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SHAGGY and DISHEVELLED exert opposite effects on *wingless* and *decapentaplegic* expression and on positional identity in imaginal discs

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SUMMARY

The finding that Wingless (WG) and Decapentaplegic (DPP) suppress each others transcription provides a mechanism for creating developmental territories in fields of cells. Here, we address the mechanism of that antagonism. The *dishevelled* (*dsh*) and *shaggy* (*sgg*) genes encode intracellular proteins generally thought of as downstream of WG signaling. We have investigated the effects of changing either DSH or SGG activity on both cell fate and *wg* and *dpp* expression. At the level of cell fate in discs, DSH antagonizes SGG activity. At the level of gene expression, SGG positively regulates *dpp* expression and negatively regulates *wg* expression while DSH activity suppresses *dpp* expression and promotes *wg* expression. Sharp borders of gene expression correlating precisely with clone boundaries

suggest that the effects of DSH and SGG on transcription of *wg* and *dpp* are not mediated by secreted factors but rather act through intracellular effectors. The interactions described here suggest a model for the antagonism between WG and DPP that is mediated via SGG. The model incorporates autoactivation and lateral inhibition, which are properties required for the production of stable patterns. The regulatory interactions described exhibit extensive ability to organize new pattern in response to manipulation or injury.

Key words: *Drosophila* development, *shaggy* gene, *zeste white 3* gene, *dishevelled* gene, *armadillo* gene, *wingless* gene, *wingless* signaling, pattern formation

INTRODUCTION

Surgical grafting in both amphibians and insects shows that growth and patterning are stimulated by bringing cells from different positions in a morphogenetic field into contact (French et al., 1976). Tissues from different locations are said to possess positional information (Wolpert, 1969) which is operationally defined both by the differentiated structures which a cell produces and by the interaction of that cell with its neighbors. When cells are placed in contact with cells from other locations, they often stimulate growth and organize surrounding normal cells into ectopic structures such as supernumerary limbs. This transmission of positional information to surrounding cells could be mediated via contact-dependent interactions (French et al., 1976; Bryant et al., 1981) or by response to secreted morphogens (Wolpert, 1969; Meinhardt, 1983) or both. Recent studies find that antagonistic interactions between the Wingless (WG) and Decapentaplegic (DPP) signals of *Drosophila* establish distinct territories of gene expression in leg and eye-antennal discs (Maves and Schubiger, 1995; Theisen et al., 1996; Penton and Hoffmann, 1996; Jiang and Struhl, 1996; Brook and Cohen, 1996; Johnston and Schubiger, 1996; Morimura et al., 1996). In the ventral anterior region of the leg disc, WG signaling predominates while in the dorsal anterior region, DPP signaling predominates. WG and DPP also exert powerful effects on neighboring tissues when ectopically activated (Struhl and Basler,

1993; Diaz-Benjumea and Cohen, 1994; Campbell and Tomlinson, 1995; Maves and Schubiger, 1995; Wilder and Perrimon, 1995; Zecca et al., 1995; Lecuit et al., 1996; Nellen et al., 1996) and they could play a role in establishing positional identity. The *dishevelled* (*dsh*), *shaggy* (*sgg*, aka, *zeste-white 3*) and *armadillo* (*arm*) genes encode ubiquitous intracellular components of the WG signaling pathway (Riggelman et al., 1990; Peifer et al., 1991; Siegfried et al., 1992; Klingensmith et al., 1994; Theisen et al., 1994). SGG activity causes degradation of ARM protein (Peifer et al., 1994). WG signaling activates DSH, which inhibits SGG activity (Peifer et al., 1994) resulting in accumulation of ARM protein, which transduces a signal to the nucleus. Loss of *dsh* in clones in leg discs causes patterning responses that mimic the response caused by loss of *wg* function (Theisen et al., 1994; Klingensmith et al., 1994) while loss of *sgg* generates ectopic patterns similar to those caused by ectopic expression of *wg* (Diaz-Benjumea and Cohen, 1994).

