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Pattern formation in a secondary field: a hierarchy of regulatory genes subdivides the developing *Drosophila* wing disc into discrete subregions

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SUMMARY

The legs and wings of insects and vertebrates develop from secondary embryonic fields that arise after the primary body axes have been established. In order to understand how the insect imaginal wing field is patterned, we have examined in detail the temporal and spatial expression patterns of, and epistatic relationships between, four key regulatory genes that are specifically required for wing formation in *Drosophila*. The *wing-less* protein, in a role surprisingly distinct from its embryonic segment polarity function, appears to be the earliest-acting member of the hierarchy and crucial for distinguishing the notum/wing subfields, and for the compartmentalization of the dorsal and ventral wing surfaces. The *wingless* product is required to restrict the expression of the *apterous* gene to dorsal cells and to promote the expression of the *vestigial* and *scalloped* genes that demarcate the wing primordia and act in concert to promote morphogenesis.

Key words: *scalloped*, *apterous*, *vestigial*, cell death, *Drosophila*, regulatory gene, pattern formation

INTRODUCTION

Most work on pattern formation in *Drosophila* has focused on understanding the genetic regulatory hierarchy and molecular mechanisms that pattern the embryo along the anterior-posterior (A/P) and dorsal-ventral (D/V) axes. These studies have revealed much about the mechanisms of patterning within a primary field, but comparatively little is known about the mechanisms by which secondary patterning fields (e.g., insect wings and legs, vertebrate limb buds, etc.) are generated. The urodele amphibian limb, cockroach leg and *Drosophila* wing disc are all model systems utilized to study the patterning of secondary fields (see French et al., 1976). Traditionally, patterning of these structures was studied by observing their regulative capacities following disruption by either mechanical (ablation) or genetic (temperature-sensitive alleles or clonal analysis) means. The striking observation was that all three fields regulated in analogous manners, implying that the underlying patterning systems are similarly organized in secondary fields in organisms as far diverged as insects and vertebrates (French et al., 1976; Bryant and Gardiner, 1992). Numerous models to explain the regulative behavior of these systems have been proposed (e.g. the polar coordinate model; French et al., 1976 and Bryant et al., 1981); however, the verification of these models awaits molecular evidence for the patterning mechanisms operating in any of these systems.

Of these model systems, the *Drosophila* wing disc is particularly well suited for the genetic and molecular dissection of patterning processes in a cellular field. In previous work, we have shown that the embryonic origin and subsequent development of the wing can be followed using an antibody to the *vestigial* protein (Williams et al., 1991). The disc is first visualized as a cluster of cells in germ-band-retracted embryos and forms a pouch of approximately fifty cells at the time of embryo hatching. The size of the disc increases exponentially during larval life and contains approximately 50,000 cells at the end of the third larval instar (Whittle, 1990). During metamorphosis, this pouch evaginates to form most of the epidermal (cuticular) structures of the dorsal thorax, as well as the wing.

The adult wing exhibits anterior-posterior, dorsal-ventral and proximal-distal polarity, and positional information arises at some time during wing development that distinguishes the different subregions or compartments. Two compartmental restrictions that develop within the wing disc have been defined by mitotic clonal analysis and reveal the presence of boundaries that prevent clones of cells from mixing (Garcia-Bellido et al., 1976). One clear restriction is the anterior/posterior (A/P) boundary, which is established early in embryogenesis and requires the *engrailed* gene product in the posterior compartment for its maintenance (Lawrence and Morata, 1976). The other proposed compartmental restriction is the dorsal/ventral (D/V) boundary, which is drawn along the wing margin, separating the future dorsal and ventral wing surfaces and is detected in clones during early larval life (first or second larval instar; Garcia-Bellido et al., 1976). However, the presence of a zone of non-proliferating cells along the

developing wing margin throughout the third larval instar (O'Brochta and Bryant, 1985) has led to considerable debate as to whether the detected clonal restriction is a compartment boundary or simply a region of reduced cell proliferation (Brower, 1985). In addition to these potential lineage restrictions, the late third instar disc is extensively patterned, as evidenced by the complex expression patterns identified by enhancer traps (for a review, see Whittle, 1990) and the array of cuticle structures formed when specific disc fragments are allowed to metamorphose separately (Bryant, 1978). Indeed, the latter technique has been used to make a detailed fate map of the disc such that the location of the primordia for the adult structures derived from this disc are known (Bryant, 1978).

Our approach to understanding patterning in this system is focused on the formation of the presumptive wing region of the wing imaginal disc. In terms of proximal-distal patterning, this disc forms both distal (wing) and proximal (body wall; notum) structures. The wing is a relatively recent evolutionary innovation (primitive ancestral insects lack wings) and may represent a primary division within this patterning field. Analysis of wing formation could therefore reveal basic patterning mechanisms within the larger imaginal field. A key advantage to studying this problem in *Drosophila* is the availability of numerous mutations that alter the adult cuticle (Lindsley and Zimm, 1992). Indeed, a large number of mutations have been identified that alter the development of the wing. However, only a few of these have been found to globally and specifically remove the entire structure. One well-studied gene, *decapentaplegic* (*dpp*), is required for proximal/distal patterning of all adult appendages (Posakony et al., 1990), while four other genes, which are now cloned, are specifically required for formation of the wing region of the disc (i.e. these genes do not appear to perform obviously analogous functions in other tissues). Strong mutations in three of these genes result in complete loss of the wing [*apterous* (*ap*), *scalloped* (*sd*) and *vestigial* (*vg*); see Lindsley and Zimm (1992) for mutant descriptions] while one results in a transformation of wing to notum [*wingless* (*wg*)]. All of these genes are required for other functions in development (e.g. *wingless* is a tissue-specific component of a signaling pathway that is required for embryonic and imaginal patterning; see Peifer and Bejsovec, 1992), but the effects of loss of these genes within the wing disc (during larval development; *wg* is required during embryonic development for imaginal disc formation; Simcox et al., 1989) are specific to the wing region of the disc (Baker, 1988a,b; Williams and Bell, 1988; Stevens and Bryant, 1985; James and Bryant, 1981). The proteins encoded by these genes possess structural features [e.g. homeodomain (*ap*; Cohen et al., 1992), novel nuclear protein (*vg*; Williams et al., 1991), TEA domain putative transcription factor (*sd*; Campbell et al., 1992) and *int-1* growth factor homologue (*wg*; Rijsewijk et al., 1987)] that are consistent with their playing primary roles in establishing the wing region of this disc.

The availability of molecular probes for these genes has allowed us to examine the dynamics of early regulatory gene expression in the wing disc, the correlations between regulatory gene expression and the wing fate map, and the regulatory interactions among these genes that are required to specify the wing region of the disc. The results indicate that the four genes fall into two classes that reflect either an early or late requirement in disc development. *vg* and *sd* are required later in development (during the third larval instar) for formation of the wing region of the disc and their expression patterns reflect the specification of the wing region of the disc during disc development. *ap*, in contrast, is required before wing specification to establish the proper domains of *sd* and *vg* expression and to mark the D/V boundary within the developing wing region. *wg* has the earliest requirement and influences both the notum/wing and dorsal/ventral boundaries. It plays a key role in positioning *ap* expression and promoting *vg* expression, which suggests that it sits at or near the top of the genetic regulatory hierarchy guiding wing formation. It is clear from the details emerging from the study of various developmental systems that *wingless* and its many relatives contribute to the patterning of a wide variety of invertebrate and vertebrate fields.

MATERIALS AND METHODS

Description of alleles, gene probes and antibody detection strategies

All gene and allele designations are as in Lindsley and Zimm (1992). *vg* and *ap* are not essential genes, and null alleles of *vg* (vg^{83b27R} and vg^{AlExt}; Williams et al., 1991) were used to examine *vg* defects. Homozygous *ap* enhancer trap flies show a strong *ap* phenotype (Fig.1, almost as extreme as *ap* null flies; Cohen et al., 1992) and this genotype was utilized for examination of alterations in *ap* mutants. *wg* is an essential gene, so heteroallelic combinations of two *cis*-acting *wg* hypomorphic alleles were used to generate viable *wg* mutant larvae (wg^1 and In(2L)wg^P; see Baker, 1988a). Likewise strong *sd* alleles are early larval lethals, so a pupal lethal allele (sd^{31h}) with severe pupal wing defects was used as a *sd* mutant (see Campbell et al., 1992). *ap* expression was detected utilizing the *ap* enhancer trap line. This line accurately reflects *ap* mRNA expression in embryos and late imaginal discs (Cohen et al., 1992) as a heterozygote or homozygote. *sd* expression was also detected with an enhancer trap insertion that accurately reflects wild-type *sd* expression in all tissues that have been examined (Campbell et al., 1992). Thus, analysis of β-gal expression patterns driven by either *ap* or *sd* is likely to reflect the true expression of these genes. The primary antibodies used to detect β-gal expression were either a β-gal-specific mouse monoclonal antibody (Boehringer Mannheim) or a rabbit anti β-gal polyclonal antibody. *vg* expression was detected utilizing a *vg*-specific rabbit polyclonal antibody, while *wg* protein expression was detected with either rat or rabbit polyclonal antibodies. *ac* was detected with a mouse monoclonal antibody. These primary antibodies were developed by two or three steps to either fluorescein or rhodamine conjugates utilizing standard methodology (see Skeath and Carroll, 1991 and Williams et al., 1991 for strategies).

