The Genes *raw* and *ribbon* Are Required for Proper Shape of Tubular Epithelial Tissues in Drosophila

Joseph Jack and Guy Myette

Department of Anatomy, University of Connecticut Health Center, Farmington, Connecticut 06030

Manuscript received December 2, 1996
Accepted for publication June 13, 1997

ABSTRACT

The products of two genes, *raw* and *ribbon* (*rib*), are required for the proper morphogenesis of a variety of tissues. Malpighian tubules mutant for *raw* or *rib* are wider and shorter than normal tubules, which are only two cells in circumference when they are fully formed. The mutations alter the shape of the tubules beginning early in their formation and block cell rearrangement late in development, which normally lengthens and narrows the tubes. Mutations of both genes affect a number of other tissues as well. Both genes are required for dorsal closure and retraction of the CNS during embryonic development. In addition, *rib* mutations block head involution, and broaden and shorten other tubular epithelia (salivary glands, tracheae, and hindgut) in much same manner as they alter the shape of the Malpighian tubules. In tissues in which the shape of cells can be observed readily, *rib* mutations alter cell shape, which probably causes the change in shape of the organs that are affected. In double mutants *raw* enhances the phenotypes of all the tissues that are affected by *rib* but unaffected by *raw* alone, indicating that *raw* is also active in these tissues.

SIMPLE epithelial tubes are prominent components of a variety of organs in metazoans, including the respiratory and excretory systems and glands. The elaboration of tubules and the molecular mechanisms underlying their formation are crucial to organogenesis. Insect renal or Malpighian tubules, like the tubes that constitute insect tracheae and salivary glands, form by invagination followed by tubular growth. When morphogenesis is complete, Drosophila have four Malpighian tubules, an anterior and a posterior pair. Each pair is joined in a ureter that empties into the hindgut at its junction with the posterior midgut. The events that comprise the morphogenesis of the organ occur in sequence (reviewed in SKAER 1993). Initially, one pair of buds, the primordia of the anterior tubules, pushes out from the dorsal side of the proctodeum, and another pair of buds, the primordia of the posterior tubules, pushes out from the ventral side of the proctodeum (HOCH *et al.* 1994). The buds lengthen by cell division and become tubular by maintaining a constant eight cell circumference all along their proximo-distal axis as proliferation continues. Specialized tip cells control cell division of each tubule, and division ceases if the tip cells are ablated (SKAER 1989). When the tubes reach their final cell number of just over 140 cells for the anterior tubules and ~105 cells in the posterior tubules, cell division ceases and the tubes begin a process of cell rearrangement that lengthens and narrows the tubules from their original circumference of eight cells to a circumference of only two cells (JANNING *et al.* 1986; SKAER and MARTINEZ ARIAS 1992).

The events that lead to the determination of cell type and morphogenesis of tissues can be investigated by mutational analysis. In the case of the Malpighian tubules, for instance, mutations might block the initial invagination of the tubules, branching, cell division, or the cell rearrangement that lengthens and narrows the tubules. Mutations might also interfere with the maintenance of the shape of the tubules at various stages of their morphogenesis. Mutations have, in fact, been discovered that block specific events in tubule morphogenesis. Determination and differentiation are affected by mutations of *Krüppel* and *cut* (LIU *et al.* 1991; LIU and JACK 1992; HARBECKE and LENGYEL 1995), two genes that encode transcription factors (BLOCHLINGER *et al.* 1988; PANKRATZ *et al.* 1989, 1990). The distal tip cells are determined by a mechanism similar to the determination of external sensory organs (HOCH *et al.* 1994).

Other genes required for morphogenesis of the Malpighian tubules include *wingless* and *Egfr,* both of which are required for normal cell proliferation in the tubules (SKAER and MARTINEZ ARIAS 1992; BAUMANN and SKAER 1993). In addition, the *wingless* product is also involved in either the budding of one set of tubules or the formation of pairs of buds because *wingless* mutants have only two of the normal four tubules (SKAER and MARTINEZ ARIAS 1992). HARBECKE and LENGYEL (1995) have described a deficiency mutation that blocks invagination of the tubules and a number of other mutations of specific genes that affect the diameter of the tubules in late stage embryos.

We set out to identify genes that encode cellular com-

Corresponding author: Joseph Jack, Department of Anatomy, MC-3405, University of Connecticut Health Center, 263 Farmington Ave., Farmington, CT 06030. E-mail: jack@panda.uchc.edu

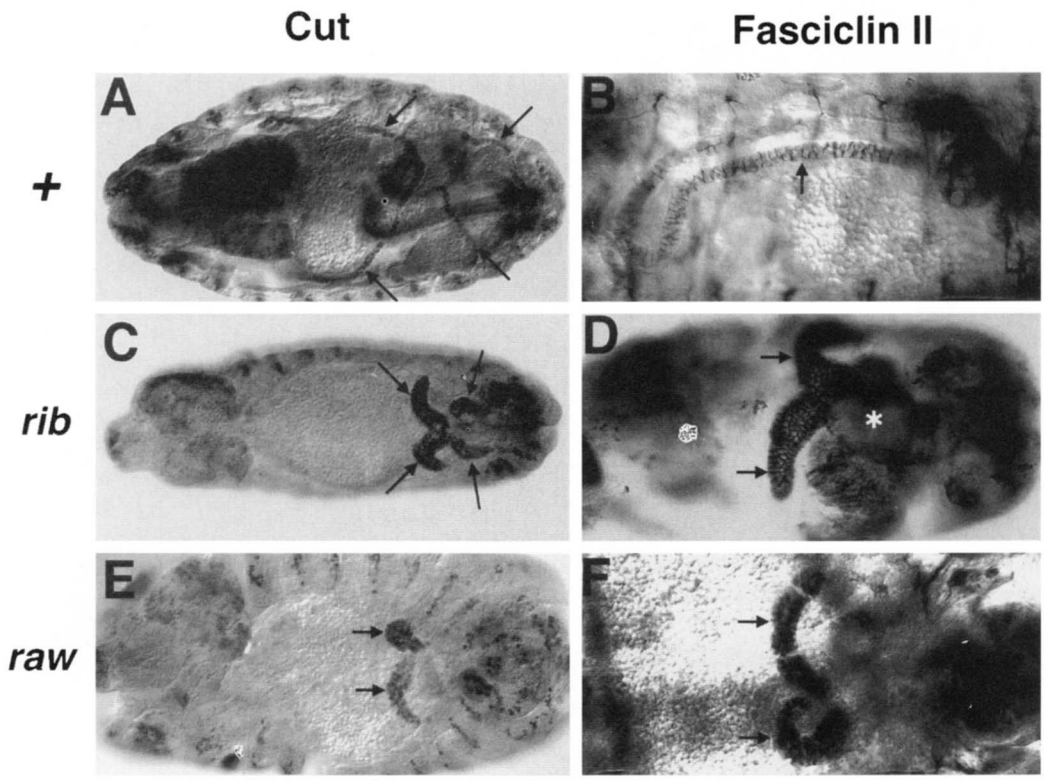

FIGURE 1.—Malpighian tubules of mutant embryos are shorter and thicker than wild-type tubules. Embryos in A, C, and E are stained with an antibody against Cut protein, which is located in the nucleus, and those in B, D, and F are stained with antibody 1D4 against Fasciclin II, which is located in the margins between cells. Anterior is to the left and dorsal to the top in all figures. Embryos are aged at 25° for 14.5–17 hr after egg laying. At this time (stage 16–17), all of the wild-type Malpighian tubules have elongated as shown in A and B. Wild-type Malpighian tubules (arrows in A and B) are two cells thick when morphogenesis is complete. (A) A view from the ventral side of a wild-type embryo. Arrows point to portions of the anterior and posterior tubules. (B) A view of an anterior tubule (arrow). Arrows in C show the four short, thick Malpighian tubules of a homozygous rib^1 mutant. Arrows in D show the anterior tubules of a rib^1 mutant, and the asterisk shows the hindgut at the point where the tubules are attached. The epidermis never closes dorsally in the mutants. Arrows in E and F show anterior tubules in homozygous raw^1 mutant embryos viewed from the dorsal side.

ponents required for morphogenesis. Mutations that block the function of proteins that act in morphogenesis of the Malpighian tubules may alter their form. Since the events of tubule morphogenesis occur sequentially, the specific event in which a gene product acts may be inferred by observation of the mutant tubules at various stages during their development. We began to identify genes whose mutations cause loss or alteration in the shape of the Malpighian tubules by surveying mutations generated in a large-scale screen for cuticle defects (JÜRGENS *et al.*; 1984; NÜSSLEIN-VOLHARD *et al.* 1984; WIESCHAUS *et al.* 1984). Our rationale was that many products that are required for proper morphogenesis, whether they are involved in mechanical functions between or within cells, or in regulatory functions, will be necessary for development of tissues other than the Malpighian tubules. The genes may be required for events that affect the embryo in such a way that the cuticle will be formed abnormally, in which case the genes are likely to have been identified in the previous searches for cuticle defect mutations.