The ability of *dsh* or *sgg* clones to organize normal cells into ectopic pattern elements could be mediated either by changes in transcription of *wg* or *dpp* or by changes in contact-mediated cell interactions that lead to intercalary regeneration. To distinguish these possibilities, we examined the effects of *dsh* and *sgg* clones in different discs with particular reference to potential activation of *wg* or *dpp* during the patterning response. We find that in leg and eye-antennal discs, SGG represses *wg* expression and promotes *dpp* expression, while

DSH represses *dpp* expression (Jiang and Struhl, 1996) and promotes *wg*. These interactions do not occur in wings. The ectopic activation and repression of *wg* and *dpp* in *dsh* and *sgg* clones suggests a model for the mutual repression of *wg* and *dpp* that centers on potentiating or inhibiting SGG activity.

MATERIALS AND METHODS

Drosophila melanogaster stocks and crosses

Flies were raised at 25°C. Genetic markers used are described in (Lindsley and Zimm, 1992). *sgg* clones detectable in adults were induced in $y\ sgg^{M1-1}\ w\ P[FRT101, w^+]/w\ P[FRT101, w^+]; P[hs-FLP38]$ larvae and *dsh* clones were induced in $y\ w^{v24}\ dsh^{75}\ P[FRT101, w^+]/w\ P[FRT101, w^+]; P[hs-FLP38]$ larvae (Chou and Perrimon, 1992). To examine imaginal discs stained for β -galactosidase activity, *sgg* clones were induced in $y\ sgg^{M1-1}\ w\ P[FRT101, w^+]/w\ P[FRT101, w^+]; CyO, wg-lacZ/P[hs-FLP38]$ larvae and *dsh* clones in $y\ w\ dsh^{VA153}\ P[FRT, ry^+]/18A\ w\ P[hs-myc, w^+]/10D\ P[FRT, ry^+]/18A; cn\ [dpp-lacZ]; MKRS\ P[hs-FLP^{99}, ry^+]$ larvae (Kassis et al., 1992; Xu and Rubin, 1993). For *hs-myc* marked *sgg* clones, female larvae of the following genotype were made: $w\ P[hs-myc, w^+]/5A\ P[hs-myc, w^+]/10D\ P[FRT, ry^+]/18A\ w\ sgg^{M1-1}\ w\ P[FRT, ry^+]/18A; cn\ [dpp-lacZ]/CyO, wg-lacZ; MKRS\ P[hs-FLP^{99}, ry^+]$. For *hs-myc* marked *dsh* clones, female larvae of the following genotype were made: $w\ P[hs-myc, w^+]/5A\ P[hs-myc, w^+]/10D\ P[FRT, ry^+]/18A\ w\ dsh^{VA153}\ P[FRT, ry^+]/18A; cn\ [dpp-lacZ]/CyO, wg-lacZ; MKRS\ P[hs-FLP^{99}, ry^+]$. Clones were generated by heat shocking appropriately staged larvae at 37°C for 30–60 minutes.

β -galactosidase staining of imaginal discs

To examine putative ectopic activation of gene expression as a result of clone induction, we induced clones in first instar larvae, allowed some larvae to grow to adulthood to determine clone frequency and examined others at third instar for ectopic pattern regulation and for ectopic gene expression (either *dpp* or *wg*). Discs were dissected in cold PBS, fixed in 4% formaldehyde PBS plus 0.1% Triton X-100 (PBT) for 10 minutes then washed three times in PBT for 5 minutes each. Discs were incubated in staining solution without X-gal for 5 minutes and then incubated at 37°C in prewarmed staining solution plus 0.2% X-gal. After appropriate color development, the staining solution was removed, discs were rinsed once in 70% EtOH and once in 100% EtOH and mounted in 80% glycerol/PBS. Staining solution: Fe/NaP (pH 7.2), 1.8 ml of 0.2 M Na₂HPO₄, 0.7 ml of 0.2 M NaH₂PO₄, 1.5 ml of 5 M NaCl, 50 ml of 1 M MgCl₂, 3.05 ml of 50 mM K₃(Fe(CN)₆), 3.05 ml of 50 mM K₄(Fe(CN)₆), H₂O to 50 ml, stored in dark at 4°C.

