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AN ANALYSIS OF INTERMEDIATE SEGMENT-POLARITY PHENOTYPES IN *DROSOPHILA* EMBRYOS USING A TEMPERATURE-SENSITIVE ALLELE OF *WINGLESS*.

BY

SUSAN JANE MINAKER

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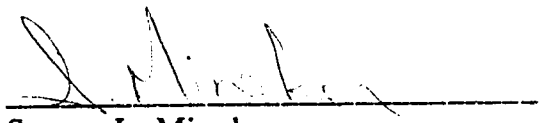

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The undersigned certify that they have read, and recommend to the faculty of graduate studies and research for acceptance, a thesis entitled: AN ANALYSIS OF INTERMEDIATE SEGMENT-POLARITY PHENOTYPES IN *DROSOPHILA* EMBRYOS USING A TEMPERATURE-SENSITIVE ALLELE OF *WINGLESS*. submitted by SUSAN MINAKER in partial fulfillment of the requirements for the degree of Master of Science.

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Date: 26th March 1991

ABSTRACT

In order to understand the generation of the segmented embryonic cuticular pattern in *Drosophila melanogaster*, a temperature-sensitive allele of the segment-polarity gene, *wingless*, was used to create a series of intermediate embryonic phenotypes in two ways. First, the embryos were raised for varying lengths of the temperature-sensitive period (TSP) (which is from germ band extension to germ band shortening) at the restrictive temperature, and second, they were raised at semi-permissive temperatures, creating a series of hypomorphs. Shifting up during the TSP is quantitatively different from shifting down. The phenotypes resulting from shifting up are similar to the series of hypomorphs. When the strength of the mutant phenotype increases in the hypomorphs, the denticle belts broaden as ectopic denticles appear on either side and within them. Polarity reversal becomes evident when the belts touch each other. In a weak *wingless* background, regions normally naked cuticle may take on a denticle-producing identity; in a strong background, most of these regions may be lost through cell death with subsequent polarity-reversal. There is evidence for cell death in at least the strong *wingless* background, so cell death in the posterior compartment was studied by observing *engrailed*-regulated expression of *E. coli* ß-galactosidase in *wingless* intermediates and comparing it to the corresponding cuticular phenotype. An extreme *wingless* phenotype causes loss of all of the initially normal expression in the epidermis beyond the prothorax; intermediate *wingless* causes loss of expression first from the odd-

numbered segments and the more posterior segments in a pattern reminiscent of the generation of the *engrailed* pattern. In recent years, the idea that gradients are involved in patterning each segment has been abandoned in favour of models for cell states and cellular interactions; however, the results presented here suggest that *wingless* is involved in the generation of a gradient, possibly through a cell signaling mechanism, rather than through a diffusable morphogen. Interactions between cell states and a gradient of information may allow fine-tuning of the segmental pattern.

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LIST OF ABBREVIATIONS

ß-gal.....ß-galactosidase

A.....anterior

A/P.....anterior/posterior

A1-A8.....Abdominal segments 1 to 8

arm.....*armadillo*

b.....*black*

blast(c).....cellular blastoderm

blast(s).....syncytial blastoderm

bw.....*brown*

ci^{D}.....*cubitus interruptus Dominant*

cn.....*cinnabar*

CNS.....central nervous system()

Cy.....*curly*

dc.....dorsal closure

Df.....deficiency

DMF.....dimethyl formamide

egb.....extended germ band

en.....*engrailed*

ftz.....*fushi-tarazu*

gbe.....germ band extension

gbs.....germ band shortening

gsb.....*gooseberry*

gst.....gastrulation

KO.....Keilin's organs

m.u.....map unit

MS.....mesothorax (also T2)

MT.....metathorax (also T3)

nkd.....*naked*

P.....posterior

PBS.....phosphate buffered saline

pc(e).....early pole cell formation

pc(l).....late pole cell formation

prd.....*paired*

PRO.....prothorax (also T1)

ptc.....*patched*

r1-r7.....row 1 to row 7 of denticles

ry.....*rosy*

sha.....*shavenoid*

st2.....stage 2

T1-T3.....thoracic segments 1 to 3

TS.....temperature-sensitive

TSP.....temperature-sensitive period

wg.....*wingless*

X-gal.....5-bromo-4-chloro-3-indolyl ß-D-galactoside

INTRODUCTION

The genetic control of development in the fruit fly, *Drosophila melanogaster*, has been extensively studied and the combination of genetic and molecular analyses has generated a vast amount of information about the genes and processes required to turn a single fertilized egg cell into a complex organism composed of numerous cells, or groups of cells, each with a specialized identity and function, yet organized in a cooperative manner.

The zygotic nuclei of the *Drosophila* egg undergo several synchronous divisions without cytoplasmic division. After the seventh nuclear division, most of the nuclei migrate to the periphery of the egg cell, forming the syncytial blastoderm, where they undergo four more rounds of division before cell membranes form between them to give rise to the cellular blastoderm. As the syncytial blastoderm is forming, 2-3 nuclei migrate to the posterior pole where they undergo several more rounds of division to become the pole cells, which are precursors of the germ line (Campos-Ortega and Hartenstein, 1985). Shortly after cellularization of the blastoderm, the process of gastrulation rearranges the single cell layer of the blastoderm to establish the three germ layers of the gastrula: the ectoderm, the mesoderm, and the endoderm (Bownes, 1982). The mesoderm and the overlying ectoderm form the germ band (Turner and Mahowald, 1977), which extends around to the dorsal side of the embryo in a process known as germ band extension. After the germ band has fully extended, the first morphological evidence of metamerization

appears with the formation of grooves along the germ band; these metamers will eventually form six head (Jürgens, et. al.. 1986), three thoracic, and eight abdominal segments. The germ band then proceeds to retract back to its original position, followed by dorsal closure (the germ band extends laterally in both directions around the embryo to meet at the dorsal surface (Bownes, 1982)) and head involution to give rise to a larva that externally displays three thoracic and eight abdominal segments; the eighth abdominal segment is fused with three more abdominal segments, which are morphologically unidentifiable, as well as the unsegmented telson to form the tail region of the embryo (Jürgens, 1987).

One of the more intriguing, and certainly much studied questions in *Drosophila* development is how the series of segmental repeats found along the anterior-posterior axis of the animal are formed. These segments can be most clearly discerned in the larval cuticle, which displays a series of denticle belts on the ventral side of the body. The denticle belts are associated with each of the thoracic and abdominal segments and lie at the anterior border of each segment, with the border being defined morphologically as the major line of longitudinal muscle attachment sites (Szabad, et. al., 1979), resulting in a visible groove in the epidermis of the larva. Another border, which is not morphologically apparent in the larva, exists within each of these segments to separate the anterior and posterior compartments. A compartment is a region of the body that is derived from one set of 'founder' cells; descendants of these cells will not mix with cells from an adjacent compartment in the wildtype animal.

These compartments have been defined by clonal analysis in the imaginal discs (Garçia-Bellido, et. al., 1973, 1976), but not conclusively in the larval segments. Although gynandromorph studies revealed a clonal restriction at the segmental borders in the larval epidermis (Szabad, et. al., 1979), a clonally restricted border within the larval segments was not detected. The presence of discrete compartments within the segments, that is, anterior and posterior compartments, is assumed from the compilation of much genetic evidence. The gene, *engrailed*, is required to restrict cells to the posterior compartment in wing discs (Morata and Lawrence, 1975) as well as in the adult abdomen (Kornberg, 1981). Expression of this gene is restricted to the posterior cells of the discs (Hama, et. al., 1990; Brower, 1986) and of each embryonic segment at the extended germ band stage (Hama, et. al., 1990; Karr, et. al., 1989; Kornberg, et. al., 1985; Fjose, et. al., 1985; DiNardo, et. al., 1985; Weir and Kornberg, 1985). Recently, Hama, et. al. (1990), showed that the posterior border of *engrailed*-regulated ß-galactosidase expression lies between the first two rows of abdominal denticles in the larva, agreeing with the previous placement of the segmental border (Szabad, et. al., 1979). They also showed that the anterior border of ß-galactosidase extends to, but not beyond, the Keilin's organs of the thoracic segments. The Keilin's organs are located at the compartmental boundary, as defined by the borders of the regions that are affected by genes of the bithorax complex (Struhl, 1984). Thus *engrailed* expression is restricted to a posterior compartment in each segment.

These compartmental borders define the borders of parasegments (Martinez-Arias and Lawrence, 1985). During germ band extension, the grooves that appear as the first visible sign of metamerization define the boundaries of the parasegments (Ingham, et. al., 1985; Martinez-Arias and Lawrence, 1985), each of which includes one posterior compartment and the anterior compartment from the adjacent segment. Later, metamerization is reorganized into the segments that are seen in the larval epidermis. Therefore, the parasegmental boundary lies at the posterior edge of the anterior compartment and the segmental boundary lies at the posterior edge of the posterior compartment (see Figure 30): the embryo can be thought of as consisting of a series of alternating anterior and posterior compartments.

The primordia of the embryonic segments can be mapped onto the blastoderm; when cells at this stage are damaged, such as by an ultraviolet laser microbeam (Lohs-Schardin, et. al., 1979), damage at a specific location on the blastoderm can be associated with localized defects in the larval cuticle (Lohs-Schardin, et. al., 1979). This suggests that, by the time the blastoderm has cellularized, cell fate is 'determined'; that is, the cells have become committed to a particular developmental pathway and cannot change their fate to replace the damaged cells. From this type of experiment, a blastoderm fate map can be produced, and has been done for the thoracic and abdominal segments (Lohs-Schardin, et. al., 1979) as well as for the head (Jürgens, et. al., 1986) and the tail (Jürgens, 1987) regions of the embryo.

Since there are several more cell divisions between formation of the cellular blastoderm, when determination of larval cells has occurred, and eventual cell differentiation, the determined states must be heritable so that the progeny of a determined cell will not later undergo a fate change. The discovery of compartments and the heritability of cell fate led to the proposal of a selector gene hypothesis (Garçia-Bellido, 1975), where a selector gene would differentiate between two states by being on in one compartment and off in the other: *engrailed* would appear to be the selector gene for the posterior compartment. Each compartment would subsequently be subdivided according to this binary code by several more selector genes so that a tissue, or an embryonic segment, would ultimately consist of a mosaic of cell states, each of which is defined by a unique combination of selector genes. This combination of selector genes would be required for maintenance of the determined states through several more cell divisions until the cells differentiate.

The process of development in the *Drosophila* embryo that leads to the final segmented pattern seen in the larva requires a hierarchy of genes, with the genes in each level of the hierarchy requiring the genes in the previous level to initiate or modify their expression (reviewed by Ingham, 1988).

Initially, the coordinate genes, which have a maternal effect, are required to establish the asymmetry of the embryonic axes. Mutations in the genes required for the anterior/posterior axis cause a deletion of structures at one of the termini, which is frequently

accompanied by duplication of the remaining terminus. For example, mutations in the gene, *bicoid*, delete head and thorax structures and replaces them with an inverted duplication of the telson (Frohnhöfer and Nüsslein-Volhard, 1986). The product of the *bicoid* gene has been found to be a graded morphogen along the anterior-posterior axis (Driever and Nüsslein-Volhard 1988a,b) and is required for determination in the anterior end of the embryo (Frohnhöfer and Nüsslein-Volhard, 1986). The information provided by the coordinate genes regulates the expression of the gap genes, which subdivide the embryo into several large regions along the anterior-posterior axis. Mutations in the gap genes cause deletions of continuous regions of the embryo, consisting of up to eight segments (Nüsslein-Volhard and Wieschaus, 1980), and are expressed in overlapping contiguous blocks along the embryo (Lehmann and Frohnhöfer, 1989). The gap genes spatially organize the expression of the pair-rule genes into a series of stripes in order to generate the parasegmental boundaries. Mutations in the pair-rule genes delete alternate segments of the embryo (Nüsslein-Volhard and Wieschaus, 1980). Alternate parasegmental boundaries require different pair-rule genes; for example, *fushi-tarazu* (*ftz*) is required to form the even-numbered parasegments (Lawrence and Johnston, 1989; DiNardo and O'Farrell, 1987; Howard and Ingham, 1986) and *paired* (*prd*) is required for the odd-numbered parasegments (DiNardo and O'Farrell, 1987). Furthermore, *fushi-tarazu* and *even-skipped* (*eve*) appear to be required to allocate cells to parasegments, with the anterior borders of *fushi-tarazu* expression demarcating the anterior border of the even-numbered parasegments and *even-skipped* demarcating the

anterior border of the odd segments (Lawrence and Johnston, 1989; Lawrence, et al, 1987). The *fushi-tarazu* and *even-skipped* pair-rule genes are required to establish the expression of the segment-polarity genes, *engrailed* and *wingless* (Ingham, et. al., 1988), which are subsequently required for proper patterning within each segment. As well as regulating gene expression in the next level of the hierarchy, the gap, pair-rule, and segment-polarity genes also regulate each other (*eg*: Jäckle, et. al., 1986; Harding, et. al., 1986; Martinez-Arias, et. al., 1988), probably to refine and maintain each other's domains of expression.

In mutants of the segment-polarity class of genes, a portion of each segment is missing and replaced with a duplication of the remaining part in mirror-image symmetry (Nüsslein-Volhard and Wieschaus, 1980). In mutants for one group of these genes, naked cuticle from the posterior region has been deleted and replaced with denticles from the anterior region, to give an embryo covered in denticles. Four of these genes have been cloned and their transcript expression pattern determined: *armadillo* (*arm*) transcripts are expressed uniformly over the embryo (Riggleman, et. al., 1989); whereas *gooseberry* (*gsb*) (Côté, et. al., 1987; Baumgartner, et. al., 1987), *cubitus interruptus Dominant* (*ci*^{D}) (Orenic, et. al., 1990; Eaton and Kornberg, 1990), and *wingless* (*wg*) (Baker, 1987) transcripts are expressed in a spatially restricted and periodic manner, although not in the same manner as each other, despite the similarities in mutant phenotypes (see Table 8).

The gene, *wingless*, has attracted much attention due to the fact that its protein product is homologous to the murine mammary oncogene, *int-1*. The first allele of *wingless* to be discovered was an adult viable allele (wg^1) (Sharma, 1973), which causes a wing to notum transformation (Sharma and Chopra, 1976). Several lethal alleles, including one temperature-sensitive one, (Babu, 1977; Nüsslein-Volhard and Wieschaus, 1980; Nüsslein-Volhard, et. al., 1984) give the embryonic phenotype described above (Baker, 1987).

Several lines of evidence suggest that *wingless* is involved in cell-cell communication. The mutant phenotype is not cell autonomous in mosaics in imaginal discs (Morata and Lawrence, 1977) or in embryonic tissue (Wieschaus and Riggleman, 1987). Mutations of *wingless* affect regions of the segment in which transcripts of this gene have not been detected (Baker, 1987): *wingless* transcripts, which first appear during blastoderm at the anterior pole and in the posterior region, accumulate in 14 stripes at a position corresponding to the posterior edge of the anterior compartment in each segment, but mutations affect the more posterior region in which *engrailed* is expressed, as well as the more anterior region consisting of naked cuticle. Although *wingless* and *engrailed* are expressed in non-overlapping regions, wildtype *wingless* function is required to maintain the normal expression of *engrailed* (Martinez-Arias, et. al., 1988; DiNardo, et. al., 1988). Further evidence for the role of *wingless* in cell-cell communication is that the protein contains sequences characteristic of a secreted protein (Rijsewijk, et. al., 1987) and has been seen in vesicles outside of the cells that express *wingless* (van

den Heuvel, et. al., 1989). Also, *wingless* is homologous to the murine mammary oncogene, *int-1* (Rijsewijk, et. al., 1987), which has been shown to enter the secretory pathway (McMahon and Moon, 1989). Therefore, *wingless* may be involved in inter-cellular signaling in order to allow coordinated pattern formation in the developing organism.

Numerous explanations for the generation and patterning of segmental repeats have been proposed. The idea of gradients has long been entrenched in the field of insect development (reviewed by French, 1988). Wolpert (1969) put forward the theory of positional information in which each cell in a developing tissue would read a value from the gradient based upon its location within that tissue. In order to generate a repeated pattern, a gradient of a morphogen could exist within each segment. If there is a source at one border and a sink at the other border, then there would be an abrupt change from high to low levels of the gradient at the segmental border (Locke, 1959, 1960), forming a 'sawtooth-like' wave along the anterior/posterior axis and implying that the segmental border must serve as a physical barrier to the diffusion of the morphogen. After surgical removal of parts of the segment, a smoothing of the gradient could account for the regeneration of the missing pattern elements as has been seen (Wright and Lawrence, 1981). However, the segmental border could not be expected to regenerate in a similar manner, but surgical experiments in *Oncopeltus* have demonstrated that the border does behave as any other reiterated pattern element in the segment, and can be

regenerated after its removal (Wright and Lawrence, 1981). Therefore, if a gradient is responsible for the patterning of the insect segments, the transition from one segment to the next could be smoother, such as with the waves suggested by Russell (1985), so that there is not a sharp transition at each border, and the border can then behave as any other iterated pattern element along the axis. This model requires the superimposition of two waves, out-of-phase with each other, in order to generate an unique positional value at each point along the anterior/posterior axis of the segment. With this type of wave, there is not a need to independently set up the gradient in each segment; such waves can be initiated at any one point along the embryo, possibly through a reaction-diffusion mechanism (Turing, 1952).

Another outcome of the surgical experiments by Wright and Lawrence (1981) was the demonstration that an excision of greater than one-half of the segment resulted in a duplication of the remaining pattern elements in reverse polarity rather than a regeneration of the missing ones, as was seen when less than one-half of the segment was removed. This phenomenon is also seen when imaginal discs or insect appendages are cut into two pieces: the smaller fragment duplicates and the other regenerates (summarized in French, et. al., 1976). In order to explain these results and the generation of polarity-reversal, a model that assigns an angular positional value in a polar coordinate system was proposed (French, et. al., 1976). In this model, each position in the tissue is represented by a circumferential value and intercalation would occur via the

shorter of two possible routes between two abnormally juxtaposed angular values. If the positional values along the anterior/posterior axis of a segment represent a set of angular values that are repeated in every segment, then the aberrant juxtaposition of two values by surgery, or mutation, would again result in intercalation via the shortest possible route (Russell, 1985; Wright and Lawrence, 1981), so that the mirror-image duplication would occur when greater than one-half of the segment (or imaginal disc) is deleted; otherwise, regeneration occurs.

In opposition to gradient models for pattern formation within each segment, a cell state model was proposed by Meinhardt (1986) where at least three cell states exist within a segment. The three states, referred to as S (segmental border), A (anterior compartment), and P (posterior compartment), would allow polarity to be set up within a segment, and a precise location for the segmental border to be defined. Deletion of any one of these states as a result of mutation would leave two states in which polarity reversal could occur due to the juxtaposition of two states in an order opposite that of wildtype. In this case, deletion of greater than one-half of the segment is not required in order to cause polarity-reversal. Deleting two states would result in an apolar embryo. Each of these states could be defined by a specific segment-polarity gene that acts as a selector gene for that state, such as *engrailed* for the P state and *wingless* for the A state.

It is unclear whether each cell state simply has a specific selector gene assigned to it. Martinez-Arias, et. al. (1988) extended the three cell state model to four cell states, which interact with each other to assign and maintain their identities, in order to explain the interactions among the segment-polarity genes, *engrailed*, *wingless*, *patched* (*ptc*), and *naked* (*nkd*). Mutations in one of these genes could lead to either a loss of one or more cell states, to the re-definition of a cell state, or the ectopic induction of a cell state as a result of the anomalous juxtaposition of cells states. Again, polarity-reversal could result from the anomalous juxtaposition of cell states. Certainly, there are several complex interactions that are required to set up and maintain patterning within each segment.

When mutations that cause extreme alterations in a pattern are analysed, such as *wingless*-lethal alleles, it can be difficult to discern how that pattern change occurred. It can be extremely useful to have several mutations of varying strengths to help analyse how the loss of a particular gene can lead to the pattern change. When one looks at only the strong embryonic lethal phenotype of *wingless* as well as the wildtype expression pattern, it can be seen that, somehow, loss of this gene's normal function has affected a region of the segment that is much larger than the region in which it is detectably expressed. It can be difficult to determine if this mutation has altered some variable of a wave function (Russell, 1985) or if it has altered a cell state, leading to changes in neighbouring regions due to improper gene interactions (Martinez-Arias, et. al., 1988). Weaker mutations of the gene might show what sort of mechanisms are used to set up the

wildtype pattern. If a mutation in a segment-polarity gene affects the amplitude of a morphogenic wave, and pattern elements within the segment are dependent on a certain level of the morphogen, then a decrease in the amplitude, such as what might be produced by a hypomorphic mutation of the gene, could result in a 'stretching' of the pattern elements within the segment, before the gradient is 'flattened out' by a more extreme mutation to produce either cell death or a change in cell fate, producing the polarity-reversals seen. If the gene is normally required to define one cell state independently of another cell state, then a hypomorphic mutation would probably affect only the region defined by that gene, although this would not necessarily be the case for *wingless*, which affects a region beyond its domain of expression. If, as Martinez-Arias, et. al. (1988) suggested, *wingless* is required to set up or maintain the cell states in neighbouring regions, and the effect on all of the regions that *wingless* is required for is equal, then the 'stretching' of pattern elements along the axis would not necessarily be expected. Rather, one might expect discrete pattern changes, such as the ectopic formation of structures or the loss of certain structures due to possible threshold requirements for the gene, rather than the displacement of them, as might be expected by changes in a gradient. With a cell state type of mechanism, one might expect there to be one or more thresholds at which changes in pattern occur, rather than a continuous change as the level of gene activity is gradually lowered.

The purpose of this study was to analyse how the *wingless* mutations cause the pattern changes seen. In order to accomplish this, intermediates of *wingless* were generated using the temperature-sensitive allele wg^{IL114}: by either shifting the embryos homozygous for this allele during the temperature-sensitive period, or by raising them for the duration of embryogenesis at semi-permissive temperatures. For reasons that will be discussed later, the second method was used to analyse the changes in the ventral cuticular pattern of the embryo. The changes were quantified by using measurements that were made along the anterior-posterior axis as described in the materials and methods. Also, by using a *lac-Z* reporter gene for *engrailed*-expressing cells, an analysis of the effect of intermediate *wingless* backgrounds on the posterior compartment was done. From these results, a model for the mode of action of *wingless* within the segment will be discussed, involving cell states to define the region of the segment in which *wingless* is expressed and the polarity of the pattern in the segment. Superimposed on this is a form of a wave that is generated by *wingless* in order to allow maintenance of the determined pattern within the segment.

MATERIALS AND METHODS

Drosophila Strains

The following strains of *Drosophila melanogaster*, with relevant second chromosome markers, were used in this study:

b cn bw/b cn bw

Df(2L)NL/SM5 (Tiong and Nash, 1990): Df(2L)NL is a deficiency of the polytene region 27E2-28C2, which includes the *wg* locus, and was used to determine that the wg^{IL114} is an amorph at the non-permissive temperature. The stock was obtained from S. Tiong and D. Nash.

ry Xho25/CyO: *ry Xho25* (Hama, et. al., 1990) carries an insert of a fusion between *engrailed* regulatory regions and the *E. coli lac-Z* gene (coding for the enzyme, ß-galactosidase) on the second chromosome, in the endogenous *engrailed* gene. This insertion causes an *engrailed* mutation that has low penetrance for the embryonic phenotype when homozygous, but fails to complement other *engrailed* mutations. Expression of *E. coli* ß-galactosidase (ß-gal) is presumably regulated by the endogenous *engrailed* regulatory regions, and is expressed in the same pattern, both spatially and temporally, as the wildtype *engrailed* gene (Hama, et. al., 1990). Therefore, *lac-Z* can be used as a reporter gene for engrailed expression. This insert will be referred to as $en^{lac\text{-}Z}$. The gene,

engrailed, maps to 62.0 on the genetic map and to 48A on the polytene map. The stock was obtained from T. Kornberg.

sha wg^{IL114}: *shavenoid* (*sha*) is a mutation that causes the ventral denticles to be small and sparse. Since it is linked to *wingless*, it can be used as a marker to identify weak *wingless* embryos. It maps to 62.0 on the genetic map. The stock is maintained over one of the *Curly* balancers (see below for balancer information).

sha: *shavenoid* (*sha*) also maintained over a *Curly* balancer.

wg^{IL114} *cn bw*/CyO: wg^{IL114} is a temperature-sensitive allele of the segment-polarity gene, *wingless*, which maps to 30.0 on the genetic map and to 28A1-2 on the cytological map. The strain was obtained from E. Wieschaus.

wg^{IL114} *c px sp*/SM6a: a stock created by P. Onofrechuk (1986) in this laboratory.

The second chromosome balancers used with the above stocks are as follows:

CyO= IN(2LR)O, *Cy* dp^{1vi} *pr* cn^2

SM5= IN(2LR)SM5, al^2 ds^{55} *Cy* lt^v cn^2 sp^2

SM6a= IN(2LR)SM6a, al^2 *Cy* dp^{1vi} cn^2 sp^2

Other markers of concern in this study were:

bw= brown: brown eye colour (2-104.5; 59D9-11)

cn= cinnabar: bright-red eye colour (2-57.5; 43E3-14)

Cy= Curly: curled-up wings (2-6.1)

Descriptions of other markers can be found in Lindsley and Grell (1968). All genes used are on chromosome 2. All stocks were maintained at room temperature (21±1°C) on standard medium (Table 1).

A)Standard Medium (pH 7.4)

10 g agar
100 g sucrose
100 g brewer's yeast
100 mL chloramphenicol (1 g/L stock solution)
10 mL propionic acid
4.3 g sodium phosphate dibasic
2.7 g sodium phosphate monobasic
water to 1 litre

B)Apple Juice Medium

1.2 g agar in 1 L commercial apple juice

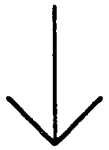
Table 1) Constituents of media fed to *Drosophila melanogaster*.

Construction of the *en*$^{lac\text{-}Z}$, *wg*^{IL114} Double Mutant

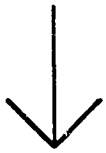
In order to construct a stock that is doubly mutant for wg^{IL114} and *en*$^{lac\text{-}Z}$, the crossing scheme outlined in Figure 1 was used.

Wildtype males obtained in generation 3 (G3) were assumed to carry *en*$^{lac\text{-}Z}$ since a double cross-over in the G2 females would be required to create *cn*$^{+}$ *en*$^{+}$ *bw*$^{+}$. The probability of this occurring is 0.045 x 0.425 (see Figure 2). Therefore, approximately one in fifty of the wild-type males collected in G3 would not carry the insert. Of these males, 27.5% should also carry the *wg*^{IL114} allele (Figure 2). These wildtype males were then pair-mated to *wg*^{IL114} and crosses in which no wildtype adults appeared were saved. A double mutant line was created by selecting phenotypically *Curly* (class a) males and virgin females from these crosses. The presence of the insert in two these lines was then verified by staining for ß-galactosidase and ensuring that it was expressed in an *engrailed*-like manner. These two lines have been maintained as stocks named *en*$^{lac\text{-}Z}$*wg*^{IL144}/CyO (#1 and #2). It should be noted that the original *ryXho25* stock is mutant for the endogenous *rosy* gene, in order to select for the presence of the insert which carries the wildtype allele of this gene. The inheritance of the endogenous *rosy* gene was not controlled for; therefore, the alleles at this locus in the double mutant stocks are unknown.

G1) *en*$^{lac\text{-}Z}$/*CyO* ♂♂ X *wg*^{IL114} *cn bw*/*CyO* ♀♀



G2) *en*$^{lac\text{-}Z}$/*wg*^{IL114} *cn bw* ♀♀ X *b cn bw*/*b cn bw* ♂♂



G3) *wg*$^{?}$*en*$^{lac\text{-}Z}$/*b cn bw* ♂ X *wg*^{IL114} *cn bw*/*CyO* ♀♀

G4) GENOTYPE	PHENOTYPE
a) *wg*$^{?}$*en*$^{lac\text{-}Z}$/*CyO*	Curly
b) *b cn bw*/*wg*^{IL114} *cn bw*	cinnabar, brown
c) *b cn bw*/*CyO*	Curly, cinnabar
d) *wg*$^{?}$*en*$^{lac\text{-}Z}$/*wg*^{IL114} *cn bw*	wildtype or dead

Figure 1) The crossing scheme used to generate *wg*^{IL114} *en*$^{lac\text{-}Z}$ double mutants.

