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The *wingless* product is required for cell proliferation in the Malpighian tubule anlage of *Drosophila melanogaster*

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Summary

Cell division in the Malpighian tubules of *Drosophila melanogaster* depends on the presence of a specialised cell at the tip of each tubule (Skaer, H. le B (1989) *Nature* 342, 566-569). Here we show that cell division also depends on the normal expression of the segment polarity gene, *wingless*. The pattern of *wingless* RNA and protein in developing tubules is consistent with a requirement for *wingless* for cell division. Analysis of the temporal requirement for *wingless* using a temperature-sensitive allele confirms that the normal expression of *wingless* is necessary during cell proliferation in the Malpighian tubules. Over-expression of the gene, induced in a stock containing the *wg* gene under the control of a heat-shock promoter, results in supernumerary cells in the tubules. We discuss the role of *wingless* in the cell interactions that govern cell division in the Malpighian tubules.

Key words: *Drosophila*, *wingless*, cell division, cell interactions.

Introduction

The development of Malpighian tubules, a simple excretory epithelium associated with the hindgut in insects, results from a sequence of cell activities which includes the specification of primordial cells, their proliferation, rearrangement and final cell differentiation. The coordination of these processes, which are common to the generation of many epithelia (see Fleming, 1992), depends on intercellular communication. In the epidermis, the cellular interactions that regulate pattern formation occur concurrently with proliferation and morphogenetic movements of the cells (Campos-Ortega and Hartenstein, 1985; Foe, 1989). Thus the study of regulatory cell interactions is complicated by the interplay between these processes. In the Malpighian tubules, however, the patterning of the epithelium takes place in stages, thus allowing an analysis of the signalling events involved in the regulation of each step in the sequence (Skaer, 1992a). Further study of one stage has shown that proliferation of the primordial cells is controlled by an interaction between an identified cell (the tip cell) and its neighbours, since ablation of the tip cell prevents further cell division in the tubule of which it is a part (Skaer, 1989).

One approach towards a fuller understanding of the nature of this interaction is the isolation of mutants that disrupt intercellular signalling. The gene *wingless* is a member of the segment polarity class and encodes a secreted product (Rijsewick et al., 1987), which is thought to act as a signal for the establishment of cell identity in the epidermis (Baker, 1988; Martinez Arias et al., 1988; Bejsovec and Martinez Arias, 1991; reviewed in Ingham, 1991). Here we demonstrate that mutants at the *wingless* locus show profound abnormalities in the development of the Malpighian tubules, including the absence of cell division in the primordia. This phenotype suggests that the *wg* product might also play a role in the signalling system underlying the regulation of cell division by the tip cell.

In this paper, we explore the temporal and spatial requirements for *wingless* to support normal embryogenesis of the Malpighian tubules. We demonstrate that *wingless* is required for all postblastodermal divisions in the proctodeum and Malpighian tubules and show that this requirement corresponds with the expression of *wg* in the tubule primordia and in a subset of the cells later in development. The importance of the *wg* product in regulating cell proliferation in the tubules is underlined by the demonstration that extra divisions can be induced in the tubules by extending the expression of *wg* under the control of a heat-shock promoter. These data allow us to analyse the possible role of the *wg* protein in the cell interactions that regulate the generation of Malpighian tubules.

Materials and methods

Fly stocks

The following strains were used: wild type, Oregon R; two enhancer trap lines, one in which the P element is regulated by the *wg* promoter (Kassis et al., 1992) and the other by the *fasc II* promoter (Grenningloh et al., 1991; A31, Ghysen and O'Kane, 1989), a null allele of *wg*, wg^{cx4} *b pr/CyO* (Baker, 1987); a ts allele of *wg*, wg^{IL114} *cn bw sp/CyO* (Nüsslein-Volhard et al., 1984), encoding a protein defective in secretion (Gonzalez et al., 1991); a strain in which *wg* is expressed under the control of a heat-shock

promoter, *HS-wg*/TM3, *hb lac z* (Nordermeer et al., 1992); and *stg*^{7B69}/TM3 (Jürgens et al., 1984).

In situ hybridisation, immunocytochemistry and embryo staining

Eggs were collected on agar/apple juice plates (Wieschaus and Nüsslein-Volhard, 1986) and kept at 25°C. They were dechorionated in bleach and embryos were staged from gastrulation.

In situ hybridisation was carried out on sectioned embryos using ^{35}S probes from a *wingless* cDNA as described in Baker (1987) and Martinez Arias et al. (1988).

Immunostaining for *wg* was performed using two antibodies (van den Heuvel et al., 1989 and Gonzalez et al., 1991), which gave similar results. Antibodies against β-galactosidase were a gift of C. Doe and immunostaining of β-galactosidase as well as incubation for *LacZ* activity followed standard protocols (Ashburner, 1989).

Flat preparations of embryos were made as described in Bate (1990). Toluidine blue staining is described in Truman and Bate (1988). Incubation in BrdU and subsequent staining of embryos was carried out as previously described (Skaer, 1989). Counts of Malpighian tubule cell numbers were made from toluidine-blue-stained flat preparations of embryos, which were selected as gastrulae and aged at 25°C before dissection.

Temperature-shift experiments

Embryos laid overnight by *wg*^{IL114} *cn bw sp/CyO* flies were dechorionated, gastrulae selected and maintained on damp blotting paper for known periods of time before shifting into Ringer (Roberts, 1986) prewarmed at 25°C. All operations prior to the temperature shift were carried out at 17.5°C. Temperature-shifted embryos were left to develop at 25°C for a further 7-8 hours before dissection to make flat preparations and staining with toluidine blue as described above. In each experiment, some embryos were allowed to hatch to check for the viability of heterozygous and homozygous balancer embryos.

Downshift experiments were carried out in exactly the same way except that the laying of eggs and preparation of embryos was carried out at 25°C before shifting to 17.5°C and the period of incubation before subsequent dissection was 18-25 hours. As before some embryos were left to hatch as controls.

Embryogenesis at the permissive temperature (17.5°C) takes twice as long as at 25°C (Wieschaus and Nüsslein-Volhard, 1986). In the description of temperature-shift experiments, all developmental times are given, for the sake of comparison, as those for 25°C.

The numbers of cells in each tubule were counted using a 63× objective on a Zeiss Standard microscope.