Antibody staining of marked clones

Wandering third instar larvae in which *dsh* or *sgg* clones were induced were heat shocked at 37°C for 1 hour and allowed to recover for 1 hour. Female larvae were then picked and discs were dissected in cold PBS, fixed in 4% formaldehyde PBS plus 0.1% Triton X-100 (PBT) for 30 minutes then washed 5 times in PBT for 5 minutes each. The discs were incubated in myc cell line supernatant diluted 1:1 and rabbit anti- β -galactosidase diluted 1:10,000 in PBT overnight at 4°C. The discs were washed for 2 hours in PBT and then incubated in fluorescently labeled secondary antibodies: Cy3-conjugated anti-mouse and FITC-conjugated anti-rabbit from Jackson laboratories, diluted 1:200 in PBT for 1.5 hours at room temperature. In some instances, a DNA dye, DAPI or TO-PRO, was included in one of the post-absorption washes to label all cells. The discs were washed again for 2 hours at room temperature and then mounted in 75% glycerol, 50 mg/ml n-propyl Gallate and observed with a Bio-Rad MRC 1024 scanning confocal microscope.

Mounting adult structures

Adult structures were dissected in 70% EtOH, dehydrated briefly in isopropanol, placed in Gary's magic mountant (Struhl and Basler, 1993) and photographed using a Nikon Optiphot.

RESULTS

The spatial requirements and organizing potential of *dsh* and *sgg* are complementary

The regions of leg discs in which *dsh* and *sgg* clones organize neighboring normal cells into ectopic pattern elements are distinct and complementary (Fig. 1). Clones of *dsh* in the anterior/ventral third of the disc exert a strong dorsalizing influence in that they organize a new dorsoventral axis and, while the mutant cells produce only dorsal structures, they cause surrounding normal cells to adopt new fates intermediate between ventral and dorsal (Klingensmith et al., 1994; Theisen et al., 1994; Jiang and Struhl, 1996). This frequently results in bifurcated or triplicated legs (Fig. 2A,B). Clones that occur close to the proximal edge of the leg disc, in the region that gives rise to the coxa, can produce complete secondary legs including a distal tip as indicated by the presence of claws (Fig. 2B)(Jiang and Struhl, 1996). Clones located more distally can lead to converging pattern triplications (Fig. 2A). Clones of *dsh* that occur in the posterior compartment induce the formation of extra rows of mutant bristles but do not organize surrounding normal cells into ectopic pattern elements (Fig. 1). Clones of *dsh* in the dorsal third of the leg have no effect on pattern (Fig. 1). In all clones, the *dsh* mutant cells never give rise to ventral pattern elements regardless of clone location and, when located anteroventrally, they behave as dorsal organizers by recruiting neighboring normal cells into new patterns in which *dsh* cells occupy dorsal positions and normal cells are recruited for positions intermediate between ventral and dorsal.