We examined the Malpighian tubules of mutant embryos at stages 16 and 17, when the cell division and cell rearrangement in the tubules would normally have been completed. Mutations of a number of the genes were observed to prevent the normal development of the tubules (to be published separately). Mutations of two of these genes, *raw* and *ribbon,* alter the shape of the tubules and prevent the final step in tubule morphogenesis, the cell rearrangement that causes the tubules to narrow and lengthen. These genes have not previously been reported to affect internal organs. Mutations of both also affect a variety of other tissues, including especially tubular epithelia.

MATERIALS AND METHODS

Drosophila stocks: All of the analysis of mutant phenotypes of the *rib* and *raw* genes was done using two alleles of each of gene. The $rib^1 = rib^{IK}$ and $raw^1 = raw^{IG}$ alleles were obtained from the Bloomington Drosophila Stock Center. The alleles $rib^2 = rib^{IIB44}$ and $raw^2 = raw^{IIP08}$ were a gift of CHRISTIANE NÜSSLEIN-VOLHARD from the Tübingen Stock Center. The phenotypes of both alleles of each gene were similar.

Antibody staining: Antibody staining was done as previously described (LIU *et al.* 1991). Anti-Cut antibody clp2 (BLOCHLINGER *et al.* 1988) was diluted 1:2000. Anti-Crumbs antibody, kindly provided by ELISABETH KNUST (TEPAß *et al.*

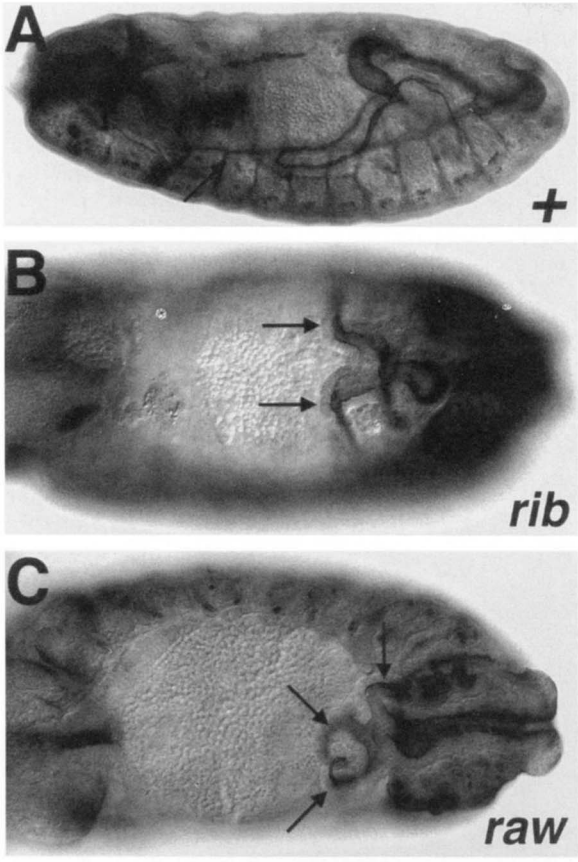

FIGURE 2.—The lumens of mutant Malpighian tubules are wider than wild type. Stage 16–17 embryos are stained with the anti-Crumbs antibody. The apical surface of the tubules and other ectodermal epithelia stain with the antibody. (A) A view of the hindgut and an anterior and posterior tubule of a wild-type embryo. (B and C) Dorsal views of a rib^1/rib^1 and raw^1/raw^1 mutant embryos, respectively, showing tubules (arrows) and parts of the hindgut.

1990), was diluted 1:50. Monoclonal antibody 22C10 (ZIPURSKY *et al.* 1984), a gift of DENNIS BALLINGER, was diluted 1:1000. Monoclonal antibody ID4 (GRENNINGLOH *et al.* 1991), kindly provided by COREY GOODMAN, was diluted 1:10.

To obtain embryos in which organogenesis, head involution, and dorsal closure were nearly complete (stages 16 and 17), embryos were aged for 14.5–17 hr at 25° before being fixed and processed for staining. At this time the Malpighian tubules of wild-type animals were completely elongated, and dorsal closure and head involution were complete. $rib^1/Df(2R)P34$ embryos were aged for 24–27 hr at 18° to obtain fully developed embryos and for 15–18 hr at 20° to obtain embryos at stages 13 and 14 before elongation of the Malpighian tubules. Stages were determined by inspection of wild-type embryos that are laid with the mutant embryos.

RESULTS

***raw* and *rib* mutations alter the shape of the Malpighian tubules:** Representative mutations of genes identified in previous screens (JÜRGENS *et al.* 1984; NÜSSLEIN-VOLHARD *et al.* 1984; WIESCHAUS *et al.* 1984; EBERL and HILLIKER 1988) were surveyed to determine whether any of the mutations cause alteration of the normal morphology of the Malpighian tubules. Because Cut protein is located in the nuclei of a select group of embryonic cells including the nuclei of the Malpighian tubule cells (BLOCHLINGER *et al.* 1988), an antibody against the Cut protein was used in the initial survey to stain mutant embryos so that their tubules could be easily observed. Mutations of two genes, *raw* and *rib*, were found to alter the shape of the tubules.

Normal Malpighian tubules have a uniform circumference along their length. The circumference of the tubes is eight cells during the time the cells are dividing, after which the cells rearrange producing tubes with a circumference of two cells. The mechanism by which the diameter of the tubes is held constant as growth occurs is not known. Embryos homozygous for mutations of either of the genes *raw* or *ribbon* (*rib*) have Malpighian tubules whose circumference is greater than normal near the proximal end giving the tubules a gourd-like shape (Figure 1). The lumen of the mutant tubules is greatly enlarged compared to the normal lumen. This is seen most clearly when the embryos are stained with an antibody against the Crumbs protein (Figure 2), which is localized to the apical region of the lateral cell margins (KNUST *et al.* 1993).

In addition, the *rib* and *raw* mutant Malpighian tubules do not undergo the final cell rearrangement that lengthens and narrows normal tubules from eight cells to two cells in circumference. However, the enlargement of the circumference of the tubules is observable, at least in *rib* mutant embryos, before cell rearrangement would normally narrow and elongate the tubules (Figure 3). In some cases the altered shape of the tubules is apparent soon after the tubules bud out from the hindgut, at least by stage 12 (Figure 3, A and B). However, the mutant phenotype is variable at this stage and is frequently similar to wild type. By stage 14, when cell division has lengthened the tubules, but before cell rearrangement has begun, the broadened lumen is apparent in many mutants (Figure 3, C and D) although the phenotype is still somewhat variable. Therefore, the alteration in the shape of the tubules is not solely a result of the failure of the tubules to elongate. Rather, *rib* and *raw* must be required in the process that establishes the diameter of the Malpighian tubules. On the other hand, the direct involvement of the *rib* and *raw* products in cell rearrangement is less certain. The mutations could block the process indirectly through effects they have on the tubules before rearrangement.