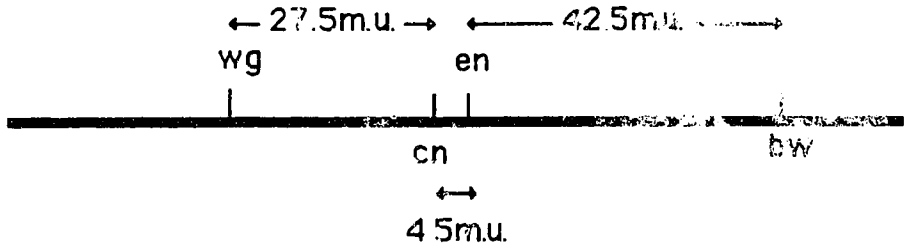


Figure 2) The arrangement of *wg*, *cn*, *en*, and *bw* on chromosome 2. The G2 females in Figure 1 carried $en^{lac\text{-}Z}$ *in trans* to the other markers

Egg Collection

Eggs were collected on either standard medium or apple juice medium (Table 1) for a period of one hour unless otherwise indicated. The apparatus consisted of a 100 ml plastic beaker with holes punched in the bottom for ventilation. A plastic petri dish filled with approximately 10 ml of medium was held on to the beaker with an elastic band. If necessary, a small amount of live yeast paste was spread on the medium to help induce the flies to lay eggs. After removing the adult flies, the embryos were aged for the appropriate amount of time, at the appropriate temperature. The age of the embryos was determined from the mid-point of the egg lay. The temperature of the incubator was determined at the beginning of the egg lay, at the end of the egg lay, and at the end of the developmental period. The same thermometer was used for all experiments in order to reduce the sources of variability.

Cuticle Preparation

The embryos were incubated at the appropriate temperature until all surviving individuals hatched (this took 1-3 days depending on the temperature); the remaining dead ones were collected, placed on double-sticky tape, and rolled with a dissecting needle until the chorion broke and the embryo could be removed to mounting medium (1 part 95% ethanol to 9 parts 85% lactic acid). When possible, the vitelline membrane was manually removed; however, removal of the membrane from extreme *wingless* embryos proved to be difficult since the fragile nature of these embryos often resulted in them being destroyed upon devitellinization. Weak *wingless* intermediates often hatch, so an attempt was made to catch them before they would hatch, or they were picked off of the medium as newly hatched first instar larvae. A #1 coverslip was placed over the embryos and the slide was placed on a 40°C slide warmer overnight to allow the embryos to 'clear': the lactic acid dissolves the soft internal tissues, leaving a flat transparent cuticle preparation that could be viewed under a compound microscope, using phase-contrast optics. In order to preserve the preparations, the coverslip was sealed with clear nail polish: these preparations can last for an extremely long time.

Microscopic Measurements

In this study, measurements along the anterior/posterior axis for various cuticle pattern elements in the thoracic and abdominal

segments were taken as illustrated in Figure 3. Measurements were taken on a WILD M20 compound microscope with phase-contrast optics at 400X magnification, where one unit on the ocular micrometre equals 1.01 μm. Measurements were expressed in these units, which approximately equal micrometres.

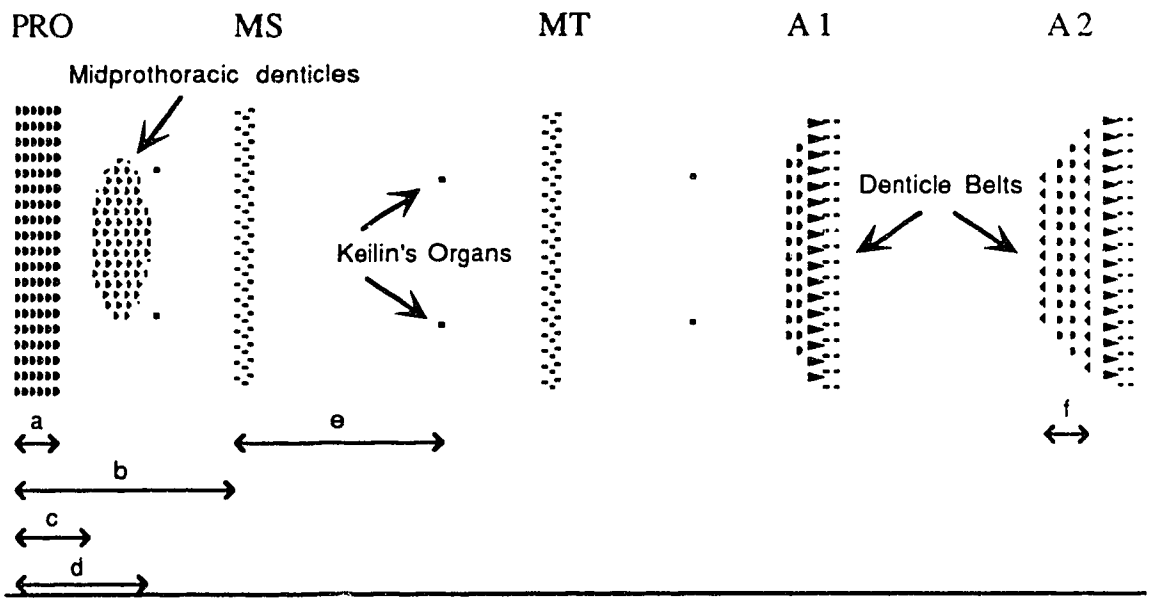


Figure 3) Measurements of ventral cuticle pattern elements along the anterior/posterior axis. Anterior is to the left. **a**= length of denticle belt, **b**= length of the segment, **c**= distance to anterior boundary of the denticles in the middle of the prothorax (the mid-prothoracic denticles), **d**= distance to posterior boundary of the the mid-prothoracic denticles, **e**= distance to the Keilin's organs of each thoracic segment, and **f**= distance between the two anterior-pointing rows of denticles in abdominal segments A2-A8. The vertical lines represent the segmental borders (Szabad, et al 1979).

Staining for ß-galactosidase Expression

In order to visualize the presence of ß-galactosidase, embryos were stained with X-gal (5-bromo-4-chloro-3-indolyl ß-D-galactoside), which stains the embryos blue after the enzymatic reaction. The following protocol was adapted from Bellan, et. al. (1988) for staining non-devitellinized embryos *en masse*. Constituents of the solutions used are in Table 2.

The embryos were collected as described above and placed into baskets constructed from cut-off Eppendorf tubes with fine nylon mesh melted onto the bottom. They were washed with water and then dechorionated in 3% sodium hypochlorite (household bleach diluted by 50%) for 1-2 minutes. They were then washed again with water 2-3 times, blotted dry and placed into the fix solution which was shaken in a 1:2 ratio with heptane. The heptane permeablizes the vitelline membrane to the formaldehyde fixative. They were fixed at room temperature for 20 minutes with periodic shaking. Next, the embryos were washed 2-3 times in phosphate-buffered-saline (PBS) with 0.3% Triton-X100 until the heptane was removed and then placed in the staining solution+X-gal at 37°C for $1^1/_2$ hours, or until they were sufficiently stained. When staining was completed, they were washed in PBS+Triton-X 2-3 times and then placed into the staining solution without X-gal. At this point, they can be stored in Eppendorf tubes at 4°C. In order to view and photograph the embryos, they were placed on a slide in the staining solution and

covered with a coverslip raised by either a ring of petroleum jelly or by coverslips. Using the coverslips to make a well for the embryos allowed gentle rolling to orient the embryos as desired for photography.

A)Fix Solution

0.1 M Pipes, pH6.9
2.0 mM EGTA
1.0 mM $MgSO_4 \cdot 7H_2O$
37% formaldehyde

B)PBS (Ashburner, 1989)

130 mM NaCl
7.0 mM $Na_2HPO_4 \cdot 2H_2O$
3.0 mM $NaH_2PO_4 \cdot H_2O$

C)Staining Solution

10.0 mM $NaH_2PO_4 \cdot H_2O/NaHPO_4 \cdot H_2O$ (pH 7.2)
150.0 mM NaCl
1.0 mM $MgCl_2 \cdot 6H_2O$
3.1 mM $K_4[Fe^{II}(CN)_6]$
3.1 mM $K_3[Fe^{III}(CN)_6]$
0.3% Triton X-100
0.08% X-gal (stored frozen in DMF at 8%)

Table 2) Constituents of Solutions Used to Stain Embryos with X-gal. The final concentration of the X-gal was reduced from 0.2% to 0.08% when it was found that the higher concentration resulted in over-staining of the embryos. This was especially apparent when low numbers of embryos (<50) were being stained.

Photography

Cuticle preparations were photographed under the WILD M20 compound microscope with phase-contrast optics, using 35mm Panatomic-X 32 black and white film. Embryos stained with X-gal were photographed using either bright-field or Nomarski optics. The same film type as above was used.

The film was developed with Kodak D-76 developer and printed with Dektol developer. In both cases, Kodak Rapid-fixer was used.

RESULTS

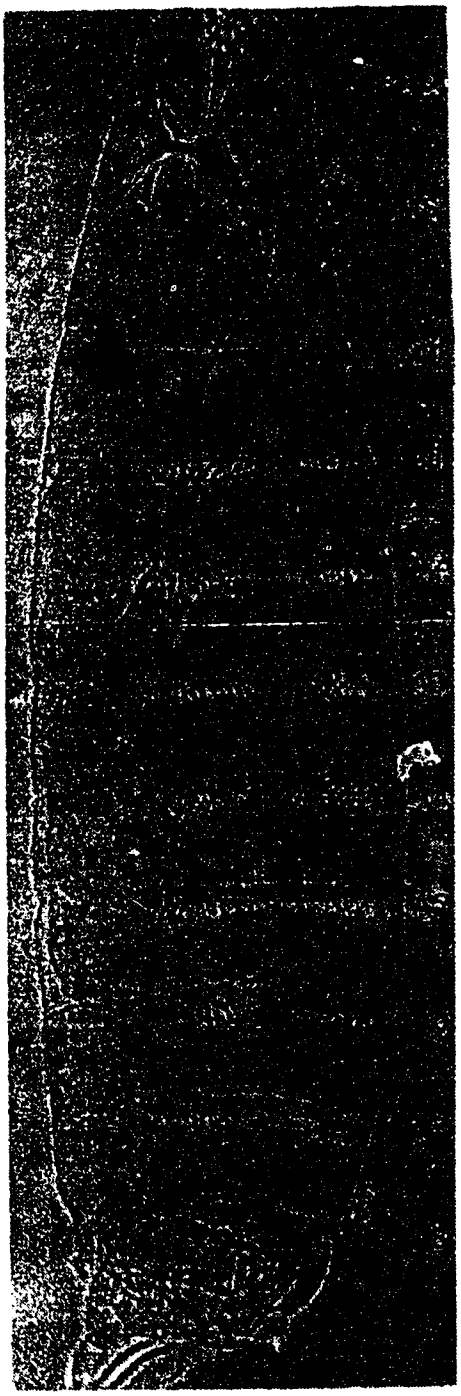
The Wildtype Cuticular Pattern

The following is a description of the features relevant to this study that are found on the ventral surface of the wildtype larval cuticle (refer to Figure 4). A more complete description can be found in Campos-Ortega and Hartenstein (1985). The anterior region of the larva contains the head segments followed by the trunk region consisting of three thoracic segments and eight abdominal segments, each of which has a belt of denticles consisting of several rows in the anterior region of the segment. The segmental border lies between the first and second rows of denticles[1]. Therefore, the first row of denticles demarcates the posterior edge of each segment by this definition. There are three more abdominal segments and an unsegmented telson, which appear to fuse together with the eighth abdominal segment during embryogenesis to form the tail region (Jürgens, 1987). The eighth abdominal segment appears to define the anterior edge of the tail region, since *tailless* mutants eliminate this segment along with the more posterior tail region (Jürgens, et al, 1984, Jürgens, 1987).

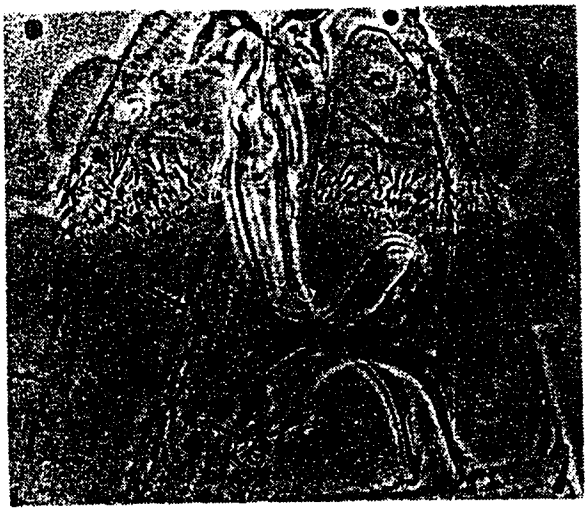
The three thoracic denticle belts consist of denticles that all point posteriorly. The denticles of the prothoracic (PRO) belt are arranged

[1]The segment border has been defined as the major line of muscle attachment sites in the abdominal segments A2 to A8 (Szabad, et al 1979, pg. 259; Campos-Ortega and Hartenstein, 1985, pg. 117).

in 4-5 rows; whereas there are only 3-4 rows in the mesothoracic (MS) and metathoracic (MT) belts and these are much finer than the denticles of the prothorax or of the abdominal segments. The first abdominal segment, with 4-5 rows, also contains denticles that all point posteriorly. The remaining abdominal segments have two rows of anterior pointing denticles in the first and fourth positions of the wildtype belt. The fifth row consists of large posterior-pointing denticles and the remaining two or three rows are finer denticles (of similar size to the meso- and metathoracic denticles) that are also posterior-pointing (see Figure 17f). The remaining portion of each of segments A1 to A7 consists of only naked cuticle, whereas the denticle belt of segment A8 lies immediately anterior to the anal pads. Each of the three thoracic segments has a pair of Keilin's organs which lie in the middle of the naked cuticle, straddling the boundary between the anterior and posterior compartments (Struhl, 1984). The prothorax has, in addition to the anterior belt of denticles, a patch of denticles in the middle of the segment, lying between the anterior belt of denticles and the Keilin's organs (Figure 4b).



a



b

Figure 4) The wildtype ventral cuticle of the *Drosophila* larva. Anterior is oriented to the top in this and subsequent figures, unless otherwise noted. **a)** The whole embryo with three thoracic and eight abdominal segments. **b)** The thoracic segments, showing Keilin's organs (KO) and the mid-prothoracic patch of denticles (arrow). Magnification: 182X (a) 437X (b).

The *wingless* Cuticular Pattern

In embryos homozygous for alleles of *wingless* that are embryonic lethal, approximately three-quarters of each abdominal segment from the posterior region of the segment, consisting of the naked cuticle and part of the denticle belt, is missing and replaced with a mirror-image duplication of the remaining pattern to form a 'lawn of denticles' (Baker, 1988b). Traces of a repeat pattern can be seen within this 'lawn'; denticles in the anterior part still have polarity, and the denticles that remain can be tentatively identified as the middle three or four rows of the belt, which are duplicated in reverse polarity (see Figure 5a). At the posterior end of this 'lawn', it is difficult to discern individual segments, since the denticles are more randomly oriented, and a repeat pattern is difficult to distinguish (Figure 5c). Part of the region of each segment that is deleted in these embryonic lethal alleles is the segmental border (Nüsslein-Volhard and Wieschaus, 1980; Baker, 1988b). There is also a belt of denticles that is thought to be thoracically derived due to its position anterior of the 'lawn' (Figure 5b). The identity of this belt will be discussed later.

Homozygotes for the temperature-sensitive allele, wg^{IL114}, give a phenotype at the restrictive temperature of 25°C that is similar to that of the lethal alleles (Baker, 1988b). At the permissive temperature of 18°C, first instar larvae hatch with a wildtype cuticular pattern; however, they do not develop to adulthood (Baker, 1988b).

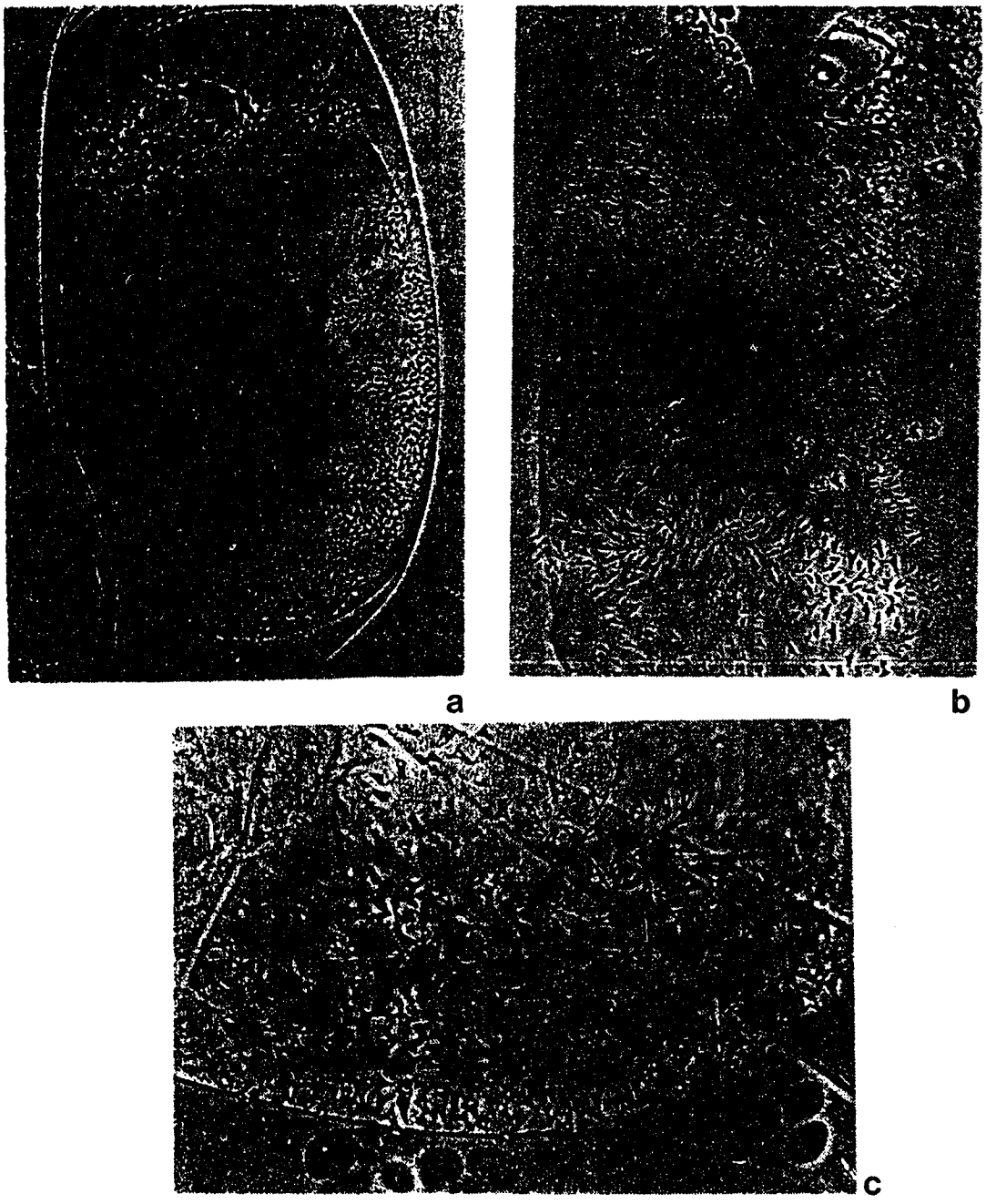


Figure 5) The extreme *wingless* phenotype: wg^{IL114}/wg^{IL114} raised at the restrictive temperature. **a)** A metameric repeat is indicated in the anterior portion of the 'lawn' of denticles (bracket) (raised at 29°C). **b)** Naked cuticle separates a belt of thoracic denticles from the lawn (arrow) (raised at 25°C). **c)** Random polarity of denticles in the posterior region of the lawn (raised at 29°C). Magnification: 182X (a), 437X (b), 728X (c).

Temperature-Shift Experiment

When an experiment was done in this laboratory by M. Auld (unpublished results) to determine the temperature-sensitive period (TSP) for *wingless*, several interesting intermediate *wingless* phenotypes were observed in embryos shifted during the TSP. It was thought, then, that a closer analysis of these intermediates could reveal the mechanism by which *wingless* generates the segmented pattern seen in the embryo. The first part of this study involves the analysis of these previously prepared embryos in order to determine the TSP and to describe the intermediate phenotypes that were generated.

To determine when during embryogenesis *wingless* is required, embryos were shifted at various identified stages; these stages and the abbreviations used for them are summarized in Table 3. The embryos were collected over an extended period of time (4-12 hours) from a stock carrying wg^{IL114} and the larval marker, *shavenoid* (*sha* wg^{IL114}/Curly balancer, see Materials and Methods) at either the permissive temperature of 18°C or the restrictive temperature of 29°C. After manual dechorionation, they were mounted on a slide under halocarbon oil (to prevent dehydration of the living embryos) and individually staged. They were then shifted to the reciprocal temperature of either 18°C or 29°C. After a period of 72 hours at 18°C or 48 hours at 29°C (developmental times decrease at higher temperatures), the unhatched larvae or embryos were moved to a new slide according to the stage that they were at when

shifted, rinsed with heptane to remove the halocarbon oil, and mounted as described in the Materials and Methods.

Abbre-viation used	Stage	Description	Approxi-mate timing at 25°C (hours)
st2	stage 2 (early cleavage)	Clear crescent between the vitelline membrane and cell membrane at posterior end of the embryo.	0-1.5
pc(e)	early pole cell formation	Pole cells fill the posterior crescent. Minimal migration of nuclei to the periphery of the egg.	1.5-2
pc(l)	late pole cell formation	Crescent filled with many small pole cells. Nuclei have migrated to periphery.	2-3
blast(s)	syncytial blastoderm	Cell membrane invagination around peripheral nuclei. No cellular basement membrane.	3-4
blast(c)	cellular blastoderm	Basement membrane complete.	4-5
gst	gastrulation	Cephalic furrow begins to form.	5-5.5
gbe	germ band extension	Pole cells pushed to dorsal surface. Germ band extends around dorsal surface.	5.5-6.5
egb	extended germ band	Germ band remains fully extended. Segmentation begins to be evident on the germ band.	6.5-8
gbs	germ band shortening	Segmentation clearly seen. Dorsal space left as germ band retracts.	8-9.5
dc	dorsal closure	Midgut and other gut structures evident. Some definitive structures evident.	9.5-12
later	later stages	Definitive structures present. Gut differentiated and motile.	12-20

Table 3) The stages of *Drosophila* embryogenesis and abbreviations used for the temperature-shift experiment.

To properly describe the intermediates seen when the shift was during the TSP, it was important to determine which segmentation defects were actually due to the inhibition of the *wingless* product and which were more likely to be due to other factors, such as

temperature shock during a sensitive period of embryogenesis or to a mechanical shock caused by the dechorionation required to stage the embryos. This objective can be accomplished by comparing defects that occurred in the genotypically *wingless* embryos (as identified by the presence of *shavenoid*) to those seen in the non-*shavenoid* embryos resulting from the same cross. These non-*shavenoid* embryos may be balancer homozygotes or *sha wg*/balancer heterozygotes.

Defects that were frequently seen in the non-*shavenoid* embryos were fusions of two or more denticle belts and deletions of part or all of particular belts. The fusions were sometimes complete with no naked cuticle between two belts; otherwise only part of the belts were fused (see Figure 6). This partial fusion could occur anywhere along the width of the belts. These defects occurred very frequently when the embryos were shifted during certain stages (see Table 4); syncytial blastoderm appears to be quite sensitive to segmentation defects when the embryos were shifted up. The frequency of defects in non-*shavenoid* embryos was particularly high when the embryos were shifted to the higher temperature. Because of the high frequency of defects that occurred in the non-*shavenoid* embryos, defects that occurred more frequently in the *shavenoid* embryos than in the non-*shavenoid* embryos at any one stage were considered as likely to be due to *wingless*, and are classified as such in Table 4.

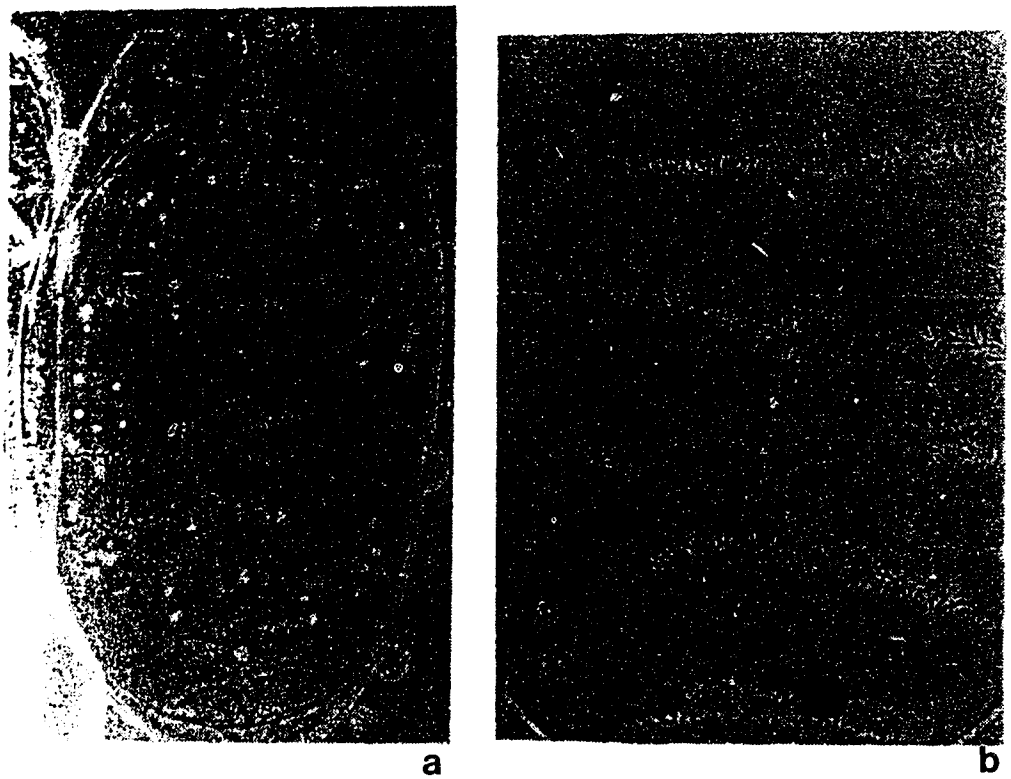


Figure 6) Defects commonly seen in wildtype embryos that were temperature-shifted during development. The shift was from 29°C to 18°C during the extended germ band stage. **a)** The whole embryo. **b)** Segments A3 to A8. Magnification: 182X (a), 364X (b).

stage	total	non-*shavenoid* normal	non-*shavenoid* abnormal	*shavenoid* normal	*shavenoid* *wingless*	*shavenoid* abnormal
CONTROLS						
18°C	240	5	7(2.92)	2	0 (0)	1(0.42)
29°C	240	22	1(0.42)	0	40(100)	0(0.00)
SHIFT DOWN						
st2	174	14	12 (6.90)	4	0 (0)	3(1.72)
pc(e)	94	11	12(12.77)	3	0 (0)	3(3.19)
pc(l)	84	3	14(16.67)	4	0 (0)	3(3.57)
blast(s)	114	12	25(21.93)	5	0 (0)	8(7.02)
blast(c)	34	6	2 (5.88)	3	0 (0)	3(8.82)
gst	93	9	7 (7.53)	7	0 (0)	8(8.60)
gbe	102	15	10 (9.80)	12	12 (50)	3(2.94)
egb	75	9	2 (2.67)	1	12 (92)	0(0.00)
gbs	63	2	0 (0.00)	2	14 (88)	0(0.00)
dc	104	6	2 (1.92)	0	20(100)	1(0.96)
later	84	12	0 (0.00)	0	5(100)	0(0.00)
SHIFT UP						
st2	124	17	41(33.06)	0	17(100)	0(0.00)
pc(e)	69	19	19(27.54)	0	8(100)	0(0.00)
pc(l)	46	6	21(45.65)	0	8(100)	0(0.00)
blast(s)	78	22	23(29.49)	0	9(100)	0(0.00)
blast(c)	50	19	5(10.00)	0	13(100)	0(0.00)
gst	58	28	5 (8.62)	0	11(100)	0(0.00)
gbe	94	56	4 (4.26)	0	19(100)	0(0.00)
egb	76	36	0 (0.00)	0	8(100)	0(0.00)
gbs	86	38	2 (2.33)	0	16(100)	0(0.00)
dc	81	32	1 (1.23)	14	0 (0)	0(0.00)
later	37	18	0 (0.00)	3	0 (0)	0(0.00)

Table 4) Determination of the temperature-sensitive period for *wingless*. The total represents the total eggs collected, including those that hatched and were not mounted for cuticle preparations. Numbers in parentheses for abnormal columns give the percentages of total embryos shifted that showed defects. Numbers in parentheses for the *wingless* column give the percentages of classifiable *shavenoid* embryos that showed a *wingless* phenotype, and are graphed in Figure 8. For a list of abbreviations, see Table 3.

