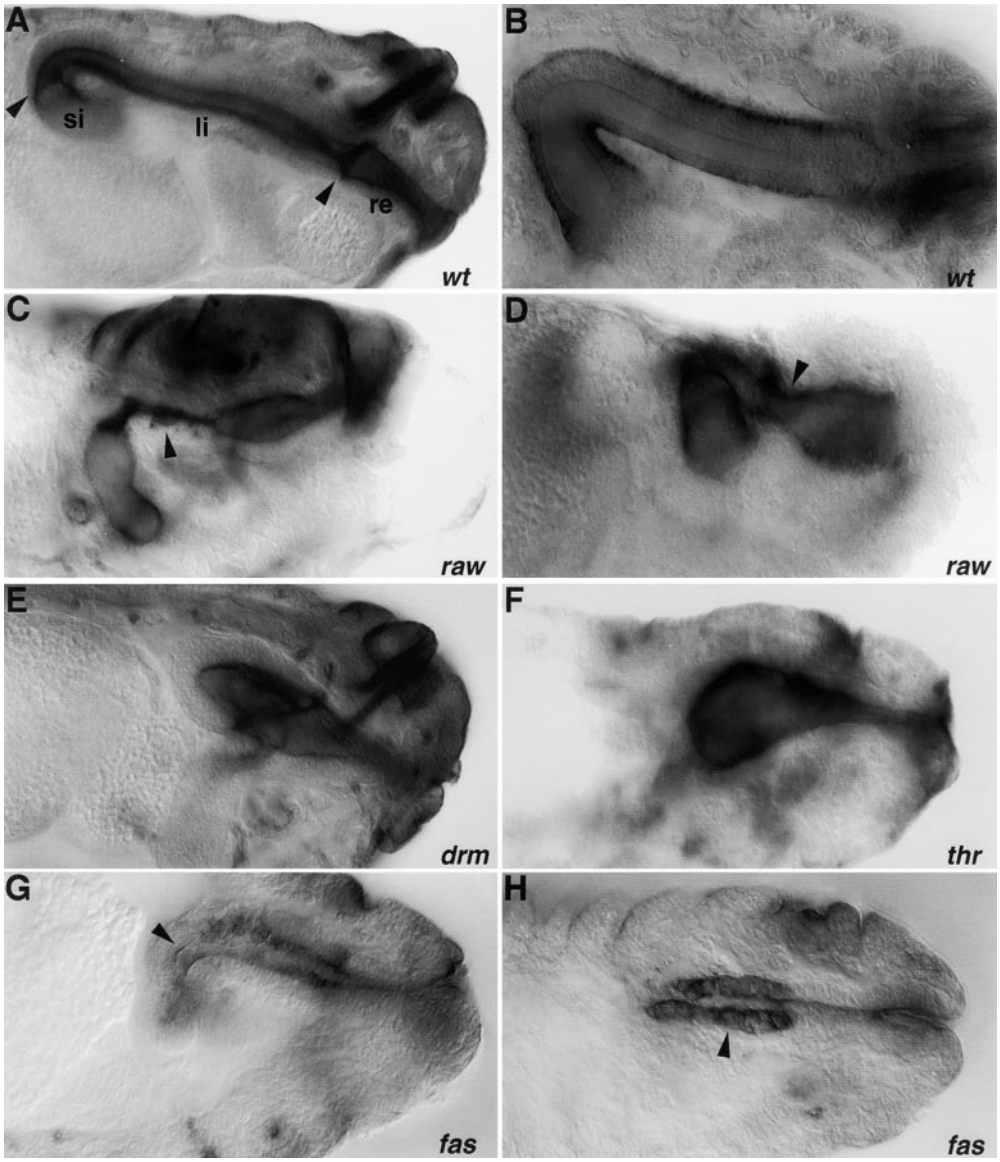

Figure 2.—Mutations affecting hindgut morphogenesis. Embryos of stage 16 or older stained with anti-Crb (A, C, and E–H), or anti-Connectin (B and D). (A) The wild-type hindgut can be divided, from anterior to posterior, into three parts—small intestine (si), large intestine (li), and rectum (re)—that are delineated by rings of Crb staining between si and li, and between li and re (arrowheads; Hoch and Pankratz 1996). (B) The hindgut epithelium is surrounded by visceral mesodermal cells (labeled with anti-Connectin). In *raw*, the large intestine appears to be collapsed, indicated by the extremely narrow lumen of the large intestine (arrowhead in C). However, the visceral mesoderm surrounding the entire hindgut epithelium, including the collapsed large intestine (arrowhead in D) remains intact. The hindgut is short and wide in *drm* (E) and *thr* (F). In *fas*, the small intestine appears reduced in size and misshapen (arrowhead in G); in addition, the cells of large intestine epithelium lack polarity, indicated by their circumferential staining by anti-Crb (arrowhead in H).