***rib* and *raw* mutants are defective in dorsal closure and morphogenesis of the central nervous system and midgut:** In addition to their effect on the Malpighian tubules, *raw* and *rib* mutations affect some other morphogenetic events in similar ways. In their initial description of mutations affecting cuticle pattern, NÜSSLEIN-VOLHARD *et al.* (1984) reported that *rib* and *raw* mutants are defective in dorsal closure. The epidermis fails to complete its dorsal movement, leaving a sizable

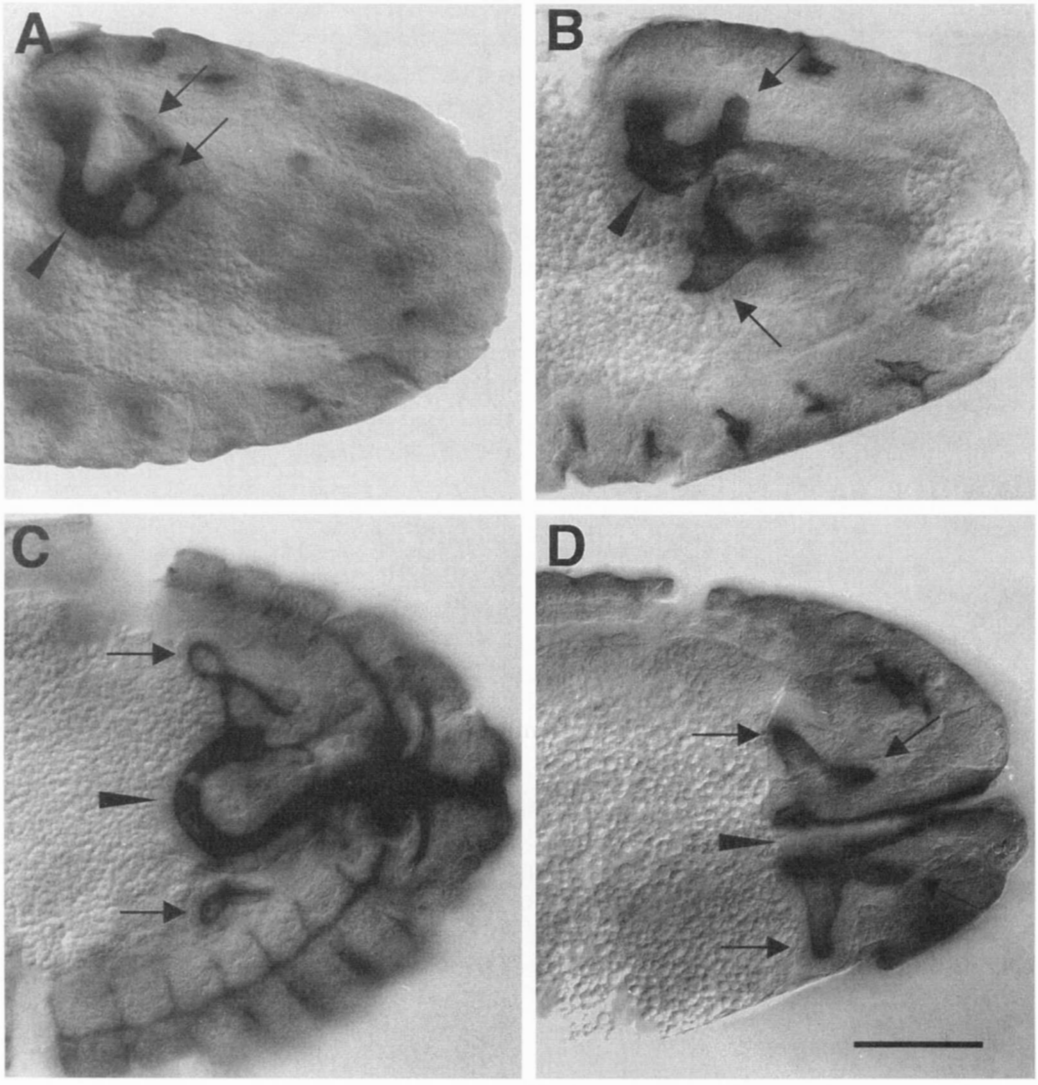

FIGURE 3.—Malpighian tubules of *rib* mutants are often shorter and wider than wild type before the tubules are fully developed. A and C are rib^{+}/rib^{1} or $rib^{+}/Df(2R)P34$, and B and D are $rib^{1}/Df(2R)P34$ embryos that exhibit relatively extreme rib Malpighian tubules phenotypes for their developmental stages. The embryos in A and B are stage 12 embryos with short tubules. The embryo in C is at stage 14. The Malpighian tubules are not yet elongated but they have grown in length by cell division and the hindgut has assumed the characteristic sigmoid shape. Only the anterior tubules and the ureter of the posterior tubules are in the focal plane. The embryo in D is stage 13 or 14. The lumens of the Malpighian tubules are wider than those of normal tubes, which would be similar to those of the embryo in C. The hindgut is shorter than normal and is straight. Arrows mark Malpighian tubules and arrowheads mark hindgut. Scale bar, 50 μm for all panels.

dorsal opening from ~40 to 60% of the egg length from the posterior end (40–60% EL) (Figure 1, C and E).

We observed two other defects common to mutants of both genes. One is the failure of the CNS to retract. In normal embryos, the CNS retracts bringing the posterior tip of the CNS from a point just ventral to the anus in stage 16 embryos to a point ~40% EL in stage 17. Figure 4A shows a wild-type embryo in which the CNS is in the process of shortening. In *rib* and *raw* mutants, the CNS remains the full length of the embryo (Figure 4, B and C).

The other defect common to mutants of both genes is the lack of constrictions in the midgut. Normal embryos have three constrictions that subdivide the midgut into four chambers (Figure 4A). The midgut of *rib* mutants remains a single undivided sack (Figure 4B), and the midgut of *raw* mutants has incomplete constrictions (arrowheads in Figure 4C).

***rib* mutations cause defects in a variety of organ systems and morphogenetic events that are not affected by *raw* mutations:** One of the major events of gastrulation, the internalization of head structures, is blocked by mutations in the *rib* gene. In normal embryos, the head segments involute orally in a gradual process that internalizes all of the larval head structures. In *rib* mutants little if any of this process occurs. The failure of head involution in *rib* mutants can be seen in comparing the anterior ends of the embryos shown in Figure 1, A and C, Figure 4, A and B, or Figure 5, A and B.

In addition, mutations of *rib* alter the shape of most tube-shaped organs in a manner similar to their effect on the Malpighian tubules. The salivary glands and hindgut of *rib* mutants are both affected. Normal salivary glands are paired, tubular organs, each consisting of a more distal secretory portion composed of columnar cells (Figure 5A) and a more proximal duct composed of small, flat cells. The ducts from the two glands join to form a common segment. The formation of the salivary gland is reviewed in CAMPOS-ORTEGA and HARTENSTEIN (1985) and SKAER (1993). Each gland and duct forms by invaginating from the ventral-lateral labial and maxillary segments, respectively, without cell division after the beginning of invagination. The two

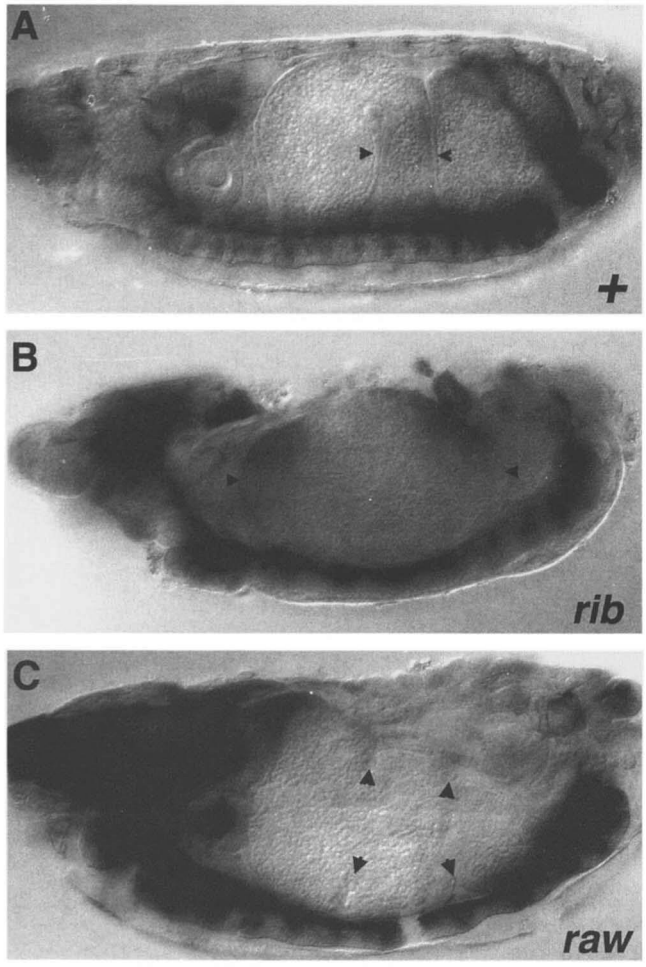

FIGURE 4.—The CNS of *rib* and *raw* mutant embryos fails to retract and the constrictions in their midguts are defective. Stage 16 embryos are stained with monoclonal antibody 1D4, which recognizes fasciclin II. (A) Wild-type embryo in which the CNS has shortened to ~80% EL and constrictions can be seen in the midgut (arrowheads). (B) Homozygous rib^1 embryo in which the CNS does not shorten and the midgut does not constrict. The arrowheads mark the anterior and posterior boundaries of the midgut. Head segment can be seen not to be involuted. (C) Homozygous raw^1 embryo. The CNS has not shortened and constrictions in the midgut are rudimentary (arrowheads).

separate ducts each move toward the midline and eventually fuse and continue to invaginate to form a portion of the duct common to the two glands. The duct remains open to the outside until head involution carries it into the stomodeum where it opens onto the floor of the pharynx.