Dissection and staining of second and early third instar larvae

Adult flies were allowed to lay eggs on yeasted molasses agar caps for defined time intervals (4 hour collections if precise staging was important). The entire cap was placed in a molasses agar

100 mm Petri plate (a hole to accommodate the cap was cut in the agar), given a generous amount of live yeast paste and incubated at 25°C. Abundant yeast and uncrowded conditions are essential for optimal timed stagings. Staging was performed by both total incubation time and optical examination of larval anterior spiracles and mouthhooks. Larval markers were utilized to facilitate identification of larvae of appropriate genotypes. *ap*, *vg* and *wg* stocks were balanced with the dominant *Black cell* (*Bc*) marker (associated with the In(2LR)*Gla* balancer) to facilitate identification of homozygous mutant larvae (absence of black cells throughout the larvae). The sd^{31H} stock was marked with *yellow* (*y*) and balanced with *Bascn*, which allowed mutant *sd* male larvae to be identified via the presence of yellow larval mouthhooks. The abdomen of selected early larvae were grasped with fine tweezers and one side of the cuticle (approximately mid-lateral) was pinched and held with a second set of tweezers. These tweezers were pulled rostrally until the larval head was cleanly removed. With practice this generated a dissected larval head that is either split longitudinally or inverted, either of which are satisfactory for antibody or X-gal staining. If the entire head is removed intact, grasping the inside of the mouthhooks facilitates simple eversion with the second set of tweezers. Eversion (or splitting) is essential for allowing antibody access, but is not necessary for X-gal staining. All dissections were performed in depression wells (containing PBS), and accumulated in a PBS-containing microtiter well. Fixation, antibody staining and washing were performed in microtiter wells as described previously (Skeath and Carroll, 1991). Many dissected larvae may be stained per well (at least twenty) without noticeable loss of antibody staining; however, extra care must be taken to recover tiny larvae lost from the baskets during washing! The larvae were mounted in 50 mm Tris (pH 8.8) containing 10% glycerol and 0.5 mg/ml p-phenylenediamine (to prevent quenching) as in Skeath and Carroll (1991). Individual discs were neither visible or individually dissected. Rather the larvae were fully inverted and the cuticle shredded away from the head with fine tungsten needles. The larvae were viewed under fluorescence on a Zeiss Axiophot; switching to bright field allowed identification of mouthhook and anterior spiracles to check staging of the larvae to which discs were still attached. Favorable disc preparations were viewed on a Zeiss/IM35 equipped with a Bio-Rad MRC600 Lasersharp Confocal system (LSCM). Optical projections of through focus sections were used, to ensure accurate representation of expression patterns. Late disc complexes were stained and photographed as in Williams et al. (1991) and Paddock et al. (1993).

Other techniques

Acridine orange staining was performed by the method of Spreij (1971), as modified by Masucci et al. (1990) and imaged using the LSCM. Xgal stainings and mountings of imaginal discs were performed as in Ghysen and O'Kane (1989) and viewed with Nomarski optics on a Zeiss Axiophot, using a Dage VE1000 video camera system and the work station of the confocal microscope for further image processing.

RESULTS

Mutant phenotypes of four wing-determining genes

The aim of this study is to define how the wing is specified in the developing wing disc by the analysis of the developmental roles of and interactions between four genes (*ap*, *sd*, *wg* and *vg*) involved in wing formation. A detailed description of the alleles and gene probes utilized in this study is given in Materials and Methods. The phenotypes of adult flies mutant for *ap*, *sd*, *vg* or *wg* are shown in Fig. 1. Flies homozygous for severe hypomorphic or null *ap* alleles show a complete loss of the wing, retaining only a stump of wing hinge (or an amorphous mass of tissue; Fig. 1E). The phenotype of null *vg* flies is similar with a total elimination of wing structures and retention of only a small fragment of the wing hinge region (Fig. 1B). Certain genetic backgrounds can modify this phenotype and frequently produce flies that exhibit wing-to-notum transformations (Fig. 1C). Strong *sd* alleles are early larval or pupal lethal whereas weak alleles produce viable adults, which show strong wing reductions (Fig. 1F) essentially identical to those shown by *vg* hypomorphic flies. Strong *wg* alleles are embryonic lethal, whereas hypomorphic *wg* alleles exist that cause wing-to-notum transformations (Fig. 1D). This is likely to represent a true loss-of-function phenotype, since mitotic clonal analysis utilising an *armadillo* null allele (another member of the *wg* signaling pathway) also revealed wing-to-notum transformation (Peifer et al., 1991). While it is obvious that each gene is required globally for wing formation, one cannot deduce from these adult mutant phenotypes when and how each gene influences wing determination. To gain more direct information, we have examined the expression and regulation of each gene throughout the growth and development of the wing disc to arrive at a specific model of how the wing is patterned.

Correlations between the late larval expression patterns of the wing regulatory genes, *vestigial*, *apterous* and *wingless*, and the wing fate map

In order to determine the relationship between the domains of *vg*, *ap* and *wg* gene expression and the fate of cells expressing each product, we examined the expression pattern of each gene in the late third instar wing disc, a point when the growth of the disc is complete, but before the cellular rearrangements that occur during pupation have taken place. A fate map has been constructed for this stage (Fig. 2E; Bryant, 1978), which allows us to correlate spatial coordinates of gene expression with the fate of each subregion. Each gene expression pattern and certain overlapping domains of expression highlight distinct primordia. The *vg* protein (and *sd*, see Fig. 5B) demarcates those cells that will give rise to the wing proper (Fig. 2A: Williams et al., 1991). *vg* is not expressed uniformly throughout the presumptive wing region. Maximal levels of protein expression occur in a stripe of cells along the length of the future wing margin, and protein levels decrease in a graded fashion into the dorsal and ventral wing blade primordia (Fig. 2A). The *vg* expression in the most proximal portion of the disc (arrow, Fig. 2A) may not correspond solely to wing structures and could include some future notum structures (Fig. 2E). Unlike the rest of the presumptive wing, this region is not lost in *vg* (or *sd*) null mutant discs. It is possible that the *vg* expression in this region is a consequence of the manner in which *vg* gene expression is activated along the entire dorsal-ventral boundary of the disc (J. A. W., unpublished observations: see also Discussion).

The *ap* pattern is restricted to those cells whose fate is to form dorsal structures (both notum and dorsal wing; Fig.

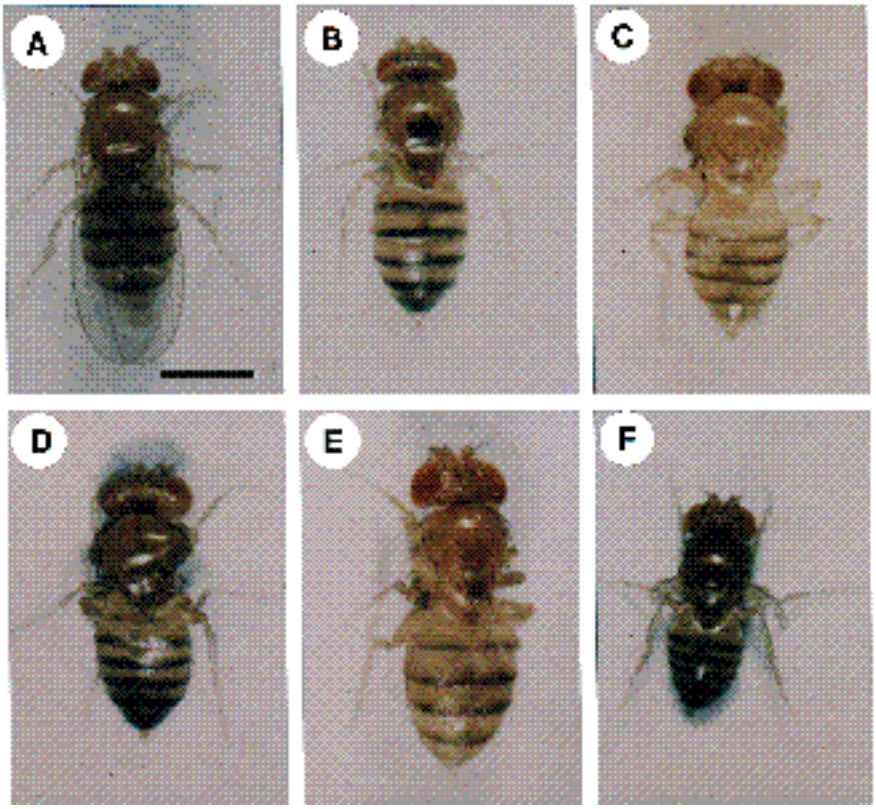

Fig. 1. Adult cuticular defects associated with *ap*, *sd*, *vg* or *wg* alleles. A wild-type fly is shown in A, and can be compared to a homozygous *vg* null mutant (vg^{83b27R}, B and C), *wg* hypomorphic mutant (wg^{1}/In(2L)wg^{P}; D) a strong *ap* homozygous mutant (ap^{K568}; E) and *sd* hypomorphic mutant (sd^{58}/*sd* deficiency; F), mutant flies. An example of a *vg* mutant fly that lacks the wing is shown in B, while a partial notum duplication seen in some *vg* stocks is shown in C. The sd^{58} allele is a *cis*-acting control mutant, and is less severe than stronger *sd* alleles (which are pupal or larval lethal; Campbell et al., 1992). Scale bar, 1 mm.