Like *dsh* clones, *sgg* clones produce different regulatory responses depending upon the location of the clone (Figs 1, 2C,D). Clones of *sgg* in any location produce the characteristic ventral-type bristles (Fig. 2C). Dorsal *sgg* clones produce ventral-type bristles and they exert a strong ventralizing influence and cause bifurcations and triplications (Fig. 2D). Unlike *dsh*, proximal clones of *sgg* (i.e. coxa, trochanter and femur) do not organize complete ectopic appendages but frequently produce small outgrowths, which rarely distalize past the next more distal leg segment boundary (data not shown) (Diaz-Benjumea and Cohen, 1994). However, *sgg* clones in both the tibia and tarsi, can form complete secondary axes as well as triplications (Fig. 2D). The dorsal/ventral polarity of the ectopic outgrowths is opposite for the two genes (compare Fig. 2A,B to 2D). Lateral *sgg* clones in either the anterior or posterior compartments (Fig. 1) produce extra rows of bristles and sometimes induce the formation of extra rows of non-mutant bristles (Fig. 2C). In contrast to *dsh* mutant cells, which never form ventral structures, cells that lose *sgg* are transformed to the extreme ventral-most fate normally exhibited by the *wg*-expressing cells (Fig. 2C). These cells never adopt the intermediate fates (over 1300 bristles scored in 106 clones), which are proposed to be dependent on paracrine *wg* signaling (Struhl and Basler, 1993). Ventral *sgg* clones that fall within the *wg* expression domain have no effect on patterning (Fig. 1). Thus, *dsh* and *sgg* clones can organize surrounding cells into ectopic patterns only when they occur in certain regions of the discs and the organizing ability does not require proximity to the anterior/posterior (A/P) boundary. In addition, both the polarity of ectopic pattern caused by *dsh* and *sgg* clones and the regions in which clones stimulate ectopic patterning are complementary.

***dsh* and *sgg* are also required in complementary domains in the eye-antennal disc**

We examined the effect of *dsh* and *sgg* clones in the eye-antennal disc with particular reference to the domains of *wg* expression. To relate the topology of *wg* expression in the eye-antennal disc to the corresponding adult structures, the expression of *wg* in the mature disc (Fig. 2F) is compared with *wg* expression in the mature head (Fig. 2I). In the eye disc, *wg* is expressed in a reversed 'C' pattern bordering the anlage of the eye proper and two additional domains in the dorsal head anlage (Fig. 2F). In the adult, these regions correspond to the head cuticle adjacent to the eye, half moons medial to the ocelli (Fig. 2I) and the region of the ptilinum (Royet and Finkelstein, 1996).

The opposing effects of *dsh* and *sgg* are evident in both the eye and antennal regions of the eye-antennal disc. Clones of *dsh* that occur in the region that normally produces head cuticle can cause ectopic ommatidia, ocelli or sensilla to develop (e.g. Fig. 2J). Loss of *dsh* in the eye field affects polarity but not cell fate (Theisen et al., 1994). Conversely, *sgg* clones in the eye field produce cuticle and large sensory bristles characteristic of cells at the dorsal margin of the eye (Fig. 2G,H). *sgg* clones elsewhere in the head can cause loss of ocelli or formation of extra macrochaetes (data not shown). As in legs, both *dsh* and *sgg* clones in the eye disc can cause wild-type tissue to be recruited into the ectopic pattern suggesting that the interaction of the clones with neighboring tissue can exert an organizing influence on adjacent non-mutant cells.

The antenna is analogous to a leg disc that has been rotated by 180° (Struhl, 1981), so that *wg* marks dorsal and *dpp* marks ventral, which is reversed compared to the leg (Baker, 1988; Masucci et al., 1990). Clones of *dsh* that occur in *wg*-expressing regions of the first antennal segment frequently cause duplications of all three antennal segments including the arista (not shown). Clones of *sgg* that occur below the arista, in a region where *wg* is not expressed, produce duplications of structures characteristic of the *wg*-expressing cells that lie above the arista such as ectopic protruding bulbs covered with trichomes (not shown). Thus, in antennae, *dsh* is a ventral antennal organizer and *sgg* a dorsal antennal organizer consistent with the territories of *dpp* and *wg* expression. In the eye-antennal disc, as in the leg discs, *dsh* and *sgg* exhibit complementary effects on cell fate and positional identity.