The salivary glands of *rib* mutants are bulb-shaped rather than tubular (Figure 5B). As in wild type, the mutant glands appear to be divided into secretory and duct segments, because the lumen is more narrow in the section of the gland that connects to the outside. However, in most of the mutant embryos the ducts do not move to the ventral midline and fuse but remain open to the outside of the embryo. Through most of their development, the salivary glands maintain a tubular shape, only beginning to be bulb shaped toward the end of organogenesis in stage 16. Therefore, expression of *rib* is apparently necessary for tissues to maintain, as well as achieve, their shape.

The hindgut of *rib* mutants is similarly shortened and widened. The normal hindgut when fully developed is a straight tube from the anus to 45% EL, at which point it bends backward and to the right to join the posterior midgut (Figure 5C). The hindgut of *rib* mutants is dramatically shortened and widened, forming a bulb rather than a tube (Figure 5D). The malformation of the hindgut becomes obvious fairly early in the morphogenesis of the organ (Figure 3, A and B) and can be seen throughout the development of the organ (Figure 3, C and D).

The shape of the tracheae, the system of branched tubes that allows the exchange of gases between insect tissues and the atmosphere, is also altered by *rib* mutations. The normal development of the tracheal system [reviewed in MANNING and KRASNOW (1993)] begins with the invagination of paired clusters of cells that are laterally placed in segments T2 to A8. The invaginations subsequently branch and grow out, maintaining a tubular morphology. Some branches fuse with branches from clusters in adjacent segments to form continuous tubes, the dorsal and lateral trunks, that extend from the head to the posterior end of the embryo (Figure 5E). In *rib* mutants the clusters invaginate and form tubes that run dorsoventrally in each segment, and some rudimentary horizontal branches form. However, the branches usually do not grow out (Figure 5F). When they do grow out, they never contact branches from neighboring segments although they sometimes extend as far as two segments away from their origin (Figure 5G). The tracheae that form also remain considerably larger in diameter than wild-type tracheae. Thus, *rib* mutations have a similar effect on a variety of different tubular organs in Drosophila embryos, increasing the diameter of the tubes in each case.

The effect in some tissues is more extreme if the rib^1 mutation is heterozygous with a deficiency that deletes the gene than in rib^1 homozygotes, indicating that the rib^1 mutation is not a null mutation. The salivary glands are somewhat rounder and less variable in phenotype in $rib^1/Df(2R)P34$ embryos than in rib^1 homozygotes (Figure 5H). The rib^1/rib^1 gland in Figure 5B is a fairly extreme example of the genotype, while the $rib^1/Df(2R)P34$ gland in Figure 5H is typical of most salivary glands of this genotype. The tissue that exhibits the most extreme difference in phenotype between homozygous rib^1 and $rib^1/Df(2R)P34$ is the tracheal system. A range of phenotypes is observed in the $rib^1/Df(2R)P34$ mutants, but the tracheae are usually at least slightly more mutant than rib^1 homozygotes, and in the most extreme cases the tracheae form bags with no tubular morphology (Figure 6). Fully developed Malpighian tu-

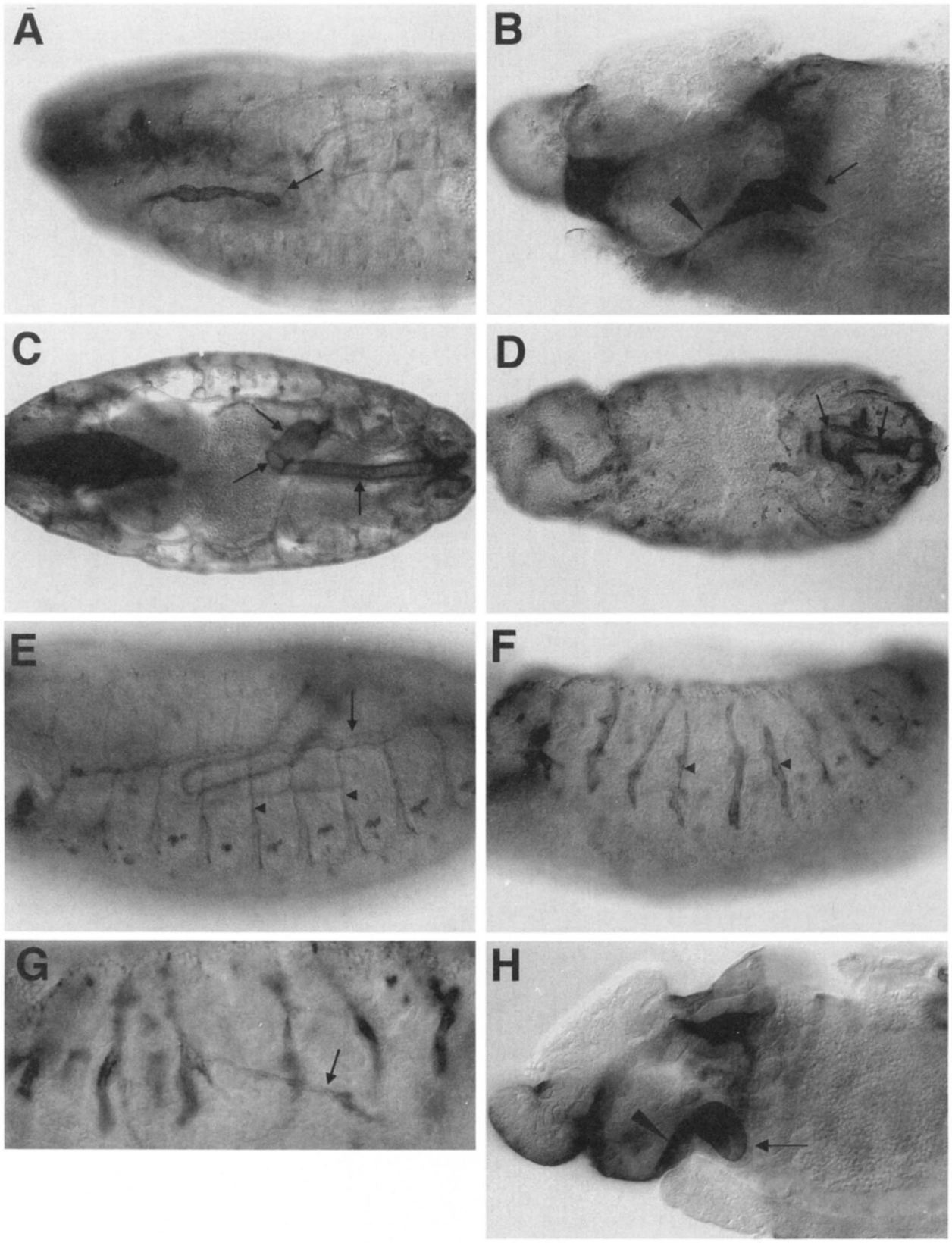

FIGURE 5.—Mutations of *rib* cause malformation of epithelial tubes. Stage 16 embryos are stained with anti-Crumbs antibody. Embryos in A, C, and E and are wild type, and those in B, D, F, and G are homozygous rib^1. The antibody recognizes a protein in the lateral cell membrane near the apical surface and, therefore, highlights the lumens of the tubular tissues that are stained. (A) A normal, cylindrical salivary gland (arrow). (B) The secretory portion (arrow) and duct (arrowhead) of a mutant salivary gland. The secretory portion is wider and has a much larger lumen than normal. Unlike wild-type salivary glands, whose ducts join at the midline and empty into the pharynx, the ducts of the mutant glands do not join, and they open to the outside of