2B). Superimposition of the *vg* and *ap* patterns and a comparison with the fate map demonstrates a striking correlation between the expression of these two genes and the fate of disc subregions (Fig. 2D,E): the red cells that express only *vg* will form the future ventral surface of the wing (and hinge region), the yellow cells that express both *vg* and *ap* will form the dorsal surface of the wing and the green cells that express only *ap* will form dorsal notum. Thus, it appears that two subdivisions of the wing disc are revealed by the domains of *vg* and *ap* gene expression alone: the wing region is highlighted by the pattern of *vestigial* and the dorsal/ventral boundary by the edge of the *ap* pattern where it bisects the *vg* domain.

The *wg* pattern also highlights the dorsal/ventral boundary, as a brilliant stripe of *wg* expression runs along the presumptive wing margin where the future dorsal and ventral surfaces of the wing meet (Fig. 2C; and Baker, 1988b). As we shall see later, however, this late pattern of *wg* is misleading with respect to the time and place where *wg* has its most dramatic impact on wing formation.

The relationship between domains of gene expression and mutant phenotypes

vg and *sd* demarcate the wing primordia and are required for development of the wing proper

The *vg* and *sd* genes are expressed in identical patterns (Fig. 5A,B) and label the presumptive wing region of the imaginal disc (Fig. 2A). These expression patterns and the complete removal of the wing observed in *vg* and *sd* mutant discs (see below) suggest that both genes encode components essential for formation of the wing region of this disc. These figures also demonstrate that the wing region of the disc exists as a domain of gene expression in the late larval disc.

apterous is expressed only in dorsal cells but is required for formation of the entire wing

ap is expressed in all cells of the dorsal region of the disc (Fig. 2B). Double labeling with *achaete* (which marks bristle precursor cells that flank the wing margin) indicates *ap* expression extends precisely to the wing margin (data not shown), and therefore extends to the border of the putative dorsal compartment of the disc and is excluded from cells of the ventral compartment. Mitotic clonal analysis will have to be utilized rigorously to determine if the border of *ap* expression is exactly coincident with the entire length of the D/V boundary in a cell-by-cell manner. Since *ap* is not expressed in the ventral wing pouch, it is not clear why this region is lost along with the rest of the wing in *ap* mutants, since this region is lost from the disc as early as the third instar (Fig. 5E).

The late *wg* pattern and *wg* mutant phenotype do not correlate

The late *wg* expression pattern is highlighted by a stripe along the presumptive wing margin, and a patch of expression in the presumptive notum region (Fig. 2C). Although *wg* clearly prefigures the future wing margin, the global wing-notum transformations seen in *wg* mutant discs

are difficult to reconcile with the observed limited pattern of expression in late third instar discs.

As we show below, a more complete picture of wing formation comes from understanding the dynamics of gene expression and the regulatory interactions between the genes. In the case of *wingless*, the wing-to-notum phenotype can better be explained by the pattern of *wingless* expression in very early discs and by the effects of mutations in *wingless* on the expression of the other three wing-determining genes.

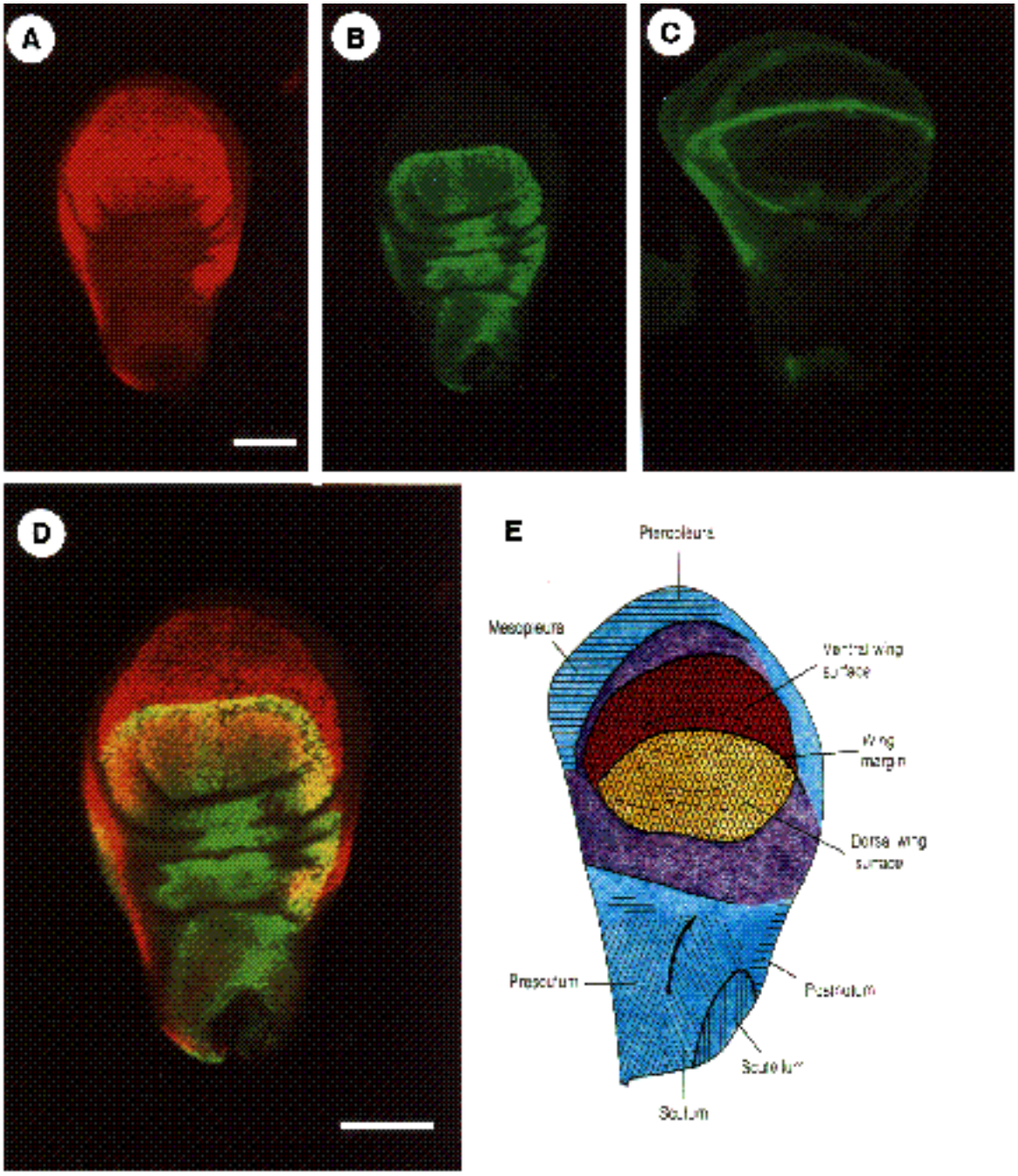

Fig. 2. The expression patterns of wing regulatory genes demarcate distinct regions of the imaginal fate map. The late third instar expression patterns of *vg* protein (A), and *apterous* (B) and *wingless* (C) enhancer traps driving β-galactosidase expression. (D) The *vg* (red) and *ap* (green) patterns overlap in the dorsal compartment of the wing (yellow), the boundary of *ap* expression is the dorsal/ventral boundary and future wing margin. (E) Wing fate map from Bryant (1978) has been color-coded to highlight the ventral (red) and dorsal (yellow) wing surfaces, corresponding to the domains defined by the *vg* and *ap* expression in (D). Parts of the wing base are not illustrated on the fate map: these regions probably correspond to the lateral regions that express *vg* protein (A). The most proximal portion of the lateral *vg* expression may form notal structures (see text). In A, B, C and D, scale bar, 100 μm.