rectum is demonstrated by the presence of a tube of visceral mesodermal cells surrounding this region, as indicated by staining with anti-Connectin (Figure 2D). In *thr* embryos, the hindgut is shorter and broader than normal (Figure 2F); this incomplete elongation appears similar, and may be related in cause, to the incomplete extension of Malpighian tubules seen in the same embryos.

Two defects are seen in the hindgut of *fas* embryos: (1) the small intestine is reduced and misshapen (Figure 2G), and (2) the cells of the large intestine are stained circumferentially with anti-Crb (Figure 2H). Since Crb is normally found only on the apical surface of epithelia (the lumen of the hindgut in this case), the uniform surface staining of cells in the *fas* large intestine indicates that these cells lack apical-basal polarity.

The *drumstick* mutation is unique among the loci identified in this screen, as *drm* mutant embryos show defects only in hindgut elongation (Figure 2E) and in proventriculus formation (Figure 3B, see next section). The dramatically shorter but wider hindgut in *drm* mutant embryos suggests a failure of convergent extension (cell rearrangement) in hindgut epithelium.

Foregut morphogenesis: Invagination of the foregut primordium (the stomodeum) starts at the beginning of stage 10 (reviewed by Skaer 1993). The earliest invaginated portion, which becomes the most posterior part of the esophagus, bulges out during stage 13 to form a bubble-shaped structure ("keyhole"; Pankratz and Hoch 1995); this region then undergoes folding morphogenesis during stages 14–16 to form the three-layered, heart-shaped proventriculus (Pankratz and Hoch 1995; Figure 3A). Anteriorly, this structure connects via the narrow tube of the esophagus to the pharynx.

Five of the mutants identified in the screen have altered foregut morphogenesis. In *barr* embryos, both formation of the pharynx and folding morphogenesis of the proventriculus are incomplete (Figure 3C). In *wal*