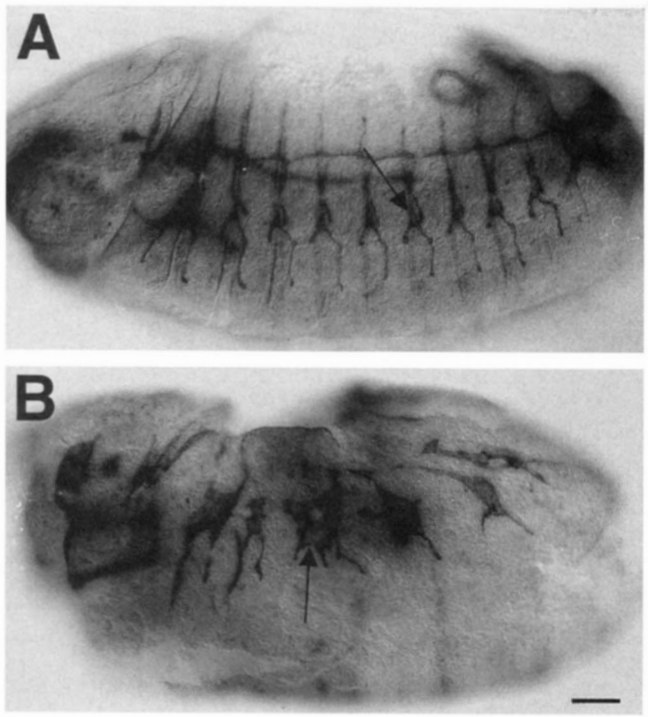

FIGURE 6.—Tracheae of *rib*^{1}/*Df(2R)P34* embryos lack tubular morphology. The embryo in A is at stage 14 and of the genotype *rib*$^{+}$/*rib*^{1} or *rib*$^{+}$/*Df(2R)P34*. The embryo in B is of the genotype *rib*^{1}/*Df(2R)P34* and is approximately the same age as the embryo in A although it does appear to be somewhat germband elongated. The tracheae appear to have branches, but the segmental tube appears to be a flattened area with no tubular morphology. The tracheal phenotype of *rib*^{1}/*Df(2R)P34* embryos is variable, with that shown in B being typical of the most extreme. Others seen at this stage have a similar tracheal phenotype but are germband shortened. Scale bar, 50 μm.

bules and hindgut have a phenotype in *rib*^{1}/*Df(2R)P34* embryos similar to that of *rib*^{1}/*rib*^{1} embryos.

In addition to the effects of *rib* on tubulogenesis, the PNS of *rib* mutants is disorganized. None of the cells of the nervous system is obviously missing in *rib* mutants, but the position of cells is altered. The PNS of wild-type embryos is made up of four clusters of sense organs in each hemisegment: one dorsal cluster, one lateral cluster, and two ventral clusters (CAMPOS-ORTEGA and HARTENSTEIN 1985; BODMER *et al.* 1989), shown in Figure 7A. In the PNS of *rib* mutant embryos, the clusters of sense organs are indistinct and compacted dorsoventrally. The two ventral clusters and the lateral cluster of sense organs are often combined into a single large cluster in the mutants, and the dorsal clusters of sense organs, which are stretched out dorsoventrally in wild type, are compacted into a tight bunch in mutants (Figure 7B). The disorganization of cells is observed clearly in the group of five chordotonal organs in the lateral cluster. In wild-type embryos the five chordotonal organs are positioned very precisely in a row (arrow in Figure 7C). The five chordotonal organs of *rib* embryos are clustered but positioned randomly rather than in a straight row (arrows in Figure 7D). Another phenotype that is occasionally observed is that the bundles of axons that run from the clusters of sense organs to the CNS are missing or misdirected. For instance, two axon bundles in Figure 7B (arrowheads) veer out of the normal path of axons from the dorsal cluster of sense organs past the lateral cluster. In some *rib* mutant embryos the axon bundles are absent altogether.

Cell shape is altered in mutants: The shape of cells is altered by *rib* mutations in at least some of the tissues affected by the mutations. In the hindgut, *rib* mutant cells are less columnar than wild-type cells. The apical surface of the hindgut cells can be observed using an antibody against the Crumbs protein. The Crumbs protein is located in the apical region of the lateral cell membranes of ectodermally derived epithelia (TEPAß *et al.* 1990; GRAWE *et al.* 1996). Therefore, staining with an antibody against the protein outlines the apical surface of the epithelial cells. The lumens of the Malpighian tubules are too small to view the surfaces of the cells easily, but the outlines of the lumenal surfaces of cells of the hindgut are easily visible. The apical surface of the cells in wild type is fairly uniform, while that of the mutant cells varies. However, the smaller mutant cells are as large as wild type, while the larger mutant cells have a surface area two to three times the size of wild type (Figure 8). The number of cells in the circumference of the hindgut is also larger in *rib* mutants than in wild type. The width of the hindgut in the *rib* mutant is six cells in the plane of focus (Figure 8B) compared to three cells in the plane of focus in wild type (Figure 8A). Normal hindgut cells are wedge-shaped with an apical surface smaller than the basal surface because the inside diameter of the structure is smaller than the outside diameter, but the mutant cells are less wedge-shaped than wild type because the mutant hindguts have more cells in their circumference than normal and the cells have a larger apical surface.

The alteration in the shape of the apical surface of the hindgut cells may be either a cause or a result of the change in shape of the hindgut. If a factor other than the shape of cells causes the hindgut to conform to its normal shape, the cells of the hindgut might then be forced into a wedge shape. However, the height of the hindgut cells is altered in *rib* mutants. The hindgut

the epidermis. (C) A dorsal view of a normal hindgut (arrows). (D) A much shorter and wider mutant hindgut. (E) The main trunk (arrow) and segmental branches (arrowheads) of the tracheal system of a wild-type embryo. (F) The wider segmental tracheal segments of mutants (arrowheads). The segments fail to branch to form the horizontal segments that form the dorsal and lateral connections between the segmental branches. (G) A higher magnification view of one tracheal segment that branches. The branch extends over a distance of two segments without fusing with another branch. (H) A salivary gland of a *rib*^{1}/*Df(2R)P34* embryo showing the bulbous secretory portion (arrow) and the narrow duct (arrowhead).