Heat-shock experiments

A heterozygous *HS-wg*/TM3, *hb lac z* stock (made by P. Johnston from a stock donated by G. Struhl) was used and the homozygous *HS-wg* embryos were identified by their failure to stain for *LacZ* activity. Dechorionated embryos from an overnight lay at 25°C were selected at the beginning of germ band shortening (7.75-8 hours AEL). They were shifted from room temperature (22-25°C) into prewarmed PBS at 36°C and incubated at that temperature for 20 minutes every 2 hours, the intervening periods being spent at room temperature. This treatment was repeated 3 times. The heat-shock regime was found to slow development so that after 6 hours, embryos had reached stage 14-15 (approx. 12 hours AEL). Some embryos were dissected just before cuticle deposition (14-15 hours AEL) and stained for *LacZ* activity (Ashburner, 1989) in order to identify those homozygous for *HS-wg*. Those embryos that stained for *LacZ* activity were discarded as subsequent staining with toluidine blue can obscure the *LacZ* reaction product. The remaining preparations were then stained with toluidine blue, as described above, in order to count the number of cells in their Malpighian tubules. Counts were made separately for the anterior and posterior pairs of tubules. The remaining embryos were left to complete embryogenesis in order to examine their cuticle morphology to confirm the effectiveness of the heat-shock regime. Cuticle preparations were made as described in van der Meer (1977).

Control embryos were genetically identical to the experimental stock except that they did not carry the heat-shock construct or the *hb lac z* insert.

Results

Normal development of Malpighian tubules

The Malpighian tubules arise as four separate primordia from the posterior ectodermal intucking of the gut, the proctodeum. Immunostaining of embryos from the enhancer trap line A31 (Ghysen and O'Kane, 1989, in which *LacZ* is regulated by the *fasc II* promoter (Grenningloh et al., 1991)), reveals β-galactosidase in cells of the Malpighian tubules throughout their development and can therefore be used to highlight the Malpighian tubules during embryogenesis (Fig. 1). Staining can first be observed in the extended germ band, initially in a subset of cells on the ventral side of the proctodeum (Fig. 1A) and by 5 hours after egg laying (AEL) (stage 10; Campos-Ortega and Hartenstein, 1985) as two groups of cells spreading out from the ventral midline of the proctodeum. From 5.30 hours AEL two protruberances can be seen pushing out from the proctodeum (Campos-Oretega and Hartenstein, 1985), which subdivide immediately after they evert so that by 5.75-6 hours AEL cells labelled for β-galactosidase can be resolved as four separate buds: the Malpighian tubule primordia (Fig. 1B). The number of cells in each primordium increases by cell division during the next 4 hours (Figs 1C, 2). Cells moving through the cell cycle can be labelled during S phase by the incorporation of BrdU, a thymidine analogue, which can then be stained immunocytochemically. Staining specific to the Malpighian tubules rather than the hindgut can be identified only after the tubule primordia have everted, approx. 6-6.5 hours AEL. From this time until the end of the extended germ band stage (7.75 hours AEL), cells in S phase are restricted to a lateral patch on the posterior side of each Malpighian tubule (Fig. 3C).

At 7.75 hours AEL (early in stage 12), just as germ band retraction begins, a prominent cell becomes apparent at the tip of each tubule and remains in this characteristic position as the tubule grows by further division (Fig. 1C). By 8 hours AEL, cells throughout the length of the developing tubules incorporate BrdU. The number of cells in S phase diminishes soon after this, cells ceasing to cycle earlier in the proximal region of the tubules, while more distally, cells closer to the tip cell continue to incorporate BrdU (Skaer, 1989). This pattern of staining is seen earlier in two of the tubules, at 8.25-8.5 hours AEL, while cells in the other pair continue to cycle for a further 30 minutes. Cell division follows S phase so that the mature number of cells is reached in all four tubules by the end of stage 13 (10-10.5 hours AEL; Fig. 2); the cells at the distal end of the tubules being the last to divide. The difference in the