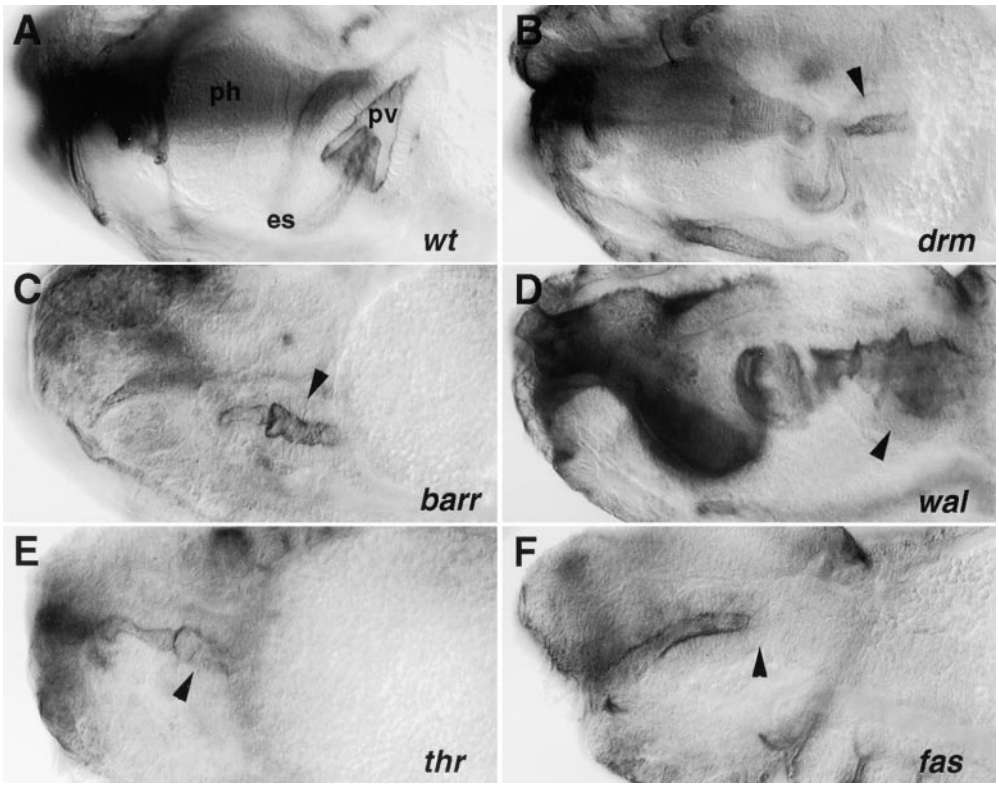

Figure 3.—Mutations affecting foregut morphogenesis. Stage 16 embryos stained with anti-Crb. (A) The wild-type foregut consists of three parts: pharynx (ph), esophagus (es), and proventriculus (pv). (B) In *drm*, proventriculus morphogenesis is defective, while the pharynx and esophagus appear to be normal. (C) In *barr*, proventriculus morphogenesis and pharynx formation are defective. (D) In *wal*, the esophagus and proventriculus appear hypertrophied and corrugated. (E and F) Foregut is short in *thr* and *fas*; pharynx, esophagus, and proventriculus fail to complete morphogenesis in *thr*, while the proventriculus is missing in *fas*. The arrowheads in B–F indicate the defective proventriculus structures.

embryos, the esophagus is wider than normal and its epithelium highly corrugated; no proventriculus is evident (Figure 3D). In *thr* and *fas* embryos, the esophagus is shorter and the proventriculus is defective (Figure 3, E and F); in particular, in *fas* embryos, the keyhole that constitutes the beginning of proventriculus formation does not form (Figure 3F).

The defect in proventriculus formation in *drm* embryos is quite different from that seen in the other mutants. First, it is not associated with defects in head involution or esophagus formation, both of which appear normal in *drm* embryos. Second, the region of the esophagus that would form the proventriculus becomes elongated, but does not initiate either keyhole formation or folding morphogenesis (Figure 3B).

Other epithelial morphogenesis: With the exception of the *drm* mutation, which has a very restricted phenotype, all of the mutants identified display defects in the development of multiple epithelial structures. The organs affected include the midgut, salivary glands, and tracheae, and the morphogenetic processes affected include germ band retraction, dorsal closure, and head involution. These defects are shown in Figure 4 and are summarized in Table 1.

DISCUSSION

By screening a collection of second chromosome lethals, we have identified six loci that affect Malpighian tubule development and one locus that affects hindgut development. Five of the loci identified were known previously, and two are novel. The previously known genes all affect multiple aspects of epithelial development. Of the genes in this group that have been molecularly characterized, all are expressed globally and encode proteins required in many cell types at many stages: the EGF receptor (*flb*), a cell adhesion molecule (*fas*), and proteins required for chromatid segregation (*barr*, *thr*; Schejter and Shilo 1989; D'Andrea *et al.* 1993; Philp *et al.* 1993; Bhat *et al.* 1996; Lekven *et al.* 1998). For these known genes, we provide here the first description of their effects on Malpighian tubule development (*barr*, *fas*, and *thr*), as well as their effects on hindgut (*fas*, *raw*, and *thr*) and foregut (*barr*, *fas*, and *thr*) development. Of the two novel genes identified, *wal* has global defects in addition to affecting Malpighian tubule, hindgut, and foregut development, while *drm* is unusual in that it is required only for morphogenesis of the hindgut and foregut, and does not appear to affect other aspects of morphogenesis.