***dsh* acts upstream of *sgg* in discs**

There is a dichotomous relationship between *sgg* and *dsh*, with *dsh*⁺ driving cells toward a ventral positional identity and *sgg*⁺ driving cells toward a dorsal positional identity in the leg. To determine the nature of the interaction between *dsh* and *sgg*, we made mitotic clones that are mutant for both genes. Double mutant clones produce cuticle structures characteristic of *wg*-expressing cells and they cause pattern reorganization similar to that produced by cells mutant for *sgg* alone. For example, double mutant clones in the leg adopt ventral fates, and when they occur dorsally they lead to pattern regulation (Fig. 2E). Double mutant

clones in the eye cause production of head cuticle within the eye (compare Fig. 2G to 2K). Both in terms of the effect on the differentiation of the cells and the location of the clone required to initiate patterning, loss of *sgg* is epistatic to loss of *dsh* in double mutant clones. Similarly, embryos derived from *sgg,dsh* double mutant germ-line clones or *sgg* single mutant germ-line clones exhibit the same loss of denticle phenotype showing that *sgg* is epistatic to *dsh* in embryos (Siegfried et al., 1994).

Loss of *sgg* stimulates *wg* and represses *dpp* expression

To test whether changes in SGG activity affect cytokine expression, we induced *sgg* clones in first instar larvae and examined the imaginal discs in late third instar larvae for *wg* expression and for morphological evidence of pattern regulation. Discs with extensive ectopic pattern and growth were observed and they exhibited a range of ectopic *wg* expression from undetectable to easily detectable levels (Fig. 3A-C). To determine the relationship between *wg* expression and the clone boundary, we used double fluorescent labeling and confocal microscopy to mark clones and detect *wg* expression. The extent of a *sgg* clone is revealed by lack of the *myc* epitope while ectopic *wg* expression is noted by *lacZ* staining. All *sgg* clones in dorsal positions cause ectopic growths in which the mutant tissue rounds up and appears to minimize contact with non-mutant cells (Fig. 4A-D,F). This correlates with the adult phenotypes of dorsal *sgg* clones where the mutant cells are often displaced

Fig. 1. The positional requirements and organizing potential of *dsh* and *sgg* are complementary. Clones of *sgg* and *dsh* are plotted on the fate map of the leg disc according to their location on the leg and whether they cause supernumerary legs, extra row(s) of bristles around the circumference, or have no effect on patterning or fate (for *sgg* and *dsh* $n=58$). Positions around the leg are determined with reference to the 8 distinct bristle rows around the circumference of the tarsus and other characteristic structures (Hannah-Alava, 1958). *sgg* and *dsh* exert their maximal effect when they occur in opposite regions of the leg disc. *sgg* clones that occur in the dorsal region lead to ectopic legs while *dsh* clones must occur in the anterior ventral region to cause ectopic legs. Similarly, *dsh* clones in the dorsal region have no effect while *sgg* clones in the ventral region (bristle row 1) have no effect. Clones in other locations, cause extra row(s) of bristles. Wherever they occur, *sgg* cells all adopt the phenotype the ventral-most bristles. Clones of *dsh* adopt a dorsal phenotype. Since the dorsal-most bristle rows exhibit a less distinctive phenotype than the ventral rows, we cannot determine the fate of *dsh* cells as precisely as *sgg* cells. ●, supernumerary appendage; ○, no effect on fate or rows; ⊕, extra rows, altered fate. Note: we use the original numbering system (Hannah-Alava, 1958) for bristle rows on the legs.