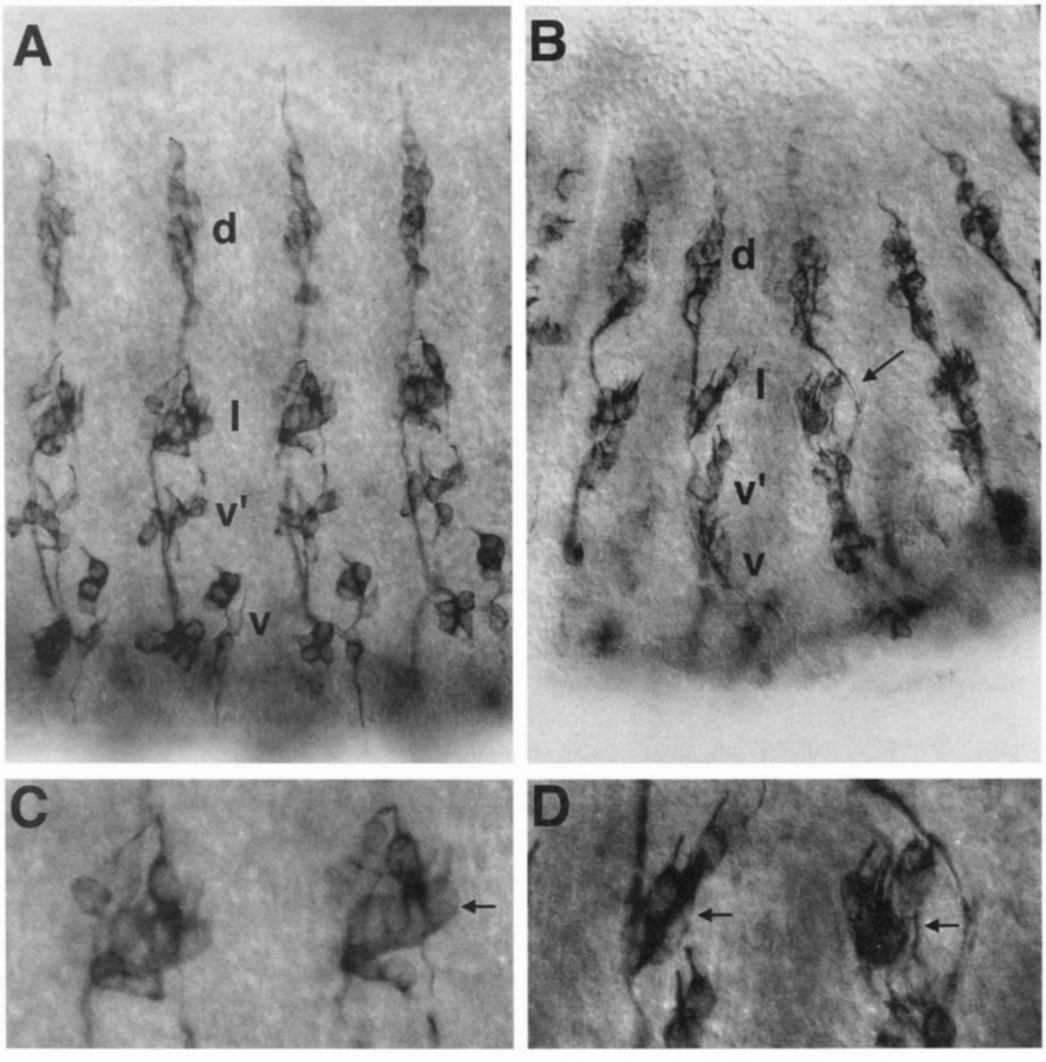

FIGURE 7.—The peripheral nervous systems of *rib* mutant embryos are disorganized. The peripheral nervous system in four segments of a normal, early stage 17 embryo (A) and homozygous rib^1 mutant embryo at a similar age (B) is stained with the monoclonal antibody 22C10. The dorsal (d), lateral (l), and two ventral (v and v′) clusters of sense organs are labeled. The neurons of each mutant cluster are more compacted within the cluster and less stereotypically positioned than the wild-type neurons. In many cases the axon bundles take a different route in the mutants than in wild type (arrows in B). (C and D) An enlarged view of the pentascolopidial chordotonal organs of two segments. In wild-type embryos (C) the five neuronal cell bodies are arranged one next to the other (arrow). In *rib* mutant embryos (D) the five cell bodies are clustered in no apparent order (arrows).

cells are taller in wild type than in *rib* mutants as indicated by the greater width of the hindgut wall in wild type than in mutants (Figure 8). Clearly the cells of *rib* mutants are more cuboidal than the normal columnar epithelial cells of the hindgut. The altered height of the cells in the hindgut is less easily explained as a consequence of shaping of the tissues by other factors, suggesting that the mutations may directly affect either the shape of the cells or the adhesion between the cells, which in turn alters the shape of the tissues.

Phenotype of *raw* and *rib* double mutants: Because the Malpighian tubule phenotype of the *raw* and *rib* mutants is essentially identical, the products of the two genes could function in the same process or pathway at least in the tubules. If both gene products function in the same process and either the *raw* or *rib* mutation is completely inactive, the phenotype of double mutant embryos should be identical to that of either the *raw* or *rib* mutants alone. If the phenotype of double mutants is more extreme, it might reveal more about the nature of the changes that alter the shape of the Malpighian tubules or other tissues that are affected by the mutations. Therefore, we constructed a raw^1 rib^1 second chromosome and examined homozygous embryos from the cross raw^1 rib^1/*CyO* × raw^1 rib^1/*CyO*. Since the *CyO* balancer chromosome prevents most crossovers, about a quarter of the progeny embryos are raw^1 rib^1 homozygotes.

The Malpighian tubule phenotype of the double mutants is similar to the phenotype of the individual mutants (Figure 9A, compare to Figure 1C), consistent with the possibility that the two genes affect the same process in the tubules. Unexpectedly, however, the phenotype of the double mutants is more extreme in tissues that appear to be unaffected in embryos carrying the *raw* mutation alone. The salivary glands and tracheae of the double mutants are both smaller than those of *rib* mutants (*cf.* Figure 5B to 9B and 5F to 9C). In particular, the salivary glands, which are simply misshapen in *rib* mutants and unaffected in *raw* mutants, are rudimentary in the double mutants. In addition, the PNS, which is mildly disorganized in *rib* mutants and ostensibly normal in *raw* mutants, is grossly disorganized in double mutants (*cf.* Figure 7B with 9D). Nearly the entire PNS of each segment is clustered along the dorsal edge of the epidermis of the double mutant embryos

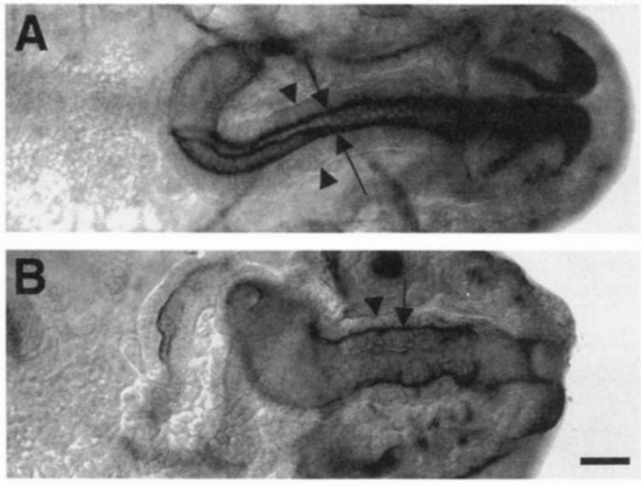

FIGURE 8.—The shape of cells in mutant hindguts is different from wild-type hindguts. Early stage 17 *rib*^{1}/*CyO* (A) and homozygous *rib*^{1} embryos (B) are stained with anti-Crumbs antibody. The stain shows the shape of the cell at the apical surface. The apical surface of the cells in the mutants (B) is larger than that of wild type (A), and the distance between the apical surface (arrows) and the basal surface (arrowheads) is smaller in the mutants than in wild type. So, the hindgut cells, which are normally columnar, are more cuboidal in the mutant hindgut. Scale bar, 16 μm.

(arrows in 9D), and axons are not apparent. In some segments clusters of neurons are located in lateral positions (arrowheads in 9D), but in no case are specific elements of the PNS recognizable.

Although the effects of *raw* mutations are not observed in the salivary glands or tracheae in single mutants, the *raw* gene product clearly has an activity that affects those tissues, either directly or as a secondary effect of its activity in another tissue, since the effect of the mutation is observed in the tissues in combination with a *rib* mutation. A *raw* phenotype may not be apparent in the tissues either because both the *raw*^{1} and *raw*^{2} alleles are too leaky for the phenotype to be manifested there, because inactivation of the *raw* gene is simply insufficient to confer a mutant phenotype on all of the tissues in which the gene is expressed, or because maternal product of the *raw* gene is sufficient for these tissues to have a wild-type morphology. In any case, the fact that both genes are clearly active in most of the same tissues supports the hypothesis that the products of the two genes function in the same or related processes.

DISCUSSION

We describe two genes, *raw* and *ribbon,* that are required for the Malpighian tubules and some other simple epithelial tubes to assume their normal shape. The greater width and diminished length of the mutant tubules becomes apparent once the progenitors begin to lengthen into tubes. But the effect of *raw* and *rib* mutations is not limited to the Malpighian tubules. The genes are also required for the normal shape of other simple epithelial tubes and for proper arrangement of cells in some nontubular tissues as well. Salivary glands, hindgut, and tracheae, all tubular epithelia, are shortened and widened in *rib* mutants, similarly to the Malpighian tubules. In their examination of genes that are required for posterior gut development, HARBECKE and LENGYEL (1995) noted that most mutations that affect the hindgut also affect the Malpighian tubules. So, the fact that both hindgut and Malpighian tubules are similarly affected by *rib* mutations is consistent with observations of other genes and could reflect the derivation of the Malpighian tubules from the hindgut primordium. In both *raw* and *rib* mutants, some or all of the constrictions of the midgut are eliminated. HARBECKE and LENGYEL (1995) also observed a number of mutations that reduced or eliminated midgut constrictions and noted that mutations that reduce the midgut epithelium reduce or eliminate the constrictions.