The dynamics of regulatory gene expression and the specification of major boundaries in the developing wing disc

vg and *sd* mark the developing wing in early discs

Since the *vg* protein is expressed in the embryonic primordia of the wing disc (Williams et al., 1991), it is possible that early larval discs also express *vg*. Indeed, it turns out that staining with the *vg* antibody, which in embryos and third instar larvae specifically stains wing and haltere discs, has allowed us to identify wing discs in as early as newly molted second instar larvae (data not shown, and Fig. 3). This has greatly facilitated the identification of the early disc expression patterns of *ap*, *sd* and *wg*, using a double

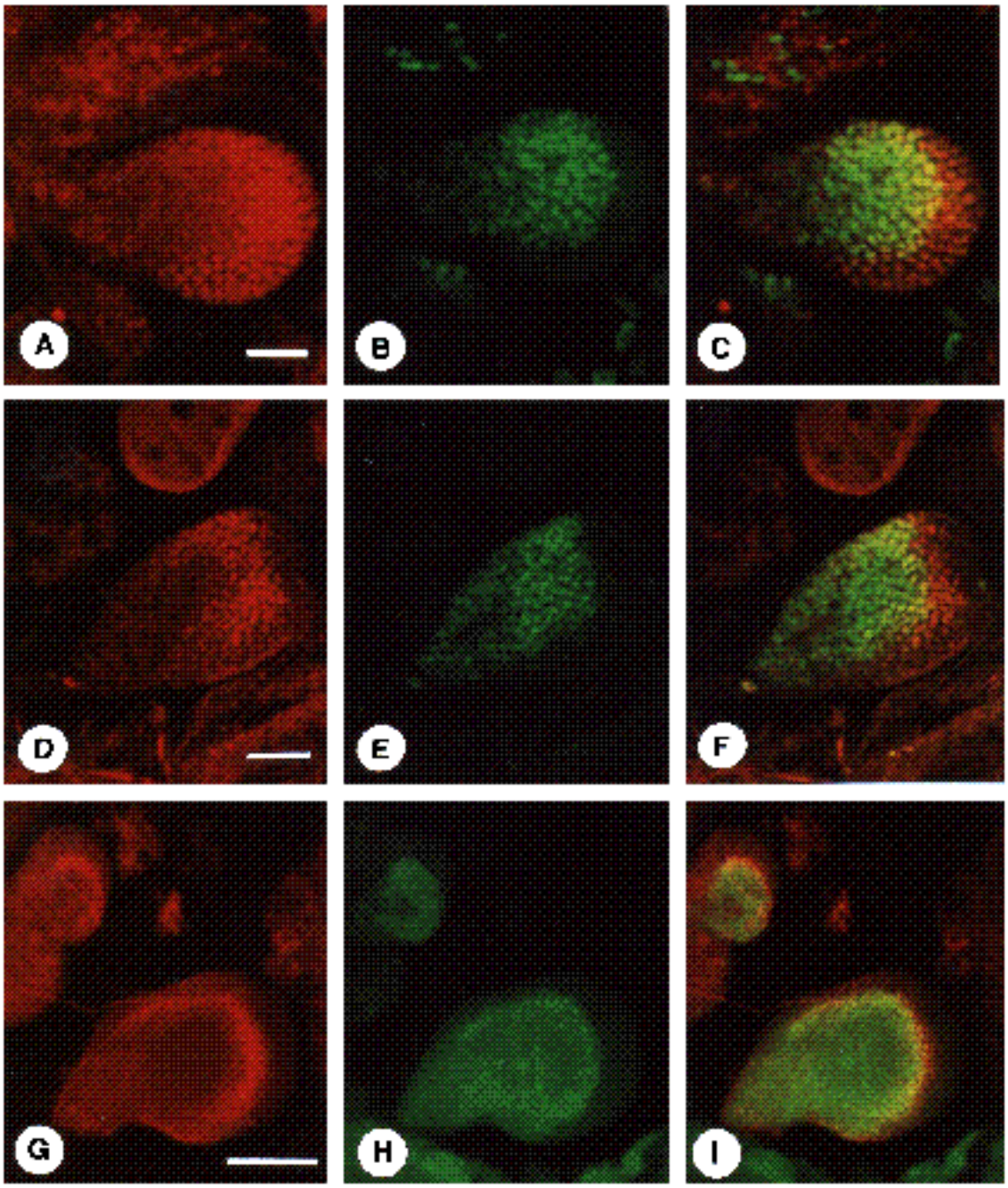

Fig. 3. Development of the *vg* and *ap* expression patterns during the growth and development of the wing disc. Middle (A-C) and late (D-F) second instar and early third instar (G-I) wing discs stained with either *vg* (A, D and G), *ap* (B, E and H) or both (C, F and I) are shown. The early *vg* pattern retreats from the distal (A) edge of the wing to just the presumptive wing (G), while the *ap* pattern is always restricted to just dorsal cells with a stable boundary at the dorsal/ventral boundary (B, E, H). The *vg* protein becomes more concentrated at the D/V boundary and decreases symmetrically in the dorsal and ventral directions. The smaller structure at the upper left in G-I is the haltere disc that exhibits homologous patterns of *vg* and *ap* expression. In A, B and C, scale bar, 10 μm; in D, E and F, scale bar, 25 μm; in G, H and I, scale bar, 50 μm.

fluorescence labeling strategy to identify the otherwise nearly invisible young wing discs. From correlation of the *vg* patterns with those of the other regulatory genes, we have determined that various subregions and boundaries are specified progressively and depend upon interactions among some of the four regulatory genes.

The mature *vg* expression pattern develops in roughly two stages. We first detect low level ubiquitous expression in early second instar discs at the time when the discs contain approximately 100 cells. The expression eventually becomes elevated at approximately the early-mid second instar (Fig. 3A; disc size is less than 200 cells when elevation of *vg* expression is first detected) to give strong staining throughout the disc. This expression is then repressed distally through the remainder of the second instar (Fig. 3D) and finally resolves to form a well-defined stripe by the early third instar (Fig. 3G). This stripe clearly is non-uniform and drops off in intensity both proximally and distally (Fig. 3G), similar to the graded distribution seen in late third instar discs (Fig. 2A, 5A). Double labeling with antibody to *vg* and the *sd* enhancer trap indicates that *sd*-driven *lacZ* expression closely matches *vg* pattern in early discs (data not shown). The *sd* pattern resolves into a stripe more slowly than *vg*, but this probably reflects the slow kinetics of β-galactosidase degradation rather than differential regulation of the two genes. Thus, *sd* and *vg* expression appears identical, in both early and late imaginal discs. However, double labeling experiments with a *sd*-specific antibody (when available) will be necessary to determine if the *sd* enhancer trap does accurately reflect *sd* gene expression in early discs. Examination of *vg* expression throughout the third instar, and later, indicates that the graded *vg* stripe (Fig. 3G) largely corresponds to the early wing region of the disc. This suggests that the wing region exists as a specific domain of gene expression by the early third instar discs, but is not detected as a clearly localized domain at earlier stages (as assayed by *sd* and *vg* expression).

Early *ap* expression reveals the D/V boundary in the second instar wing disc

ap expression is not detected in early second instar discs, but is activated in its apparently mature pattern in early-mid second instar discs (Figs 3B, 4E). This initial activation of *ap* is likely to reflect accurately the initial pattern of *ap* expression, and certainly cannot be due to perdurance of β-galactosidase. While the number of cells subsequently increases enormously in the wing disc, the *ap* pattern remains relatively unaltered with respect to the ventral notum and wing boundaries throughout later larval development (Fig. 3E,H). The early expression of *ap* in a dorsally restricted pattern indicates that a D/V boundary exists in early second instar discs, even before the developing disc field contains 200 cells. Interestingly, *ap* expression never outlines the developing wing (as revealed by *vg* and *sd* expression), which implies that the loss of the wing in *ap* mutants is caused by indirect effects on cells not expressing *ap*. The expression pattern indicates that *ap* specifies a dorsal-specific component which is indirectly necessary for wing formation. Comparison of *ap* and *vg* expression (Fig. 3I) demonstrates that the early third instar *vg* stripe (i.e. the early wing region) and the resolving *vg* stripe in the second instar (Fig. 3F) is centered precisely on the D/V boundary, which is also the border of *ap* expression. This is the same spatial relationship between *ap* and *vg* that is observed in late discs.