Fig. 2. *dsh* and *sgg* clones have complementary effects on positional identity and patterning. (A) A *dsh* clone located in the anterior ventral region of the second leg disc leads to a converging triplication. The induction of a clone with dorsal positional identity part way along the ventral P/D axis leads to ventral cells confronting dorsal cells on both sides of the clone and consequent symmetrical duplication of pattern elements (note two apical bristles specified by normal cells on either side of the clone). Symmetrical ectopic patterns such as these produce converging triplications that truncate as they extend distally (Girton, 1981). Note how the *dsh* clone narrows and disappears in the more distal parts of the ectopic pair of legs. Arrowheads indicate the proximal and distal extent of the clone (yellow bristles) and a dashed white line marks the dorsal border of the clone along the tarsus. This clone occurs in a location approximately opposite to that of the *sgg* clone shown in D with respect to the circumference of the leg. (B) A *dsh* clone located in the proximal anterior ventral region of the coxa causes a complete ectopic leg to form (yellow bristles; arrowheads). The cells of the *dsh* clone only participate in making dorsal structures; yet they organize a distally complete ectopic leg. (C) A *sgg* clone induced during the second instar on the tarsus of a second leg in the posterior compartment. The yellow (lighter colored) bristles (arrowheads) arise from cells homozygous for the *sgg* mutation. Note that the morphology of the yellow bristles is similar to the stout peg-like bristles produced by the ventral-most *wg*-expressing cells (e.g. arrows). (D) A dorsal *sgg* clone induced during first instar in the tarsus of a third leg disc leads to extensive pattern regulation and supernumerary appendages. The *sgg* mutant cells (marked by yellow, arrowheads) only participate in forming ventral pattern elements; yet, they cause surrounding normal tissue to replicate two extra legs with dorsal elements (e.g. the claws) and intermediate lateral elements. (E) A *sgg dsh* double mutant clone in dorsal region behaves like *sgg* clones alone. Note the ventral-like bristles on the outgrowth (arrowheads). (F) Expression of *wingless* in the eye-antennal disc revealed by in situ hybridization. Note that *wg* is expressed in a patch in the region of the future dorsal head connected by a long thin stripe along the posterior margin of the presumptive eye to a second patch in the ventral cuticle below the eye. (G,H) *sgg* clones in the eye lead to cuticle deposition and the elaboration of large bristles (arrowhead) similar to those found in the dorsal *wg*-expressing region of the head (arrow in I). The pattern of ommatidia is disrupted in the region of the eye anterior to the clone in G or 'downstream' of the morphogenetic furrow. (I) The topological relationship between *wg* expression in the disc and structures in the adult head is revealed by staining a *wg-lacZ* enhancer trap line (Kassis et al., 1992). Note the high level of expression in the dorsal region of the head adjacent to the eye (arrow). This region produces very characteristic bristles, the verticals. (J) A *dsh* clone in the dorsal head leads to ommatidia developing in regions normally giving rise to cuticle (arrowhead). Since the *dsh*^{v26} chromosome is marked with yellow and white, the ectopic ommatidia are white. Note that loss of *dsh* and *sgg* have the opposite effects on positional identity in both leg and eye discs (Compare G and H to J). (K) A *sgg dsh* double mutant clone in the eye (arrowhead) looks similar to clones of *sgg* alone (compare G to K).

distally in ectopic outgrowths. With respect to *wg* expression, two classes of dorsal *sgg* clones were observed. Dorsal clones near the A/P compartment boundary lead to ectopic *wg* expression that always fills the entire clone and is never observed

in surrounding wild-type cells (Fig. 4B,D). In a single collection of staged larvae that were heat shocked together, 22 clones were produced of which four of four that were located near the A/P compartment boundary ectopically expressed *wg* while none of

Fig. 3. *sgg* and *dsh* clones in imaginal discs stained for *wg* and *dpp* expression, respectively. All discs are dorsal up and anterior to left. (A) A *sgg* clone in the dorsal region of a leg imaginal disc has generated ectopic growth and pattern regulation (arrow) but has activated little if any detectable ectopic *wg* expression. (B) A *sgg* clone in the dorsal part of the leg disc has led to extensive growth and pattern regulation (arrow) and ectopic activation of *wg* is just detectable. (C) A *sgg* clone in the extreme dorsal region of the leg disc results in a large ectopic growth and exhibits extensive ectopic expression of *wg* (arrow). A clone such as this might lead to ectopic structures similar to those in Fig. 2D. (D) A *dsh* clone at the anterior ventral edge of the leg disc stained for *dpp* expression. Note the ectopic activation of *dpp* expression associated with the ectopic pattern (arrow).

the others expressed *wg*. It is unclear why *wg* activation was not observed in *sgg* clones induced in a *Minute* background (Diaz-Benjumea and Cohen, 1994). We clearly see activation of *wg* in *sgg* clones near the dorsal A/P border. Dorsal *sgg* clones that are distant from the A/P boundary do not ectopically activate *wg* (Fig. 4B-D), indicating that proximity to the compartment boundary influences *wg* activation. Similarly, *sgg* clones located either in the posterior compartment or anterior ventral positions exhibit no detectable *wg* expression (Fig. 4A,D).