Efficacy of screen: Although we screened specifically for Malpighian tubule defects, all of the mutants that we identified (with the exception of *drm*) display defects in multiple epithelial structures (*e.g.*, tracheae, salivary glands), and/or in processes involving epithelia, such as midgut constriction, germ band retraction, dorsal closure, and head involution. Four of these six mutants with multiple epithelial defects were previously identified in a screen for embryonic lethals affecting cuticular differentiation (*fas*, *flb*, *raw*, and *thr*; Nüsslein-Volhard

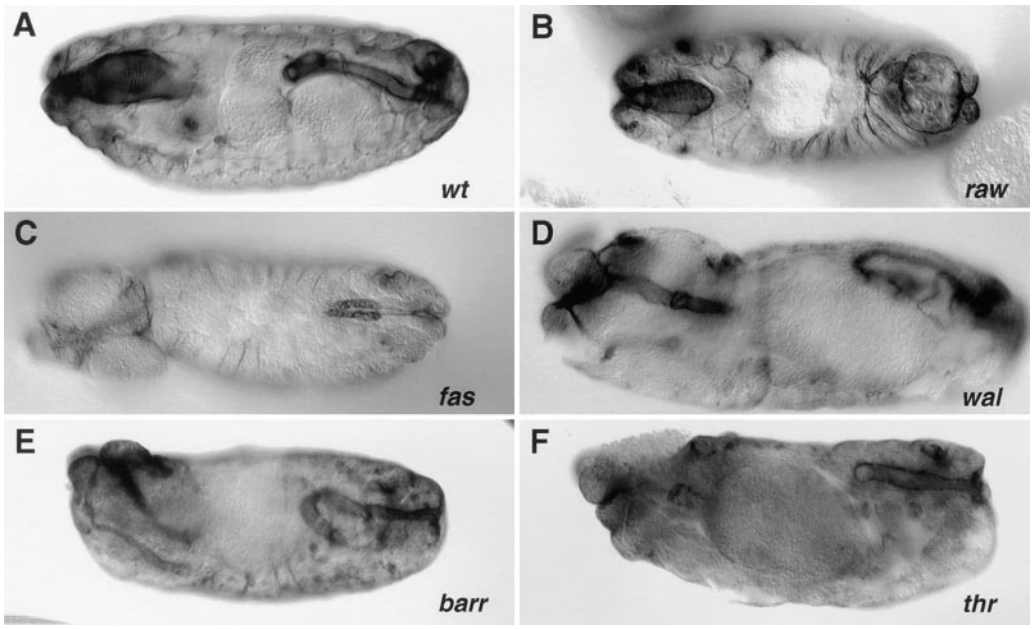

Figure 4.—Global defects in formation and/or maintenance of epithelial structures in mutants identified in the screen. Stage 16–17 embryos of wild type (A) and mutants (B–F) stained with anti-Crb. The most common defect is head involution, which is defective to different degrees in *fas*, *wal*, *barr*, and *thr* (C–F); there is also a lack of midgut constrictions in these mutants. Dorsal closure is not completed in *raw* and *fas* (B and C). Finally, germ band retraction is incomplete in *raw* (B).

et al. 1984). This suggests that a major fraction of embryonic lethals affecting Malpighian tubule development do so as part of more general effects on a variety of epithelial tissues; such mutations are likely to be represented in the existing collections of mutants identified by their epidermal defects. The fact that the *barr* and *wal* genes, both of which have cuticular defects, were not identified in earlier screens for epidermal defects, however, indicates that more genes affecting global epithelial development remain to be discovered. Since the 1800 *P*-element lines screened here contain mutations in only about 40% of the estimated 2000 essential genes on the second chromosome (Török *et al.* 1993), the screen we carried out was not saturating. Consistent with this notion, there was only 1 gene (*thr*) for which we obtained two mutant alleles.

We identified only one mutation (*drm*) that specifically affects the gut; this mutation by itself is not embryonic lethal. Thus, future screens for novel mutants with defects specific to Malpighian tubule and/or hindgut should not be based on embryonic lethality. Instead, such screens could use antibody staining of fixed embryos or direct observation of larvae expressing markers such as green fluorescent protein.

Novel genes identified: *wal:* Mutations in this gene affect many processes of epithelial morphogenesis, including migration of the midgut endodermal epithelia over the yolk and subsequent midgut constrictions, cell recruitment from the Malpighian tubule primordia into buds, proventriculus formation, formation of the dorsal tracheal tubes, and head involution. As we did not observe obvious manifestations of cell death (such as holes seen in the cuticles of *fas*, *barr*, and *thr* embryos) in *wal* embryos, *wal* is likely to be required not so much for epithelial maintenance, but rather for a process common to the morphogenesis of multiple epithelia, such as cell rearrangement.