Novel and dynamic patterns of *wg* expression during wing disc development

The *wg* expression pattern was assayed in both a *wg* enhancer trap line and with a *wg*-specific antibody. Both patterns of expression are essentially identical in late discs (Fig. 4A,B) except for the slightly broader domain of *wg* protein expression, which is expected since *wg* encodes a secreted product (Van den Heuvel et al., 1989). The spatial coordinates of *wg* enhancer trap expression in the second instar are radically different from the later pattern, and is localized ventrally throughout the second instar (Fig. 4C,D). Expression is first detected in a cap comprising less than 10 cells in early-mid second instar discs (Fig. 4C). This is approximately the time *vg* expression is elevated and *ap* expression is activated (Fig. 4E). The *wg*-specific antibody reveals that protein expression is regulated more dynamically than *lacZ* expression. The sequential development of the late *wg* protein pattern from the early ventral cap is shown in Fig. 4F,I,L (and with respect to *ap* expression in Fig. 4E,H,K). In mid-early third instar larvae, the *wg* expression pattern is similar to the late pattern (compare Fig. 4B and L); indeed, the future *wg* margin stripe is first apparent at this stage. *wg* expression in the early disc roughly complements that of *ap*, and is highest in the ventral region of the disc (Fig. 4G,J,M). This may indicate a requirement for *wg* in the ventral region of the disc, and suggests that both *ap* and *wg* may be required for D/V polarity in the early disc. As is the case with *ap*, at none of the stages examined does *wg* prefigure the developing wing, which argues that *wg* does not directly specify the wing region of the disc and that loss of the entire wing in *wg* mutants may be a secondary effect of its requirement in establishing D/V polarity. As we shall see below, the dynamics of *wg* expression are directly related to the progressive specification of restrictions within the wing.

The *ap* and *wg* genes are required earlier in disc development than *sd* and *vg*

Cell death and morphological abnormalities in early mutant discs

In order to approximate the time of action of the *sd*, *vg*, *ap* and *wg* genes, the morphology of imaginal discs from each of these mutants was examined. By the late third instar, wing discs from all of the mutants are morphologically abnormal and exhibit a complete loss of the wing region (Fig. 5C-F). In order to examine earlier stages, the *ap* enhancer trap was introduced into *sd*, *vg* and *wg* mutant backgrounds and the morphology, *ap* expression patterns and regions of cell death in second and early third instar discs were viewed by Nomarski images of the X-gal-stained tissues and by staining with acridine orange. Second instar and early third instar *vg* and *sd* discs appear morphologically normal; the first detectable defect in these mutants is

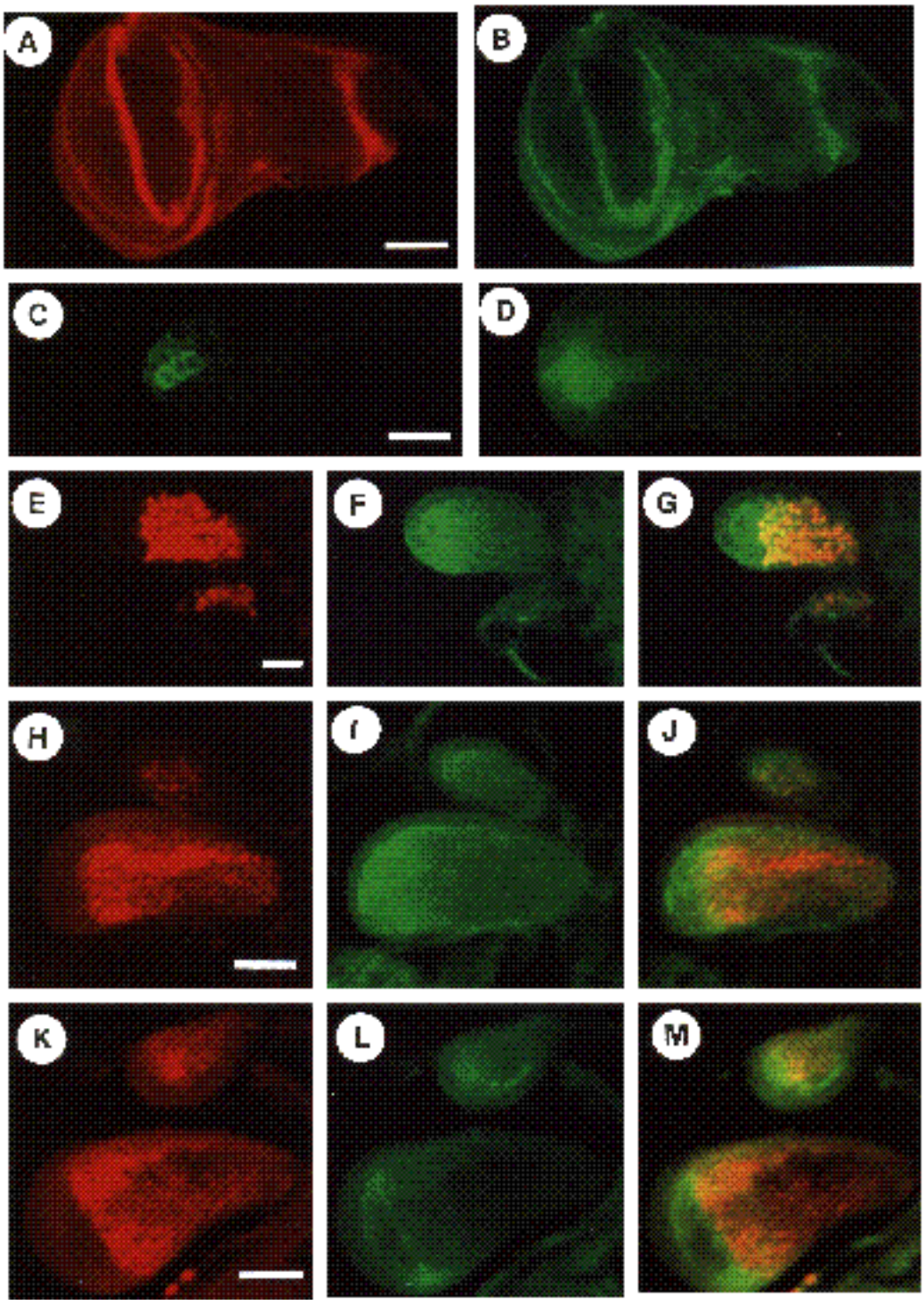

Fig. 4. The novel pattern of *wg* protein in early discs and the dynamics of *wg* expression during disc growth. Single images of a double-labeled late third instar wing disc expressing (A) *wg* enhancer trapand (B) *wg* protein. Early-middle (C) and late (D) second instar expression from the *wg* enhancer trap is indicated below.Development of the *wg* protein (F, I and L) expression pattern (with respect to *ap*; E, H and K; and merged, G, J and M) is indicated for a middle second instar disc (E-G), an early third instar disc (H-J) and a mid-early third instar disc (K-M). The *wg* pattern begins in a few cells at the distal tip of the early disc (C, left) and remains largely restricted to the ventral region of the disc (D, F, I and L) in a pattern that is complementary to the domain of *ap* expression (compare E, H and K with G, J and M). The presumptive margin stripe doesn't emerge until the mid-early third instar (L) where it persists (A, B). In A and B, scale bar, 100 μm; in C and D, scale bar, 25 μm; in E, F and G, scale bar, 10 μm; in H, I and J, scale bar, 25 μm; in K, L and M, scale bar, 50 μm.