In eye-antennal discs, *sgg* clones can also ectopically activate *wg* expression in some locations (Fig. 5A,B). In the antenna, *wg* is ectopically activated only in *sgg* clones located ventrally near the A/P boundary (Fig. 5B). Clones of *sgg* that occur in the eye field, as opposed to the head anlage of the eye disc, also ectopically activate *wg* and they stimulate ectopic growth and patterning (Fig. 5A). In both the eye and the antenna, as in the leg, ectopic *wg* is expressed in all clonal cells and is not expressed in wild-type cells adjacent to the clone when marked clones are examined by confocal microscopy (not shown). This implicates an autonomous link between loss of *sgg* and ectopic activation of *wg* expression.

We next asked whether *dpp* expression was repressed in *sgg* clones. When *sgg* clones occur in regions where *dpp* is normally

expressed in leg or eye-antennal discs, they interrupt *dpp* expression autonomously. For example, a *sgg* clone that occurs in the dorsal region of *dpp* expression in the leg completely eliminates *dpp* expression in the entire clone (Fig. 4F). Similarly, *sgg* clones in eye discs completely eliminate *dpp* expression in the morphogenetic furrow (Fig. 5C). Notably, *dpp* expression in neighboring normal cells is unaffected by the presence of the clone (e.g. see the clone border in Fig. 5C). Thus, the ectopic WG produced by clones is not sufficient to repress *dpp* expression in neighboring cells despite the antagonism between WG and DPP in normal patterning (Theisen et al., 1996). Since *dpp* is required for the formation of eye tissue (Spencer et al., 1982; Heberlein et al., 1993; Ma et al., 1993), loss of *dpp* expression in *sgg* clones in the eye is consistent with *sgg* clones producing large growths of cuticle in the adult eye (Fig. 2G,H). Interestingly, *sgg* clones have no effect on *dpp* expression in the wing disc. For example, a *sgg* clone that lies in the band of *dpp* expression that runs along the A/P boundary of the wing (Raftery et al., 1991) has no effect on *dpp* expression (Fig. 4 G-I). Thus, *sgg* activity is required for *dpp* expression in the leg and eye-antennal discs but not in wing discs.

Loss of *dsh* function has opposite effects on *dpp* and *wg* expression

To determine if ectopic activation of *dpp* is involved in the response to *dsh* clones, we examined discs with *dsh* clones for activation of *dpp* and for morphological evidence of ectopic growth and pattern regulation. Discs in which ectopic growth and pattern are morphologically evident exhibit ectopic *dpp* expression (Fig. 3D). To determine the relationship of *dpp* expression to the clone boundaries, we used double fluorescent labeling and confocal microscopy to mark *dsh* clones and examine *dpp* expression. Clones in the anterior ventral third of the leg disc, which is sensitive to *dsh* activity (Fig. 1), always exhibit extensive ectopic expression of *dpp-lacZ* (Fig. 6A,C,D). In clones that occur close to the A/P boundary, ectopic *dpp* expression fills the clone indicating an autonomous effect on activation within the clone (Fig. 6) (Jiang and Struhl, 1996). However, in clones that lie away from the boundary, ectopic *dpp* expression is seen in some but not all *dsh* mutant cells (Fig. 6C,D) and ectopic *dpp* is induced non-autonomously in neighboring normal cells (Fig. 6D). These clones are also always associated with clear ectopic growth. These observations correlate well with the adult clonal analysis where anterior/lateral and ventral clones induce large regulatory responses (Figs 1, 2A,B). Clones of *dsh* in posterior or dorsal locations do not exhibit detectable ectopic expression of *dpp* (Fig. 6A,B). For example, in nineteen clones, four of four ventral/anterior clones located near the A/P boundary were filled with ectopic *dpp* while none of fifteen clones in the dorsal or posterior regions expressed *dpp*.