In addition to their effects on tubular epithelia, *raw* and *rib* are required for proper organization of cells in the nervous system and for axonal pathfinding. In both *raw* and *rib* mutants the CNS is disorganized and fails to contract late in embryonic development. In *rib* mutants the PNS is disorganized, and axons sometimes fail to form or are misrouted. Furthermore, mutants of both are defective in dorsal closure, and mutants of *rib* are defective in head involution as well. While the phenotypes of *raw* mutations are restricted to the Malpighian tubules, CNS, and dorsal closure in embryos mutant for *raw* alone, *raw* mutations enhance all of the *rib* phenotypes in double mutant embryos, suggesting that *raw* is functional in all the same organs as *rib.* The lack of a phenotype in *raw* mutant tissues could be the result either of leakiness of the *raw* alleles, of maternal effect, or a combination of the two. Consistent with the possibility that maternal effect is a factor in determining the *raw* phenotype, all of the phenotypes that are observed (misshapen Malpighian tubules, failure of dorsal closure, failure of the CNS to retract) occur late in embryonic development, perhaps when maternal product begins to wane.

Changes in the shapes of the cells or disturbance of the adhesion between the cells could, among other possibilities, cause the abnormal shape of some organs and disorganization of cells in others. In *rib* mutants at least, the shape or size of some cells is clearly different from wild type. The lumenal surface area of hindgut cells of *rib* mutants is larger than normal, and the height of the cells is reduced (Figure 8). Some changes in shape or size of the cells in the mutants could be either a cause or an effect of the difference in the shapes of the tissues. However, the changes in the height of the cells in hindguts of mutants would not be expected to be caused by changes in the shape of the tissue. The changes in height of cells could be caused either by changes in the cytoskeleton of the cells or by alterations in the number or positions of adhesive junctions. Thus,

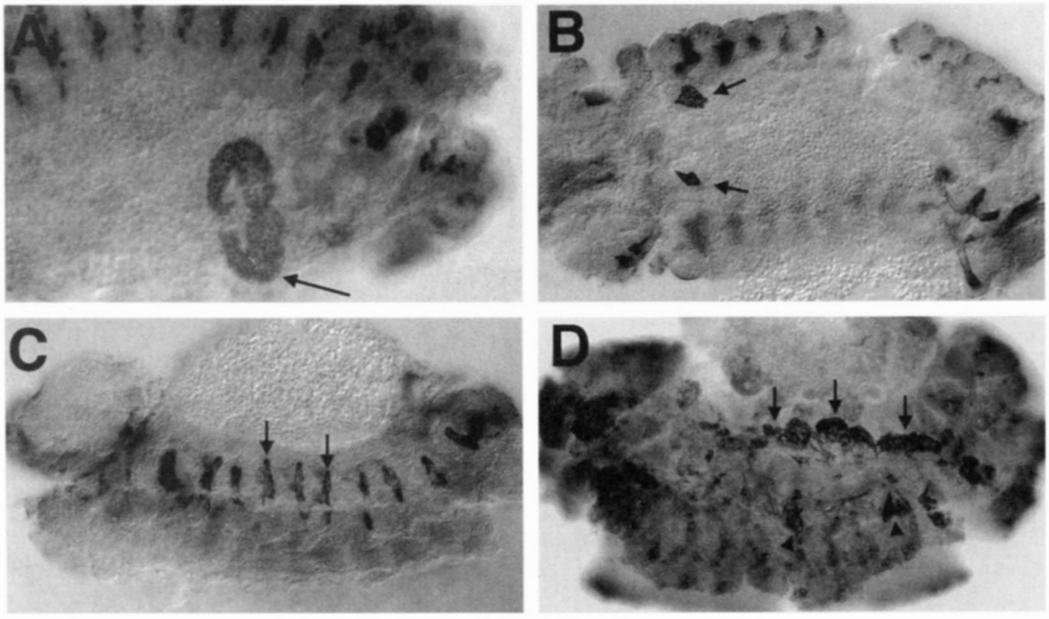

FIGURE 9.—*raw rib* double mutants have a more extreme phenotype in the salivary glands, tracheae, and PNS but not in the Malpighian tubules. raw^1 rib^1 homozygous embryos at stage 16–17 are stained with anti-Cut antibody (A), anti-Crumbs antibody (B and C), and Mab 22C10 (D). The Malpighian tubules (arrow in A) are similar in size and shape to embryos homozygous for either of the mutations alone. The double mutant salivary glands (arrows in B) and tracheae (arrows in C) are much smaller than the *rib* homozygotes. The PNS is much more disorganized in the double mutants (D) than in *rib* mutants. Most of the PNS neurons in each segment are clustered together at the dorsalmost region of the epidermis (arrows). However, in a few segments, some neurons are located just above the most lateral extent of the CNS (arrowheads).

the *raw* and *rib* products might have either adhesive or cytoskeletal functions.

Defects in head involution and dorsal closure, events that are defective in *raw* and *rib* mutants, have been associated with mutations that prevent the cell shape changes of normal development. In each case, the genes that are affected encode cytoskeletal proteins or proteins that regulate the cytoskeleton. *zipper* (*zip*), for instance, encodes nonmuscle myosin II heavy chain, a component of the cytoskeleton required for cells to undergo the shape changes that propel both dorsal closure (YOUNG *et al.* 1993) and the striking elongation of the leg imaginal discs during metamorphosis (CONDIC *et al.* 1991; GOTWALS and FRISTROM 1991; FRISTROM and FRISTROM 1993). Other mutant phenotypes reported for *zip* and common to *rib* mutants, in addition to the failure of dorsal closure, are defects in head involution and neuronal pathfinding (CÔTÉ *et al.* 1987; ZHAO *et al.* 1988). Another gene *l(2)giant larvae* (*l(2)gl*) is also required for the cell shape changes that cause dorsal closure, and mutants are defective in both dorsal closure and head involution (MANFRUELLI *et al.* 1996). *l(2)gl* encodes a cytoskeletal component that associates with nonmuscle myosin II heavy chain (STRAND *et al.* 1994a,b). Mutants of some alleles of the gene *coracle* (*cora*) are defective in both dorsal closure and head involution (FEHON *et al.* 1994). *cora* encodes D4.1, the Drosophila homologue of the human 4.1 protein (FEHON *et al.* 1994), which associates with spectrin and actin in the cytoskeleton of erythrocytes (DISCHER *et al.* 1993, 1995; WINARDI *et al.* 1995). Although *cora* has not been reported to be required for proper cell shape in Drosophila, the D4.1 protein is a component of the cytoskeleton localized to septate junctions (FEHON *et al.* 1994), and in humans, mutations of the gene cause abnormal erythrocyte morphology (CONBOY *et al.* 1991, 1993).

The similarity of the *raw* and *rib* phenotypes to the phenotypes that have been reported for these three genes that encode cytoskeletal components and are required for proper cell morphology gives some support to the hypothesis that the activity of the *raw* and *rib* products is connected to cytoskeletal function. If mutants of *zip*, *l(2)gl*, or *cora* have other, more specific phenotypic similarities to *raw* and *rib* mutants, such as alterations in the shapes of tubular epithelia, the hypothesis that *raw* and *rib* mutations cause cytoskeletal defects would be further supported.

Defects in adhesion or the regulation of adhesion in *raw* and *rib* mutants are another possible cause of the failure of cells to associate properly, and defects in the ability of cells to shift and move in developing tissues could account for the apparent disorganization of some cells and misassociation of others. Whatever the specific cellular defects are, the products of *raw* and *rib* may be directly involved, or they may play a regulatory role. In either case, an understanding of the requirement of the product of these genes for the generation of tubular morphology will be useful in understanding the mechanism by which tubular morphology is generated.

We thank ROBERT KOSHER, JOSEPH GRASSO, STEVE HELFAND and ROBERT REENAN for critical reading and helpful comments on the manuscript. This work was supported by a Faculty Research Award to J.J. from the University of Connecticut Health Center.

LITERATURE CITED

BAUMANN, P., and H. SKAER, 1993 The Drosophila EGF receptor homologue (DER) is required for Malpighian tubule development. Development **Suppl.:** 65–76.

BLOCHLINGER, K., R. BODMER, J. JACK, L. Y. JAN and Y. N. JAN, 1988 Primary structure and expression of a product from *cut*, a locus involved in specifying sensory organ identity in *Drosophila*. Nature **333:** 629–635.

BODMER, R., R. CARRETTO and Y. N. JAN, 1989 Neurogenesis of the peripheral nervous system in Drosophila embryos: DNA replication patterns and cell lineages. Neuron **3:** 21–32.

CAMPOS-ORTEGA, J. A., and V. HARTENSTEIN, 1985 *The Embryonic Development of Drosophila melanogaster.* Springer-Verlag, Berlin.