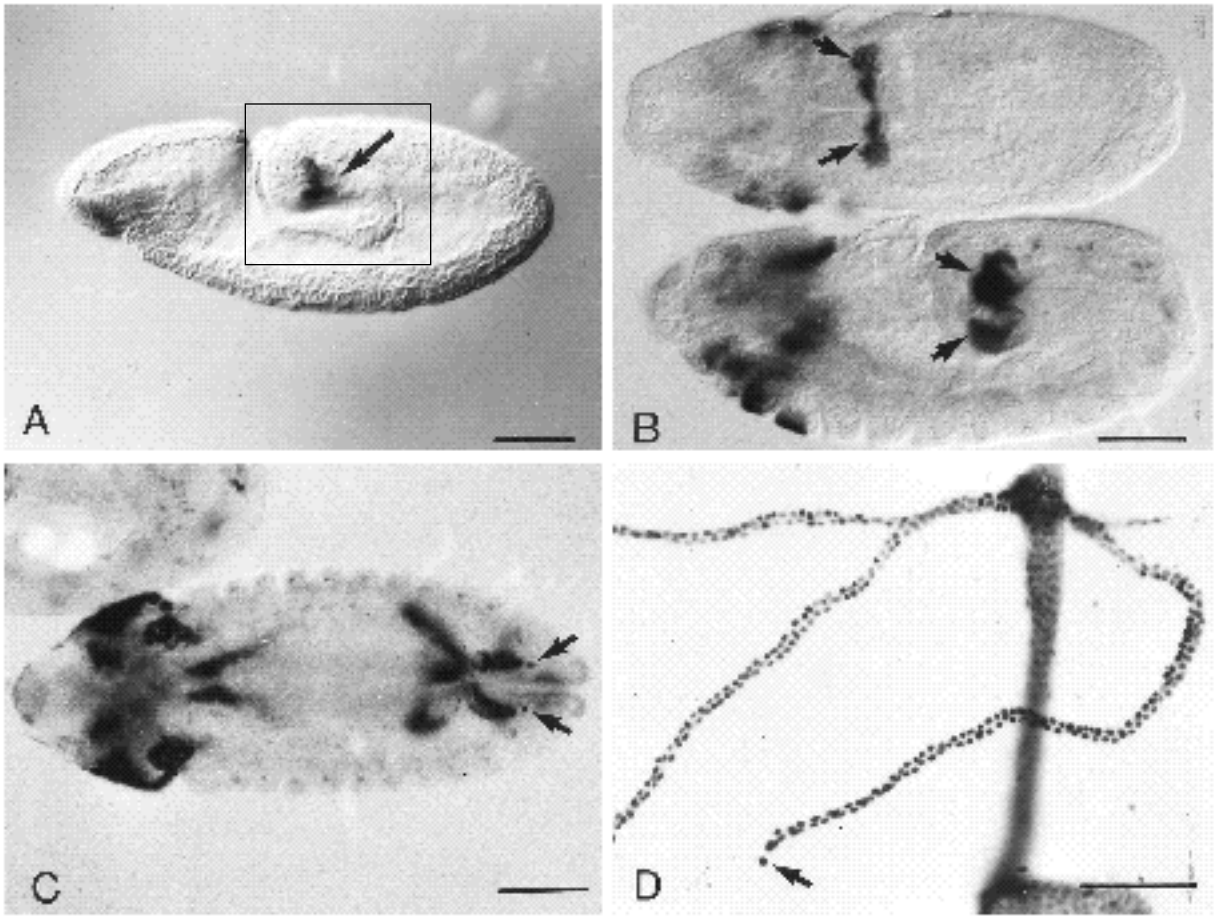

Fig. 1. Normal development of Malpighian tubules revealed by staining for β-galactosidase in the enhancer trap line A31 (Ghysen and O'Kane, 1989). (A) Early extended germ band, showing cells on one side of the proctodeum staining (arrowed). (B) Two embryos showing the four primordia as separate groups of cells (arrowed) at late extended germ band and at the beginning of germ band retraction. (C) Shortened germ band viewed from the dorsal side. Note the tip cells of the posterior tubules (arrows). (D) Tubules dissected from a 12 hour embryo. Cell division is complete and the tubules are elongating but the tip cells can still be seen (arrow). Scale bars: 500 μm. The frame drawn on Fig. 1A indicates the area of the embryo illustrated in the panels of Fig. 4.

cycling pattern of the two pairs of tubules is reflected in the final cell numbers; the posterior pair contain approx. 105 cells and the anterior pair approx. 140 cells (Fig. 2 and Janning et al., 1986). Thus it appears that the greater number of cells in the anterior tubules results from an extension of the period over which cells divide in the anterior compared with the posterior tubules (see Fig. 2). Following cell proliferation, the cells enter the first of a series of endomitotic cycles, which spread through the tubules from the proximal to the distal cells and result in a progressive increase in polyteny and consequently in cell size (Poulson, 1950; Maddrell et al., 1985; Smith and Orr-Weaver, 1991). From 10-11 hours AEL (early stage 14), rearrangement of the cells produces rapid elongation of the tubules, with the tip cells remaining in their prominent position (Fig. 1D).

The first easily identified sign of cell differentiation in the tubules is the appearance during stage 17 (from 16-17 hours AEL) of the excretory product, uric acid, in the lumen of the posterior pair of tubules before hatching (Fig. 3A) and a little later in the anterior tubules, in the first instar larva.

Phenotype of wingless *mutants*

We were not able to use the P element transformant A31 to examine tubule morphology in *wingless* mutant embryos, as there is no expression of β-galactosidase in the tubules

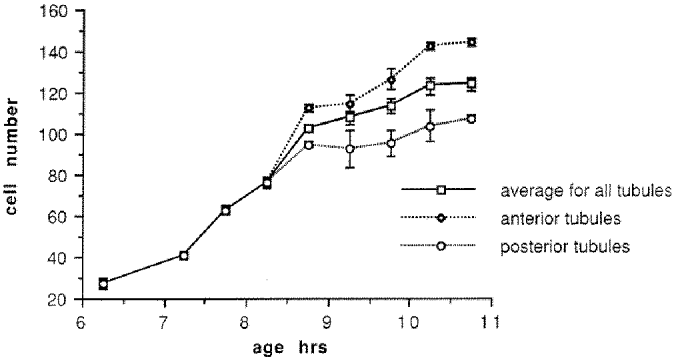

Fig. 2. Graph to show the number of cells in the Malpighian tubules at timed stages during embryogenesis, given as means ± s.e. Before 8 hours AEL the anterior and posterior pairs of tubules cannot be distinguished.