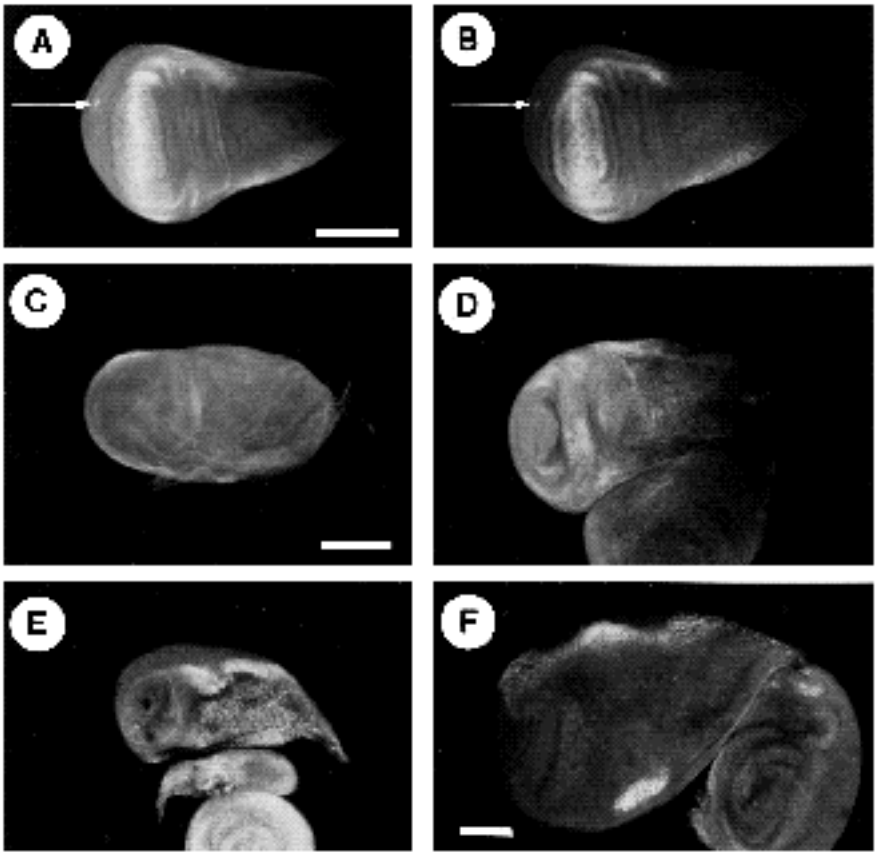

Fig. 5. Morphological alterations in late wing discs mutant for *ap*, *sd*, *vg* and *wg*. The identical expression of *vg* and *sd* is demonstrated by a disc double-labeled for *vg* (A) and *sd* (B). Note the identical expression of both *sd* and *vg* in a small cluster of cells in the pleura region of the disc (arrow). Wing discs from *vg* (C), *sd* (D), *ap* (E) and *wg* (F) mutants stained with *vg* are shown below. Localized *vg* expression (in the small lateral regions of probable notal origin; see arrow in Fig. 1A) is seen in *ap* and *wg* discs. No specific staining is observed in *vg* or *sd* discs (C and D; the fluorescence seen in these discs is background, detected at the high laser setting on the confocal microscope utilized in attempting to identify any specific staining in these discs) even though the flanking lateral notal regions are not removed in these mutants (J. A. W., unpublished results). Early *vg* expression (in the second instar, before removal of the wing region of the disc; see below) is also absent in the *vg* and *sd* null mutants (data not shown) indicating that no vg protein is produced in these mutants in either early or late discs. In the case of *vg*, both *vg* null alleles used in this study are protein nulls in embryos and imaginal discs (data not shown). In A and B, scale bar, 100 μm; in C, D and E, scale bar, 50 μm; in F, scale bar, 25 μm.

a jaggedness at the border of *ap* expression (the future wing margin) which is observed in late second instar discs and throughout the third instar (data not shown). In contrast, *wg* and *ap* discs are clearly smaller and morphologically abnormal in the late second instar, significantly earlier than *sd* or *vg* morphological defects become apparent (Fig. 6A-D). In addition, *ap* expression is abnormal in *ap* and *wg* wing discs as early as the middle second instar (Fig. 6C,D and see below). These observations indicate that *ap* and *wg* may be required earlier than *sd* and *vg*.

The timing of cell death in mutant discs also suggests an early requirement for *wg* and *ap*. Previous analyses of cell death in *vg*, *sd* and *wg* mutant wing discs has revealed the presence of cell death in the wing region of *vg* and *sd* discs throughout third instar development, but no cell death was detected in *wg* mutant discs (James and Bryant, 1981). In wild-type discs, *sd* mutants and *vg* mutants, we observed very little cell death in the wing disc during second instar development until a 'burst' of cell death occurs at the second to third instar molt. Thus, no significantly increased levels of cell death are detected in *vg* and *sd* discs, relative to wild type, until the third instar. In *wg* discs, no cell death is detected throughout the second instar; however, elevated cell death is detected at the edges of the presumptive wing region at the second to third instar molt (Fig. 6B acridine-stained cells; and Fig. 6D, blebbing cells above the focal plane of *ap* expression). Cell death is not increased in the central wing region of *wg* mutant discs throughout second or early third instar development (or later; James and Bryant, 1981). Thus, the abnormal morphology in newly hatched third instar *wg* discs cannot be due to cell death, and must be the result of some other patterning defect. In contrast, extensive cell death was observed throughout the second half of the second instar in *ap* mutant discs (Fig. 6A,C) and is clearly visible even in ventral cells that do not express *ap* (Fig. 6C, the blebbing cells outside the region stained with B-gal). In addition, the D/V border is jagged in late second instar *ap* mutant discs, perhaps the result of the earlier cell death that we have observed. Since *ap* discs undergo increased levels of cell death in the second instar and *ap* and *wg* discs are clearly morphologically abnormal, well before *vg* or *sd* mutant defects (or cell death) become apparent, it is likely that *wg* and *ap* are required and act before the *vg* and *sd* genes.

The genetic regulatory hierarchy guiding wing formation

wingless acts to regulate the boundary of *apterous* expression, the position of the D/V boundary, and *vestigial* expression

In order to determine whether the temporal and spatial relationships between regulatory gene expression and the morphological abnormalities in discs and adult wings reflected a temporal and spatial hierarchy of interactions between these genes, we examined the expression of each gene in mutant discs of each genotype. We showed earlier that the *wg* expression pattern in the second instar wing disc roughly opposes the *ap* pattern (Fig. 4) and thus may help to define the ventral region of the disc and/or to set the position of

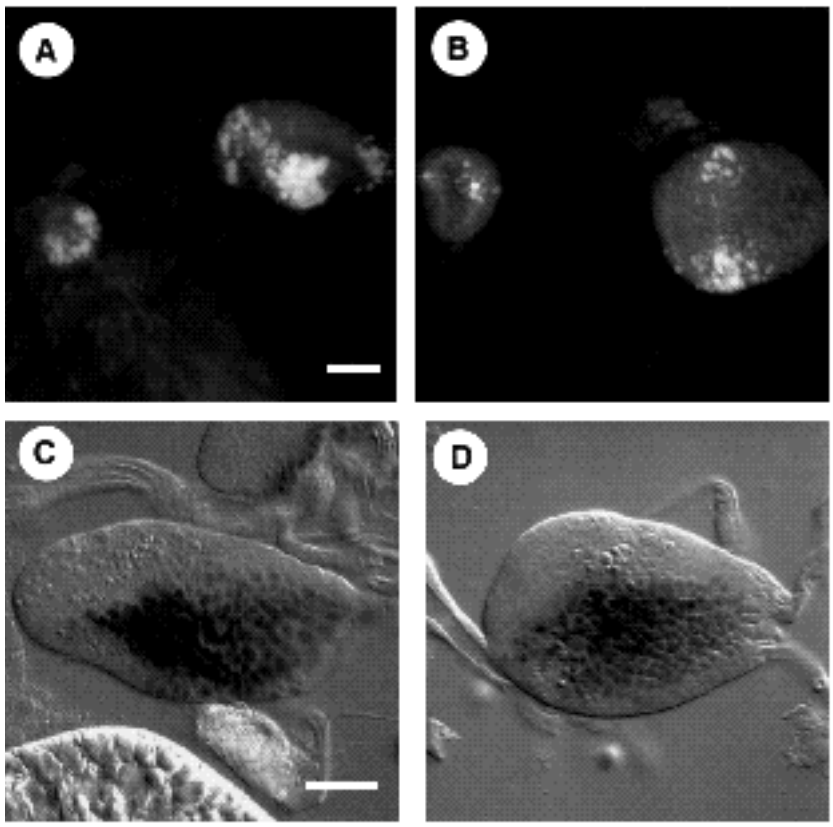

Fig. 6. Early *ap* and *wg* discs undergo cell death. Acridine orange visualized dying cells are shown in late second instar wing discs from *ap* (A) or *wg* (B) mutants. Nomarski optics of Xgal-stained late second instar wing discs from *ap* (C) or *wg* (D) mutants are shown below. In both cases, the *ap* expression pattern is marked with X-gal, as a homozygous enhancer trap in *ap* and as a linked heterozygous marker in *wg*. Dying cells are visualized as brightly staining nuclei in the acridine orange stains, or as 'blebbing' cells above or below the focal plane of X-gal staining with Nomarski optics. In A and B, scale bar, 10 μm; in C and D, scale bar, 25 μm.

ap expression and the D/V boundary. Indeed, we find that *ap* expression expands into the ventral region in *wg* mutant discs (Fig. 7B,D). Thus, *wg* appears to be required early to set the limit of *ap* expression and the location of the D/V boundary properly, which also expands ventrally in *wg* mutants. The variability of the observed expansion of *ap* expression is probably due to the fact that the *wg* alleles are hypomorphic; in fact only reduced levels of *wg* protein are detected in these *wg* mutant discs (data not shown) indicating that a complete removal of *wg* expression could result in more extreme defects.

Proper expression of *vg* protein requires both *wg* and *ap* function and the dynamics of *vg* expression are similar in early discs of both *wg* and *ap* mutants. In the second instar, the early elevated ubiquitous phase of *vg* expression is normal, and *vg* is initially restricted from the distal tip of the disc. However, rather than becoming restricted to a stripe defining the presumptive wing in the early third instar, *vg* expression is lost in both mutants (Fig. 7A,C). Thus, both *ap* and *wg* are required for retention of *vg* expression flanking the D/V boundary and for formation of the wing region.

It is important to stress that the effect of *wg* on *ap* or *vg* expression cannot be due to cell death. This is because, as indicated above, cell death is only detected in *wg* discs at the second to third instar molt and is limited to the lateral edges of the disc (Fig. 6B,D). In addition, since no cell death has been observed in third instar *wg* discs (data not shown and James and Bryant, 1981), the observed expansion of *apterous* expression in the central region of the disc (or loss of *vg* expression from this region) cannot be due to cell death. Since the notum is a dorsal derivative of the disc, the observed expansion of the dorsal region in *wg* mutant discs may account for the wing-to-notum transformations seen in *wg* adults.