It was also important to describe the phenotype of the *shavenoid* mutation, without any possible contribution from *wingless*. This was

accomplished by observing embryos from a *shavenoid* stock that does not carry *wingless*. As previously mentioned, *shavenoid* causes a 'shaven' appearance to the denticles; they are smaller and thinner than wildtype ones and they are sparsely distributed (Figure 7). In the more posterior segments, there appears to be a space between the first row of denticles and the remaining rows. This is probably due to the second row being absent rather than to an anterior shift in the placement of the first denticle row. In a *sha, wg*$^{+}$ embryo (Figure 7), there are two anterior-pointing rows, with very few denticles between them; in wildtype embryos, there should be two posterior-pointing rows between them. There may be remnants of the second row, which would be posterior-pointing in wildtype embryos. The third row, although sparse, is present. This phenotype was originally thought to be part of the intermediate *wingless* phenotype; however, it can be ruled out as it also appears in non-*wingless* embryos.

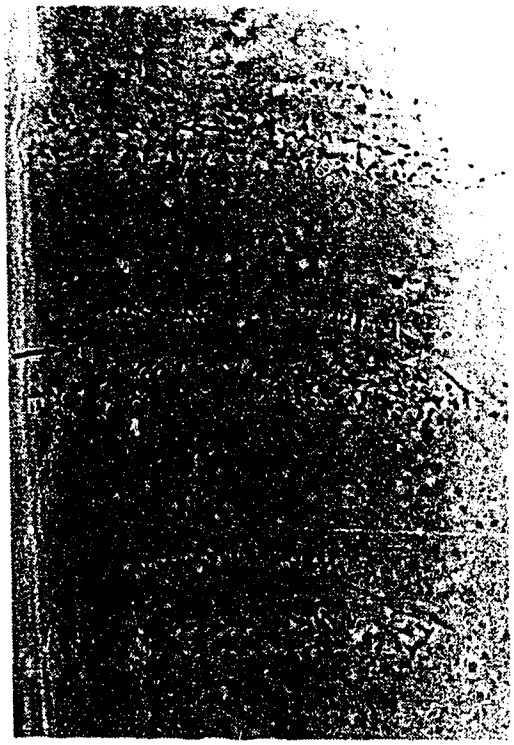


Figure 7) The *shavenoid* phenotype in a *sha wg*$^{+}$/*sha wg*$^{+}$ embryo. Segments A6 to A8 are shown. Note the apparent space after the first row of denticles (arrow) which may contain remnants of the second row (see text). Magnification: 364X.

The Temperature Sensitive Period of *wingless*

Table 4 shows that *shavenoid* embryos that were shifted down prior to germ band extension displayed a wildtype cuticular pattern; shifting them down after this stage failed to rescue the embryos and resulted in a mutant phenotype. This then defines the beginning of the requirement for *wingless* as being at germ band extension. This requirement ends by the time the germ band has shortened, as can

be seen from shifting up the embryos (Table 4). A fully wildtype phenotype was seen only when the up-shift was done after germ band shortening. Those shifted up prior to the shortened germ band stage displayed a weak *wingless* phenotype, which became stronger as the shift was made earlier in embryogenesis. The weakest phenotype that could be distinguished in the *shavenoid* embryos was a broadening of the most posterior denticle bands when shifted up during germ band shortening (Figure 6b). The results of the TSP experiment are summarized in the graph in Figure 8.

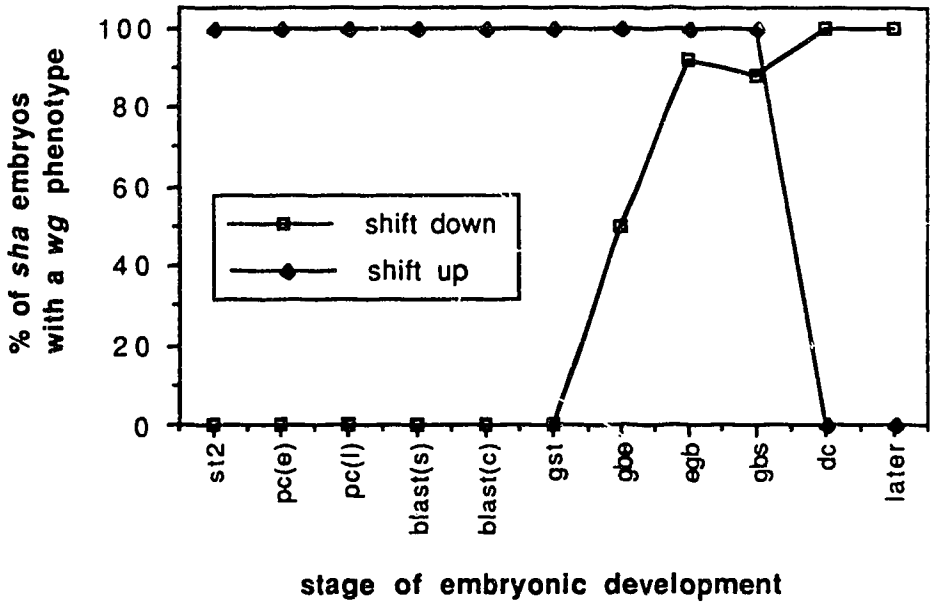


Figure 8) The temperature-sensitive period of *wingless* is from germ band extension to germ band retraction. The data used is from Table 4.

The Intermediate Phenotypes

Temperature-shift experiments using wg^{IL114} have been previously described by Baker (1988b), who shifted timed embryos rather than individually staged ones. He found that shifting down during the TSP resulted in different intermediates than shifting up during this time; this was also seen in this experiment. The following is a description of these intermediate phenotypes in the abdominal segments. Because the fine denticles of the thoracic segments are difficult to see in *shavenoid* embryos, these segments were not analysed.

Shift up experiment

When the embryos were shifted up, that is, when normal *wingless* function was inhibited during embryogenesis after the stage of the shift, the denticle belts occupied a progressively larger proportion of each segment as the shift occurred earlier in embryogenesis, and this can be seen in Figure 9. It can also be seen in the graph in Figure 10a, which shows the proportion of each abdominal segment that is covered by denticles at the various stages of shift. A gradient of effect along the anterior-posterior axis was also apparent. When the embryos were shifted during germ band shortening, a broadening of the posterior-most segments was seen without polarity reversal (Figure 9d). When the embryos were shifted up at the extended germ band stage, polarity reversal became apparent in the posterior-most segments (Figure 9c, segments A6 to A8); whereas the more anterior segments (A1 to A5 in Figure 9c) do not yet display fusion of the denticle belts or polarity-reversal. The graph in Figure 10b

compares segment A1 to segment A7. All of the embryos shifted up prior to full extension of the germ band have A7 completely fused; whereas some of the embryos still have naked cuticle in A1 when the shift was made during germ band extension, resulting in a value of less than 1 for the average proportion of this segment covered by denticles.

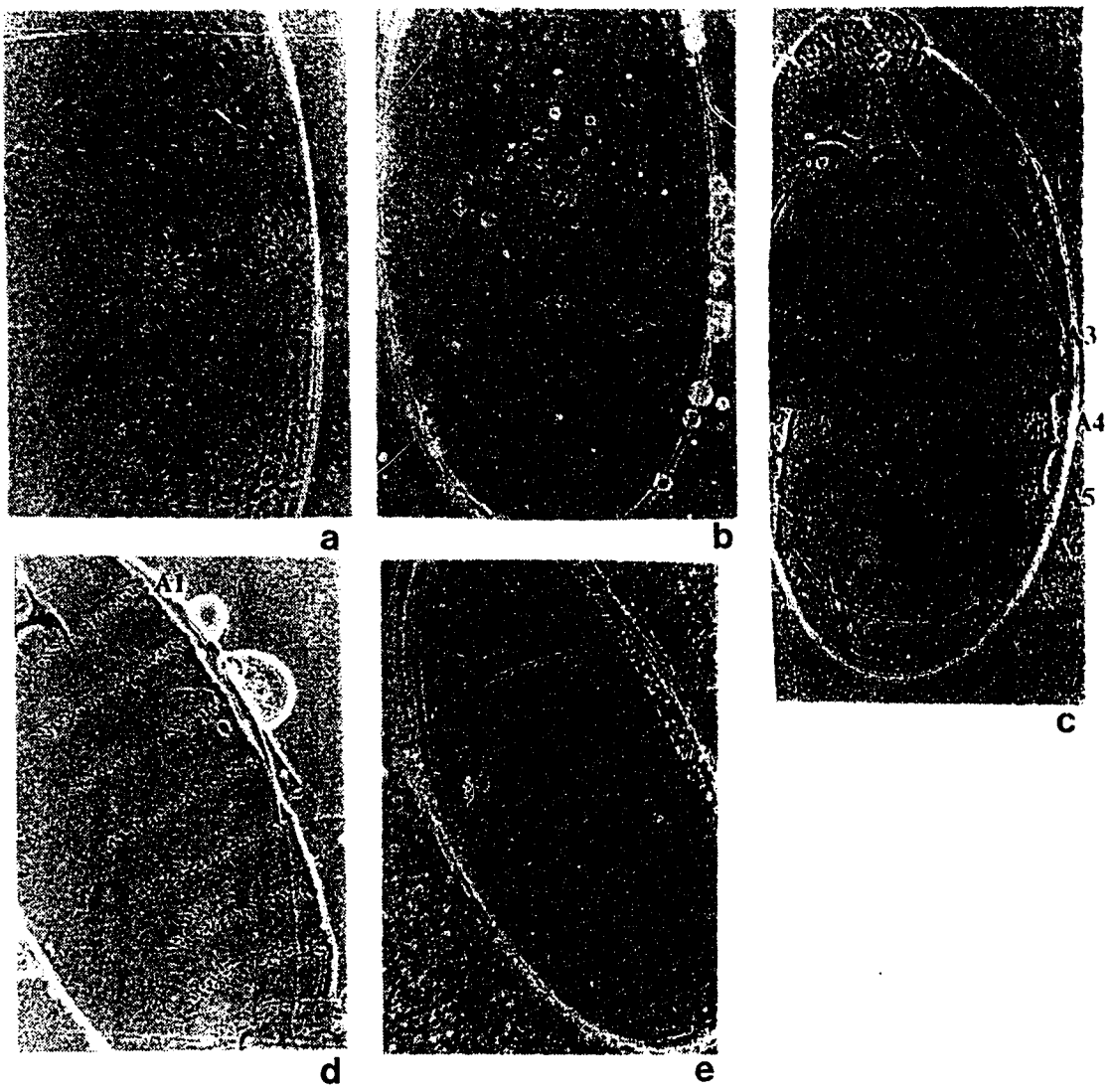


Figure 9) The series of intermediate *wingless* phenotypes produced in the shift up experiment. Stages of shift are as follows: **a)** gastrulation (photograph shows the anterior end), **b)** germ band extension, **c)** extended germ band, **d)** germ band shortening, **e)** dorsal closure. Magnification: 312X (a), 156X (b-e).

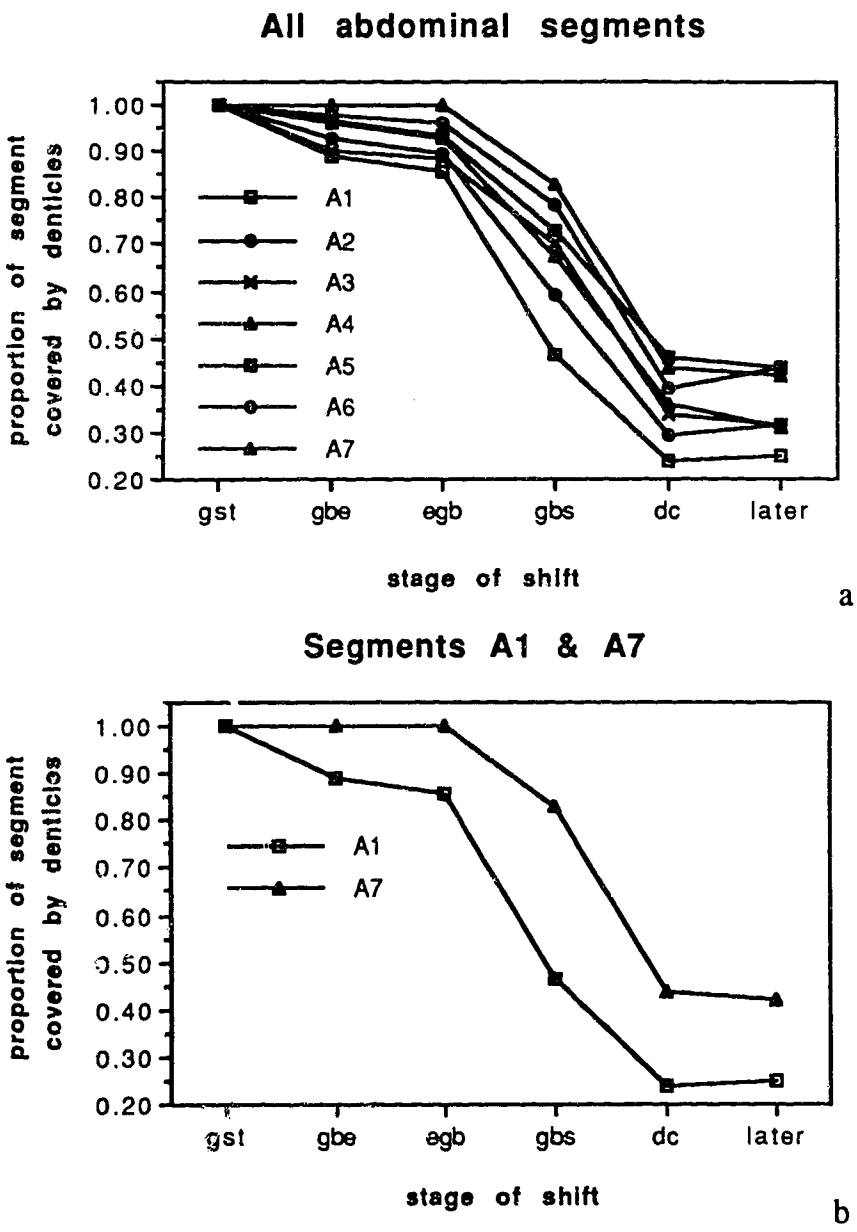


Figure 10) The proportion of each abdominal segment that is covered by denticles as a function of the stage of the shift up. Embryos shifted after dorsal closure are phenotypically wildtype.

Shift down experiment

Shifting the embryos down did not give the clear progressive broadening of the denticle belts as did shifting them up. In this case, normal *wingless* activity, which was missing during early embryogenesis, might be restored at the time of the down-shift. Baker (1988b) noticed that there was an 'all-or-nothing' effect: either the denticles occupied a proportion of the segment comparable to the wildtype without polarity-reversal or they occupied all of the segment and displayed polarity reversal. They did not appear to occupy an intermediate proportion of the segment as was seen in the shift up experiment. He also described this effect as happening in some segments but not others, with the choice of segments showing the *wingless* phenotype being random within each embryo, and with fusions occurring independently in the left or right half of each segment.

I found the intermediates resulting from the shift down experiment difficult to analyse. Since fusions that occur in only half of the segment are common in the non-*shavenoid* embryos (Figure 6), it was difficult to conclude that the ones seen in *shavenoid* embryos were actually due to *wingless*, especially when they were shifted during germ band extension. This is a stage when intermediate phenotypes occur, but a high proportion of segmentation defects are also seen among the non-*shavenoid* embryos (Table 4) and denticle belt fusions occur as frequently as they do in the *shavenoid* embryos (Table 5). It is likely, though, that the fusions that occurred in only some segments within a single embryo (Figure 11c) are due to

wingless, since the fusions seen in non-*shavenoid* embryos are usually associated with other defects, such as deletions of part or all of the other denticle belts (see Figure 6). If these fusions are indeed due to *wingless*, it appears from this experiment that the fusions were more likely to occur in the posterior segments, especially segments A4, A5, and A6 (Table 5).

To decide whether there was an 'all-or-nothing' effect, I determined the proportion of each of segments A1 to A7 that was covered by denticles in those segments where fusion had not occurred. Figure 12 demonstrates that there is actually a broadening of the denticle belts as the shift down was made later. This was most apparent in the anterior segments, A1 to A3 (Figure 12a); however, there does appear to be a broadening of the denticle belts in the more posterior segments as well (Figure 12b).

segment	frequency of fusion	
	sha$^-$	sha$^+$
A 1	3	0
A 2	0	0
A 3	2	2
A 4	4	6
A 5	8	5
A 6	6	2
A 7	0	1
Total embryos	26	25

Table 5) The frequency of fusions in abdominal segments A1 to A7 when shifted down during germ band extension. Included are fusions that run along the entire segment or just for part of it.

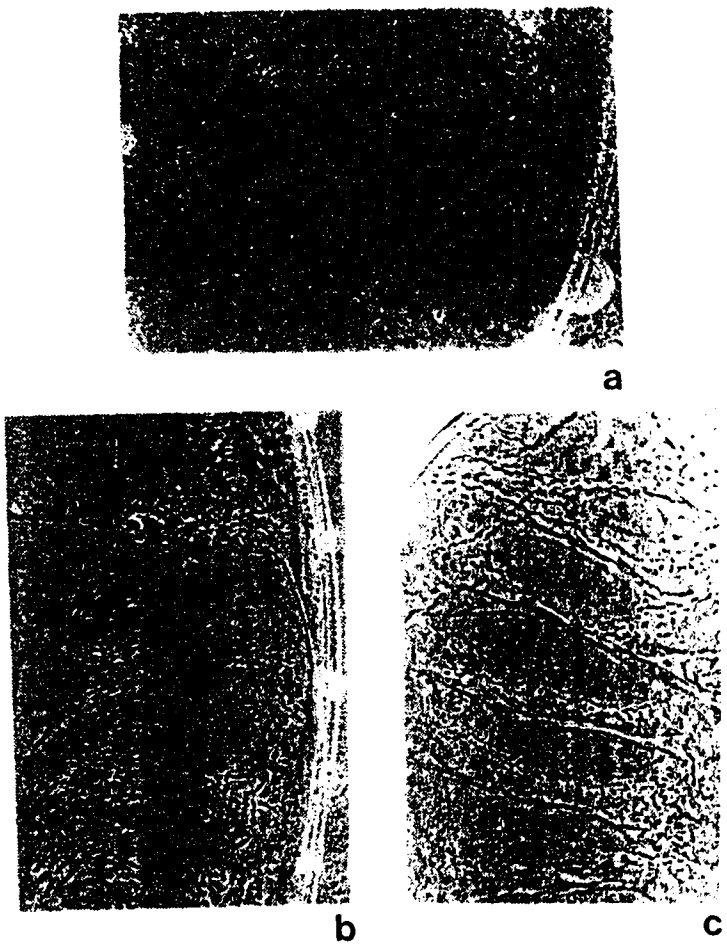
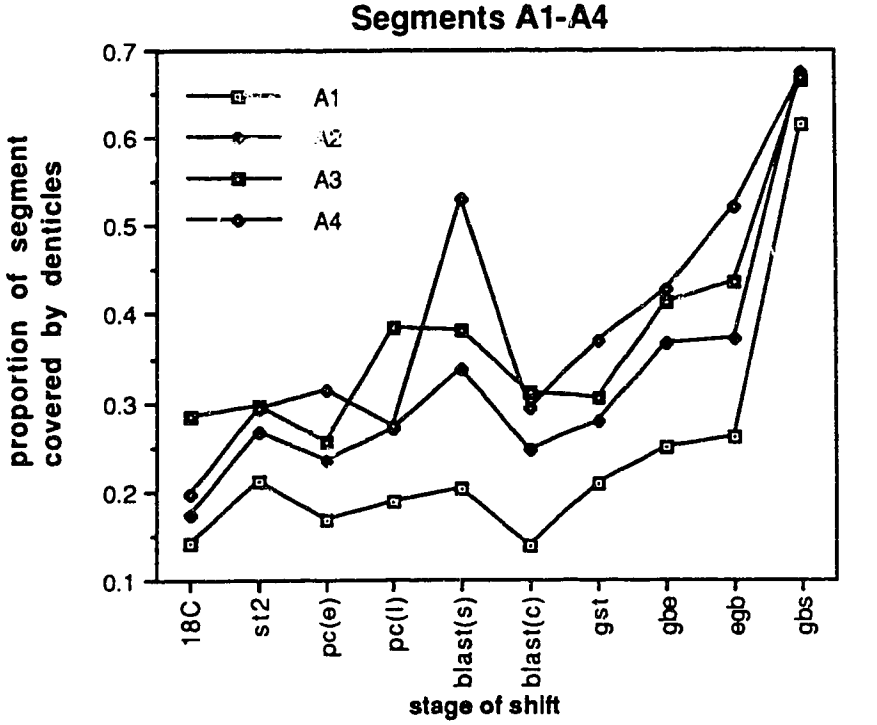
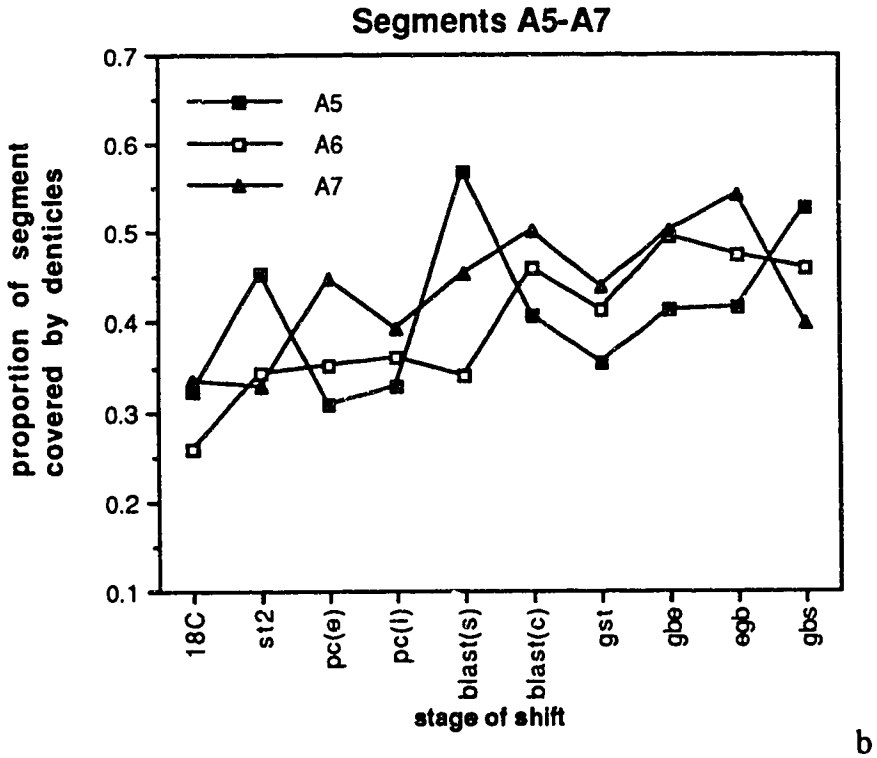


Figure 11) Intermediate *wingless* phenotypes produced in the shift down experiment. **a)** A shift down before the end of germ band extension yields a wildtype phenotype. **b,c)** Two different intermediates produced when the shift was down during the extended germ band stage. Magnification: 312X.



a



b

Figure 12) The proportion of each abdominal segment that is covered by denticles as a function of the stage of the shift down.

Another difference between up and down shifts is that, for the up shift, most of the embryos display a similar phenotype when shifted at any given stage while for the down shift, there was a range of phenotypes at any one stage, as illustrated by Figures 11b and 11c, which were both shifted down during the extended germ band. The possible implications of this will be discussed later.

This experiment revealed interesting *wingless* phenotypes, which could be used to study the effect of this gene in more detail. However, because of the high frequency of segmentation defects that occurred as a result of physical disturbances to the embryos, a more detailed analysis of the intermediate *wingless* phenotype was difficult. Also, because larvae with a weak *wingless* phenotype can be viable and only unhatched larvae or embryos were mounted, weak *wingless* embryos that did not also carry non-*wingless* segmentation defects were probably under-represented. Another problem arose with the use of *shavenoid* to identify *wingless* embryos. Because *shavenoid* causes the denticles to appear sparse and fine, it was often difficult to determine where the limits of the denticle belts were. This marker provided a useful way to identify *wingless* embryos and to give a general description of the *wingless* phenotype; however, it hindered a more quantitative description. I suspected that the ectopic denticles that would be produced by *wingless* may be removed by the effects of *shavenoid*.

wingless Intermediates At Semi-Permissive Temperatures

Rather than setting up the temperature-shift experiment again, in order to produce a larger number of embryos and to select the viable weak *wingless* larvae to analyze, an alternate method was chosen to study the *wingless* intermediates. When genotypically *wingless* embryos are raised at semi-permissive temperatures, they show intermediate phenotypes. In this case, the environmental conditions could be kept much more constant than in the shift experiments: there was no temperature shock and the embryos did not need to be handled in any way during development, especially during the temperature-sensitive period. As a result, variations in the cuticular pattern of the wild-type controls were much reduced as can be seen among the embryos from a *sha wg*/Cy stock (Table 6). Because the frequency of defects not due to *wingless* is reduced, I found that the *shavenoid* marker was not necessary to identify *wingless* embryos. Instead, the following analysis was done with *wingless* homozygotes derived from the wg^{IL114} *c px sp*/SM6a stock. Without *shavenoid*, it was much easier to determine the placement of the denticles in the *wingless* embryos.

temperature (°C)	total	non-*shavenoid*		*shavenoid*	
		normal	abnormal	normal*	abnormal
†17.9	163	98	8(4.91)	43	1(0.61)
†18.9	86	44	3(3.49)	38	0(0.00)
†19.9	40	28	1(2.50)	10	0(0.00)
†20.2	64	21	4(6.25)	23	0(0.00)
†20.8	150	34	5(3.33)	97	1(0.67)
21.8	34	1	0(0.00)	25	0(0.00)
22.8	43	3	0(0.00)	37	0(0.00)
23.8	104	3	2(1.92)	96	0(0.00)
24.8	49	34	1(2.04)	10	0(0.00)
25.0	31	0	1(3.23)	26	0(0.00)
29.0	36	3	3(8.33)	29	0(0.00)

Table 6) The frequency of defects found among embryos from a *sha wg*/Cy stock raised at various temperatures. Numbers in parentheses represent the percentages of total embryos that showed non-*wingless* segmentation defects. The total represents the total embryos mounted. †In these cases, some hatched first instar larvae as well as unhatched ones were mounted. Since most larvae that crawled away were assumed to have normal segmentation, the frequency of abnormal segmentation among the sample is likely to be an overestimate; whereas, in Table 4, the total represents the total larvae handled, including those that hatched and were not mounted for cuticle preparations. *This column includes both embryos with wildtype segmentation, and those with *wingless* segmentation.

If the temperature-sensitive allele results in abolition of gene activity at the restrictive temperature, then this type of experiment should produce a series of hypomorphic phenotypes that can be used to determine how the gene functions in order to produce the observed wildtype pattern. Traditionally, this has been done with a series of hypomorphic mutations; however, several problems can arise. For example, the genetic background can vary among mutant stocks and affect or modify the phenotype produced by the mutation.

This can be resolved somewhat by repeated back-crossing of each stock to equalize the backgrounds; however, it is impossible to remove all differences in genetic background. Also, it can be difficult to order a series of hypomorphic mutations for a pattern formation gene where a quantitative assay of gene activity is difficult. By utilizing a loss-of-function temperature-sensitive mutation, a hypomorphic series can be created from the same stock, even from the same parents, by raising the animals at the semi-permissive temperatures, and thus eliminating any differences in the genetic background. The only difference between the different *wingless* phenotypes would be due to different levels of *wingless* activity as a result of the temperature difference, unless there are other undetected temperature-sensitive mutations in the stock.