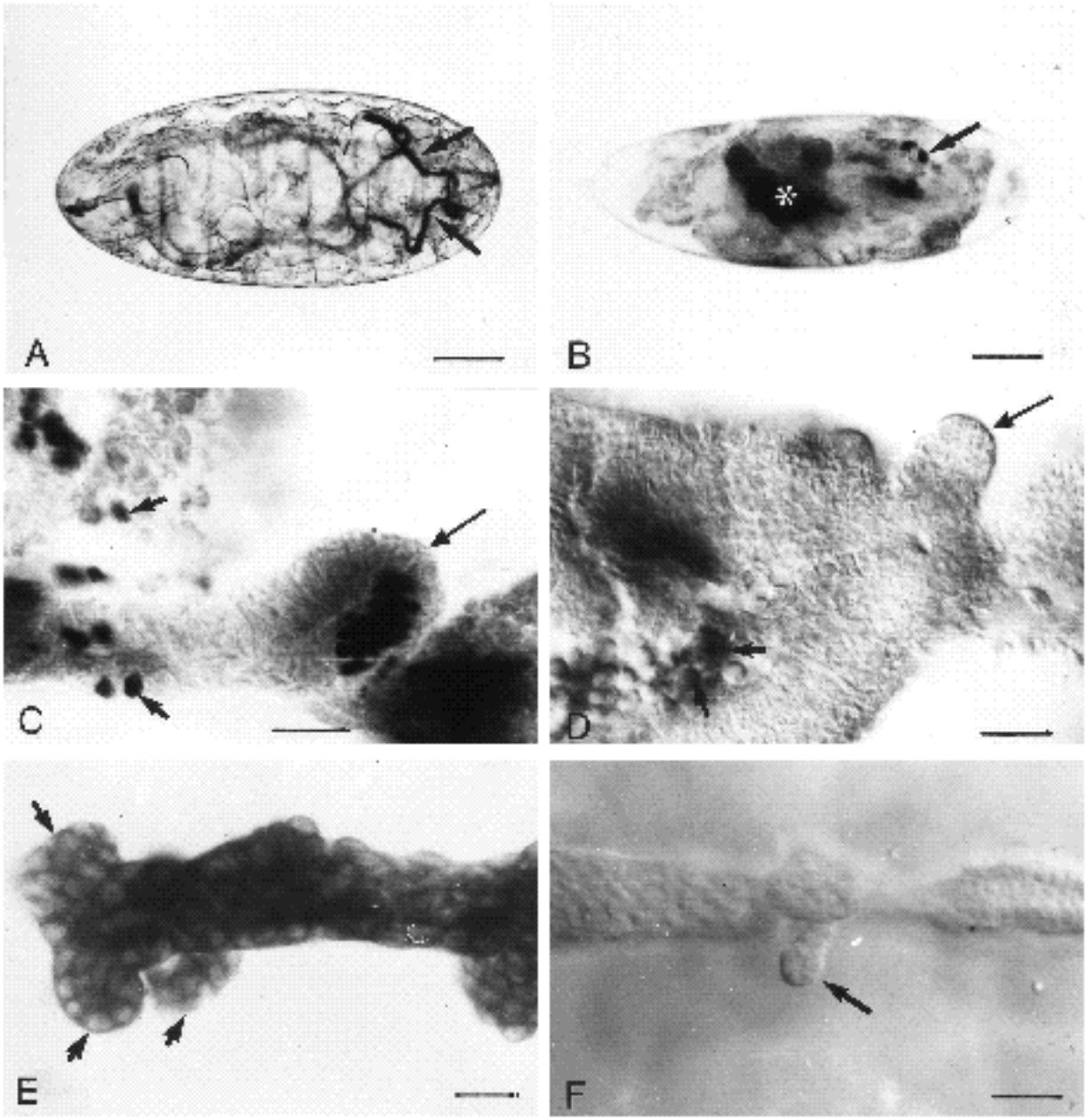

Fig. 3. (A-B) Bright-field micrographs of (A) a wild-type and (B) a wg^{cx4} embryo to show uric acid in the mature tubules (arrowed). The asterisk in B indicates undigested yolk in the mutant embryo. (C-D) Tubules dissected from wild-type (C) and wg^{cx4} (D)embryos and stained for incorporated BrdU after a 30 minutes exposure to the nucleotide, (C) starting 7.75 hours AEL and (D) approx. 6 hours AEL. Large arrows indicate the Malpighian tubules and small arrows point to cells in the midgut that have incorporated BrdU (out of focus in D). Comparison of C with D shows that in embryos null for *wg* cells in the hindgut and Malpighian tubules no longer cycle at a time when cells in the wild-type embryo are still dividing. (E-F) The hindgut and Malpighian tubules (arrows) dissected from *stg* (E) and wg^{cx4} (F)embryos; (E) stained with toluidine blue; (F) stained for β-galactosidase in a strain in which A31 has been recombined with wg^{cx4}. F shows that β-galactosidasein this construct is no longer expressed in the Malpighian tubules in *wg* embryos, although the pattern of staining is unaltered elsewhere in the embryo (data not shown). Scale bars: A-B, 500 μm; C-D, 100 μm; E-F, 200 μm.

of *wg* embryos carrying the A31 construct (Fig. 3F). However, *wg* mutant embryos dissected as flat preparations (Bate, 1990) reveal two major defects in Malpighian tubule development: two instead of four primordia appear and these two primordia remain as clusters of about 20-25 small cells (Fig. 3F). Later in embryogenesis, these primordia elongate to produce two rudimentary tubules with a prominent cell at the distal tip. Small patches of uric acid appear in the mutant Malpighian tubules towards the end of embryogenesis (Fig. 3B).

Labelling embryos mutant for *wg* at the beginning of stage 12 (7.5-8 hours AEL) with a 30 minute pulse of BrdU reveals that cells in the Malpighian tubule primordia are not replicating their DNA (cf. Fig. 3C and D). Further, in

situ hybridisation using a probe for *string*, a gene whose product is required for entry into mitosis and accumulates in cells as they pass through G_2 (Edgar and O'Farrell, 1989), shows that in *wg* mutants expression of *stg* in the proctodeum is absent (data not shown), indicating that there is no cell division in this region of the mutant embryo after gastrulation. In line with this observation, there are fewer cells in the hindgut of embryos mutant for *wg* (120-140 cf. 820 (Harbecke and Janning, 1989)).

Furthermore, the phenotype of the Malpighian tubules in embryos mutant for *stg*, in which all postblastodermal cell divisions fail (Edgar and O'Farrell, 1989), resembles their appearance in *wg* mutants: the tubules remain as clusters of about 20 cells (Fig. 3E). However, in mutants for *stg*, all four tubule primordia appear. As in embryos mutant for *wg*, the rudimentary tubules elongate with an apparent tip cell at the distal end and uric acid is produced later in embryogenesis.

In summary, it appears that *wingless* function is required for two distinct processes in tubule development; for the cellular activities underlying normal eversion of the four primordia from the proctodeum and for cell proliferation in the tubules.

Expression of wingless *in Malpighian tubules*

Localisation of *wg* mRNA by in situ hybridisation on sections of *Drosophila* embryos shows that expression in the proctodeum appears initially during extension of the germ band, on the ventral side (which at this stage of development lies closer to the dorsal side of the embryo, Fig. 4A) and can be clearly observed in the primordia of the Malpighian tubules as they evert from the proctodeum (Fig. 4C). As the tubules grow, *wg* expression is associated with the posterior side of each tubule (Fig. 4E), a pattern that persists until about 8 hours AEL.

The *wg* protein can be detected in the Malpighian tubules, although at a lower level than in the epidermis, CNS or in other parts of the gut. It is present in the proctodeum of early extended germ band embryos (4.5 hours AEL) but, like the RNA, is restricted to the ventral side. As the tubules evert from the proctodeum, all the cells in the primordia contain the protein, with a greater concentration on the luminal side of the cells (Fig. 4B). After eversion, this luminal pattern of staining persists but the protein is found only in cells on the posterior side of the tubules, extending along the whole length of each tubule (Fig. 4D,F). Like the RNA, *wg* protein disappears from one pair of tubules approx. 8.5 hours AEL, persisting in the other pair for a further 30 minutes. After approximately 9 hours, AEL *wg* protein cannot be found in the Malpighian tubules.