The late-acting pro-wing genes *vg* and *sd* regulate each other

The *vg* and *sd* wing expression patterns are essentially identical during all of the stages of wing development that we examined. The possibility of cross-regulation between these genes was investigated by examination of *vg* and *sd* expression in *sd* and *vg* mutants, respectively. Although low level *vg* expression is retained, all elevated *vg* expression is eliminated in both early and late *sd* discs (Fig. 5D, and legend). Since embryonic *vg* expression is unaltered in this mutant, and in another strong early larval lethal *sd* allele (sd^{47m}; data not shown), it appears that the observed dependence of *vg* expression upon *sd* is a wing-specific requirement. The *vg* and *sd* embryonic expression patterns are quite different (see Campbell et al., 1992 and Williams et al., 1991), so *sd* appears to be a specific activator of *vg* expression only in the wing disc. Conversely, *sd* expression is not detected in second or early third instar discs from a *vg* null mutant. Since early *sd* expression is low (even in wild-type discs) and the *vg* mutant discs cannot be easily identified (double labeling with *vg* was not possible), it is difficult to determine if early *sd* expression is just reduced or totally absent. However, late *sd* wing disc expression is clearly eliminated in a *vg* null mutant (data not shown). These results argue that *sd* and *vg* may be spatially regu-

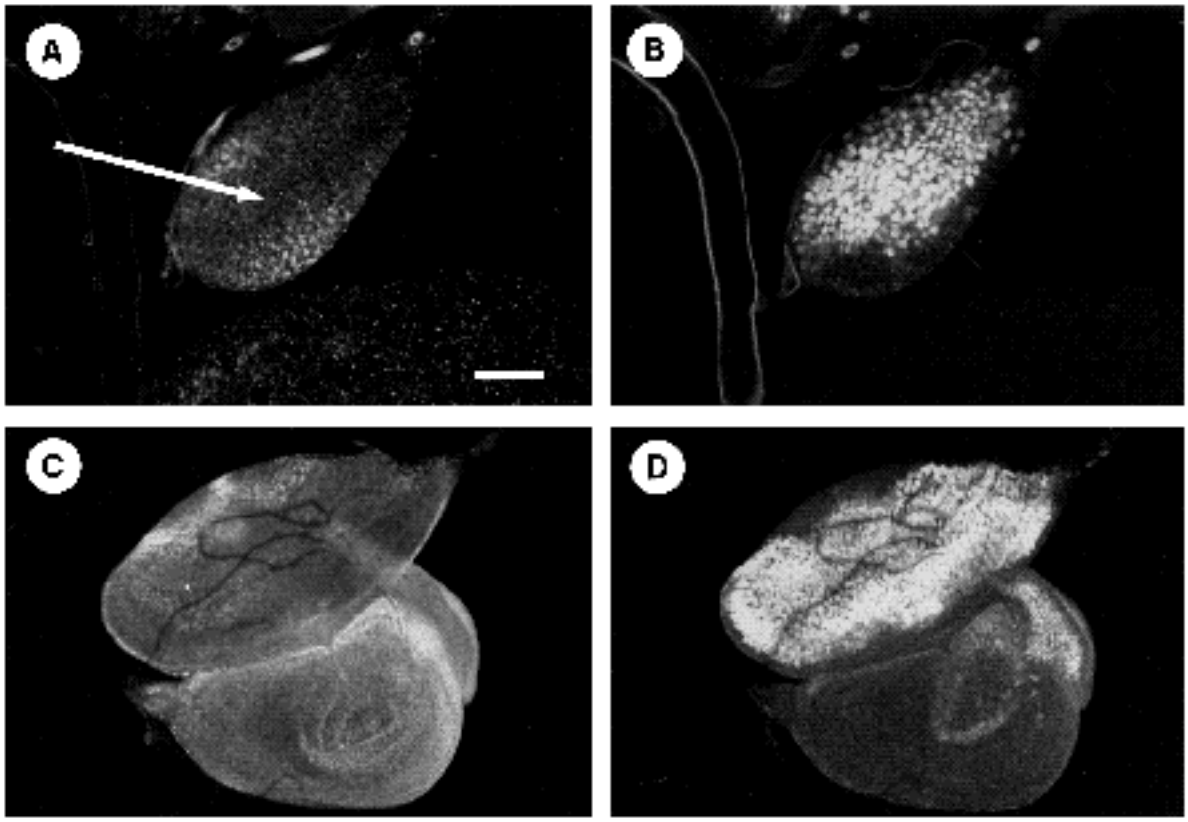

Fig. 7. *wg* regulates the ventral boundary of *ap* expression and is required for *vg* expression. Either *vg* (A and C) or *ap* (B and D) expression in double-labeled early (A and B) or late (C and D) third instar wing discs is shown. *vg* expression is variably retained in lateral regions of these discs (A and C); the variability is probably due to the hypomorphic nature of the *wg* mutants. In the early disc, *vg* expression is retained flanking the region where *vg* expression is normally maximal (see arrow in A; most early third *wingless* discs have undetectable levels of *vg* expression, the less extreme disc chosen was shown since it illustrates where the wing region would normally be present). *ap* expression is clearly expanded into the ventral region of the disc, and is most extremely expanded in the regions where *vg* expression is extinguished (compare A and B). The same relationship is observed in the late third instar disc; *ap* expression is expanded ventrally to the tip of the disc (C), but is not expanded laterally into the region where *vg* expression is retained (compare C and D). Again, the variable (and reciprocal) expansion of *ap* and loss of *vg* are likely to be due to the hypomorphic nature of the *wg* alleles. In A-D, scale bar, 25 μm.

lated in a similar manner and probably act to maintain the expression of each other in the developing wing disc.

DISCUSSION

We have examined the developmental roles of and interactions between four genes that are globally required for wing formation. By determining their temporal and spatial patterns of expression throughout the growth and development of the wing disc, correlating domains of gene expression with the wing fate map, identifying the stage when mutant discs first exhibit defects and demonstrating the dependence of some genes on the proper function of others, an initial picture of the genetic hierarchy guiding the subdivision of the wing disc has emerged. In addition, we have demonstrated two key and surprising functions of the *wg* protein in establishing dorsal/ventral polarity and distinguishing the presumptive wing from notum. By opposing *ap* expression, *wg* acts to distinguish the ventral from dorsal regions of the wing surface. And, as a positive regulator of *vg* and *sd* expression, *wg* promotes the development of the wing from the surrounding notum.

vestigial and *scalloped* are 'pro-wing' genes

Genetic analysis has identified only a few genes that are globally and specifically required for wing development (Lindsley and Zimm, 1992). It is likely that additional genes exist that affect the global development of the wing; however, the paucity of adult viable alleles of essential genes and the difficulty of performing systematic mitotic clonal analyses have thus far prevented their identification. Of the four genes that we have concentrated on, two (*vg* and *sd*) have striking expression patterns that demarcate the wing region of the mature wing disc, and each gene is required to maintain the expression of the other. Their expression in early third instar discs indicates that the wing region exists as a specific domain of *vg* and *sd* gene expression at this time and is subsequently lost completely in *vg* or *sd* mutants. These results indicate that these two genes are

'pro-wing' genes, required specifically to form the wing region of the disc. The identification of *sd* as a putative transcription factor (TEA domain protein; Campbell et al., 1992) and *vg* as a novel nuclear protein (Williams et al., 1991) is consistent with the idea that these genes are transcription control genes required for the differentiation and morphogenesis of the wing region of the wing disc in the third larval instar. Loss of either gene leads to extensive cell death and loss of the wing region of the disc during third instar development (James and Bryant, 1981), presumably due to an inability to pattern this region properly.

Inspection of the overlapping *vg* (or *sd*) and *ap* late third instar expression patterns clearly demonstrates that these two genes differentially mark the dorsal and ventral surfaces of the wing; *vg* expression specifically labels the ventral wing surface while *vg* and *ap* expression mark the dorsal wing blade (Fig. 2). This raises the intriguing possibility that these genes regulate dorsal/ventral identity in the wing by controlling the expression of structural genes in cells of each wing surface. Certain position-specific (PS) proteins are known that are expressed in either the dorsal (PS1) or ventral (PS2) wing surfaces (Brower et al., 1984). It is possible that genes such as those encoding PS1 or PS2 are differentially regulated by *vg* and *ap*.