Another advantage of creating intermediates with semi-permissive temperatures is that variability among the embryos is reduced. In the shift experiment, there must be differences in the degree of development among the embryos grouped into any one stage, since development is continuous and not broken down into the discrete stages that are used for classification, so that the variability among those embryos is a function of the degree of precision with which one can distinguish between stages of development. This is not the case with the embryos that were grown at any particular semi-permissive temperature; any fluctuation in temperature during development would affect all the embryos in the group in a similar manner.

In this section, I will describe the intermediate phenotypes produced at the semi-permissive temperatures for both the thoracic and the abdominal segments in order to try and understand how *wingless* is involved in the generation of the periodic repeat pattern seen in these segments. It is important, however, to first establish that the allele used is indeed a hypo- or amorphic allele, and that incrementally increasing the temperature from permissive (<18°C) to restrictive (>25°C) actually lowers the level of *wingless* activity to give hypomorphic intermediates.

Determination that wg^{IL114} is an Amorph at the Restrictive Temperature

To determine that wg^{IL114} results in a loss of *wingless* activity at the restrictive temperature and is therefore an amorphic mutation, the phenotype of an individual homozygous for the allele wg^{IL114} was compared to one heterozygous for the allele and for a deficiency of the region (see Materials and Methods) at three different temperatures. If the allele is a hypomorph, that is, a reduced amount of the product causes the phenotype seen, then the homozygote should have a less extreme phenotype than does the heterozygote. If it is an amorph, the phenotypes of the two genotypes should be the same. Therefore, as the temperature increases, and the level of activity decreases, to approach an amorphic state, the difference between the homozygote and the heterozygote should decrease. Figure 13 shows the results from this experiment. At 21.6°C, the phenotype of the wg^{IL114}/Df(2L)NL heterozygote is clearly more

severe than that of the wg^{IL114} /wg^{IL114} homozygote: in the heterozygote, fusion of all of the segments and polarity reversal is evident in most embryos raised at this temperature; whereas in the homozygote, the bands have broadened and fusion only occurs in the posterior-most segments of a few embryos. At 23.0°C, the difference between the two genotypes is less extreme and at 25.6°C, the extreme *wingless* phenotype is seen for both genotypes, suggesting that, at this temperature, the wg^{IL114} allele behaves as an amorph. These results have been depicted in a graph in Figure 14, to show the difference in the proportions of segments A1 and A7 that are covered by denticles. At 21.6°C, 74% of A1 is covered by denticles in the homozygote, whereas in the deficiency heterozygote, 94% of segment A1 is covered by denticles. This difference is also apparent for segment A7.

A true amorphic state for *wingless* would be in a homozygote for a deficiency of the region. Df(2L)NL/Df(2L)NL individuals display a typical *wingless* phenotype that may be a little more extreme than for any of the other genotypes. One difference is that there is not a clear separation of the thoracically-derived denticles from the 'lawn' by naked cuticle, as was seen in the wg^{IL114} homozygotes at the restrictive temperature; rather, this region is filled with fine, apparently randomly oriented, denticles (Figure 20b).

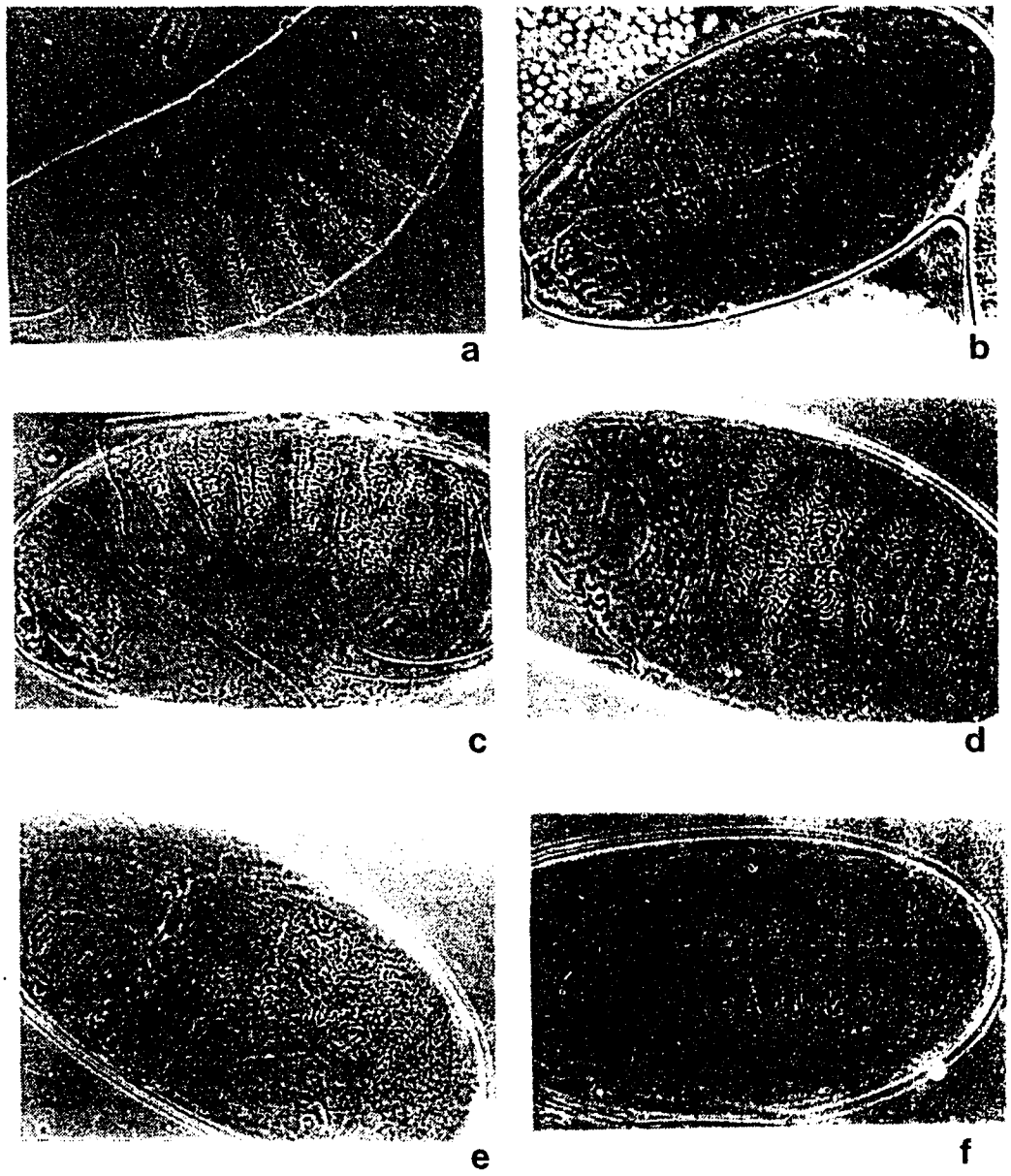


Figure 13) Qualitative determination that wg^{IL114} is a loss-of-function allele at the restrictive temperature. Anterior is oriented to the left. The two genotypes, wg^{IL114}/wg^{IL114} **(a, c, e)** and wg^{IL114}/Df(2L)NL **(b, d, f)** are compared at 21.6°C **(a, b)**, 23.0°C **(c, d)** and 25.6°C **(e, f)**. Magnification: 130X (a,b), 156X (c-f).

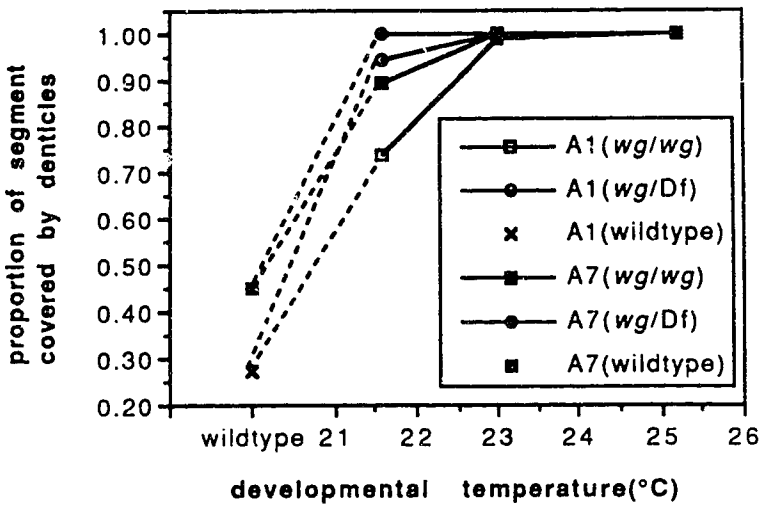


Figure 14) Quantitative determination that wg^{IL114} is a loss-of-function allele at the restrictive temperature: the proportion of segments A1 and A7 covered by denticles as a function of temperature in wg^{IL114}/wg^{IL114} homozygotes and wg^{IL114}/Df(2L)NL heterozygotes. They are compared at intermediate temperatures in order to determine that wg^{IL114} results in a loss-of-function phenotype at the restrictive temperature. The ratios for the wildtype controls were determined in embryos raised at 19.8°C for this and subsequent graphs.

Analysis of the Pattern Repeat

In order to closely analyse the effect that intermediate levels of *wingless* have on the segmental pattern, changes in various cuticle markers on the ventral surface of the larvae were determined in the series of intermediates. These markers included the denticles that define the anterior region of all thoracic and abdominal segments, the Keilin's organs, which lie in the middle of the naked cuticle and define the anterior/posterior compartment (Struhl, 1984), and the

patch of denticles that lies between the anterior denticles and the Keilin's organs of the prothorax. For the abdominal segments, there are no ventral cuticle markers posterior of the denticle belts, about three-quarters of these segments are occupied by naked cuticle. It was also of interest to study changes within the denticle belts. Since most of the rows in the wildtype abdominal belts A2 to A7 can be distinguished from each other due to differences in size and polarity, changes in these rows, and the positions of them, can be described. These rows will be referred to as r1 to r7, as described in Table 7. The changes seen are similar for all of these abdominal belts, although equivalent changes occur at higher temperatures in the more anterior segments than in the more posterior segments, due to the gradient of effect along the anterior/posterior axis, which is clearly evident at 21.8°C (see Figure 15c). Therefore, in the embryos grown at any one semi-permissive temperature, there exists a series of intermediate phenotypes that increase in severity along the anterior/posterior axis, in addition to the series that is seen for any one denticle belt as the temperature is increased.

row	relative size	polarity
row 1 (r1)	medium	anterior-pointing
row 2 (r2)	medium	posterior-pointing
row 3 (r3)	medium	posterior-pointing
row 4 (r4)	medium	anterior-pointing
row 5 (r5)	large	posterior-pointing
row 6 (r6)	small	posterior-pointing
row 7 (r7)	small	posterior-pointing

Table 7) Size and polarity of the denticles in the abdominal belts, A2-A7 (refer to Figure 17a for a photograph of the wildtype denticle belt).

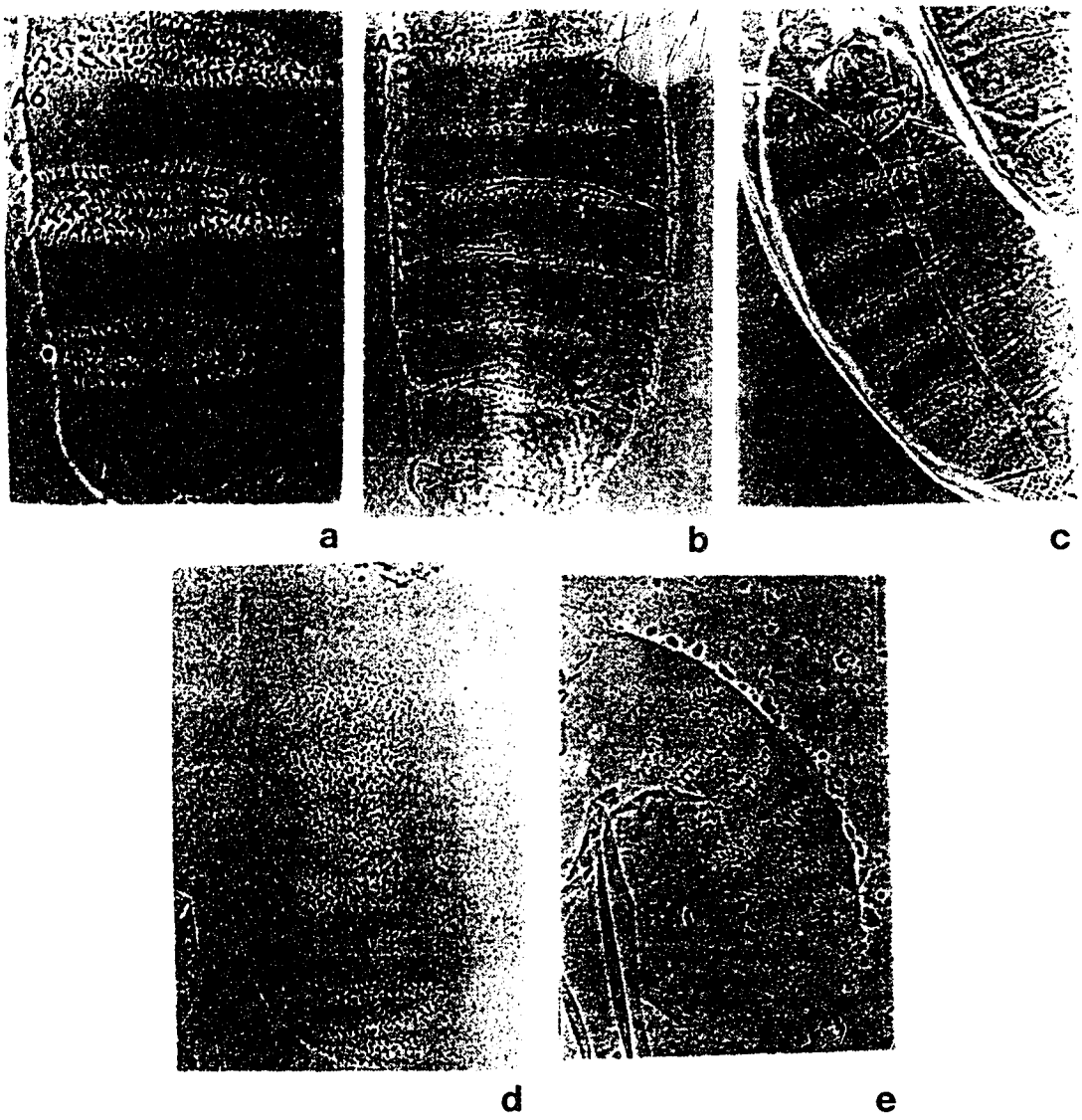
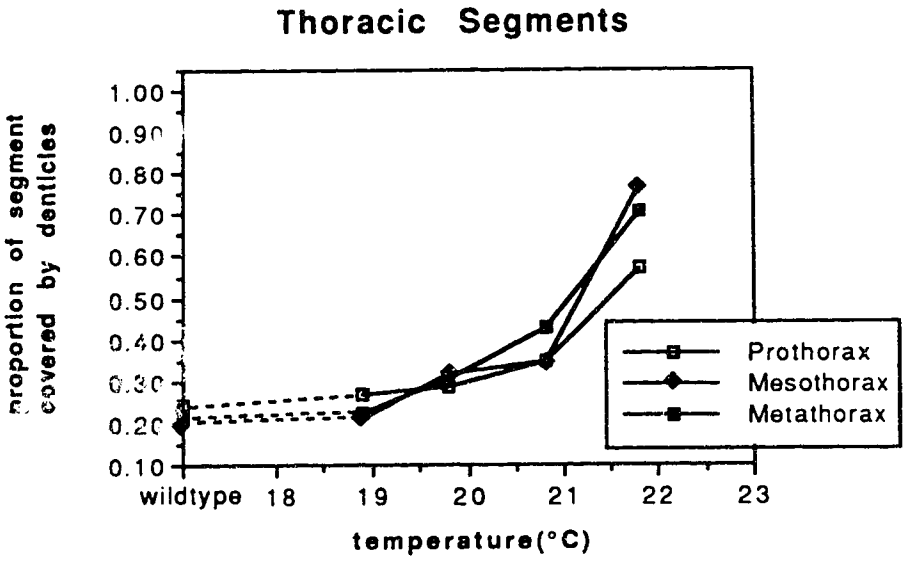


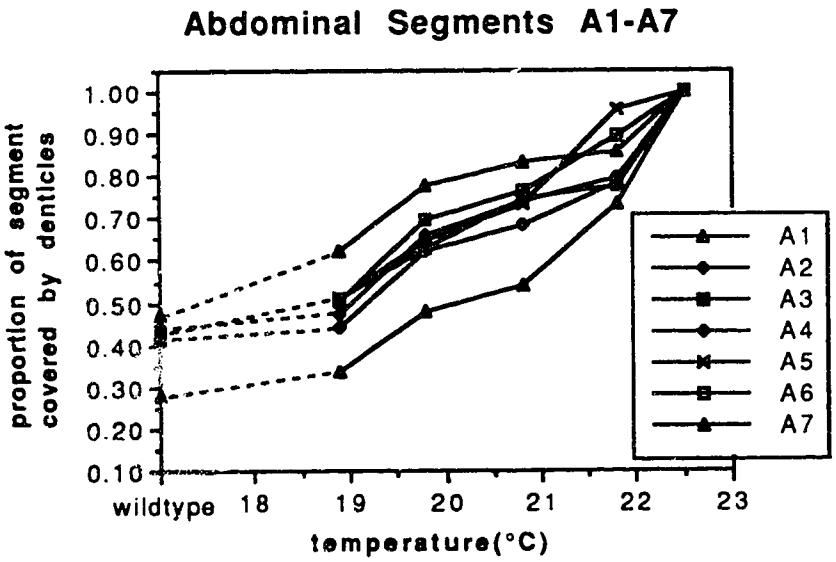
Figure 15) The series of intermediates produced when embryos are raised at semi-permissive temperatures. Developmental temperatures: a) 19.8°C, b) 20.8°C, c) 21.8°C, d) 23.8°C, e) 24.8°C. Magnification: 312X (a), 156X (b,e), 130X (c,d).

Changes in the denticle belts

As the developmental temperature is increased from the permissive temperature of 18°C, the denticles occupy a progressively larger proportion of each segment in the wg^{IL114} homozygotes (Figures 15, 16). This effect is first apparent in the more posterior segments as can be seen in Figure 15c. Initially, the belts appear to broaden without polarity-reversal, but at higher temperatures, as the denticle belts begin to fuse, polarity reversal becomes evident (Figure 15d). This is very similar to the situation that was seen in the shift up experiment.



a



b

Figure 16) The proportion of each thoracic and abdominal segment that is covered by denticles in wg^{IL114}/wg^{IL114} homozygotes as a function of developmental temperature.

The first change seen is the appearance of ectopic denticles on either side of the wildtype belts as can be seen for A7 at 18.9°C (Figure

17b). These new denticles are located near the ventral mid-line, and are usually apparent only in the most posterior segments at this temperature, that is, A7 and A8. The number of these ectopic denticles increased as the temperature increased, spreading out both laterally and anterior-posteriorly (Figure 17c), particularly on the anterior side. Not only do the extra denticles appear on either side of the belt; frequently, part of an additional row appears between the two anterior-pointing rows within the belt (Figure 17d). When the proportion of the segment that is occupied by the region between the two anterior-pointing rows of abdominal segments was determined for segments A3 and A7, it was found that this distance increased with temperature (Figure 18). When the extra denticles became apparent within the belt, the number of ectopic denticles anterior of row 1 appeared to decrease (Figure 17d). With a further increase in temperature, the anterior-pointing row 1 can no longer be seen; rather, there are three or more rows of posterior-pointing denticles in the anterior region of the belt. The more posterior rows, rows 4 to 7, appear normal at this temperature for A2 (21.8°C, see Figure 17e), and the number of ectopic denticles posterior of row 7 has not significantly increased. Therefore, it seems that regions of the segment that are naked cuticle in the wildtype become filled with denticles in the weak *wingless* background but are subsequently lost in a stronger *wingless* background, possibly due to cell death. Also, the belt might 'stretch-out', resulting in denticles being secreted by new cells that appear within the belt. These ideas will be discussed later.

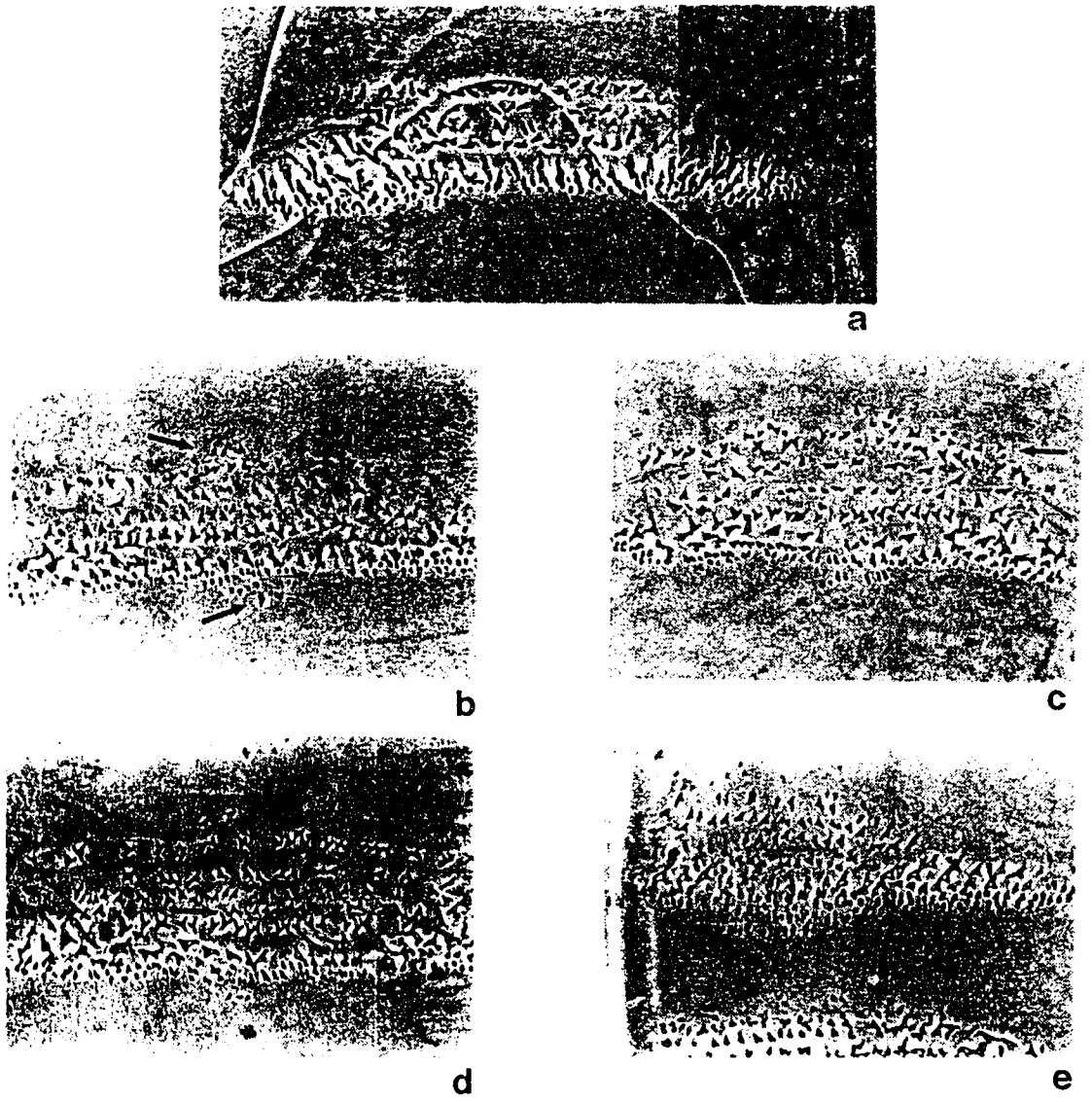


Figure 17) Changes in the abdominal denticle belts as the developmental temperature is increased. **a)** The wildtype belt. Note the 6-7 different rows designated r1 to r7 as described in Table 7. **b)** Ectopic denticles on either side of the belt. **c)** Spreading of the ectopic denticles. **d)** Part of an extra row between r4 & r5. **e)** r1 is undetectable. Magnification: 520X. Identification of belts and developmental temperatures: a) *wg*$^{+}$ A4 at 19.8°C, b) A5 at 18.9°C, c) A7 at 19.8°C, d) A7 at 19.8°C, e) A2 at 21.8°C. (Figure 17 continued on the next page).

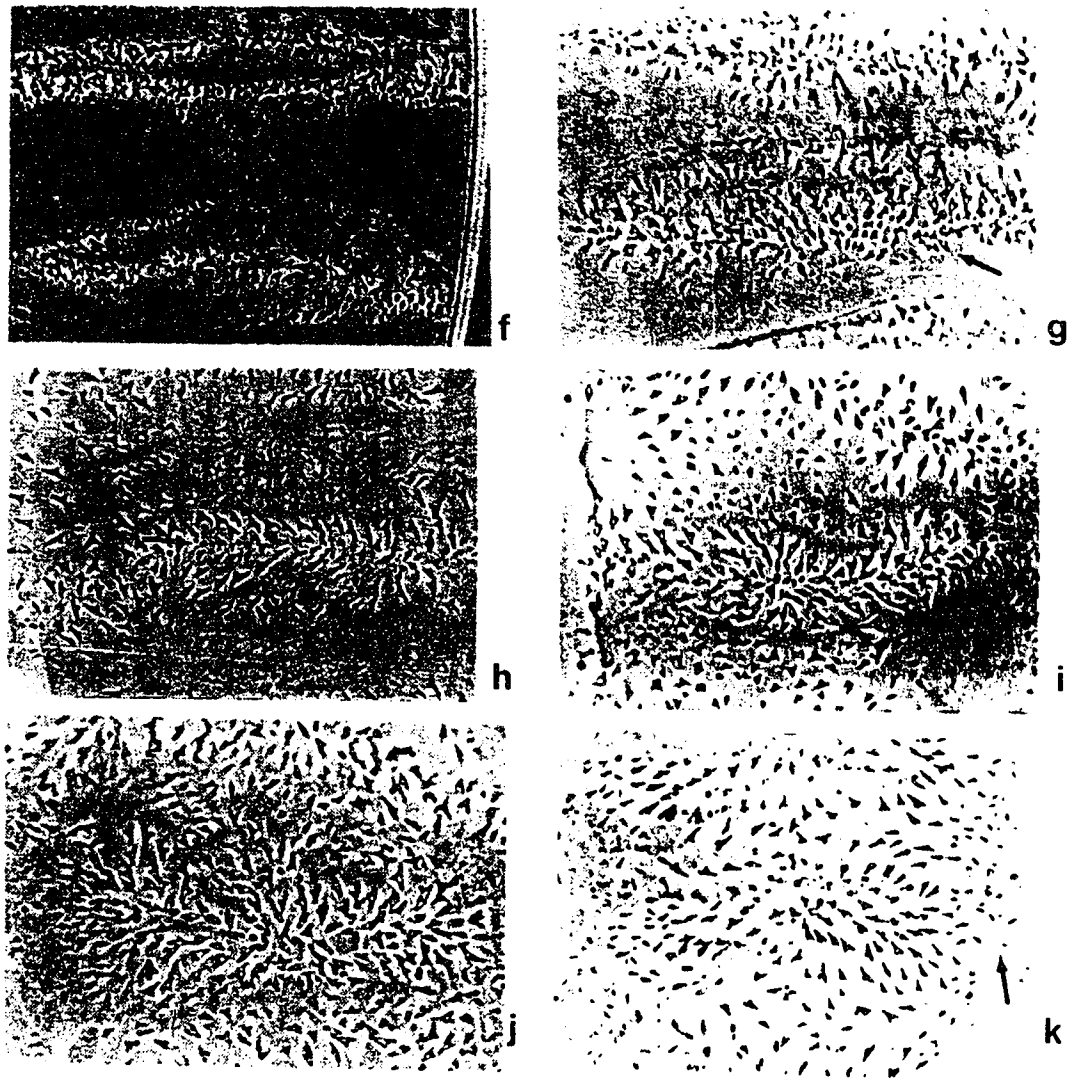


Figure 17) (continued from previous page). **f)** heavy r5-like denticles appear in the posterior region of the belt. Note that they point posteriorly. **g)** The new heavy denticles point anteriorly (arrow). The smaller denticles in the middle of the repeat (between the heavier rows) point posteriorly (small bracket). **h)** The two rows of smaller denticles in the middle now mirror-image each other. **i-k)** The small denticles in the middle disappear and denticles pointing towards the mid-line flank the sides of the lawn (arrow in **k**). **k)** The heavy r5-like denticles predominate in the repeat in the *wingless* deficiency. Magnification: 520X. Identification of belts and temperatures: f) A6 at 21.8°C, g) A2 at 22.9°C, h) A2 at 23.8°C, i) A2 at 24.8°C, j) A2 at 29.0°C, k) A2 in the deficiency homozygote.