Further details of the expression of *wg* in the Malpighian tubules can be deduced by preparing flat preparations of embryos from a P-element insertion line in which β-galactosidase is expressed under the control of the *wg* promoter (Kassis et al., 1992). The pattern revealed by staining embryos from this line shows that, while all the Malpighian tubule cells express β-galactosidase as they evert (Fig. 5A), the staining becomes faint on the anterior side of the tubules (Fig. 5B) and in the tip cells (Fig. 5C) during germ band shortening. Taking into account that in such constructs the perdurance of β-galactosidase is often longer than that of the normal gene products (Hiromi and Gehring, 1987), this pattern of staining suggests that the expression of both *wg* and β-galactosidase becomes restricted to cells on the posterior side of the tubules as development proceeds and, further, that *wg* expression in the tip cells stops earlier than in this posterior group of cells.

Requirement for wingless *in the Malpighian tubules*

In order to establish the period during embryogenesis when functional *wg* product is required for normal development of the Malpighian tubules, we have used the temperature-sensitive (ts) allele wg^{IL114} (Nüsslein-Volhard et al., 1984). Embryos homozygous for this allele exhibit a completely wild-type phenotype when raised at 17.5°C, producing viable larvae, whereas at 25°C they show a *wg* null phenotype (Baker, 1988; Bejsovec and Martinez Arias, 1991). Thus the Malpighian tubules of embryos raised at 25°C appear as two clumps of 20-25 small cells protruding from the hindgut.

Experiments in which embryos were raised at the permissive temperature until timed stages of development before transfer to the restrictive temperature (upshift; see Materials and Methods), show that the later the shift, the milder the resulting phenotype in the Malpighian tubules (Fig. 6). Shifting to the restrictive temperature prior to 3.75 hours AEL results in the null *wg* phenotype. In mutant embryos shifted after 3.75 hours, all four primordia are present. However, shifting to the restrictive temperature between 3.75 and 7.5 hours AEL limits the final number of cells in the tubules. As the shift in temperature is performed later in development, the number of cells in each tubule approaches closer to the wild-type number. In embryos shifted after 7.5 hours AEL, the number of cells approximates to the wild type.

Downshift experiments, in which embryos are raised at the restrictive temperature before being shifted to the permissive temperature at timed stages in development, show the reverse of the upshift experiments: the later the temperature shift, the more extreme the *wg* phenotype (Fig. 6). Embryos shifted at 3.75 hours AEL, or earlier, show a wild-type Malpighian tubule morphology. Shifting after this stage results in only partial rescue of the mutant phenotype, so that, although four tubules evert, there is a reduction in the final number of cells in the tubules; the later the time of the shift, the smaller the number of cells. Embryos shifted early after gastrulation and through germ band extension have four Malpighian tubules. Embryos with two rather than four tubules are found only when the temperature shift is later than 6.5 hours AEL.

The effects of shifting temperature on the levels and distribution of the *wg* product are not immediate. The delay in loss of *wg* function after raising the temperature depends on the half life of the functional protein that remains. Studies using inhibitors to protein synthesis show that during embryogenesis the half life of the *wg* product is approx. 20 minutes (A. M. A. and F. Gonzalez, unpublished observations). The gain of function can be studied by antibody staining: the normal distribution of *wg* product has been shown to be re-established 20-30 minutes after embryos are shifted down to the permissive temperature