An interesting question is 'does the wing exist as a domain of gene expression before the resolution of *vg* and *sd* expression? If a more primary tissue-specific pro-wing gene than *sd* or *vg* exists, mutational analysis has yet to identify it. Indeed, both *ap* and *wg* alter the disc earlier than *vg* or *sd*, but this appears to be the consequence of D/V polarity defects rather than reflecting earlier specification of the wing region. It seems likely that accumulation of *vg* protein accurately reflects the initial specification of the wing, and thus the wing is first identifiable as a domain of gene expression in the early third instar. The graded expression of *vg* protein (Figs 3, 4) may be required to specify coordinates within the wing, with the margin region expressing maximal *vg* protein, and the interior of the wing expressing lower levels. The *vg* expression patterns can be correlated with the fact that hypomorphic *vg* alleles progressively delete wing tissue from the margin, implying that higher levels of *vg* are required to make the wing margin than are required to make the wing blade (Williams and Bell, 1988). Analysis of the effects of ectopic expression of *vg* (and *sd*) in the wing blade should help to address this possibility.

The *ap* and *wg* genes are required for D/V compartmentalization in second instar discs

The D/V compartmental boundary, which is drawn along the wing margin, separates the dorsal and ventral wing surfaces (Brower, 1985). This boundary is first clonally detected during early larval life in first or second instar development (Garcia-Bellido et al., 1976). However, the presence of a zone of non-proliferating cells along the developing wing margin throughout the third larval instar has led to considerable debate regarding whether the detected clonal restriction is a compartment boundary or simply a region of reduced cell proliferation (Brower, 1985), and speculation that the actual in vivo timing of the formation of mitotic clones in the clonal analysis studies is considerably later than initially assumed. The visualization of this boundary in the early second instar via the *ap* expression pattern argues that it is a true compartmental restriction, since *ap* is compartmentally localized well before the zone of non-proliferation is detected (O'Brochta and Bryant, 1985). In addition, the timing of the onset of *ap* expression in the early-mid second instar disc correlates well with initial predictions from clonal analysis about the timing of the restriction, arguing that *ap* may mark the dorsal compartment. Although further analysis is required to confirm that the D/V boundary is a true clonal restriction, it is clear that the dorsal compartment exists as a domain of gene expression in mid-early second instar discs (before the disc size is 200 cells) and may be considered a compartment in this context (Brower, 1985). Analysis of *ap* and *wg* mutants indicates that proper compartmentalization is essential for subsequent disc patterning. Loss of either *ap* or *wg* is associated with D/V pattern abnormalities in late second instar discs; the D/V boundary is irregular in both *ap* and *wg* mutants, and is expanded ventrally in *wg* mutants. These results and the early *ap* and *wg* expression patterns indicate that *ap* and *wg* are primarily required for establishment of the D/V boundary; *wg* appears to help set the proper location of this boundary and may provide an important ventral region component, while *ap* presumably provides an essential dorsal-specific component. It is clear that *wg* cannot affect *ap* expression directly since *wg* is an extracellular component of a signalling pathway. Indeed, since wing-to-notum transformations have been observed in clones of cells mutant for other identified members of this signalling pathway (*armadillo, dishevelled* and *porcupine*; see Peifer and Bejsovec, 1992), it is likely that some downstream target of the *wg* signal regulates *ap* directly.

D/V compartmentalization is essential for wing formation

In the early third instar larva, *vg* and *sd* are expressed at discernably higher levels in stripes that are centered along the entire length of the D/V boundary (Fig. 3G). Earlier *vg* expression is ubiquitous (in the wing disc) and is progressively restricted to this stripe via repression of *vg* expression in the remainder of the disc (compare Fig. 3D,G). This is consistent with a model that the D/V restriction is a necessary prerequisite to wing formation, and that the wing is 'specified' from the D/V boundary. The loss of *vg* expression in *ap* and *wg* mutants which show D/V defects is also consistent with this model, and argues that wing formation is a sequential event, requiring the establishment of the D/V boundary (*ap* and *wg* functions) and subsequent wing specification (*vg* and *sd* functions). Indeed, a *vg* region control element (which is essential for normal wing patterning) has been identified that directs reporter gene expression specifically in a strip of cells directly spanning the D/V boundary in third instar wing and haltere discs, but not in the remainder of the wing region of these discs. This expression is independent of *vg* or *sd* function (J. A. W., unpublished results) and implies that this regulatory element interprets information along the D/V boundary of developing wing and haltere discs. These recent results may also explain why small regions of potentially notal identity

express *vg* (see arrow, Fig. 2A); since these cells flank the base of the D/V boundary, they presumably reflect the fact that *vg* is initially activated along the entire length of the D/V boundary, not all of which may form wing tissue. The mechanism by which *vg* and *sd* (or the *vg* control element) expression is retained in cells flanking the D/V boundary is unknown; however, the analysis of this process may yield valuable information about how pattern may be generated by local discontinuities between adjacent cell populations.

Compartments and secondary patterning fields

Following mechanical damage or genetically induced cell death, discs or disc fragments undergo epimorphic pattern regulation. This regulation may duplicate existing structures, or regenerate missing structures by intercalation, depending on the particular fragment that undergoes regeneration. Analysis of the regenerative capacity of a variety of wing disc fragments and analogous results with other systems has led to a number of loose rules of epimorphic regulation (French et al., 1976), and most models of secondary field patterning attempt to predict underlying pattern systems that could account for the observed regulative behavior of different disc fragments. Several such models predict multiple radial and angular positional determinants to exist within the field, e.g. the polar coordinate model (French et al., 1976, Bryant et al., 1981). However, these models require several rules to explain certain regulatory events, such as the full circle rule of distal regeneration. Moreover, candidate positional morphogens have not been clearly identified. In addition, the observation that distal regeneration in wing discs requires that the removed region spans the A/P boundary (Schubiger and Schubiger, 1978) is difficult to reconcile in terms of these models, which predict multiple angular determinants. In general, these models work well to explain the regulatory behavior of mature fields, but do not clearly predict the underlying mechanisms through which a mature pattern is set up.

Meinhart (1982) and Karlsson (1984) have proposed models that represent feasible molecular mechanisms by which pattern may be sequentially assigned to developing fields of cells. These models predict that cell determination boundaries (e.g. compartment boundaries) act as organizing regions for secondary fields. They propose that pattern is highest along compartment boundaries. Distal regeneration requires the presence of compartment boundaries in the healing tissue, while pattern duplications occur when the damaged tissue does not contain compartment boundaries. These models predict that normal proximal-distal patterning within the disc would also require these compartmental restrictions. The expression patterns of two wing disc patterning genes (*dpp*; Masucci et al., 1990 and *patched*; Phillips et al., 1990) define stripes, which are aligned with the A/P boundary; presumably the A/P restriction is required at some stage for establishment of these expression patterns. We have shown the presence of an early D/V boundary at least in terms of a domain of gene expression, which appears to set up the specific wing patterns of *sd* and *vg*. These results are consistent with the idea that pattern may be generated from compartment boundaries, and that both A/P and D/V oriented patterns are necessary for normal proximal-distal patterning. For example, the absence of either *dpp* (A/P oriented), *sd* or *vg* (D/V oriented) result in phenotypically similar losses of distal wing structures.

wg may function analogously in other imaginal discs

Larval *wg* function is not restricted to the wing disc and is required for normal patterning in most imaginal discs (Baker, 1988b). We did note in the course of these studies that the halteres, the other *Drosophila* flight appendages, appear to be patterned in the same way as the wing (Fig. 3G-I), which is not too surprising since mutations in all four genes also affect the halteres and this structure is a developmental homolog of the wing. We also note that the *wingless* transcript is localized to a small apically localized quadrant (in the anterior-ventral region; Baker, 1988b) of late third instar leg discs; although no relationship between this pattern and the late wing disc pattern is obvious, there is a striking similarity to the *wingless* expression pattern in the second instar wing disc. Thus, it is possible that the *wg* function in the leg disc is analogous to the early wing disc function, and may be required to define the ventral region of the leg discs. Indeed a D/V clonal restriction has been detected in the leg discs. The restriction is established in the second instar, and has been speculated to be analogous to the wing disc D/V restriction (Steiner, 1976). This restriction is only clearly identified in the large anterior compartment of the leg, and the resulting ventral region is the zone in which *wg* is maximally expressed. Loss of distal structures is observed in adult legs from some *wg* mutants (Baker, 1988b; J. A. W., unpublished observations); this is analogous to the loss of distal (wing) structures from the wing disc. Interestingly, loss of *wg* function in the leg results in removal of ventral parts of the pattern and their replacement with mirror-image duplications of dorsal elements (Baker, 1988b). This is consistent with the idea that the function of *wg* is to specify the ventral region of the leg disc, and that the dorsal domain of the leg may expand in a *wg* mutant. Thus, *wg* function in the leg and early wing may be analogous, and involved in D/V compartmentalization and ventral specification in both patterning fields. It is startling that *wg* (and other members of the *Wnt* gene family) are involved in patterning so many structures within both vertebrate (Nusse and Varmus, 1992) and invertebrate organisms. The observation that the *Wnt-3* and *Wnt-5A* genes are expressed in the developing mouse limb bud (Nusse and Varmus, 1992) may imply that the *Wnt* family is also required to pattern vertebrate limbs, perhaps in a manner analogous to its patterning function in the *Drosophila* wing and leg.

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