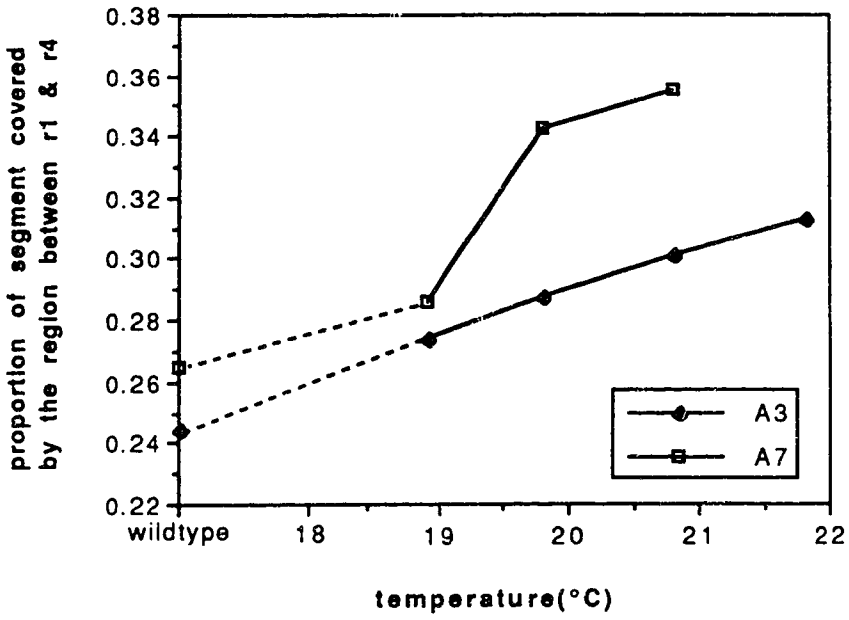


Figure 18) The effect of temperature on the distance between the anterior-pointing rows, r1 and r4, for abdominal segments A3 and A7.

On the posterior side of the belt, a few ectopic denticles, of similar morphology to row 6 and row 7, appear at the ventral mid-line, giving a distorted appearance to that region of the belt (Figures 17b to 17d). In a more extreme phenotype, when row 1 cannot be detected, heavier denticles similar to those in row 5 appear in the posterior region of the belt (Figure 17f). These denticles have a random polarity: some point posteriorly and some point anteriorly. At a higher temperature, these denticles all point anteriorly, reverse of the wildtype polarity of row 5 (Figure 17g). Therefore, at this stage polarity-reversal is starting to be evident, although not all of

the denticles in the posterior part of the repeat point anteriorly as they do in the more extreme *wingless* phenotypes (Figures 17h-k).

In segment A2 at 23.8°C, approximately two row of smaller denticles resembling those in rows 6 and 7 lie between the two rows of large denticles that resemble row 5, with the more posterior row of these small denticles in reverse polarity (Figure 17h). Both of these rows point posteriorly in Figure 17g. In the most extreme *wingless* phenotype found for the temperature-sensitive allele, these smaller denticles are rarely seen (Figure 17j). At this point, two types of denticles seem to predominate in the repeat: several rows of large denticles, characteristic of the wildtype r5 and several rows of medium denticles characteristic of the more anterior wildtype denticles, r1 to r4. Only one type of denticle is apparent in the deficiency homozygote, the large ones characteristic of r5 denticles (Figure 17k). In the extreme *wingless* background, there are also finer denticles which bound either side of the 'lawn' and point towards the mid-line (Figures 17i-k).

Although a detailed description of the apparent broadening, fusion, and polarity-reversal of the denticle belts was provided only for segments A2 to A7, the broadening of the denticle belts is apparent in all of the segments, both thoracic, and abdominal A1 to A8 as is demonstrated in Figure 16, which shows the proportion of each segment that is covered by denticles. In the prothorax, it appears that not all of the naked cuticle is deleted in the most extreme phenotype seen for wg^{IL114}/wg^{IL114} embryos (Figure 20a). The

anterior band of denticles that is separated from the 'lawn' by naked cuticle in the extreme *wingless* embryos appears to be derived from the prothorax whereas the meso- and metathorax belts are fused in with the abdominal belts (see Figure 19b). This would be consistent with the idea that the more anterior segments are less affected at any one temperature than the more posterior segments. In the complete amorph, Df(2L)NL/Df(2L)NL (Figure 20b), there is usually no naked cuticle separating the anterior belt of denticles. This region is filled in with fine denticles; however, the anterior prothoracic denticles are still distinguishable from the lawn.

Since the posterior regions of the abdominal segments do not contain any cuticular markers, the ones found in the thoracic segments, that is, the Keilin's organs and the patch of denticles in the prothorax, were used to study the effect of *wingless* levels on the posterior region of the segments.

Changes in the location of the Keilin's organs

When the position of the Keilin's organs was determined relative to the anterior boundary of the preceding denticle belt, it did not appear to move relative to this boundary, at least for the prothorax and the mesothorax (Figure 21). There did appear to be an increase in the distance between the metathoracic Keilin's organs and the anterior edge of the preceding denticle belt at temperatures over 19.8°C (Figure 21). At higher temperatures, the Keilin's organs were missing: six out of nine prothoraces, ten out of ten mesothoraces, and five out of ten metathoraces had no Keilin's organs at 21.8°C. They

are not seen at all at the higher temperatures even though, in some cases, there is still naked cuticle present in the region of the segment where the Keilin's organs would be expected (Figure 19d).

Changes in the mid-prothoracic denticles

In the prothorax, it appears that the mid-prothoracic patch of denticles first fuse with the anterior denticles (Figure 19b) before the mid-prothoracic denticles apparently disappear (Figure 19c,d). The amount of naked cuticle between the two prothoracic belts was determined and found to decrease with temperature (Figure 22), until there was no naked cuticle between them at 21.8°C, giving the fusion seen at this temperature. This fusion is probably due to the broadening of the anterior belt. The mid-prothoracic belt does not appear to broaden: when the temperature is increased, the proportion of the segment that it covers remains constant, or may actually decrease slightly, whereas the proportion covered by the anterior denticles has increased with temperature (Figure 23). At temperatures higher than 21.8°C, the mid-prothoracic denticles are not distinguishable (Figure 19d), but before they disappear, the distance between this patch of denticles and the anterior border of the prothorax increases somewhat by 21.8°C (Figure 24).

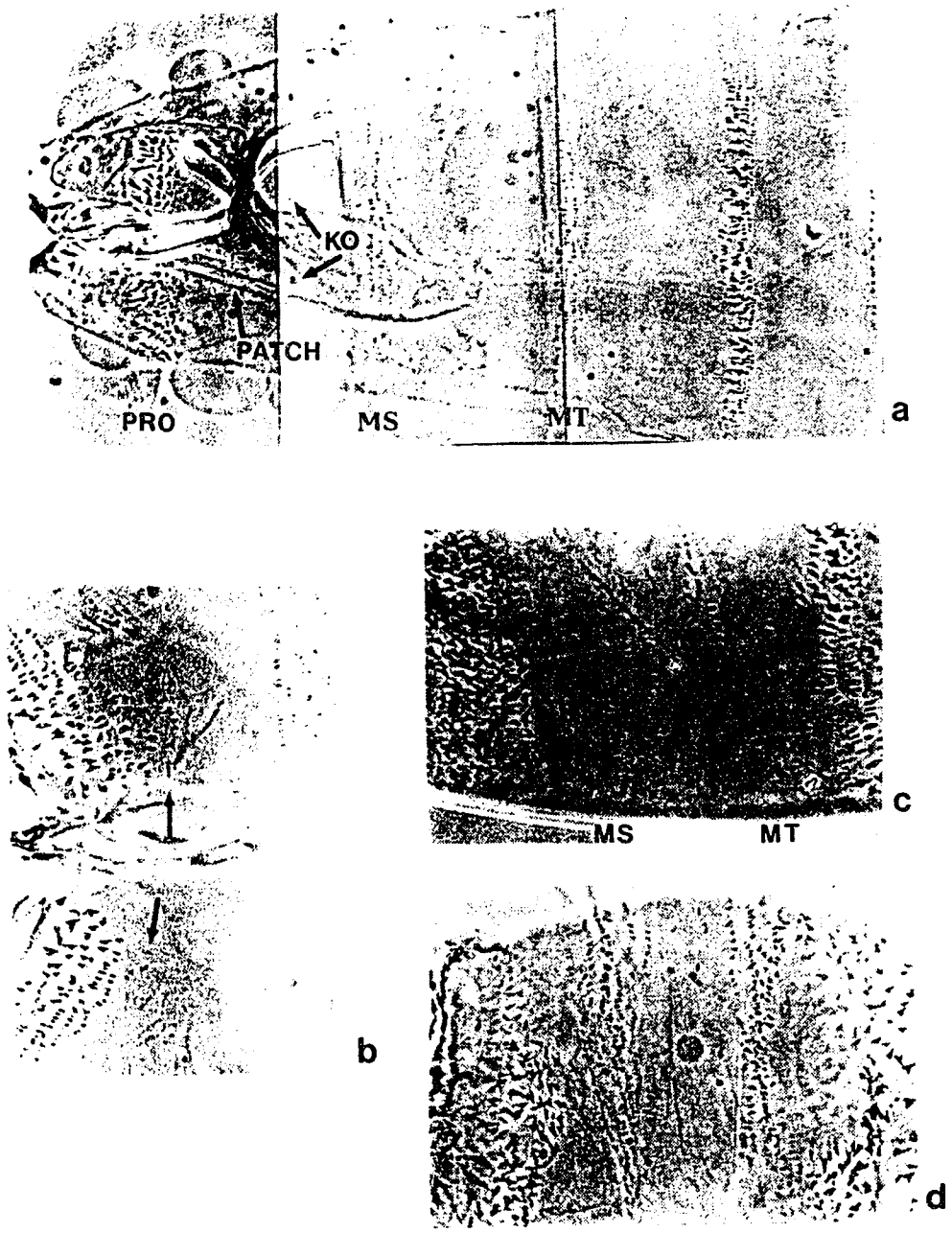


Figure 19) Changes in the thoracic pattern elements. Anterior is oriented to the left. **a)** The wildtype thorax. The bracket indicates the mid-prothoracic patch. **b)** The mid-prothoracic patch (arrows) fuses with the anterior belt at 20.8°C. **c)** Fusion between the mesothorax, metathorax and A1 at 21.8°C. Note that Keilin's organs are still present in the prothorax of this embryo (arrow). **d)** Neither mid-prothoracic denticles nor Keilin's organs are present in the prothorax at 22.8°C. Magnification: 312X (a), 520X (b-d).

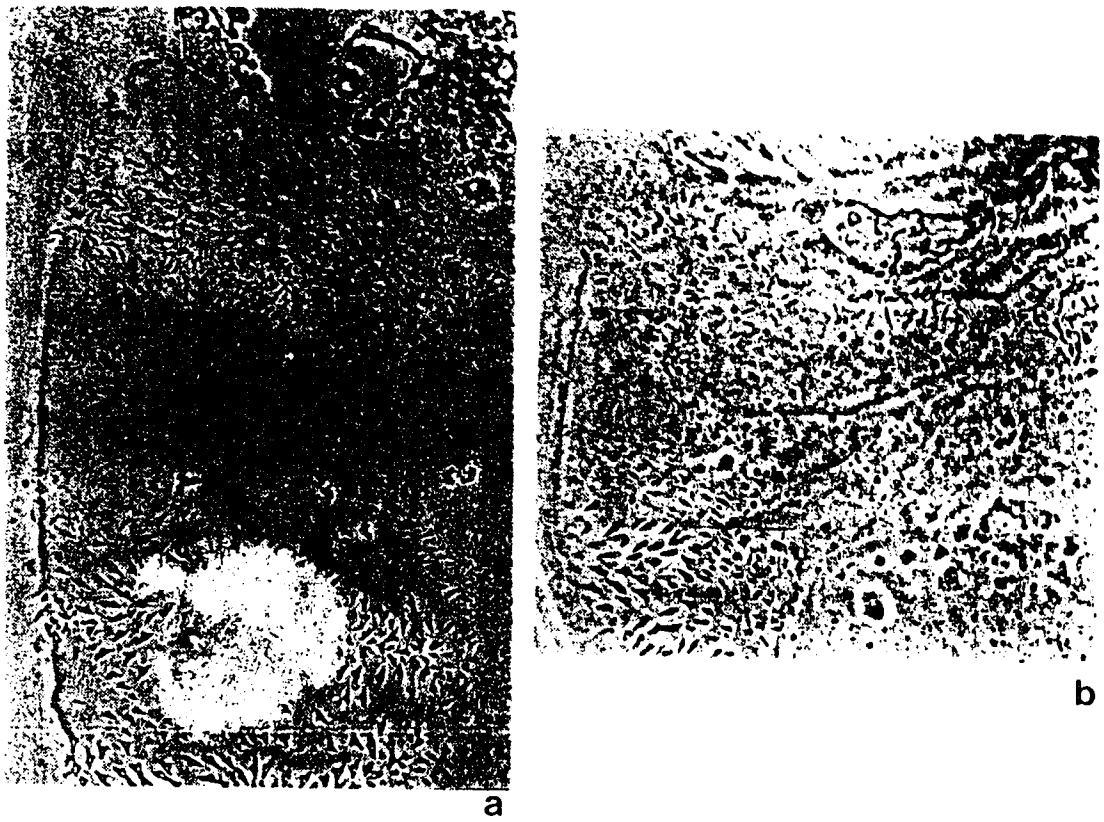


Figure 20) The extreme *wingless* thoracic phenotype. Note that there is naked cuticle separating the lawn from an anterior belt of denticles in the most extreme wg^{IL114}/wg^{IL114} phenotype (a), but not in the deficiency homozygote, where this region is filled in with fine denticles (b) (N.B. due to folds in the embryo in (b), many of the fine denticles are out of focus). Magnification: 312X. a) Raised at 24.8°C b) Raised at room temperature (~22°C).

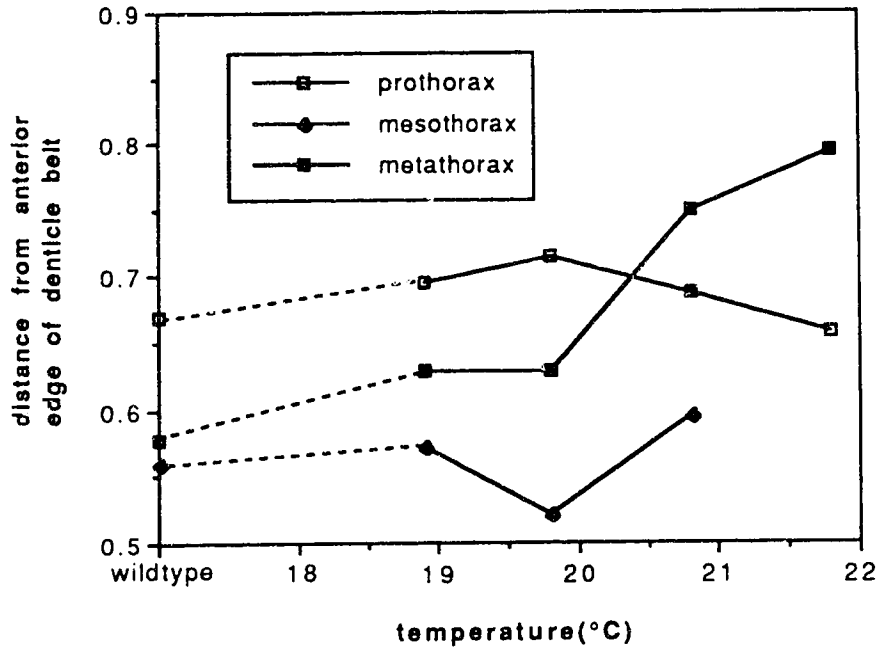


Figure 21) The effect of temperature on the position of the Keilin's organs. The position is determined relative to the anterior boundary of the preceding denticle belt and is expressed as a proportion of the entire segment.

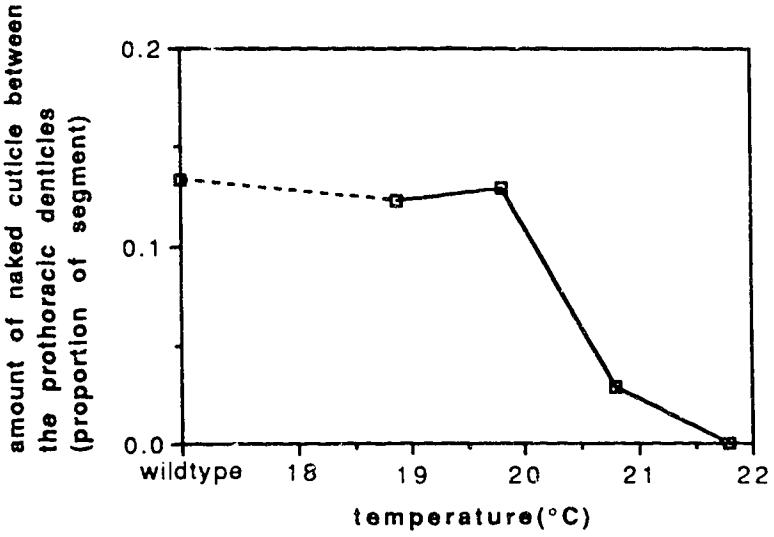


Figure 22) Fusion of the prothoracic denticles. This graph shows the amount of naked cuticle between the anterior prothoracic belt and the mid-prothoracic patch of denticles to demonstrate the fusion between the two groups of denticles.

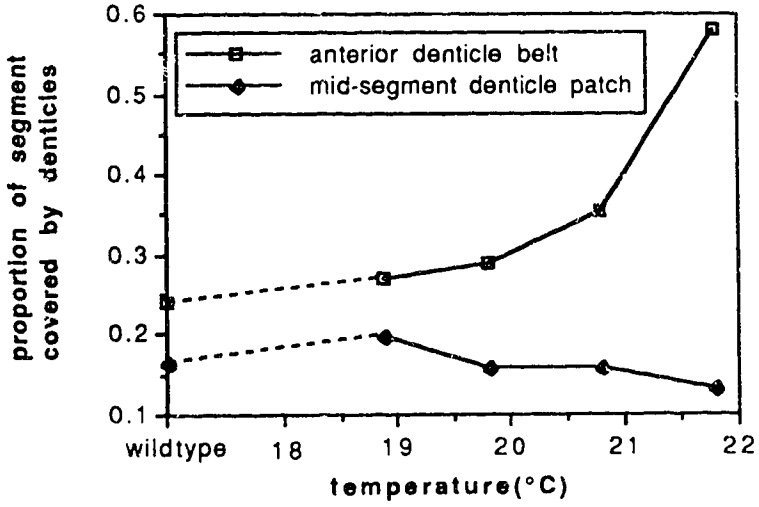


Figure 23) The proportion of the prothorax occupied by the two groups of denticles.

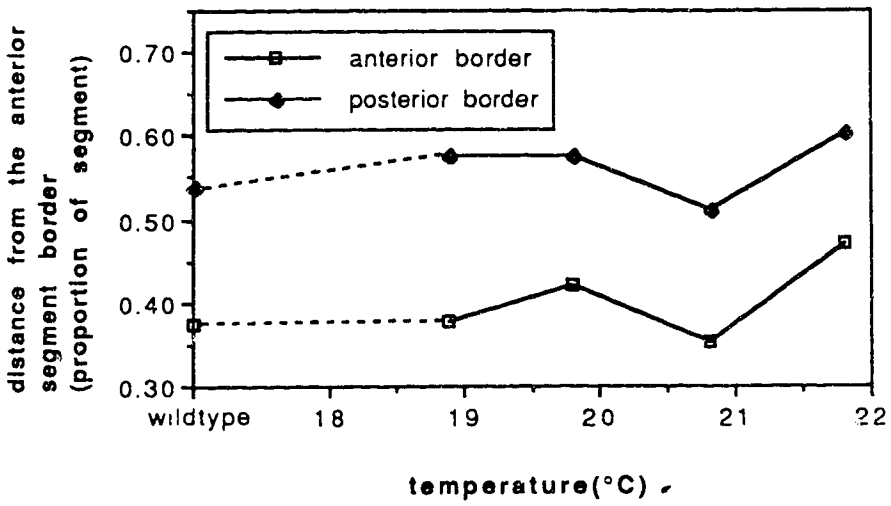


Figure 24) The position of the mid-prothoracic patch of denticles relative to the anterior boundary of the preceding denticle belt.

The Expression of *engrailed*-Regulated ß-Galactosidase in *wingless* Intermediates

The *engrailed*-expressing region, or the posterior compartment, consists of naked cuticle plus the first row of denticles in the abdominal segments (Hama, et. al., 1990). In the absence of wildtype *wingless* function, this region is missing. Cell death has been previously reported in the posterior compartment for *wingless*-like segment-polarity mutants; Perrimon and Mahowald (1987) suggested that pockets of cell death in *dishevelled* embryos may be correlated with loss of *engrailed* signal in these mutant embryos and Klingensmith, et. al. (1989) showed that dying cells in *armadillo* mutants were derived from the posterior compartment. In strong *wingless* mutants, this region is deleted, and it is the first area affected in the weaker *wingless* background with the appearance of denticles in place of naked cuticle. In *wingless* mutants, *engrailed* expression is initiated as in the wildtype; however, the signal is prematurely lost. This is not likely to be due solely to cell death in the posterior compartment because this result was seen when anti-*engrailed* antibody was used in *wingless* embryos and loss of signal was detected before cell death was apparent (DiNardo, et al, 1988). Despite the effects that mutant *wingless* has on the posterior compartment, expression of *wingless* has not been seen in this region (van den Heuvel, et al, 1989; Baker, 1987).

To study the effects of intermediate levels of *wingless* on the posterior compartment, a reporter gene for these cells was used. The

E. coli lac-Z gene has been fused to sequences from the *engrailed* promoter. A transformant strain, called *ryXho25*, has this fusion inserted 257 nucleotides upstream of the transcription initiation site for the endogenous *engrailed* gene where it is regulated with an *engrailed*-specific profile; that is, it is specifically expressed in the cells of the posterior compartment (Hama, et al, 1990). The product of this fusion, ß-galactosidase, is stable and persists later in embryogenesis than does the native *engrailed* protein in wildtype embryos (Hama, et al, 1990), so that loss of the product is more likely due to cell death rather than to a change in the regulation of the fusion gene, as would be the case for the native *engrailed* protein, which has a short half-life (Weir, et. al., 1988). When the same insert was used to study the effect of intermediate levels of *armadillo* on the posterior compartment, loss of ß-galactosidase expression was correlated with cell death (Klingensmith, et al, 1989). Therefore, although *engrailed* requires *wingless* to maintain its expression in the posterior compartment cells and loss of the *engrailed* signal is not necessarily a secondary result of cell death, the use of a reporter gene with a product that persists in the cells in which it was initially expressed, despite a change in the regulation of the gene, can allow one to study the effect of a mutation on cell death in this particular group of cells, which would normally express *engrailed*.

Homozygotes of *wingless* which carry the fusion gene were derived from a cross between the $en^{\text{lac-Z}}wg^{\text{IL114}}$ strain and the wg^{IL114} strain and raised at temperatures between 18°C and 25°C and

stained for ß-galactosidase activity as described in the Materials and Methods. As the development of these *wingless* embryos at 25°C (the restrictive temperature) proceeds beyond germ band extension, ectodermal signal is lost in patches. It is first lost from the presumptive abdominal segments (Figure 25b), with the exception of the most posterior segment, A9, where a small patch of stain persists until near the end of germ band shortening, and then from the two posterior thoracic segments. By the time the germ band has finished shortening, the only ectodermal staining that remains is in the head segments and a small dorsally located patch in the presumptive prothorax (Figure 25c). Staining in the central nervous system (CNS) remains unaffected. This is similar to what was previously seen when *engrailed* antibody was used in a *wingless* null mutant (DiNardo, et al, 1988).

Since the end of germ band shortening was the stage by which maximum loss of signal was obtained at the restrictive temperature and at which the striped pattern was the clearest in non-*wingless* embryos, this stage was used to study the staining pattern in *wingless* embryos that were raised at semi-permissive temperatures. This pattern was correlated to the cuticular pattern seen in embryos that were raised alongside of the stained embryos (see Materials and Methods).

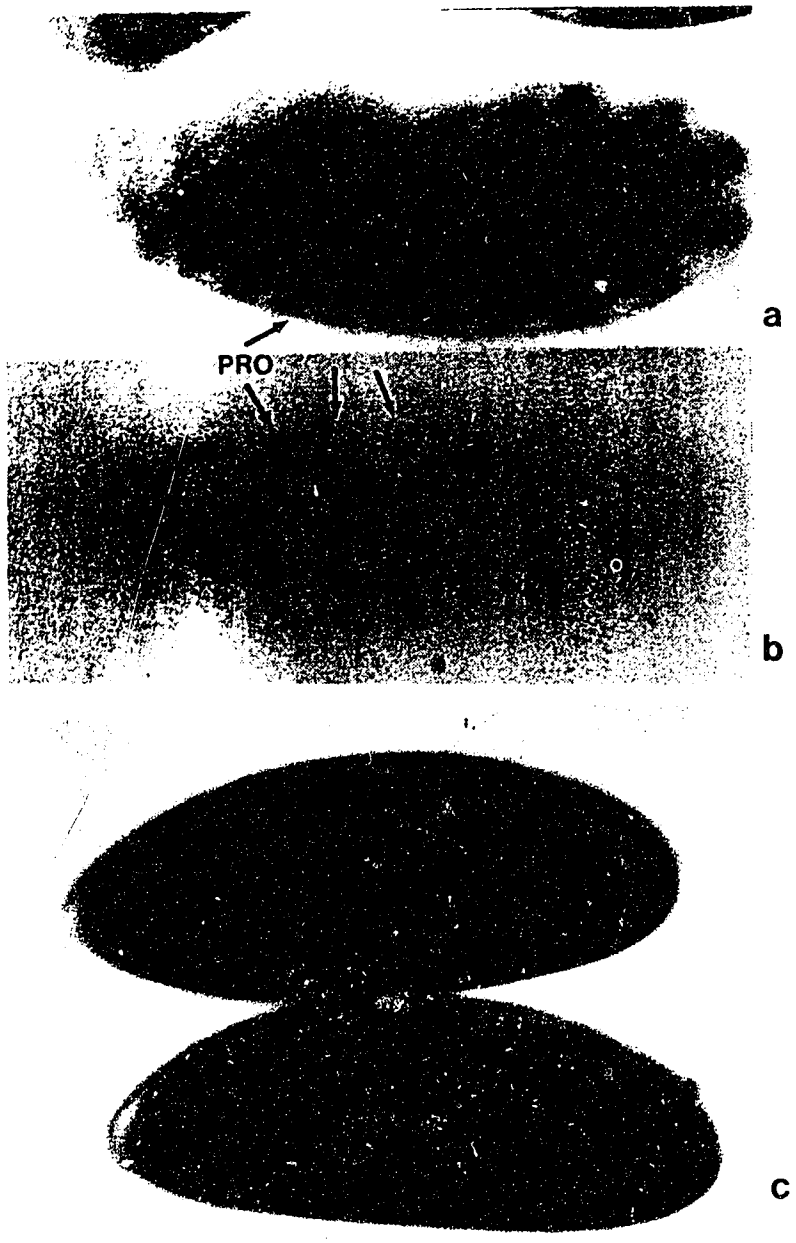


Figure 25) ß-galactosidase (ß-gal) expression in *wingless* embryos raised at 25°C. Anterior is to the left and ventral to the bottom in this and subsequent figures, unless otherwise noted. (In (c), ventral is facing out in the lower embryo). a) ß-gal expression is initiated in an *engrailed*-like pattern of 15 stripes. b) The pattern decays through gbe. Note persisting stain in the lateral regions of the thorax (arrows). c) By the end of gbs, the only remaining stain is in the CNS, the head, and a lateral spot in the prothorax (arrow). Magnification: 182X (a,c), 160X (b).