drm: This mutation is unique among the mutants identified here, as it affects only internal organs, and of these, only the foregut and hindgut. The phenotype of *drm* embryos is consistent with previous studies suggesting that there is a similarity in the genetic mechanisms controlling the development of these two structures. In particular, *fkh* is expressed in both foregut and hindgut primordia and is required for their subsequent development (Weigel *et al.* 1989). In addition, three signaling molecules, encoded by *hedgehog* (*hh*), *wg*, and *decapentaplegic* (*dpp*), are expressed in an analogous pattern in foregut and hindgut, and are required for morphogenesis of both structures (Pankratz and Hoch 1995; Hoch and Pankratz 1996). The defects in hindgut elongation and proventriculus folding in *drm* embryos may have a similar basis, as both could be interpreted as due to a common failure of cell rearrangement.

Insights into Malpighian tubule development: On the basis of work described here previously, Malpighian tubule morphogenesis can be divided into four steps or processes, some of which overlap (Figure 5). First, the tubule primordium is established. This requires the early and partially overlapping expression, at the posterior of the embryo, of *wg* and a number of genes that encode transcription factors, namely *tll*, *hkb*, and *fkh*. The activity of these latter genes, by a combination of activation and repression, initiates a program of gene activity that commits cells to the proctodeal fate (reviewed by Skaer 1993; Singer *et al.* 1996).

After completion of germ band extension, the tubule primordia undertake a morphogenetic process, bud evagination, which is initially controlled by *Kr* and *wg*. *Kr* encodes a transcription factor that is specifically ex-

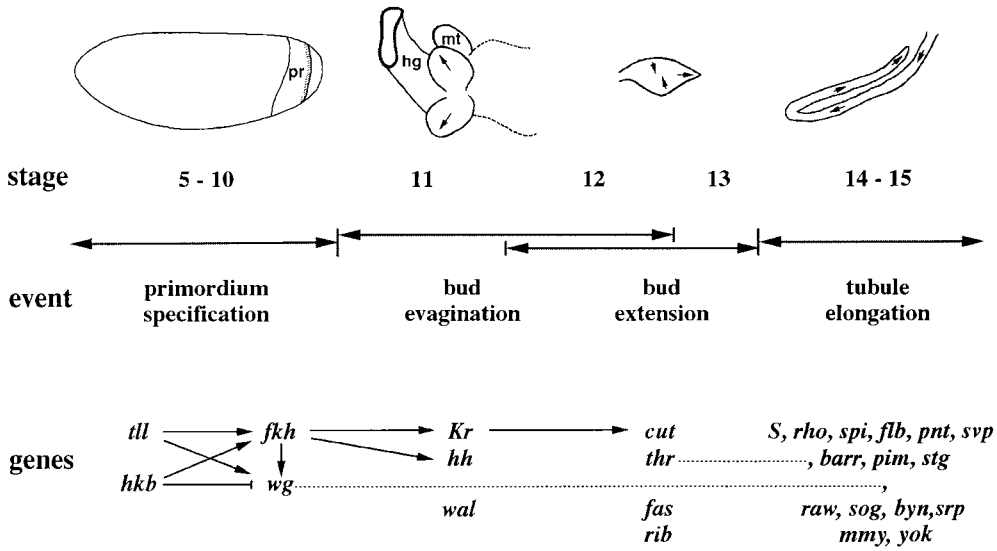

Figure 5.—Genetic control of Malpighian tubule development. Genes required for different steps in tubule development are indicated on the basis of the Malpighian tubule phenotype observed in different mutants, as described here and in references in the text. The dotted lines associated with *wg* and *thr* indicate multiple developmental steps affected in these two mutants. The known genetic hierarchy of genes controlling events of tubule development is indicated by arrows (positive regulation) and a line plus bar (negative regulation). Drawings (modified from Hartenstein 1993) above the stages represent the following, from left to right: stage 5 blastoderm embryo with the proctodeal primordium (pr) outlined and the future Malpighian tubule anlage within it shaded, evaginating tubule buds (mt) at stage 11, an extending bud during stage 13, and an elongating tubule during stage 15. The arrows in these structures indicate the direction of cell movement.

pressed in the tubule primordium and is required for bud evagination (Harbecke and Janning 1989; Gaul and Weigel 1990); *wg* is required for the eversion of a total of four buds (as compared to two in *wg* mutant) and for the subsequent development of Malpighian tubules (Skaer and Martinez Arias 1992; data not shown). As described here, a newly identified gene *wal*, which has not yet been molecularly characterized, is required for timely recruitment of cells from the proctodeum into the tubule buds, as a continuation of bud evagination. Finally, *hh* is required for a later cell recruitment from the proctodeum to form the ureters (Hoch and Pankratz 1996).