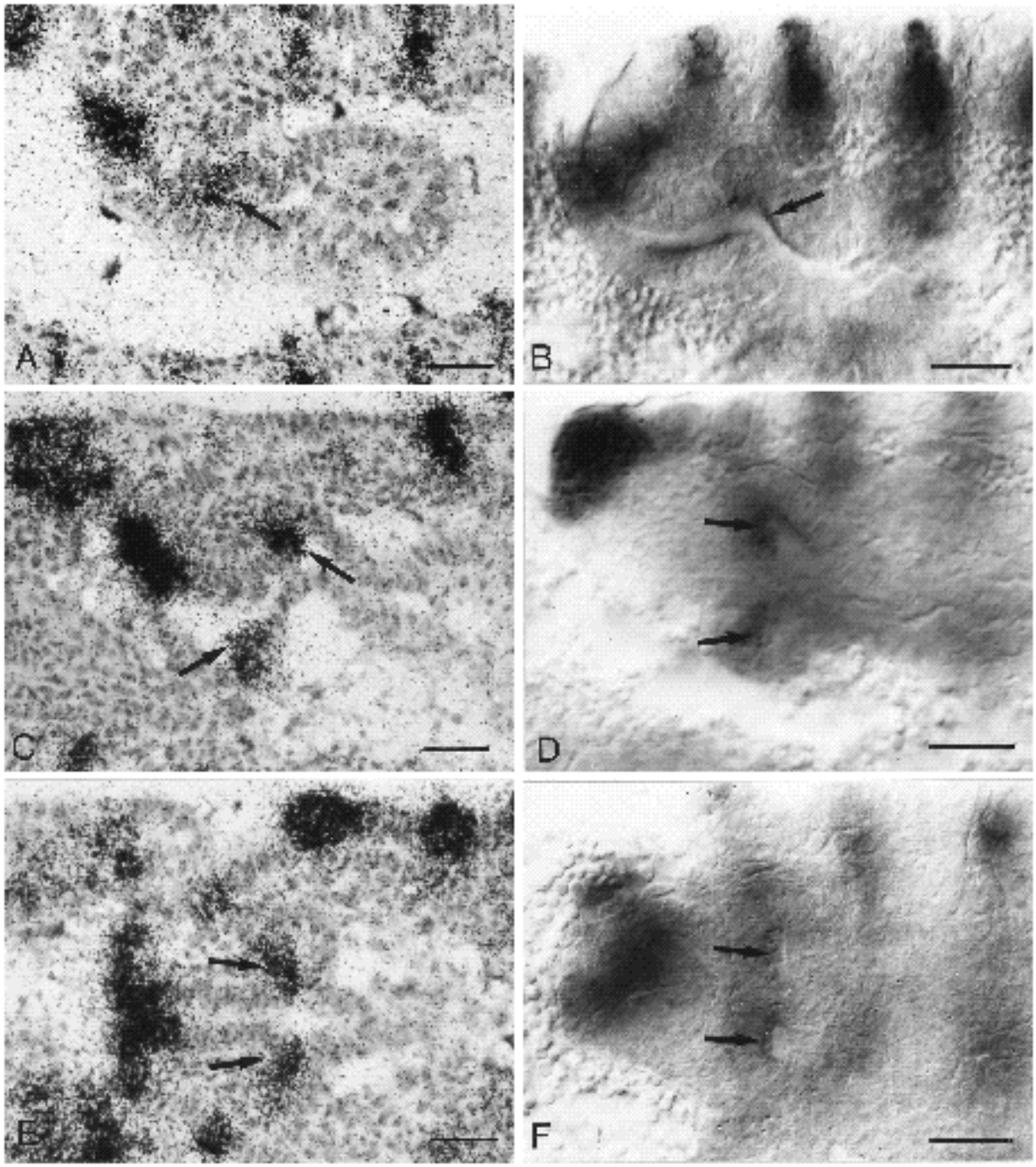

Fig. 4. Expression of *wg* in Malpighian tubules revealed by in situ hybridisation of sectioned embryos (A,C,E) and immunocytochemical staining of whole embryos (B,D,F). The area of the embryo shown in each photograph is outlined by the frame in Fig. 1A, viewed laterally in A-C and from the dorsal side in D-F. The label in each case is arrowed. (A and B) Early extended germ band (approx. 5 hours AEL); (C and D) 6-7 hours AEL; (E and F) 7.5-8 hours AEL. *wg* RNA and protein are both found predominantly on the posterior side of the Malpighian tubules (arrowed in D-F) and the protein is located on the luminal side of the cells (arrows in B,D,F). Scale bars: 200 μm.

(Bejsovec and Martinez-Arias, 1991). Taking this information into account, upshift experiments indicate that the period during which *wg* function is required for normal tubule development is until at least 8 hours AEL. Similarly, downshift experiments show that rescue of the phenotype is not discernible if the production of a functional *wg* product is initiated later than 7.5-8 hours AEL.

Extended expression of wg *in the Malpighian tubules*

The temperature shifts of wg^{IL114} embryos suggest that the *wg* protein is involved in the regulation of cell division in

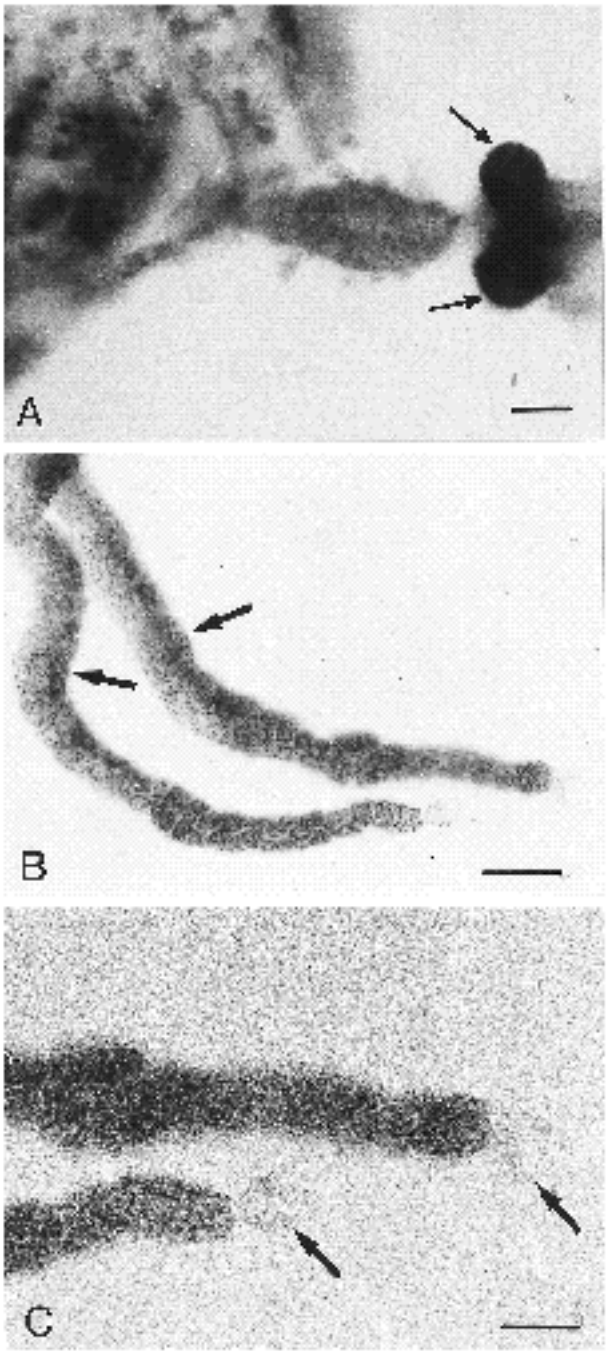

Fig. 5. Embryos stained for β-galactosidase from a line in which the enzyme is expressed under the regulation of the *wg* promotor. (A) Flat preparation of an extended germ band embryo to show staining in the Malpighian tubule primordia (arrowed). (B) Malpighian tubules dissected from an embryo 10 hours AEL. The staining is stronger on the posterior side of the tubules (arrows). (C) The same tubules at higher magnification to show that the stain in the tip cells is weaker (arrowed) than in the remaining cells of the tubules. Scale bars: A, B, 200 μm; C, 100 μm.

the Malpighian tubules. To test this hypothesis further, we have made use of a *wg* gene under heat-shock control (Nordermeer et al., 1992) to express the *wg* product in all Malpighian tubule cells at a time when, in the wild type, these cells have ceased to express *wg*. The appearance of supernumerary cells in the tubules of these embryos would confirm a role for *wg* in the regulation of cell division.

Table 1. *Number of Malpighian tubule cells in heat-shock and control embryos*

	Heat shock *wingless*	*rosy* control
Anterior tubules	164±3.5 (*n*=25)	138±1.7 (*n*=25)
Posterior tubules	104±1.0 (*n*=16)	111.0±1.8 (*n*=21)

Figures are given as means/tubule±s.e.
n=number of Malpighian tubules counted.