In a weak *wingless* background, such as that in the embryos grown at 20.0°C (Figure 26a), the stripes appear thinner than wildtype and gaps appear in the ventro-lateral region of these stripes, particularly in the odd-numbered ones, which correspond to T2 (mesothorax), A1, A3, A5, and A7 stripes (see Figure 30 for the correspondence to parasegments). Cuticle preparations at this temperature (Figure 26b) usually show an extra row of ectopic denticles anterior of the belt in the posterior segments and some extra denticles posterior of the belt, which results in a broadening of these denticle belts. Sometimes, but not often, one or two extra denticles have appeared between rows 1 and 4. At 21.2°C, the odd stripes are clearly more affected than the even stripes, and the posterior stripes more so than the anterior ones, with the exception of A8 and A9 which appear normal (Figure 27a). At this temperature, the posterior belts almost touch or are just touching each other, but polarity-reversal is not apparent (Figure 27b,c). In most cases, row 1 is still distinguishable in all of the segments. At 21.8°C, the ectodermal stripes are patchier throughout the embryo (Figure 28a). In cuticle preparations at this temperature, polarity-reversal is starting to become apparent (Figure 28b,c), although not all of the ectopic row 5-like denticles point anteriorly. Figure 29a shows staining in a stronger *wingless* background (23.2°C). At this point, there are just spots of stain remaining in the lateral regions of some segments, especially the even-numbered ones. In the larval cuticle of those animals raised at this temperature (Figure 29b,c), there is fusion of all belts and polarity-reversal is evident; the ectopic denticles posterior of the wildtype belt point anteriorly. However, there are still regions of naked cuticle in the

more lateral regions. These regions of naked cuticle might correspond to those cells with a posterior identity that have not died, and would therefore express ß-*galactosidase*.

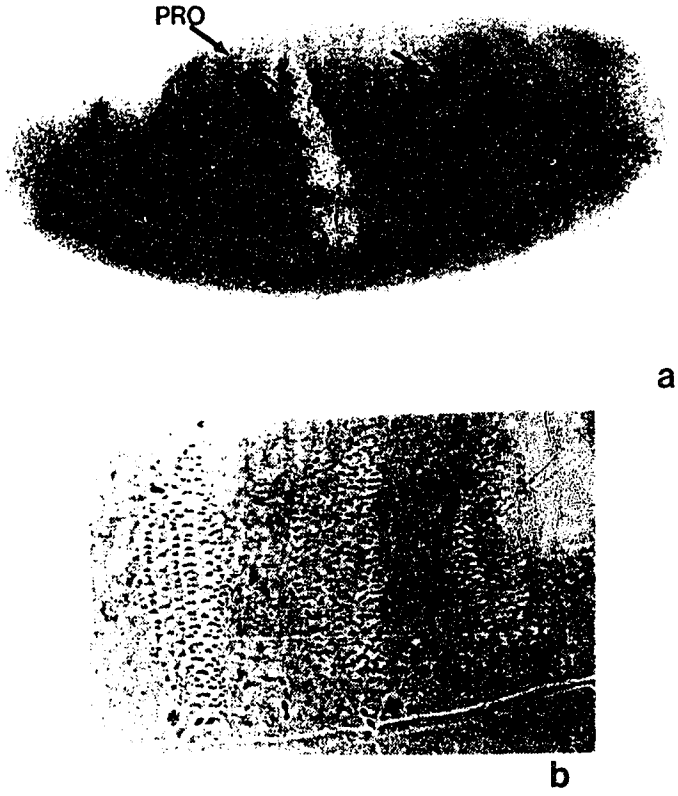


Figure 26) a) ß-galactosidase expression in *wingless* embryos raised at 20.0°C to the end of germ band shortening. The stripes thin and gaps appear in the lateral regions (arrows). b) Ectopic denticles appear in the ventral cuticle (arrow). Segments A6 to A8 are shown. Magnification: 182X (a), 260X (b).

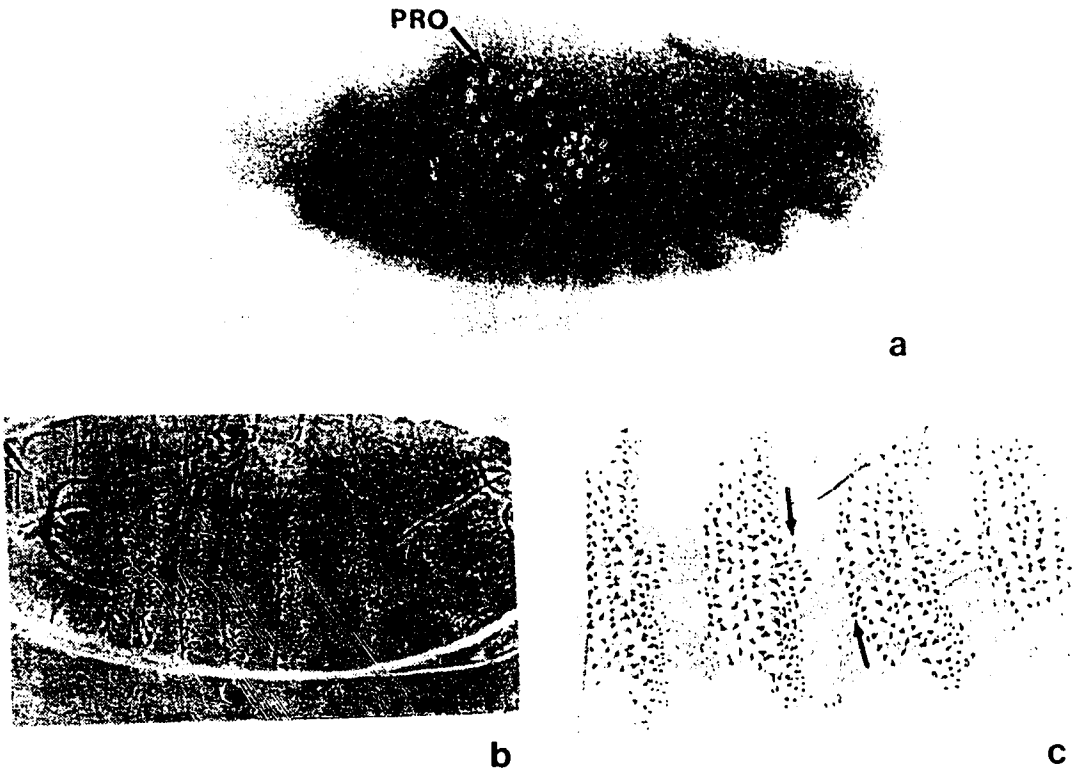


Figure 27) a) ß-galactosidase expression in *wingless* embryos raised at 21.2°C to near the end of germ band shortening. Epidermal staining is lost first from the odd-numbered segments and the posterior segments. **b,c)** Ectopic denticles appear in the ventral cuticle (arrow). c) Segments A5 to A8 are shown. Magnification: 182X (a), 130X (b), 260X (c).

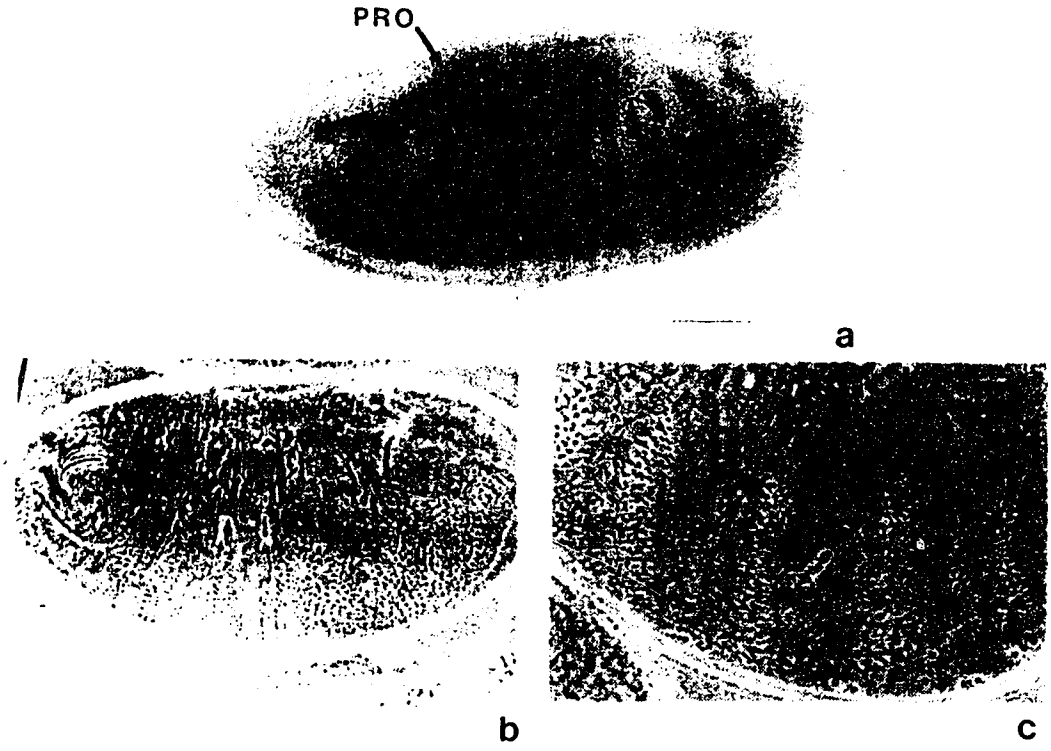
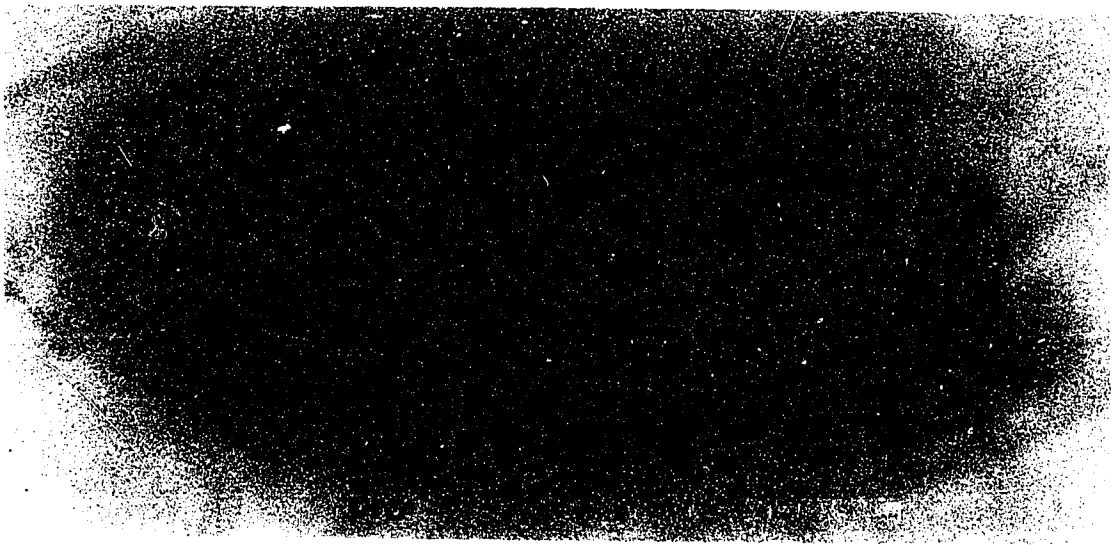
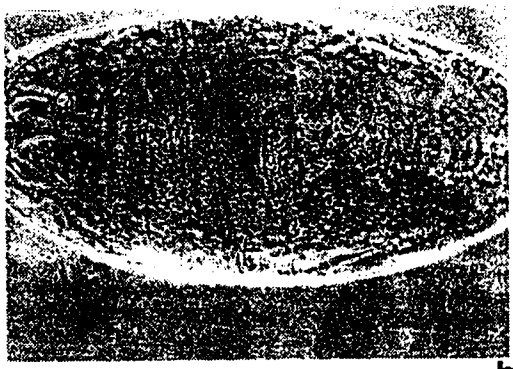


Figure 28) a) ß-galactosidase expression in *wingless* embryos raised at 21.8°C to the end of germ band shortening. b, c) Broadening of the denticle belts with polarity-reversal in cuticle preparations. c) The posterior end. Magnification: 182X (a), 130X (b), 260X (c).



a



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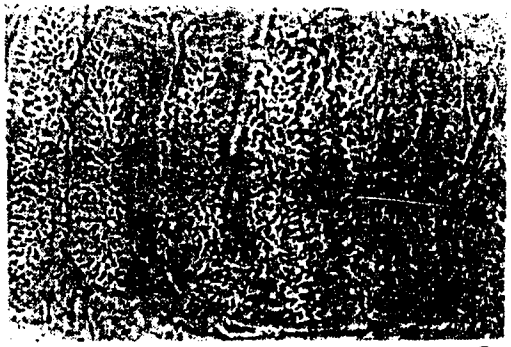

c

Figure 29) a) ß-galactosidase expression in *wingless* embryos raised at 23.2°C to the end of germ band shortening. Patches of stain remain in the lateral regions of the epidermis. **b, c)** Gaps of naked cuticle remain in the lateral regions of the ventral cuticle. c) Posterior end. Magnification: 182X (a), 130X (b), 260X (c).

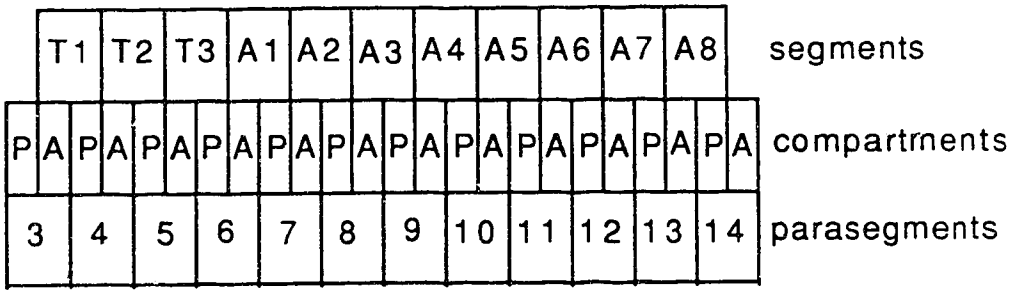

Figure 30) The relationship between segments, compartments and parasegments in the thorax and abdomen. A= anterior, P= posterior. The A and P compartments are not necessarily of equal size as implied by the diagram.

DISCUSSION

In this study, a description of the phenotypic effect of *wingless* on the larval cuticular pattern of *Drosophila melanogaster* has been provided, based on the analysis of intermediate phenotypes, in order to interpret how the extreme *wingless* mutant pattern arises, so as to provide insight into how *wingless* is involved in the generation of the segmental repeats of the larva.

The embryonic requirement for *wingless* is from germ band extension to germ band shortening as defined by the temperature shift experiment. This correlates well with the time when the gene is expressed; the periodic pattern of transcripts is seen from the cellular blastoderm stage and reaches maximum levels during germ band extension (Baker, 1987; 1988c). There is also a larval requirement for *wingless* (Baker 1988b), which extends throughout the three instar stages, and expression of the gene is seen in the larval imaginal discs (Baker, 1988a). This larval requirement was not of concern in this study.

Intermediates of *wingless* generated by two methods were used to analyse the effect of this gene on the repeat pattern. The first method involved the analysis of the intermediates that were produced when a temperature shift was made during the temperature sensitive period, so that the embryos were deprived of normal *wingless* activity either in the later part of this period after being allowed to develop with normal *wingless* function for the first

part, or deprivation was for the early part of this period, with the functional *wingless* activity theoretically being restored in the later part. It is reasonable to assume that when the shift was up, from the permissive to the restrictive temperature, in order to remove *wingless* activity in the later part of the temperature-sensitive period, the development of the embryos had progressed as in the wildtype up until the time of the shift. It is not reasonable, however to make the assumption that fully normal *wingless* activity is restored after the shift down, from the restrictive to the permissive temperature, during the temperature-sensitive period, since absence of normal activity early in development may have had some long term effect on the proper functioning of the gene later in embryogenesis, perhaps by its effect on other developmentally required genes. Therefore, the results from the shift-down experiment might be expected to be different from those of the shift-up experiment, which they were. The intermediates produced in the shift up experiment displayed a gradual increase in the proportion of segment covered by the denticle belt as the shift was made earlier; whereas in the shift down experiment, this gradual increase was not as apparent. There was much more variability in the phenotypes of the genotypically *wingless* embryos when they were shifted down at any one stage during the TSP than there was among those shifted up. For example, in some embryos, two particular denticle belts might be fused; whereas, in another embryo shifted at the same stage, those same two belts might appear wildtype (Figure 11b,c).

In the second part of this study, intermediates produced by raising embryos at semi-permissive temperatures were analysed. As previously discussed, this type of experiment would produce a series of hypomorphic phenotypes, such that a higher developmental temperature would result in an embryo with a lower level of activity from the *wingless* gene. The results from this experiment were similar to that of the shift up experiment: as the embryos were allowed to progress further into the temperature-sensitive period before being shifted to the restrictive temperature, a less extreme phenotype was produced, similar to the series produced when the level of *wingless* is increased by raising the embryos at lower temperatures. The similarity between the two experiments suggests that the intermediates produced from the shift up experiment might represent a series of hypomorphic phenotypes, and this will be discussed in light of the model to be proposed.

A detailed description of the changes within the segment was done using the intermediates from the semi-permissive temperature experiment only, since a similar analysis of the intermediates from the shift experiment was obscured by the used of *shavenoid* as a marker for *wingless* genotype, as well as by the background segmentation defects produced in embryos receiving a temperature shock. Since *wingless* affects all of the segments of the thorax and the abdomen, as can be seen in Figure 15; most likely, the changes as a result of a decrease in the level of *wingless* activity that are described here are characteristic of all segments, T1 to A8. Equivalent positions in each segment may be affected in a similar

manner, such that a change in the thoracic pattern elements would represent a similar change in the equivalent regions of the abdominal segments.

Lowering the level of *wingless* had a significant effect on the anterior region of each segment where denticles are normally formed. A 'stretching' of the belt was observed as a result of ectopic denticles appearing on either side of the belt as well as within it (Figure 17); a larger proportion of the segment had taken on an identity that leads to denticle belt formation. This expansion was at the expense of naked cuticle and of other pattern elements in the segment, such as the extra patch of denticles formed in the prothorax. This mid-prothoracic patch of denticles decreased in size somewhat before disappearing completely (Figure 23), probably through fusion with the anterior denticle belt. The Keilin's organs of the three thoracic segments disappeared as the denticle belts broadened. There did appear to be some increase in distance of the Keilin's organs of the mesothorax from the anterior boundary of the preceding denticle belt (Figure 21), as well as in distance of the mid-prothoracic patch of denticles from the anterior boundary of the prothoracic belt (Figure 24); however, this distance would also include the anterior expansion that occurred in those preceding denticle belts. The Keilin's organs of the prothorax and the mesothorax did not show this displacement (Figure 21).

Observations on the abdominal denticle belts suggested that at least the most anterior wildtype row and the ectopic denticles that formed

anterior of them at the lower temperatures became lost at the higher temperatures (Figure 17e). These denticles lie in the posterior compartment, or the E region according to the 4-cell-state model of Martinez-Arias, et. al. (1988). Cell death appeared to occur over the entire posterior compartment since cells expressing *engrailed*-regulated ß-galactosidase in a strong *wingless* background disappeared (Figure 25); thus, cell death in some regions of the segment, as well as a change in cellular identity, may contribute to the observed increase in the proportion of the segment covered by denticles.

Since the results from this study will be discussed in light of previous models proposed to explain the generation of a polarized repeat pattern, these models will be first described, followed by a description of the modifications to these models that I have suggested in order to account for these results.

Gradient Models

Locke (1959, 1960) proposed that a monotonic gradient of a diffusable morphogen exists within each repeat to explain how a position in a segment is unique from all other positions within that segment, but equivalent to a position in each of the other segments (see Figure 31a), and to explain the pattern generated when two normally distant cells come in contact with each other. This series of gradients would form a 'sawtooth' type wave along the anterior/posterior axis of the insect with an abrupt change in

positional values at the segmental borders (Locke, 1959, 1960). Disruptions in the segment would result in a smoothing of the gradient to regenerate the deleted pattern elements. However, as previously mentioned, this form of model cannot account for the regenerative behaviour of the segmental border or for the duplication versus regeneration phenomenon previously described in the insect abdomen (Wright and Lawrence, 1981).

A variant of the gradient-type model for positional information was proposed by Russell (1985) (Figure 31b). In this case, a wave of morphogen might be initiated at any one point along the longitudinal axis of the embryo and have a periodicity of one segment. In order to provide an unique positional value at each point along the segment, at least two of these waves, out of phase with each other, must be superimposed, with the relative proportions of the two morphogens providing a value for positional information. With this model, alterations in any of the parameters, such as wavelength, phase difference, or amplitude, can mimic the distortions, duplications, and deletions seen in segmentation mutants; also, with this model, the segmental border can behave as any other repeated element and thus can be regenerated under the appropriate conditions. A single monotonic gradient (Locke, 1960) cannot easily account for the generation of polarity-reversal seen in the segment-polarity mutants; however, with a double wave mechanism, complete abolition of one of the waves, perhaps by mutation in a segment-polarity gene, would leave one wave with mirror-image symmetry.

One prediction of this double wave model is that pattern elements can be distorted or displaced relative to other elements in the segment when one parameter of either wave is altered. An alteration that can produce such a distortion is a change in the amplitude of a wave. This situation might be created genetically with hypomorphic mutations. One can hypothesize that, if a gene is required to set up a morphogenic wave, either directly or indirectly, changes in the levels of the gene product or activity might accordingly change the amplitude of the wave. This situation was created in this study, so the predictions that are made are for the outcome of lowering the amplitude of each type of wave.

By lowering the amplitude of a morphogenic wave that is required for the specification of pattern elements, the pattern elements would be expected to be distorted such that those elements requiring a lower level of the morphogen would expand at the expense of those elements requiring a higher level of the morphogen. Also, the relative positions of those elements formed at intermediate levels of the gradient might be expected to move towards the position in the segment where the peak of the gradient or wave would normally occur, before they would disappear, as the amplitude is lowered below those intermediate levels.

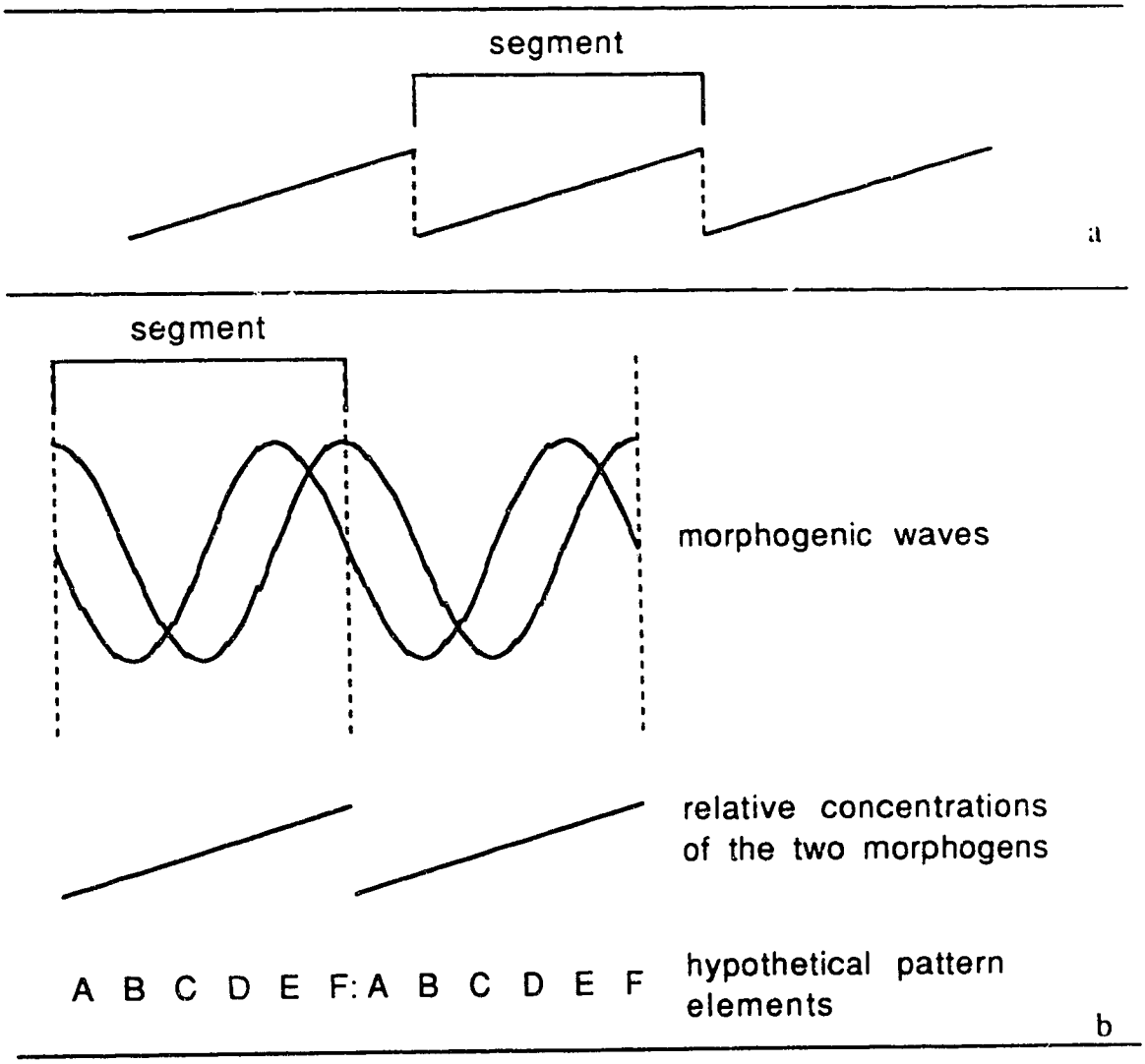

Figure 31) Gradient models for pattern formation in insect segments. **a)** an array of monotonic gradients proposed by Locke (1959, 1960). Each position in a segment can read an unique value from the gradient. **b)** two morphogenic waves superimposed upon each other can produce a series of value gradients similar to what is seen in (a) (Russell, 1985). In this case there are more parameters, which can be altered to produce deletions, distortions, and duplications in the pattern.

Cell-state Models

An opposing view of segmental pattern formation involves the interactions between neighbouring cells, without necessarily requiring diffusable morphogens; a cell's identity would be determined by its location relative to other cells rather than by the local concentration of a morphogenic gradient. A series of qualitatively different states, such as the lineage restricted compartments, along the anterior/posterior axis would confer polarity within the segment. Meinhardt (1986) pointed out that the two compartments defining the anterior and posterior regions of each segment would not be enough to give polarity; in fact, only two cell states would allow mirror-image pattern duplications to occur. His model requires a minimum of three cell states in each segment in order to define the segmental border, the anterior compartment, and the posterior compartment, with the possibility that a different selector gene (Garçia-Bellido, 1975) is required for each cell state. Removal, or transformation, of one of these three states by mutation in one of the selector genes, would leave two states where mirror image pattern duplications could occur when the states become juxtaposed in the wrong order. This model removes the requirement for a graded morphogen at this level of the developmental hierarchy, and is more consistent with the apparent non-graded expression of segment-polarity genes in discrete regions of each segment.

Martinez-Arias, et. al. (1988) suggested that a minimum of four cell states are required, to explain the observed changes in the

expression patterns of *wingless* and *engrailed* in mutants of four other segment-polarity genes: *engrailed*, *wingless*, *naked*, and *patched*. Specific interactions between a cell and its neighbours would allow the cell to maintain its determined state. When cell states are deleted, either by surgical removal or by mutation, the missing values could be intercalated between the anomalously juxtaposed cell states; this idea was previously suggested by French, et. al. (1976), who used a polar coordinate system to explain regeneration and duplication in insect imaginal discs and appendages. If cell states are transformed rather than deleted by a segment-polarity mutation, then two cell states could become juxtaposed in an order opposite to that of the wildtype, leading to mirror-image duplications in the pattern. In the model of Martinez-Arias, et. al. (1988) (Figure 32), specific interactions between neighbouring cells would allow the formation of specific pattern elements, such as segmental borders, parasegmental borders, and denticles. Some of the rules and interactions proposed are described in Figure 32. Certainly, the interactions involved are much more complex than this; however, by using these rules, some predictions can be made as to the outcome, both phenotypically and in expression patterns, when there is a mutation in any one of the genes. For example, the loss of wildtype *patched* function would allow *wingless* expression in the P region, resulting in the broadened domain of *wingless* expression that was seen in *patched* mutants (Martinez-Arias, et. al., 1988), leading to the formation of a W state adjacent to the N state. The states normally occurring between the N and W states would be intercalated, in this case, an E state; this

would lead to both the expression pattern of *engrailed* and the cuticular phenotype seen in *patched* mutants (see Figure 7 of Martinez-Arias, et. al., 1988). One might expect that a *wingless* null mutation would cause the deletion of the W state and probably the E state since *wingless* is required for the maintenance of this state. This would leave a series of alternating N and P states. As previously mentioned, two states would allow pattern duplications similar to those seen in strong *wingless* mutants.