CONBOY, J. G., J. A. CHASIS, R. WINARDI, G. TCHERNIA, Y. W. KAN *et al.*, 1993 An isoform-specific mutation in the protein 4.1 gene results in hereditary elliptocytosis and complete deficiency of protein 4.1 in erythrocytes but not in nonerythroid cells. J. Clin. Invest. **91:** 77–82.

CONBOY, J. G., R. SHITAMOTO, M. PARRA, R. WINARDI, A. KABRA *et al.*, 1991 Hereditary elliptocytosis due to both qualitative and quantitative defects in membrane skeletal protein 4.1. Blood **78:** 2438–2443.

CONDIC, M. L., D. FRISTROM and J. W. FRISTROM, 1991 Apical cell changes during *Drosophila* imaginal leg disc elongation: a novel morphogenetic mechanism. Development **111:** 23–33.

CÔTÉ, S., A. PREISS, J. HALLER, R. SCHUH, A. KIENLIN *et al.*, 1987 The *gooseberry-zipper* region of *Drosophila:* five genes encode different spatially restricted transcripts in the embryo. EMBO J. **6:** 2793–2801.

DISCHER, D., M. PARRA, J. G. CONBOY and N. MOHANDAS, 1993 Mechanochemistry of the alternatively spliced spectrin-actin binding domain in membrane skeletal protein 4.1. J. Biol. Chem. **268:** 7186–7195.

DISCHER, D. E., R. WINARDI, P. O. SCHISCHMANOFF, M. PARRA, J. G. CONBOY *et al.*, 1995 Mechanochemistry of protein 4.1's spectrin-actin-binding domain: ternary complex interactions, membrane binding, network integration, structural strengthening. J. Cell Biol. **130:** 897–907.

EBERL, D. F., and A. J. HILLIKER, 1988 Characterization of X-linked recessive lethal mutations affecting embryonic morphogenesis in *Drosophila melanogaster.* Genetics **118:** 109–120.

FEHON, R. G., I. A. DAWSON and S. ARTAVANIS-TSAKONAS, 1994 A Drosophila homologue of membrane-skeleton protein 4.1 is associated with septate junctions and is encoded by the *coracle* gene. Development **120:** 545–57.

FRISTROM, D., and J. W. FRISTROM, 1993 The metamorphic development of the adult epidermis, pp. 843–897 in *The Development of Drosophila melanogaster,* edited by M. BATE and A. MARTINEZ-ARIAS. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

GOTWALS, P. J., and J. W. FRISTROM, 1991 Three neighboring genes interact with the *Broad-Complex* and the *Stubble-stubbloid* locus to affect imaginal disc morphogenesis in Drosophila. Genetics **127:** 747–759.

GRAWE, F., A. WODARZ, B. LEE, E. KNUST and H. SKAER, 1996 The Drosophila genes *crumbs* and *stardust* are involved in the biogenesis of adherens junctions. Development **122:** 951–959.

GRENNINGLOH, G., E. J. REHM and C. S. GOODMAN, 1991 Genetic analysis of growth cone guidance in Drosophila: fasciclin II functions as a neural recognition molecule. Cell **67:** 45–57.

HARBECKE, R., and J. A. LENGYEL, 1995 Genes controlling posterior gut formation in the *Drosophila* embryo. Roux's Arch. Dev. Biol. **204:** 308–329.

HOCH, M., K. BROADIE, H. JÄCKLE and H. SKAER, 1994 Sequential fates in a single cell are established by the neurogenic cascade in the Malpighian tubules of *Drosophila.* Development **120:** 3439–3450.

JANNING, W., A. LUTZ and D. WISSEN, 1986 Clonal analysis of the blastoderm anlage of the Malpighian tubules in *Drosophila melanogaster.* Roux's Archiv. Dev. Biol. **195:** 22–32.

JÜRGENS, G., E. WIESCHAUS, C. NÜSSLEIN-VOLHARD and H. KLUDING, 1984 Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster.* II. Zygotic loci on the third chromosome. Roux's Arch. Dev. Biol. **193:** 283–295.

KNUST, E., U. TEPAß and A. WODARZ, 1993 *crumbs* and *stardust*, two genes of Drosophila required for the development of epithelial cell polarity. Development **Supplement:** 261–268.

LIU, S., and J. JACK, 1992 Regulatory interactions and role in cell type specification of the Malpighian tubules by the *cut, Krüppel,* and *caudal* genes of *Drosophila.* Dev. Biol. **150:** 133–143.

LIU, S., E. MCLEOD and J. JACK, 1991 Four distinct regulatory regions of the *cut* locus and their effect on cell type specification in *Drosophila.* Genetics **127:** 151–159.

MANFRUELLI, P., N. ARQUIER, W. P. HANRATTY and M. SÉMÉRIVA, 1996 The tumor suppressor gene, *lethal(2)giant larvae* (*l(2)gl*), is required for cell shape change of epithelial cells during *Drosophila* development. Development **122:** 2283–2294.

MANNING, G., and M. A. KRASNOW, 1993 Development of the *Drosophila* tracheal system, pp. 609–686 in *The Development of Drosophila melanogaster,* edited by M. BATE and A. MARTINEZ ARIAS. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

NÜSSLEIN-VOLHARD, C., E. WIESCHAUS and H. KLUDING, 1984 Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster.* I. Zygotic loci on the second chromosome. Roux's Arch. Dev. Biol. **193:** 267–282.

PANKRATZ, M. J., M. HOCH, E. SEIFERT and H. JÄCKLE, 1989 *Krüppel* requirement for *knirps* enhancement reflects overlapping gap gene activities in the *Drosophila* embryo. Nature **341:** 337–339.

PANKRATZ, M. J., E. SEIFERT, N. GERWIN, B. BILLI, U. NAUBER *et al.*, 1990 Gradients of *Krüppel* and *knirps* gene products direct pair-rule gene stripe patterning in the posterior region of the Drosophila embryo. Cell **61:** 309–317.

SKAER, H., 1989 Cell division in Malpighian tubule development in *D. melanogaster* is regulated by a single tip cell. Nature **342:** 566–569.

SKAER, H., 1993 The Alimentary canal, pp. 941–1012 in *The Development of Drosophila melanogaster,* edited by M. BATE and A. MARTINEZ ARIAS. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

SKAER, H., and A. MARTINEZ ARIAS, 1992 The *wingless* product is required for cell proliferation in the Malpighian tubule anlage of *Drosophila melanogaster.* Development **116:** 745–754.

STRAND, D., R. JAKOBS, G. MERDES, B. NEUMANN, A. KALMES *et al.*, 1994a The Drosophila lethal(2)giant larvae tumor suppressor protein forms homo-oligomers and is associated with nonmuscle myosin II heavy chain. J. Cell Biol. **127:** 1361–1373.

STRAND, D., I. RASKA and B. M. MECHLER, 1994b The Drosophila lethal(2)giant larvae tumor suppressor protein is a component of the cytoskeleton. J. Cell Biol. **127:** 1345–1360.

TEPAß, U., C. THERES and E. KNUST, 1990 *crumbs* encodes an EGF-like protein expressed on apical membranes of Drosophila epithelial cells and required for organization of epithelia. Cell **61:** 787–799.

WIESCHAUS, E., C. NÜSSLEIN-VOLHARD and G. JURGENS, 1984 Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster* III. Zygotic loci on the X chromosome and fourth chromosome. Roux's Arch. Dev. Biol. **193:** 296–307.

WINARDI, R., D. DISCHER, C. KELLEY, L. ZON, K. MAYS *et al.*, 1995 Evolutionarily conserved alternative pre-mRNA splicing regulates structure and function of the spectrin-actin binding domain of erythroid protein 4.1. Blood **86:** 4315–4322.

YOUNG, P. E., A. M. RICHMAN, A. S. KETCHUM and D. P. KIEHART, 1993 Morphogenesis in *Drosophila* requires non-muscle myosin heavy chain function. Genes Dev. **7:** 29–41.

ZHAO, D-B., S. CÔTÉ, F. JAHNIG, J. HALLER and H. JÄCKLE, 1988 *Zipper* encodes a putative integral membrane protein required for normal axon patterning during *Drosophila* neurogenesis. EMBO J. **7:** 1115–1119.

ZIPURSKY, S. L., T. R. VENKATESH, D. B. TEPLOW and S. BENZER, 1984 Neuronal development in the *Drosophila* retina: monoclonal antibodies as molecular probes. Cell **36:** 15–26.

Communicating editor: L. L. SEARLES