The timing of heat shock was arranged so that embryos at the beginning of germ band shortening (7.75-8 hours AEL) were induced to express *wg* continuously for a further 6 hours. Since the time taken to induce the expression of *wg* under the heat-shock promoter is approx. 20 minutes (Nordermeer et al., 1992), embryos actually expressed *wg* ectopically from 8.3 hours AEL. This was confirmed by staining heat-shocked embryos immunocytochemically (data not shown, see Nordermeer et al., 1992).

Table 1 shows the number of Malpighian tubule cells in embryos carrying the heat-shock construct and in a control stock. In embryos carrying the heat-shock construct, the anterior Malpighian tubules are made up of significantly more cells than in control embryos (P<0.01%) or in the wild type (Fig. 2 and Janning et al., 1986). In contrast, the number of cells in the posterior tubules shows no significant difference between heat-shock, control (Table 1) or wild-type stocks (Fig. 2 and Janning et al., 1986).

Discussion

wingless *is required for the establishment and proliferation of the Malpighian tubule primordia*

During normal development, the Malpighian tubules arise from the proctodeum with which they share a blastodermal anlage (Technau and Campos-Ortega, 1985), so that the early postblastodermal divisions contribute cells both to the Malpighian tubules and to the hindgut. The tubule primordia evert from the proctodeum, initially as two outpocketings which very soon subdivide to produce four buds of cells, the tubule primordia (Campos-Ortega and Hartenstein, 1985). The absence of *wingless* interferes with this process so that two rather than four primordia appear. We have shown that cells in the Malpighian tubule anlage do not proliferate in the absence of *wg*. However, the failure of cell division per se is insufficient to explain the *wg* phenotype, since four primordia appear in embryos mutant for *stg*, in which there are no postblastodermal divisions. Instead the loss of two tubules in the absence of *wg* must result from the mis-specification of cells or from abnormalities in tubule eversion.

Experiments with the ts allele (wg^{IL114}) show that *wg* is required before 4 hours AEL to form four Malpighian tubules. Interestingly, restoring *wg* function after this time can rescue the appearance of four Malpighian tubules (Fig. 6). Since the allocation of cells to a tubule fate must occur between 3 hours 10 minutes (Technau and Campos-Ortega,

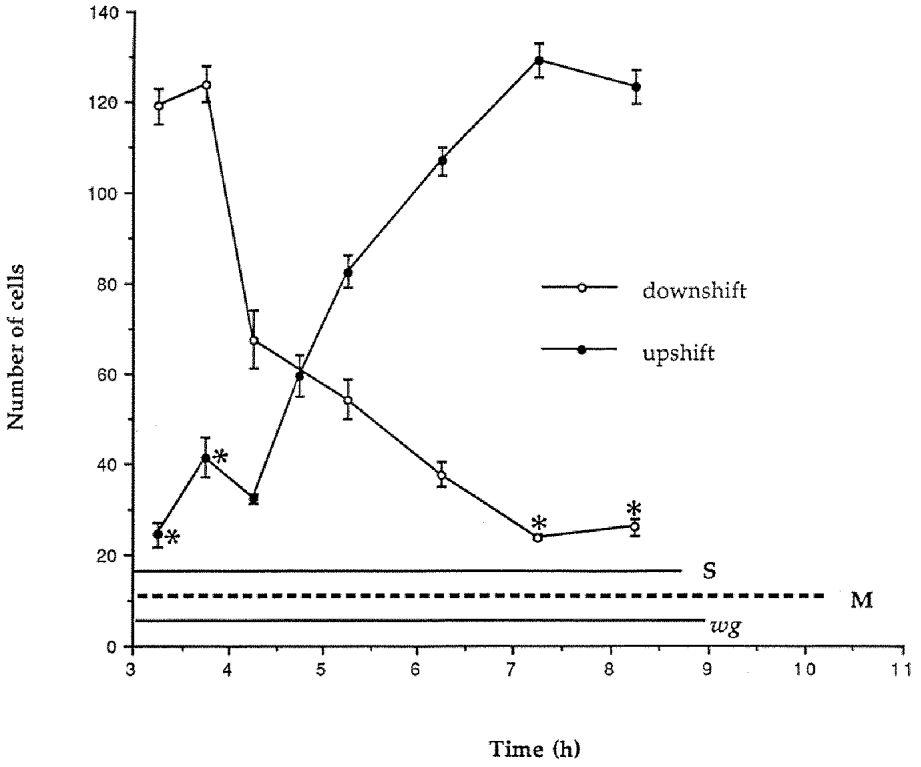

Fig. 6. Diagram to show the effects on the Malpighian tubules of shifting wg^{IL114} embryos from the permissive to the restrictive temperature (upshifts) and from the restrictive to the permissive temperature (downshifts) at different stages of development. Asterisks indicate that the embryos have two rather than four Malpighian tubules. The figures are given as the average for all four tubules as it is impossible to distinguish between the two sets of tubules until each tubule contains over 90-100 cells, as in normal development (see Fig. 2). This observation indicates that temperature shifts affect the anterior and posterior tubules in a closely parallel way. S,M, periods during embryogenesis when cells in the Malpighian tubules are in S phase (S) prior to mitosis (M). (*wg*) indicates the period when *wg* product can be detected in the Malpighian tubules during normal development.

1985) and tubule eversion at 5 hours 30 minutes, these results indicate that *wg* could be involved initially either in the allocation of cells to make one pair of tubules or in the subdivision of the initial protruberances. Since rescue of the four Malpighian tubules is possible over a period that extends for an hour beyond the time when tubule eversion can first be seen in wild-type embryos, it is more likely that *wg* is required to mediate the changes in cell adhesion, shape or motility that normally subdivide the initial primordia.

A further striking characteristic of the Malpighian tubules of embryos lacking *wg* function is that the cells of the tubule primordia fail to proliferate. In line with these observations, *wg* is expressed in the proctodeum, in the everting tubule primordia and in the growing tubules, in a pattern that is consistent with a requirement in or close to cycling cells. Further evidence that *wg* is involved in promoting cell division is provided by the demonstration that over-expression of *wg*, under the control of a heat-shock promoter, can result in extra divisions to produce supernumerary cells in the tubules.