With a cell-state model, one might expect that a threshold requirement for the genes involved in setting up the cell states would exist, so that in a series of hypomorphs for one of these genes, there would be a sharp division between those with enough activity to give wildtype functioning of the gene and those without enough so that a mutant phenotype is produced. One would not necessarily expect the graded series of intermediates that was seen for *wingless* when embryos were raised at various semi-permissive temperatures as well as in the shift up experiment. This observation is more consistent with graded levels of the gene along the segment. However, the results from the shift down experiment, where there is an apparent 'all-or-nothing' effect, suggests that there might be a discrete threshold requirement for *wingless* at some time during development.

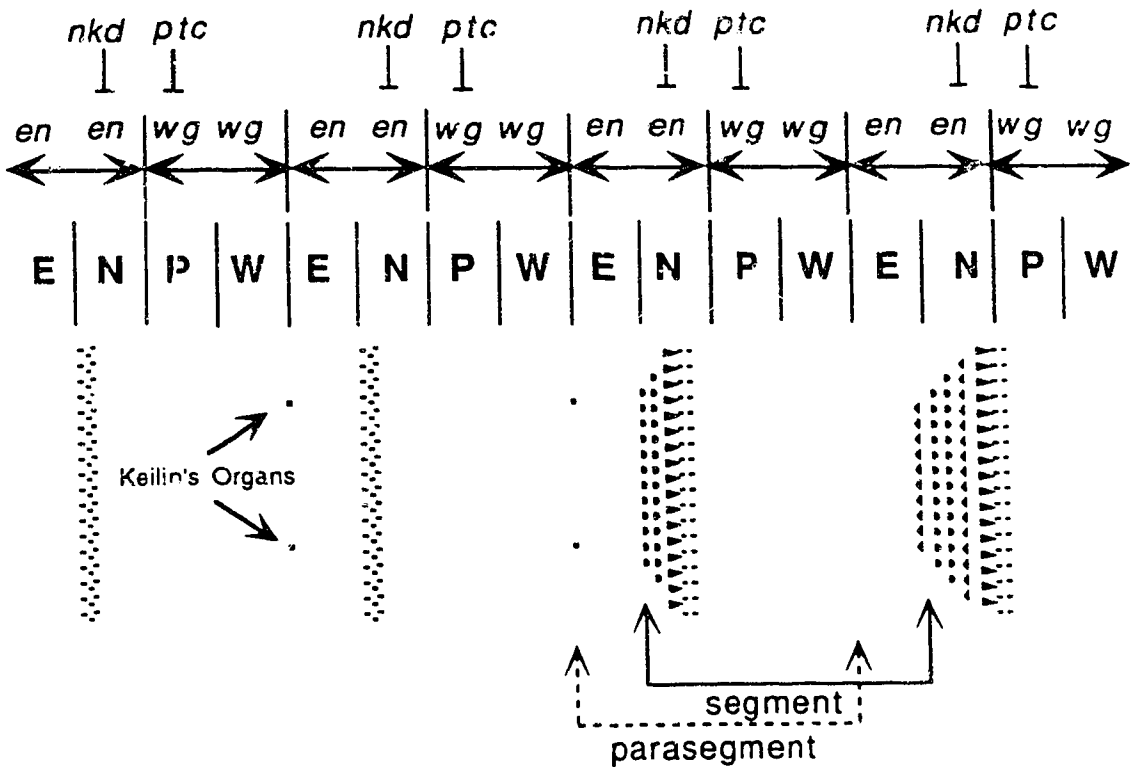

Figure 32) The four cell state model (Martinez-Arias, et. al., 1988). Some rules for this model are as follows: 1) *wingless* and *engrailed* are initially restricted to specific domains by the pair-rule genes. 2) *naked* further restricts *engrailed* expression in the N region. 3) *patched* further restricts *wingless* expression in the P region. 4) A W:E interface gives a parasegmental border. 5) An E:N interface gives a segmental border. 6) E:N, N:N, N:P interfaces give different types of denticles. 7) An ectopic N:W interface causes E to be intercalated. 8) An ectopic E:P interface causes W to be intercalated.

A Model for the Mode of Action of *wingless* in the Segment

In the following pages, a model will be described to account for the effect of intermediate levels of *wingless* activity on the embryonic segmentation pattern. This model is based upon the existence of cell states as suggested by Meinhardt (1986) and modified by Martinez-Arias, et. al. (1988). These models have been proposed in exclusion of a gradient of information, which has been predicted from the surgical experiments. However, the results presented here do suggest that the effect of *wingless* displays characteristics of a gradient, with its effects most obvious on either side of its domain of expression.

The model proposed is diagramed in Figure 33 and is defined as follows. The expression of *wingless* is restricted to one quarter of the segment (corresponding to one cell at the blastoderm stage and termed the W state in the 4-cell state model) by the action of the pair-rule genes. Through a cell signalling mechanism, this gene transmits a signal to the adjacent cells on either side of this domain of expression. The signal is subsequently transmitted to further cells along the anterior/posterior axis, but each time the signal is transmitted, it is diminished to some degree. The diminishment of this signal may be a passive process, or it may be directed by other factors in the pathway, such as through interactions with other segmentation genes. Since there are cell membranes to cross, the number of signal molecules crossing into an adjacent region may be limited, a process that could, in part, be mediated by membrane-spanning molecules, such as the one encoded by the segment-

polarity gene *patched* (see Table 8). Therefore, the cells furthest away from the *wingless*-expressing region, or the source of the gradient, receive the weakest signal. I have termed this as a gradient of '*wingless* effect', since the product of the gene itself does not necessarily get passed on to the abutting cells. It is possible that the *wingless* protein is present in these cells, at levels below detection, and it has been seen in vesicles within cells that are immediately adjacent to those producing the protein (van den Heuvel, et. al., 1989); however there is no strong evidence that the product itself is directly responsible for any effect that the gene has on these regions.

The gradient of *wingless* signal just proposed is formally similar to a diffusable morphogen, with the strength of the gene's influence being inversely proportional to the distance from the source. This model then states that the 'gradient' is initiated in each segment. This is similar to Locke's version of a simple gradient in each segment, with a source at one border and a sink at the other. In this case, though, the source does not start at the segmental border, although it may start at the parasegmental border, nor does it require a sink. The signal can also be sent in both directions so there is no need for a barrier to prevent diffusion in one direction as would be required for the simple source/sink type of gradient.

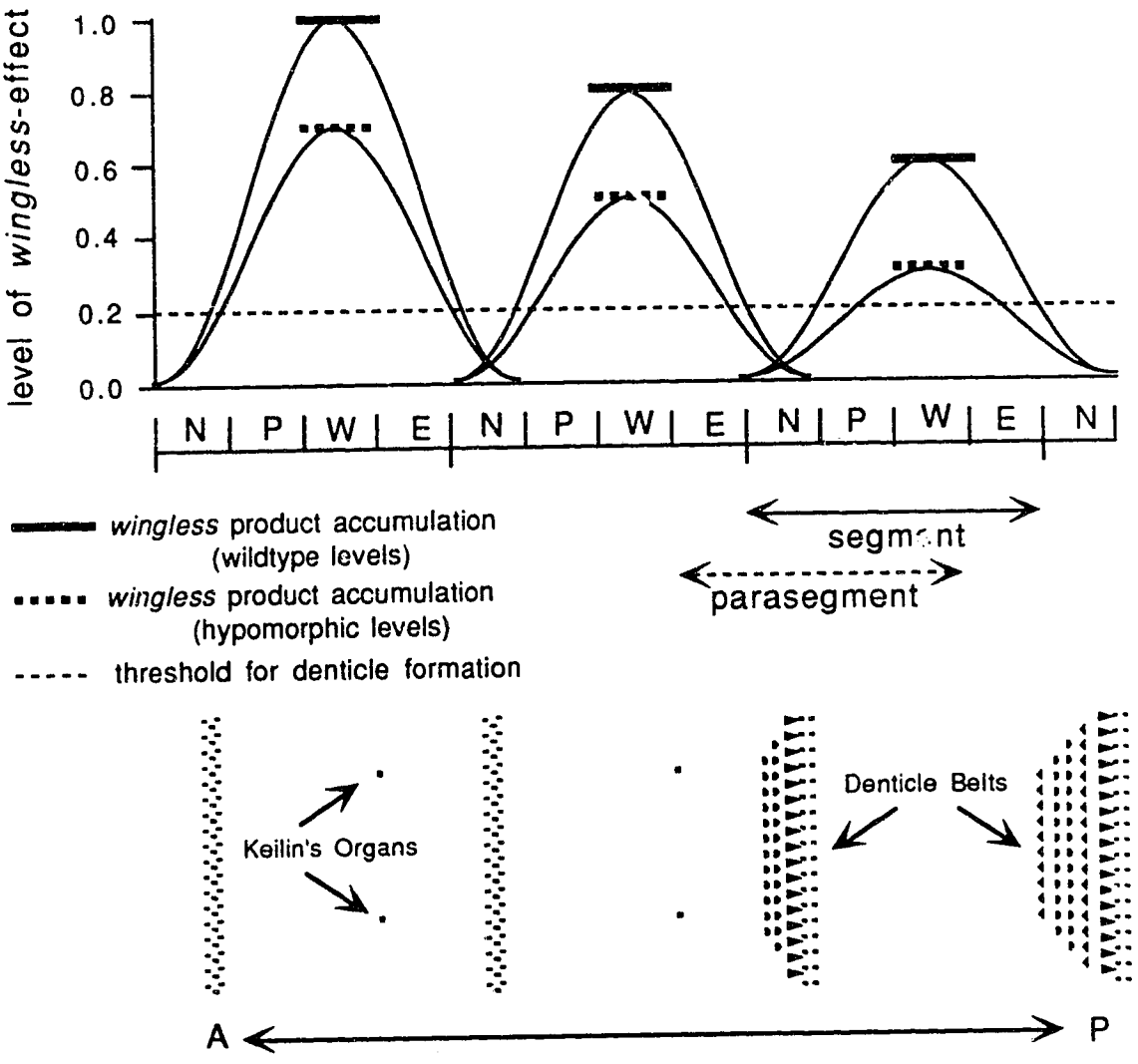

Figure 33) A model for the effect of *wingless* on the embryonic segment. W, E, N, and P are the 4 cell states defined by Martinez-Arias, et. al. (1988). A↔P is the anterior/posterior axis. The scale along the Y axis is arbitrary; the maximum level of *wingless* expression is set at 1 and other levels are arbitrary proportions of that. The threshold is a level of *wingless*-effect below which denticle formation can occur. As the source level of *wingless*-effect decreases, the proportion of each segment below this level increases. It is proposed that the initial level of *wingless* expression decreases along the anterior/posterior axis to account for the gradient of effect that was seen along the axis.

Denticles would form only in those cells in which the *wingless*-effect is below a certain threshold, which means that the cells furthest away from the *wingless* region would be capable of forming denticles. The size of the denticle-forming region would be dependent on that threshold level, as well as the level of gene expression at the source. If the source level of *wingless* is lowered, cells on either side of the wildtype belt would then be below the threshold level and be capable of forming denticles (see Figure 33), thus resulting in a broadened belt as was seen in these experiments.

Instead of a threshold being required for the suppression of denticle formation, an alternative explanation is that the gradient does not reach the denticle-forming region; which is plausible since this region is the furthest away from the *wingless*-expressing region. The appearance of denticles would then occur in the absence of any *wingless* signal. If this was the case, denticles would appear on either side of the belt when the source level was lowered since the signal would no longer reach as far, and this was observed (Figure 17), but the interior regions of the belt should not be affected, since nothing would have changed in this area with respect to *wingless* activity. Since a 'stretching' of the belts did occur and new denticles appeared within them (Figure 17d), the gradient of *wingless*-effect probably does extend along the entire segment, and there is a threshold for denticle formation.

If the suggestion by Martinez-Arias, et. al. (1988) that denticles are formed by N cells and that the different types of denticles are the

result of differing neighbours (E:N, N:N, and N:P interfaces result in different types of denticles being formed by the N cells) is the case, then a gradient would not need to extend into the denticle-forming N region in order to cause the appearance of ectopic denticles within the belt. If as suggested by my model, E and P cells adjacent to the N region are transformed to N cells (the cell state that supports denticle formation according to Martinez-Arias, et. al. (1988)) in the intermediate *wingless* background, then this could account for the appearance of the extra row of medium posterior-pointing denticles between the two anterior-pointing rows if the production of these type of denticles require N:N interfaces, since the number of cells with N neighbours would increase as the number of N cells along the anterior/posterior axis increases. This situation can occur in the absence of any *wingless*-effect in the denticle-forming region. However, this cannot explain the appearance of denticles on either side of the belt, since the number and position of the E:N and N:P interfaces (required for the formation of the specific type of denticles found at the anterior and posterior edge of the belts, respectively) relative to the denticle-forming N region would not change. Furthermore, if a segmental boundary is formed at the E:N interface, then E cells must be also secreting denticles since the first row of denticles in the abdominal segments is anterior to the segmental boundary (Hama, et. al., 1990; Szabad, et. al., 1979). For this reason, I favour the formation of denticles in response to the level of *wingless*-effect rather than strictly in response to the cell state.

If the thoracic pattern elements, which are the Keilin's organs and the mid-prothoracic patch of denticles, also arise in direct response to the level of *wingless*-effect, and require an intermediate level of this gradient in the wildtype, then, as the amplitude of this gradient is lowered, these elements would shift towards the source of the *wingless*-effect until the peak level fell below that required for these elements, resulting in the loss of these structures.

The Keilin's organs lie at the posterior boundary of the *wingless*-expressing region on the parasegmental border. If the *wingless*-effect is evenly distributed across the *wingless*-expressing region, then there should be no shift before they disappear since the gradient would immediately fall below that required for the formation of the Keilin's organs. They did not disappear immediately as would be expected if this was the case (see Figure 19). If the level of *wingless*-effect is lower at the position of the Keilin's organs than at the source, then they should shift towards the anterior boundary of the preceding denticle belt, which they did not do (Figure 21). There was evidence for a shift away from the preceding denticle belt border in the mesothorax, which would not be expected if the Keilin's organs arise in response to a level of *wingless*-effect. Possibly, it is the junction of the *wingless*-expressing region with their *engrailed*-expressing neighbours that allows a parasegmental border and thus Keilin's organs to form. Since cell death is likely occurring in the *engrailed* region when *wingless* is mutant, the *engrailed*-expressing region may be narrowed in the *wingless* intermediates (Figure 26a indicates thinning of the *engrailed*-regulated ß-galactosidase stripes)

due to only part of the region having died at this point. Therefore, there would be a decrease in the relative distance between the Keilin's organs and the next more posterior segmental border, which lies at the posterior edge of the *engrailed*-expressing region. This could account for an increase in the relative distance of the mesothoracic Keilin's organs from the preceding segmental border.

The position of the mid-prothoracic patch of denticles is difficult to ascertain in relationship to the position of *wingless* expression. The position of this structure probably correlates with the *wingless*-expressing region in the prothorax, so if it arises in response to the level of *wingless*-effect, when the level of *wingless*-effect is lowered it would be expected to immediately be transformed to naked cuticle before the region became part of the anterior denticle belt. This did not happen, so the loss of this structure is probably due to encroachment by the preceding denticle belt, which is under the influence of the *wingless*-effect gradient.

The model in Figure 33 indicates that the initial level of *wingless* expression decreases in each subsequent segment along the anterior/posterior axis, to account for the gradient of effect that was seen along this axis; this can also account for the general increase in the size of the wildtype denticle belts from the anterior to the posterior end of the embryo (see the wildtype data in Figure 16). Contrarily, the threshold level for suppression of denticle formation might be higher in the more posterior segments, to explain the increase in size of the wildtype belts along the axis. I favour the first

hypothesis since, if the threshold level was higher in each more posterior segment, one might expect that all of the segments would be equally affected as the source level of *wingless* was lowered. This was not the case; the more posterior denticle belts were the first to broaden as would be predicted from the first hypothesis.

With this new model, the role of *wingless* in cell-cell communication can allow the maintenance of the pattern within the segment, and the gradient ensures that each point along the segment has a unique identity. Positions at equivalent distances from either side of the *wingless*-expressing region might not necessarily interpret the same level of *wingless*-effect in the same way since different segment-polarity genes are expressed on either side, and interactions with unique combinations of these genes could have different effects on the action of *wingless* in these regions.

There are nine known segment-polarity genes that give a *wingless*-like phenotype when mutant; that is, part of the denticle belt is duplicated in reverse-polarity (see Table 8 for a summary of these genes). Mutations in any gene affecting the same pathway would be expected to yield a similar phenotype, so the genes in this group might interact with each other to initiate, receive, interpret, and transmit a signal across the segment. There is much heterogeneity among this group of *wingless*-like genes. Some exhibit a weaker phenotype than others; for example, deficiencies of *gooseberry* give a phenotype similar to the intermediate *wingless* seen here (compare Figure 1 of Côté, et. al., 1987 to Figure 15), whereas a strong

armadillo phenotype is similar to the strong *wingless* phenotype (Klingensmith, et. al., 1989). Some but not all, of the segment-polarity genes have a maternal effect. Also, the *wingless*-like genes are expressed in different domains of the segment and each known segment-polarity gene produces a different type of protein, suggesting that they might be different components required in a complex pathway, such as one used for intercellular signalling.

It might be expected that mutations in a gene required closer to the source of the signal, as with one needed to transmit the initial signal, would affect more of the segment than would a mutation in a gene required further away from the source. Also, a gene required over the entire segment would be expected to have an extreme effect when mutated. Transcripts from the *gooseberry* region are expressed in a spatially restricted domain, which includes *engrailed* but not *wingless* (Côté, et. al., 1987; Baumgartner, et. al., 1987). Therefore, *gooseberry* may be involved with receiving, interpreting or transmitting the *wingless* signal on the posterior side of the *wingless*-expressing domain and may only affect this region of the segment, causing the weak *wingless* phenotype seen in the deficiencies. Transcripts of *armadillo*, on the other hand, are expressed ubiquitously throughout the embryo and strong mutations give a strong phenotype. This gene is cell autonomous (Wieschaus and Riggleman, 1987; Gergen and Wieschaus, 1986) and the protein is homologous to human plakoglobin, a protein required for intercellular adhesive junctions (Peifer and Wieschaus, 1990, Riggleman, et. al., 1989). The role of *armadillo* as a cell junction

protein could explain why it is required maternally to complete oogenesis (Wieschaus and Noell, 1986). Zygotic *armadillo* protein, although ubiquitous, is found in elevated levels in the *wingless*-expressing cells as a direct response to the expression of *wingless* (Riggleman, et. al., 1990). This gene is a good candidate for one that might be required to transmit the *wingless* signal out of the *wingless*-expressing region. One segment-polarity gene that causes an opposite effect to that of *wingless* is *naked*; that is, this gene deletes denticles (Jürgens, et. al., 1984, Martinez-Arias, et. al., 1988). This gene may possibly be required in the N region, which is the furthest away from the *wingless*-expressing region, to inhibit the build up of the *wingless* signal required to repress denticle formation. This could give the *naked* gene a role analogous to that of a sink for a morphogenic gradient.

Gene	Strongest phenotype seen	Domain of expression	Type of protein	references
**armadillo (arm)*	extreme	ubiquitous elevated protein expression in the *wg* domain	Human plakoglobin homolog	Riggleman, et. al., 1989; 1990 Peifer & Wieschaus, 1990
Cell(Ce)	strong intermediate	same as ci^D?	same as ci^D?	Orenic, et. al., 1990
cubitus interruptus Dominant (ci^D)	intermediate	exclusive of *engrailed*	zinc-finger	Orenic, et. al., 1990; Eaton & Kornberg, 1990
**dishevelled (dsh)*	extreme	unknown	unknown	Perrimon & Mahowald, 1987
**fused(fu)*	strong intermediate	unknown	serine/ threonine protein kinase homolog	Préat, et. al, 1990 Busson, et. al., 1988
gooseberry (gsb)	intermediate	overlaps *engrailed*	homeobox	Cote, et. al., 1987; Baumgartner, et. al., 1987
hedgehog (hh)	extreme	unknown	unknown	Mohler, 1988
**porcupine (porc)*	extreme	unknown	unknown	Perrimon, et. al., 1989
smooth (smo)		unknown	unknown	Nüsslein-Volhard, et. al., 1984
patched (ptc)	N/A	initially ubiquitous, resolved into 2 stripes, posterior & anterior of *en*	contains membrane-spanning domains	Hooper & Scott, 1989; Nakano, et. al., 1989

Table 8) The *wingless*-like segment-polarity genes. The most extreme phenotype seen is compared to the extreme *wingless* lethal phenotype. ci^D and *Ce* may be complementing alleles of each other. *patched*, which is also involved in the segmental patterning process, and probably the signal pathway, is included in this list, although it does not produce a *wingless*-like phenotype, in order to compare its protein with the other segment-polarity genes. *These genes have a maternal effect.

In the weak *wingless* background, cells appeared to change their fates so that a larger number of them became denticle forming. There may also be some cell death occurring at this point, since stripes of ß-galactosidase expression (which probably persist after transcription of the *lac-Z* gene ceases, as discussed earlier) in the *engrailed* region become thinner at a temperature when ectopic denticles start to appear (Figure 26); however, before cell death occurs, *engrailed* transcripts cease to accumulate in the absence of *wingless* (DiNardo, et. al., 1988).

How intermediate levels of *wingless* regulate *engrailed* is unclear, although the pattern of loss for *engrailed* transcripts in *wingless* intermediates (DiNardo, et. al., 1988) was similar to that seen for ß-galactosidase expression. Perhaps, then, there is a threshold of *wingless* required for the maintenance of *engrailed* and cells that require *engrailed* expression, but can no longer maintain it because the *wingless*-effect has dropped below that threshold, die. The thinning of the ß-galactosidase stripes (Figure 26a) could be a result of the graded levels of *wingless*-effect across the region. The first row of denticles, which apparently disappeared as the level of *wingless* decreased, lie at the posterior edge of the *engrailed* region (Szabad, et. al., 1979; Hama, et. al., 1990), so it is probable that cell death is occurring here. Not only is *wingless* required to maintain the expression of *engrailed* in cells posterior to the *wingless* expressing region, it is also required to maintain the expression of *patched* in cells of the *wingless* region and of the denticle-forming (N) region. All expression of *patched* transcripts is lost in *wingless* mutants by

the end of germ band shortening except in the head segments and the prothoracic segment (Hidalgo and Ingham, 1990), as is the case for *engrailed* expression in *wingless* mutants. Whether cell death is occurring outside of the *engrailed* domain is uncertain, although the mid-prothoracic denticles disappear in the stronger *wingless* background, and these denticles would lie either within or anterior to the *wingless* domain.

Martinez-Arias and Ingham (1985) showed that the duplicated pattern elements in mutants of the *wingless*-like genes, *gooseberry*, *hedgehog*, and *cubitus interruptus Dominant*, are derived from the anterior compartment rather than being the result of transformation in the posterior compartment. This is consistent with the loss of the posterior compartment through cell death in *wingless* and *wingless*-like mutants (Martinez-Arias, 1985; Perrimon and Mahowald, 1987; Riggleman, et. al., 1989; this study). The anterior compartment is made up of denticles in the anterior region and naked cuticle in the posterior region. Cells in this naked cuticle may die, followed by cell proliferation from the denticle-containing region of the anterior compartment, possibly giving rise to cells with an opposite polarity of those from which they are derived. Certainly, this study indicates that the duplicated pattern is filled in behind the remaining wildtype pattern as *wingless* levels are lowered, although not necessarily in reverse-polarity, until the level of *wingless* is further lowered. It may be that more than one half of the segment needs to be deleted before polarity-reversal occurs, as has been previously suggested (Russell, 1985, Wright and Lawrence, 1981) and that as *wingless* is

lowered, more of the segment becomes deleted. Deletion of more than one-half of a segment is probably not a general requirement for polarity-reversals to occur, however, since *patched* mutants display polarity-reversal despite less than one half of the segment being visibly affected (see Figure 1 of Nakano, et. al., 1989). Furthermore, *patched* does not cause cell death in the developing embryos (Martinez-Arias, et. al., 1988). An analysis of cell death in regions of the anterior compartment needs to be done in *wingless* mutants; perhaps with the development of reporter genes for these regions, it will be possible to more closely study the fate of cells in these regions.

As the level of *wingless* is lowered, cells of the P region may be transformed to an N state, which normally forms denticles. Polarity reversal may not occur until most of the P region is transformed, and interactions between the transformed cells and the now adjoining W state cells has an effect. When the cells immediately abutting the *wingless*-expressing cells become transformed, a W:N interface is formed, and Martinez-Arias, et. al. (1988) suggested that an ectopic E state would be formed, based on the appearance of ectopic *engrailed* expression in *patched* mutants. By this logic, an ectopic region of *engrailed* expression might be expected in *wingless* mutants; however, the absence of *wingless* also disallows the maintenance of an E state, accounting for the fact that ectopic *engrailed* expression in *wingless* mutants has not been seen (DiNardo, et. al., 1988; this study).

Another possibility is that, as *wingless* is further lowered, cell death results in the loss of the W state before these cells can abut the N state to form a W:N interface, so that eventually only an N state remains. This would predict that in a null *wingless* mutant, only N cells are formed, resulting in an embryo with a lawn of denticles showing no polarity at all, and no differences in the type of denticles seen. In the strong *wingless* mutants, this is seen in the posterior end of the lawn (Figure 5c); however, there are still traces of polarity in the anterior region. In the deficiency homozygote, there are still traces of a repeat pattern (Figure 17k), although there are denticles completely filling the prothorax (Figure 20b). The absence of *wingless*, then, may not be sufficient to completely remove polarity in the segment, and some untransformed P cells might remain to generate a repeat pattern.

As previously mentioned, the shift up experiment was quantitatively similar to the semi-permissive temperature experiment in that the denticle belts occupied a progressively larger proportion of the segment as the shift was made earlier in the temperature-sensitive period. Although this type of experiment would not necessarily be expected to produce a series of hypomorphs, the intermediates appear similar to the hypomorphs that were produced in the semi-permissive temperature experiment. A possibility for this is that, during the TSP, levels of *wingless* are gradually built up to a maximum hypothesized in Figure 33, and if the production of *wingless* product is terminated during this time by shifting the embryo to the restrictive temperature, then a decreased maximum

level of *wingless* activity in each segment would result. Naturally, the earlier the shift is made, the less time there has been to accumulate *wingless* product, and a series of hypomorphs could be produced.

Shifting down, on the other hand, had a different result and a clear progressive broadening of the belts was not evident; also, the phenotypes at any one stage of shift were more variable. This suggests that embryos that have been deprived of *wingless* activity early in embryogenesis require a major compensation when *wingless* activity is suddenly restored, such as regeneration or duplication of the segment as suggested by Baker (1988b); thus, minor differences among the embryos at the time of the down-shift may ultimately result in major differences, depending on which side of a threshold they were on. There was some broadening of the belts, as was demonstrated in Figure 12; however, this effect may be obscured by the overlying coarser requirement for early *wingless* function. This may reflect the requirement for *wingless* to set up cell states before the gene is needed to maintain polarity along the segment. This requirement for setting up the cell states may not be dependent on maximum levels of *wingless* product accumulation; however the finer tuning and maintenance of the pattern within the segment would be dependent on the absolute levels as proposed in Figure 33.

Conclusions

Cell states and cellular interactions certainly play an important role in the patterning of insect segments; however, it appears that the finer tuning of the pattern is probably dependent on some form of a least one gradient. We do know, from the experiments that demonstrated that *bicoid* is a graded morphogen (Driever and Nüsslein-Volhard 1988a,b), that gradients of morphogens are important in providing the early cleaving embryo with whole body positional information. Certainly, after formation of the blastoderm, cell states and selector genes (Garçia-Bellido, 1975) become important, since *engrailed* is required to 'select' the cells of the posterior compartment. However, gradients or waves may be superimposed upon this scheme of cell states to set up and maintain polarity within each segment, as well as to ensure that each cell along the anterior/posterior axis of the segment has an unique identity. Such fine tuning would be difficult to provide with a limited number of discrete cell states. This study supports the presence of a gradient generated by the *wingless* gene in the insect segment, and the formation of at least one cuticular pattern element, the denticle belt, appears to be in response to this gradient. The other pattern elements studied, the Keilin's organs and the mid-prothoracic denticles do not necessarily arise in response to this gradient of *wingless*-effect; they could arise in response to local cellular interactions, or possibly in response to a different gradient.