The next step of tubule morphogenesis is bud extension. *cut* is expressed specifically in the tubules (under control of *Kr*); the tubules of *cut* embryos arrest early in the process of extension (Liu and Jack 1992; Harbecke and Lengyel 1995). Since *cut* encodes a transcription factor, the failure of tubule extension in *cut* embryos is evidence that new, tubule-specific gene activity is required for the extension process. We show here that another activity required for tubule extension is that of the *fas* gene. Known characteristics of *fas*, namely, its role in neuronal delamination and axonal pathway formation, as well as its encoding an extracellular, immunoglobulin-like molecule (Lekven *et al.* 1998), are consistent with it also playing a role in the cell rearrangement required for tubule extension. We also show that *thr*, which encodes a protein necessary for chromatid segregation, is required for bud extension. Finally, observation of gourd-shaped Malpighian tubules in *rib* embryos (Jack and Myette 1997) suggests that the *rib* gene, not yet molecularly characterized, is also required for bud extension.

Of the genes identified affecting Malpighian tubule development in this and other studies, the largest number affect tubule elongation. This might suggest that elongation is the most complex step in tubule morphogenesis; another interpretation would be that, since elongation takes place last, it is subject to cumulative effects of derangement in processes taking place earlier.

Many genes that affect cell proliferation in the tubules also affect tubule elongation. These include: genes of the EGF signaling pathway, involved in the signaling from the tip cell that stimulates cell proliferation in the tubules (*Star*, *rhomboid*, *spitz*, *flb*, *pointed*, and *seven up*; Skaer 1989; Baumann and Skaer 1993; Kerber *et al.* 1998); *wg*, which plays a required, although less well-defined role in cell division and elongation in the tubules (Skaer and Martinez Arias 1992; Harbecke and Lengyel 1995); and genes affecting mitosis throughout the embryo, namely, *barr* and *thr* as described here, and *pimples* (*pim*) and *string* (*stg*) as described previously (Skaer and Martinez Arias 1992; Harbecke and Lengyel 1995; Stratmann and Lehner 1996). The requirement for these various genes in Malpighian tubule morphogenesis is presumably due to their required roles in cell division. The localized cell proliferation controlled by the tip cell may play a specific role in tubule morphogenesis, while the global cell division requiring *barr*, *thr*, *pim*, and *stg* may simply be necessary to provide a particular number and size of cells essential for the cell rearrangement of tubule extension and elongation.

A number of genes have been identified that, although they do not appear to be expressed in the tubules themselves, have been shown to be required for tubule elongation. This is true for *sog*, which antagonizes activity of the BMP4 homolog Dpp (Francois *et al.* 1994; Harbecke and Lengyel 1995), and also for *raw*, which

encodes a novel molecule required for the cell interactions involved in dorsal closure (Jack and Myette 1997; A. Letsou, personal communication). In addition, *byn* and *srp*, which encode transcription factors required for development of hindgut and posterior midgut, respectively, seem to fall into this category. While *byn* and *srp* are expressed in the early embryo in a domain that will eventually give rise to the tubules, they are not expressed in the tubules themselves (Kispert *et al.* 1994; Rehorn *et al.* 1996; Singer *et al.* 1996). All of these results suggest that as yet undefined signaling between tubule and nontubule cells may be required for tubule elongation.

The work described here, together with previous observations, associates specific genes with specific steps in Malpighian tubule development. Identifying (by genetic and molecular techniques) the genes regulated in the Malpighian tubules by *Kr* and *cut* should contribute to our understanding of mechanisms that control bud evagination and bud extension, respectively. Characterizing the gene products of the *wal* and *rib* genes is also likely to add to our understanding of the types of molecules required for the aforementioned morphogenetic processes. Finally, increased understanding of epithelial morphogenesis of other tubular organs, namely, foregut and hindgut, should be provided by investigation into the *drm* gene.

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