Temperature-shift experiments indicate that *wg* is continuously required for cell proliferation from shortly after gastrulation until 8 hours AEL and that rescue of the mutant phenotype is possible over a similar period of embryogenesis (Fig. 6). This represents a minimum estimate for the period of *wg* requirement as the normal variation in the final number of cells in the tubules (s.d. ±20) means that the division of the last cells to proliferate (15 cells in the last hour; see Fig. 2) cannot be discerned simply by counting cells. However, as shown in Fig, 6, the periods of both *wg* requirement (8 hours) and expression (to 9 hours AEL) are shorter than the observed period of cell division in the tubules (to 10.25 hours AEL; see also Fig. 2). This suggests that *wg* activity is required early in the cell cycle to stimulate the next cell division.

Temperature-shift experiments also show that the effects of removing or restoring *wg* function in the tubules depend on the age of the embryo at the time of the shift. A change in *wg* expression has a more profound effect on the phenotype early after gastrulation, when many or all of the cells divide in the proctodeum in the wild-type embryo. Later, when only a subset of tubule cells divides during normal development (Fig. 2 and Janning et al., 1986), the removal or restoration of functional *wg* has only a slight effect on the final number of cells in the tubules (Fig. 6). Thus, even in the absence of functional wingless protein, with the consequent arrest of cell cycling in the proctodeum and the tubules, the normal programme for cell division in these tissues is retained. The restoration of functional wingless protein triggers the number of cell divisions appropriate to the age of the embryo.

In a similar way, the normal programme of cell proliferation limits the effects of extending the expression of *wg*. Although expression beyond 8 hours AEL results in supernumerary cells in the anterior tubules, there are no extra divisions in the posterior tubules. Even in the anterior pair, the production of supernumerary cells is limited; only approx. 26 out of a possible approx. 138 cells (Table 1) enter an additional round of division, indicating that factors other than the absence of wingless protein restrict the ability of cells to continue dividing. In normal development, cell proliferation stops earlier in one pair of tubules than the other. A similar discrepancy in response to the extended expression of *wg* suggests that there is a limited period after normal proliferation is complete when the presence of

wingless protein promotes entry into a further division cycle.

These observations are in line with those of Edgar and O'Farrell (1990), who found that the ectopic expression of *stg* under the control of a heat-shock promoter triggered mitosis in all cells during cycles 14-16. However, following mitosis 16, cells became unresponsive to the presence of string, suggesting that other regulatory factors become limiting at this stage of development.

Interaction of wingless *with the tip cell*

After eversion of the tubule primordia, cell division in the tubules depends not only on the normal expression of *wg* but also on the presence of a tip cell in each tubule (Skaer, 1989). Although these cells become apparent some time after the primordia form in *Drosophila*, they are evident from the time that the tubules evert in another species, *Rhodnius prolixus*, in which their role in regulating cell division in the tubules has also been demonstrated (Skaer, 1992b). One model suggested for the activity of the tip cell is that it secretes a signal, stimulating cells close to it to cycle and divide (Skaer, 1989). Several lines of evidence suggest that wingless is secreted from cells expressing it (Morata and Lawrence, 1977; Baker, 1987; Rijsewijk et al., 1987; van den Heuvel et al., 1989; Gonzalez et al., 1991) raising the possibility that *wg* is secreted by the tip cells and regulates division in neighbouring cells in the tubules. However, two aspects of the expression pattern of *wg* argue against this suggestion. Firstly, *wg* expression can be shown to decline in the tip cells before other tubule cells and, secondly, the protein is found in many cells of the tubules in a pattern indicating that *wg* might be required in or close to the cells that are moving through the cell cycle. Furthermore, there is a difference in the effects of removing either the tip cell or *wg* function on the final number of cells in the Malpighian tubules (tip cell ablation at 7.75 hours AEL, 75±4 cells (Skaer, 1989); temperature shift to remove *wg* function at the same stage, 123±4 cells). This discrepancy might be explained if the activities of *wg* and the tip cell are required at different stages in the cycle. An early requirement for *wg* (e.g. to initiate S phase) and a later requirement for the tip cell (e.g. to initiate mitosis) would result in immediate arrest of cell division after tip cell ablation, while the effects of removing *wg* function would be felt only after cells had progressed through the cycle to re-enter S phase. It should be noted that this would not preclude a more direct interaction between the tip cell and *wg*, as the early consequences of tip cell ablation would mask the later effects of perturbing this interaction.

Our studies have therefore identified two factors that are involved in regulating cell division in Malpighian tubule primordia; the expression and normal secretion of *wg* (reported here) and the activity of the tip cells (Skaer, 1989; 1992b). The response of the Malpighian tubules to *HS wg* expression suggests an interplay between the *wg* gene product and the activity of the tip cells; supernumerary cells can be induced in the tubules for only a limited period by extending the expression of *wg*. The end of this period coincides with the proposed decline in tip cell activity (Skaer, 1989), which suggests that the continued activity of the tip cell is required for the presence of *wg* to be effective. Indeed the observation that it is possible to stimulate the production of supernumerary cells in the tubules, by extending *wg* expression, indicates that in normal development it is the decline of *wg* expression, at a time when the tip cells are still active, that stops cells cycling mitotically in the tubules.

The precise nature of the interaction between *wg* and the tip cells is not yet known. However, the ultrastructure of tip cells suggests that they have a secretory function (Skaer, 1989; 1992b). This observation raises the possibility that products secreted by the tip cells interact synergistically with *wg* to stimulate a cellular response. Such interactions have been demonstrated in the activation of other growth factors such as basic fibroblastic growth factor, which stimulates a response only after it has bound to components of the extracellular matrix (Yayon et al., 1991). Analysis of products secreted specifically by the tip cells is required to understand the interaction with *wg* at a molecular level.

The mammalian homologue of wingless *also regulates cell division*

The protein encoded by the *wg* gene is a member of the *Wnt* family (Rijsewijk et al., 1987; Nusse et al., 1991), which includes *int-1*, a mammalian proto-oncogene (Nusse et al., 1984). When expressed ectopically, *int-1* stimulates cell division in mammary tissue, which can result in the production of tumours (Tsukamoto et al., 1988). We find an interesting parallel between the effects of *int-1* in mammary epithelia and those of *wg* in embryonic tissues of *Drosophila*: in both cases, expression of the gene stimulates cell division but only in certain tissues and under certain circumstances. We are currently investigating the possibility that further parallels in the modes of secretion and activity of these molecules might underlie similarities in the consequences in their expression.

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