The last decade has been an extremely productive one in providing us with a massive amount of information, and certainly a better understanding of the processes that shape the developing organism. Ever continuing advances in molecular biology techniques have provided us with very powerful tools to help refute or support the many theories that have been proposed to explain the process of segmentation and pattern generation. Certainly, as new techniques for studying molecular processes are developed, we should be able to further dissect the intriguing processes of development and pattern formation; however, a more thorough understanding can only be gained through the continued combination of studies aimed at all levels of biological organization. Also, the field of oncogenesis, which deals with cells that have reverted back to an undifferentiated, or embryonic state, will provide us with many more clues on the process of cellular differentiation and cooperation in a multicellular organism.

REFERENCES

ASHBURNER, M. (1989). *Drosophila*: A Laboratory Manual. Cold Spring Harbor Laboratories Press.

BABU, P. (1977). Early Developmental subdivisions of the wing disk in *Drosophila*. *Molecular and General Genetics* **151**: 289-294.

BAKER, N. E. (1987). Molecular cloning of sequences from *wingless*, a segment-polarity gene in *Drosophila*: the spatial distribution of a transcript in embryos. *EMBO J.* **6**: 1765-1773.

BAKER, N. E. (1988a). Transcription of the segment-polarity gene *wingless* in the imaginal discs of *Drosophila*, and the phenotype of a pupal-lethal *wg* mutation. *Development* **102**: 489-497.

BAKER, N. E. (1988b). Embryonic and imaginal requirements for *wingless*, a segment-polarity gene in *Drosophila*. *Developmental Biology* **125**: 96-108.

BAKER, N. E. (1988c). Localization of transcripts from the *wingless* gene in whole *Drosophila* embryos. *Development* **103**: 289-298.

BAUMGARTNER, S., D. BOPP, M. BURRI, and M. NOLL (1987). Structure of two genes at the *gooseberry* locus related to the *paired* gene and their spatial expression during *Drosophila* embryogenesis. *Genes and Development* **1**: 1247-1267.

BELLAN, H., U. GROSSNIKLAUS, R. K. PEARSON, C. WILSON, and W. GEHRING (1988). The Little Blue Book. Unpublished laboratory protocols.

BOWNES, M. (1982). Embryogenesis. In: Handbook of *Drosophila* Development (Ransom, ed.). Elsevier Biomedical Press.

BROWER, D. L. (1986). *engrailed* gene expression in *Drosophila* imaginal discs. *EMBO* **5**: 2649-2656.

BUSSON, D., B. LIMBOURG-BOUCHON, M-C. MARIOL, T. PRÉAT, and C. LAMOUR-ISNARD (1988). Genetic analysis of viable and lethal *fused* mutants of *Drosophila melanogaster*. *Wilhelm Roux's Archives of Developmental Biology* **197**: 221-230.

CAMPOS-ORTEGA, J. A. and V. HARTENSTEIN (1985). The Embryonic Development of *Drosophila melanogaster*. Springer-Verlag, Berlin Heidelberg New York Tokyo.

CÔTÉ, S., A. PREISS, H. J. HALLER, R. SCHUH, A. KEINLIN, E. SEIFERT, and H. JÄCKLE (1987). The *gooseberry-zipper* region of *Drosophila*: five genes encode different spatially restricted transcripts in the embryo. *EMBO J.* **6**: 2793-2802.

DINARDO, S. and P. H. O'FARRELL (1987). Establishment and refinement of segmental pattern in the *Drosophila* embryo: spatial control of *engrailed* expression by pair-rule genes. *Genes and Development* **1**: 1212-1225.

DINARDO, S., E. SHER, J. HEEMSKERK-JONGENS, J. A. KASSIS, and P. H. O'FARRELL (1988). Two-tiered regulation of spatially patterned *engrailed* gene expression during *Drosophila* embryogenesis. *Nature* **332**: 604-609.

DINARDO, S., J. M. KUNER, J. THEIS, and P. H. O'FARRELL (1985). Development of embryonic pattern in *D. melanogaster* as revealed by accumulation of the nuclear *engrailed* protein. *Cell* **43**: 59-69.

DRIEVER, W. and C. NÜSSLEIN-VOLHARD (1988a). A gradient of *bicoid* protein in *Drosophila* embryos. *Cell* **54**: 83-93.

DRIEVER, W. and C. NÜSSLEIN-VOLHARD (1988b). The *bicoid* protein determines position in the *Drosophila* embryo in a concentration-dependant manner. *Cell* **54**: 95-104.

EATON, S. and T. B. KORNBERG (1990). Repression of ci^D in posterior compartments of *Drosophila* by *engrailed*. *Genes and Development* **4**:1068-1077.

FJOSE, A., W. J. MCGINNIS, and W. J. GEHRING (1985). Isolation of a homeobox box-containing gene from the *engrailed* region of *Drosophila* and the spatial distribution of its transcripts. *Nature* **313**: 284-289.

FRENCH, V. (1988). Gradients and insect segmentation. *Development* **104**(supplement): 3-16.

FRENCH, V., P. J. BRYANT, S. V. BRYANT (1976). Pattern regulation in epimorphic fields. *Science* **193**: 969-981.

FROHNHÖFER, H. G. and C. NÜSSLEIN-VOLHARD (1986). Organization of anterior pattern in the *Drosophila* embryo by the maternal gene *bicoid*. *Nature* **324**: 120-125.

GARÇIA-BELLIDO, A. (1975). Genetic control of wing disc development in *Drosophila*. In: Cell Patterning (S. Brenner, ed.) pp. 161-182. Amsterdam, Oxford, New York: Associated Scientific Publishers.

GARÇIA-BELLIDO, A., P. RIPOLL, and G. MORATA (1973). Developmental compartmentalization in the wing disc of *Drosophila*. *Nature New Biology* **245**: 251-253.

GARÇIA-BELLIDO, A., P. RIPOLL, and G. MORATA (1976). Developmental segregations in the dorsal mesothoracic disc of *Drosophila*. *Developmental Biology* **48**: 132-147.

GERGEN, J. P. and E. F. WIESCHAUS (1986). Localized requirements for gene activity in segmentation of *Drosophila* embryos: analysis of *armadillo*, *fused*, *giant*, and *unpaired* mutations in mosaic embryos. *Wilhelm Roux's Archives of Developmental Biology* **195**: 49-62.

HAMA, C., Z. ALI, and T. KORNBERG (1990). Region-specific recombination and expression are directed by portions of the *Drosophila engrailed* promoter. *Genes and Development* **4**: 1079-1093.

HARDING, K., C. RUSHLOW, H. J. DOYLE, T. HOEY, and M. LEVINE (1986). Cross-regulatory interactions among the pair-rule genes in *Drosophila*. *Science* **233**: 953-959.

HIDALGO, A. and P. INGHAM (1990). Cell patterning in the *Drosophila* segment: spatial regulation of the segment-polarity gene *patched*. *Development* **110**: 291-301.

HOOPER, J. E., and M. P. SCOTT (1989). The *Drosophila patched* gene encodes a putative membrane protein required for segmental patterning. *Cell* **59**: 751-765.

HOWARD, K. and P. INGHAM (1986). Regulatory interactions between the segmentation genes *fushi-tarazu*, *hairy*, and *engrailed* in the *Drosophila* blastoderm. *Cell* **44**: 949-957.

INGHAM P. W. (1988). The molecular genetics of embryonic pattern formation in *Drosophila*. *Nature* **335**: 25-34.

INGHAM P. W., N. E. BAKER, A. MARTINEZ-ARIAS (1988). Regulation of segment-polarity genes in the *Drosophila* blastoderm by *fushi-tarazu* and *even-skipped*. *Nature* **331**: 73-75.

INGHAM P.W., A. MARTINEZ-ARIAS, P. A. LAWRENCE, and K. HOWARD (1985). Expression of *engrailed* in the parasegments of *Drosophila*. *Nature* **317**: 634-636.

JÄCKLE, H., D. TAUTZ, R. SCHUH, E. SEIFERT, and R. LEHMANN (1986). Cross-regulatory interactions among the gap genes of *Drosophila*. *Nature* 324: 668-670.

JÜRGENS, G. (1987). Segmental organization of the tail region in the embryo of *Drosophila melanogaster*: A blastoderm fate map of the cuticle structures of the larval tail region. *Wilhelm Roux's Archives of Developmental Biology* **196**: 141-157.

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. *Wilhelm Roux's Archives of Developmental Biology* **193**: 283-295.

JÜRGENS, G., R. LEHMANN, M. SCHARDIN, and C. NÜSSLEIN-VOLHARD (1986). Segmental organization of the head in the embryo of *Drosophila melanogaster*: A blastoderm fate map of the cuticle structures of the larval head. *Wilhelm Roux's Archives of Developmental Biology* **195**: 359-377.

KARR, T. L., M. P. WEIR, Z. ALI, and T. KORNBERG (1989). Patterns of *engrailed* protein in early *Drosophila* embryos. *Development* **105**: 605-612.

KLINGENSMITH, J., E. NOLL, and N., PERRIMON (1989). The segment-polarity phenotype of *Drosophila* involves differential tendencies toward transformation and cell death. *Developmental Biology* **134**: 130-145.

KORNBERG, T. (1981). Compartments in the abdomen of *Drosophila* and the role of the *engrailed* locus. *Developmental Biology* **86**: 363-372.

KORNBERG, T., I. SIDÉN, P. O'FARRELL, and M. SIMON (1985). The *engrailed* locus of *Drosophila*: *In situ* localization of transcripts reveals compartment-specific expression. *Cell* **40**: 45-53.

LAWRENCE, P. A., and P. JOHNSTON (1989). Pattern formation in the *Drosophila* embryo: allocation of cells to parasegments by *even-skipped* and *fushi-tarazu*. *Development* **105**: 761-767.

LAWRENCE, P. A., P. JOHNSTON, P. MACDONALD, and G. STRUHL (1987). Borders of parasegments in *Drosophila* embryos are delimited by the *fushi-tarazu* and *even-skipped* genes. *Nature* **328**: 440-442.

LEHMANN, R. and H. G. FROHNHÖFER (1989). Segmental polarity and identity in the abdomen of *Drosophila* is controlled by the relative position of gap gene expression. In: The Molecular Basis of Positional Signalling. *Development(Supplement)*: 21-29.

LINDSLEY, D. L. and E. H. GRELL (1968). Genetic variations of *Drosophila melanogaster*. *Carnegie Inst. Wash. Publ.* **627**.

LOCKE, M., (1959). The cuticular pattern in an insect, *Rhodnuis Prolixus* Stål.. *Journal of Experimental Biology* **36**: 398-406.

LOCKE, M., (1960). The cuticular pattern in an insect: the intersegmental membranes. *Journal of Experimental Biology* **37**: 459-478.

LOHS-SCHARDIN, M., C. CREMER, and C. NÜSSLEIN-VOLHARD (1979). A fate map for the larval epidermis of *Drosophila melanogaster*: Localized cuticle defects following irradiation of the blastoderm with an ultraviolet laser microbeam. *Developmental Biology* **73** : 239-255.

MARTINEZ-ARIAS, A. (1985). The development of *fused*⁻ embryos of *Drosophila melanogaster*. *J. Embryol. exp. Morph.* **87**: 99-114.

MARTINEZ-ARIAS, A. and P. A. LAWRENCE (1985). Parasegments and compartments in the *Drosophila* embryo. *Nature* **313**: 639-642.

MARTINEZ-ARIAS, A. and P. W. INGHAM (1985). The origin of pattern duplications in segment-polarity mutants of *Drosophila melanogaster*. *J. Embryol. exp. Morph.* **87**: 129-135.

MARTINEZ-ARIAS, A., N. E. BAKER, and P. W. INGHAM (1988). Role of segment-polarity genes in the definition and maintenance of cell states in the *Drosophila* embryo. *Development* **103**: 157-170.

MCMAHON, A. P., and R. T. MOON (1989). *int*-1: a proto-oncogene involved in cell signalling. In: The Molecular Basis of Positional Signalling. *Development*(*Supplement*):161-167.

MEINHARDT, H. (1986). Hierarchical inductions of cell states: A model for segmentation in *Drosophila*. *Journal of Cell Science*(*suppl.*)**4**: 357-381.

MOHLER, J. (1988). Requirements for *hedgehog*, a segment-polarity gene, in patterning larval and adult cuticle of *Drosophila*. *Genetics* **120**: 1061-1072.

MORATA, G. and P. A. LAWRENCE (1975). Control of compartment development by the *engrailed* gene in *Drosophila*. *Nature* **255**: 614-617.

MORATA, G. and P. A. LAWRENCE (1977). The development of *wingless*, a homeotic mutation of *Drosophila*. *Developmental Biology* **56**: 227-240.

NAKANO, Y., I. GUERRERO, A. HIDALGO, A. TAYLOR, J. R. S. WHITTLE, and P. INGHAM (1989). A protein with several possible membrane-spanning domains encoded by the *Drosophila* segment-polarity gene *patched*. *Nature* **341**: 508-513.

NÜSSLEIN-VOLHARD, C. and E. WIESCHAUS (1980). Mutations affecting segment number and polarity in *Drosophila* . *Nature* **287**: 795-801.

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 Developmental Biology* **193**: 267-282.

ONOFRECHUK, P. (1986). Analysis of segmentation in *Drosophila melanogaster*. *M.Sc. Thesis*, University of Alberta.

ORENIC, T. V., D. C. SLUSARSKI, K. L. KROLL, R. A. HOLMGREN (1990). Cloning and characterization of the segment-polarity gene *cubitus interruptus Dominant* of *Drosophila*. *Genes and Development* **4**: 1053-1067.

ORENIC, T., J. CHIDSEY, R. HOLMGREN (1987). *Cell and cubitus interruptus Dominant*: two segment-polarity genes on the fourth chromosome in *Drosophila*. Developmental Biology 124: 50-56

PEIFER, M. and E. WIESCHAUS (1990). The segment-polarity gene *armadillo* encodes a functionally modular protein that is the *Drosophila* homolog of human plakoglobin. Cell **63**: 1167-1178.

PERRIMON, N., and A. P. MAHOWALD (1987). Multiple functions of segment-polarity genes in *Drosophila*. *Developmental Biology* **119**: 587-600.

PERRIMON, N., L. ENGSTROM, and A. P. MAHOWALD (1989). Zygotic lethals with specific maternal effect phenotypes in *Drosophila melanogaster*. I. Loci on the X chromosome. *Genetics* **121**: 333-352.

PRÉAT, T, P. THÉROND, C. LAMOUR-ISNARD, B. LIMBOURG-BOUCHON, H. TRICOIRE, I. ERK, M-C. MARIOL, and D. BUSSON (1990). A putative serine/threonine protein kinase encoded by the segment-polarity *fused* gene of *Drosophila*. *Nature* **347**:87-89.

RIGGLEMAN, B., E. WIESCHAUS, AND P. SCHEDL (1989). Molecular analysis of the *armadillo* locus: uniformly distributed transcripts and a protein with novel internal repeats are associated with a *Drosophila* segment-polarity gene. *Genes and Development* **3**: 96-113.

RIGGLEMAN, B., P. SCHEDL, AND E. WIESCHAUS (1990). Spatial expression of the *Drosophila* segment-polarity gene *armadillo* is posttranscriptionally regulated by *wingless*. *Cell* **63**: 549-560.

RIJSEWIJK, F., M. SCHUERMANN, E. WAGENAAR, P. PARREN, D. WEIGEL, and R. NUSSE (1987). The *Drosophila* homolog of the mouse mammary oncogene *int-1* is identical to the segment-polarity gene *wingless*. *Cell* **50**: 649-657.

RUSSELL, M. A. (1985). Positional information in insects. *Developmental Biology* **108**: 269-283.

SHARMA, R. P. (1973). *wingless*: a new mutant in *Drosophila melanogaster*. *Drosophila Information Service* **50**: 134.

SHARMA, R. P. and V. L. CHOPRA (1976). Effect of the *wingless* (wg^1) mutation on wing and haltere development in *Drosophila melanogaster*. *Developmental Biology* **48**: 461-465.

STRUHL, G. (1984). Splitting the bithorax complex of *Drosophila* *Nature* **308**: 454-457.

SZABAD, J., T. SCHÜPBACH, and E. WIESCHAUS (1979). Cell lineage and development in the larval epidermis of *Drosophila melanogaster*. *Developmental Biology* **73**: 256-271.

TIONG, S. K. Y. and D. NASH (1990). Genetic analysis of the *adenosine3* (*Gart*) region of the second chromosome of *Drosophila melanogaster*. *Genetics* **124**: 889-920.

TURING, A. M. (1952). The chemical basis of morphogenesis. *Phil. Trans. R. Soc. London Ser. B.* **212**:37-72.

TURNER F. R. and MAHOWALD, A. P. (1977). Scanning electron microscopy of *Drosophila* embryogenesis. 2. Gastrulation and segmentation. *Developmental Biology* **49**: 403-416.

VAN DEN HEUVEL, M., R. NUSSE, P. JOHNSTON, and P. A. LAWRENCE (1989). Distribution of the *wingless* gene product in *Drosophila* embryos: A protein involved in cell-cell communication. *Cell* **59**: 739-749.

WEIR, M. P. and T. KORNBERG (1985). Patterns of *engrailed* and *fushi-tarazu* transcripts reveal novel intermediate stages in *Drosophila* segmentation. *Nature* **318**: 433-439.

WEIR, M. P., B. A. EDGAR, T. KORNBERG, and G. SCHUBIGER (1988). Spatial regulation of *engrailed* expression in the *Drosophila* embryo. *Genes and Development* **2**: 1194-1203.

WIESCHAUS, E. and E. NOELL (1986). Specificity of embryonic lethal mutations in *Drosophila* analysed in germ line clones. *Wilhelm Roux's Archives of Developmental Biology* **195**:63-73.

WIESCHAUS, E. and R. RIGGLEMAN (1987). Autonomous requirements for the segment-polarity gene *armadillo* during *Drosophila* embryogenesis. *Cell* **49**: 177-184.

WOLPERT, L. (1969). Positional information and the spatial pattern of cellular differentiation. *Journal of Theoretical Biology* **25**: 1-47.

WRIGHT, D. A., and P. A. LAWRENCE (1981). Regeneration of the segment boundary in *Oncopeltus*. *Developmental Biology* **85**: 317-327.

APPENDIX

Statistical Analysis of the Data

The following tables give the averages, standard deviations, and sample sizes for the data plotted in the relevant graphs. Refer to Figure 3 for the measurements (a-f) that were used in the calculations.

A) Data for Figure 10

Calculation:
the proportion of the segment covered by denticles = b/a

stage	A1	A2	A3	A4	A5	A6	A7
gbe	0.887 ± 0.221 (8)	0.929 ± 0.139 (8)	0.902 ± 0.182 (8)	0.961 ± 0.072 (8)	0.965 ± 0.066 (8)	0.977 ± 0.049 (8)	1.000 ± 0.000 (8)
egb	0.857 ± 0.221 (6)	0.929 ± 0.139 (6)	0.902 ± 0.182 (6)	0.961 ± 0.072 (6)	0.965 ± 0.066 (6)	0.977 ± 0.049 (5)	1.000 ± 0.000 (5)
gbs	0.466 ± 0.118 (16)	0.589 ± 0.136 (16)	0.701 ± 0.125 (16)	0.674 ± 0.098 (16)	0.729 ± 0.105 (16)	0.786 ± 0.120 (16)	0.828 ± 0.116 (16)
dc	0.237 ± 0.059 (11)	0.296 ± 0.038 (11)	0.339 ± 0.060 (11)	0.361 ± 0.050 (11)	0.457 ± 0.142 (11)	0.391 ± 0.083 (11)	0.437 ± 0.084 (11)
later	0.249 ± 0.033 (3)	0.314 ± 0.065 (3)	0.317 ± 0.079 (2)	0.313 ± 0.005 (2)	0.437 ± 0.015 (2)	0.439 ± 0.077 (2)	0.420 ± 0.016 (3)

B) Data for Figure 12

Calculation:
the proportion of the segment covered by denticles = b/a

stage	A1	A2	A3	A4	A5	A6	A7
18°C	0.141 ± 0.038 (3)	0.174 ± 0.048 (3)	0.285 ± 0.163 (2)	0.197 ± 0.102 (3)	0.321 ± 0.184 (3)	0.258 ± 0.056 (3)	0.334 ± 0.122 (3)
st2	0.212 ± 0.090 (4)	0.267 ± 0.100 (4)	0.295 ± 0.127 (4)	0.293 ± 0.082 (3)	0.454 ± 0.318 (3)	0.343 ± 0.140 (3)	0.329 ± 0.107 (3)
pc(e)	0.167 ± 0.000 (2)	0.233 ± 0.042 (2)	0.256 ± 0.080 (2)	0.313 ± 0.008 (2)	0.308 ± 0.130 (2)	0.352 ± 0.004 (2)	0.448 ± 0.215 (2)
pc(l)	0.188 ± 0.030 (4)	0.271 ± 0.044 (4)	0.382 ± 0.132 (4)	0.273 ± 0.097 (4)	0.327 ± 0.038 (4)	0.362 ± 0.036 (4)	0.394 ± 0.019 (4)
blast (s)	0.202 ± 0.030 (5)	0.336 ± 0.089 (5)	0.381 ± 0.107 (5)	0.530 ± 0.140 (5)	0.570 ± 0.204 (5)	0.341 ± 0.087 (5)	0.454 ± 0.060 (5)
blast (c)	0.139 ± 0.043 (2)	0.246 ± 0.006 (2)	0.312 ± 0.125 (2)	0.293 ± 0.100 (2)	0.408 ± 0.088 (2)	0.461 ± 0.001 (2)	0.502 ± 0.144 (2)
gst	0.207 ± 0.056 (6)	0.280 ± 0.041 (6)	0.304 ± 0.041 (6)	0.368 ± 0.060 (6)	0.355 ± 0.044 (6)	0.414 ± 0.112 (5)	0.440 ± 0.096 (5)
gbe	0.249 ± 0.064 (12)	0.368 ± 0.105 (13)	0.414 ± 0.076 (13)	0.427 ± 0.104 (12)	0.415 ± 0.090 (11)	0.496 ± 0.112 (10)	0.500 ± 0.138 (8)
egb	0.262 ± 0.087 (2)	0.372 ± 0.001 (2)	0.435 ± 0.092 (2)	0.521 ± 0.225 (2)	0.415 ± 0.000 (1)	0.476 ± 0.112 (17)	0.542 ± 0.000 (1)
gbs	0.616 ± 0.225 (8)	0.673 ± 0.208 (8)	0.665 ± 0.207 (8)	0.668 ± 0.283 (5)	0.527 ± 0.249 (4)	0.459 ± 0.276 (3)	0.397 ± 0.072 (2)

C) Data for Figure 14

Calculation:
the proportion of the segment covered by denticles = b/a

temperature (°C)	A1 (*wg*/*wg*)	A7 (*wg*/*wg*)	A1 (*wg*/Df)	A7 (*wg*/Df)
wildtype	0.282 ± 0.006 (5)	0.474 ± 0.022 (5)	0.282 ± 0.006 (5)	0.474 ± 0.022 (5)
21.6	0.738 ± 0.146 (15)	0.893 ± 0.110 (13)	0.944 ± 0.084 (12)	1.000 ± 0.000 (12)
23.0	0.990 ± 0.030 (10)	1.000 ± 0.000 (10)		

D) Data for Figure 16a

Calculation:
the proportion of the segment covered by denticles = b/a

temperature (°C)	prothorax	mesothorax	metathorax
wildtype	0.241 ± 0.020 (5)	0.204 ± 0.035 (5)	0.211 ± 0.042 (5)
18.9	0.272 ± 0.036 (6)	0.212 ± 0.023 (6)	0.214 ± 0.091 (6)
19.8	0.289 ± 0.032 (17)	0.341 ± 0.103 (16)	0.312 ± 0.115 (16)
20.8	0.356 ± 0.110 (10)	0.382 ± 0.114 (10)	0.432 ± 0.113 (10)
21.8	0.579 ± 0.119 (7)	0.759 ± 0.249 (11)	0.615 ± 0.260 (13)

E) Data for Figure 16b

Calculation
the proportion of the segment covered by denticles = b/a

temper-ature (°C)	A1	A2	A3	A4	A5	A6	A7
wildtype	0.282 ± 0.006 (5)	0.422 ± 0.018 (5)	0.413 ± 0.014 (5)	0.444 ± 0.023 (5)	0.421 ± 0.015 (5)	0.422 ± 0.010 (5)	0.474 ± 0.022 (5)
18.9	0.335 ± 0.109 (5)	0.440 ± 0.069 (5)	0.508 ± 0.162 (5)	0.470 ± 0.065 (5)	0.510 ± 0.106 (5)	0.502 ± 0.048 (5)	0.623 ± 0.079 (5)
19.8	0.477 ± 0.090 (14)	0.624 ± 0.099 (14)	0.629 ± 0.074 (11)	0.662 ± 0.056 (10)	0.644 ± 0.050 (9)	0.699 ± 0.113 (11)	0.780 ± 0.085 (15)
20.8	0.539 ± 0.070 (10)	0.686 ± 0.136 (10)	0.743 ± 0.130 (10)	0.738 ± 0.117 (8)	0.732 ± 0.072 (6)	0.763 ± 0.087 (6)	0.832 ± 0.063 (11)
21.8	0.732 ± 0.189 (8)	0.781 ± 0.153 (7)	0.776 ± 0.096 (7)	0.798 ± 0.138 (6)	0.956 ± 0.098 (5)	0.896 ± 0.118 (6)	0.858 ± 0.112 (6)

F) Data for Figure 18

Calculation:
the proportion of the segment covered by the region between row 1 and row 4 = f/a

temperature (°C)	A3	A7
wildtype	0.245 ± 0.018 (5)	0.265 ± 0.016 (5)
18.9	0.274 ± 0.043 (5)	0.286 ± 0.012 (5)
19.8	0.288 ± 0.025 (10)	0.343 ± 0.044 (11)
20.8	0.301 ± 0.034 (9)	0.355 ± 0.032 (10)
21.8	0.313 ± 0.063 (5)	

G) Data for Figure 21

Calculation:
the distance of the Keilin's organs from the anterior edge of the denticle belt = e/a

temperature (°C)	prothorax	mesothorax	metathorax
wildtype	0.670 ± 0.052 (5)	0.561 ± 0.039 (5)	0.579 ± 0.023 (5)
18.9	0.695 ± 0.010 (5)	0.572 ± 0.021 (4)	0.628 ± 0.128 (4)
19.8	0.714 ± 0.052 (8)	0.522 ± 0.088 (11)	0.629 ± 0.054 (11)
20.8	0.687 ± 0.064 (6)	0.596 ± 0.061 (6)	0.750 ± 0.090 (7)
21.8	0.658 ± 0.025 (2)		0.795 ± 0.120 (5)

H) Data for Figure 22

The data for this graph was generated by subtracting the averages used for plotting the proportion of the prothorax covered by anterior denticles (Figure 16a) from the averages used for plotting the position of the anterior border of the prothorax (Figure 24); that is:

the amount of naked cuticle between the two groups of prothoracic denticles (proportion of the segment) = average(c/a) - average(b/a)

I) Data for Figure 23

Calculation:

the proportion of the segment covered by mid-prothoracic denticles = (d-c)/a

temperature (°C)	mid-prothoracic patch
wildtype	0.165 ± 0.071 (5)
18.9	0.198 ± 0.018 (4)
19.8	0.160 ± 0.033 (7)
20.8	0.158 ± 0.075 (6)
21.8	0.132 ± 0.048 (3)

The curve for the anterior prothoracic denticle belt is the same as in Figure 16a.

J) Data for Figure 24

Calculations:

the distance of the mid-prothoracic patch of denticles from the anterior border of the segment

Anterior border = c/a

Posterior border = d/a

temperature (°C)	Anterior Border	Posterior Border
wildtype	0.375 ± 0.035 (5)	0.540 ± 0.044 (5)
18.9	0.377 ± 0.029 (4)	0.575 ± 0.011 (5)
19.8	0.420 ± 0.026 (7)	0.576 ± 0.036 (8)
20.8	0.354 ± 0.051 (6)	0.512 ± 0.083 (6)
21.8	0.471 ± 0.053 (3)	0.603 ± 0.095 (2)