Clones of *dsh* in the eye-antennal discs were also examined. Taking the dorsal/ventral reversal of the antenna into account, clones in the antenna give comparable results to those observed in leg imaginal discs (data not shown) with dorsal *dsh* clones leading to ectopic activation of *dpp* expression while ventral clones do not. In the eye field, *dsh* clones have no effect on *dpp* expression in the morphogenetic furrow (data not shown), which is consistent with the observation that *dsh* clones in eyes only cause cell polarity defects (Theisen et al., 1994; Theisen et al., unpublished data). In contrast to clones in the eye field, *dsh* clones in the *wg*-expressing region of the eye disc that gives

rise to dorsal head cuticle interrupt normal *wg* expression. For example, the clone shown in Fig. 5D splits one of the domains of *wg* expression into two separate domains indicating that *dsh* mutant cells do not express *wg*. Similar interruption of *wg* expression is observed in *dsh* clones in the antenna (not shown).

DISCUSSION

sgg and *dsh* affect both *wg* and *dpp* transcription

Recent studies indicate that WG and DPP negatively regulate each other's expression (Maves and Schubiger, 1995; Theisen et al., 1996; Penton and Hoffmann, 1996; Jiang and Struhl, 1996; Brook and Cohen, 1996; Johnston and Schubiger, 1996; Held and Heup, 1996; Morimura et al., 1996). We and others

(Jiang and Struhl, 1996) find that SGG and DSH activities also affect transcription of these genes. Our results show that SGG activity positively regulates *dpp* expression (Figs 4F, 5C) and negatively regulates *wg* expression (Figs 3B,C, 4B,D, 5A,B) in a cell autonomous manner (Figs 4B,D, 5A). Conversely, DSH activity autonomously represses *dpp* expression (Figs 3D, 6A; Jiang and Struhl, 1996) and promotes *wg* expression (Fig. 5D). Changes in *wg* and *dpp* expression could be mediated by autocrine mechanisms where, for example, loss of *sgg* activates *wg* expression, which in turn suppresses *dpp* or loss of *dsh* activates *dpp* whose signaling in turn suppresses *wg*. Alternatively, both DSH and SGG could affect expression of *wg* and *dpp* by directly affecting the intracellular transcriptional machinery for these genes without the need for an intermedi-

Fig. 4. *sgg* clones in leg discs (A-D) and a wing disc (G-I). (A-F) In the leg discs, anterior is to the left and dorsal is up. All *sgg* clones are indicated by absence of myc staining (red). A DNA dye (blue) is also included in A-C to indicate the presence of tissue. (A,B) Two different focal planes of the same leg disc. One clone, located in the posterior compartment (asterisk), can be seen in both views and while it causes obvious morphological changes does not show expression of *wg* (green). (B) Two more clones can be seen in this focal plane lying immediately adjacent to one another. The clone closest to the A/P boundary (arrow) contains ectopic *wg* expression throughout the clone while a more anterior clone (arrowhead) contains no *wg* expression. Examination throughout the depth of this disc confirms that there are two clones located next to each other. (C) An extreme anterior *sgg* clone (asterisk) also shows no ectopic *wg* expression. (D) This disc contains 3 clones. A dorsal *sgg* clone (arrowhead 1) is filled with ectopic *wg* expression. The line drawn below this clone permits comparison of the clone location relative to the location of the A/P border shown in E. This first clone lies very near the A/P boundary in this proximal leg segment. A more anterior clone in the next segment, (arrow 2), does not express *wg* nor does a posterior clone, (arrow 3). (E) A double antibody stain for *dpp* expression (green) and *engrailed* expression (red) to show the path of the A/P compartment boundary between *dpp* and *engrailed*. The line marks the fold between segments where the A/P boundary shifts to the right. This panel shows the pattern of *dpp* expression (green) relative to the A/P boundary and the morphology of the discs for reference with the other discs shown. (F) A *sgg* clone (asterisk), which falls in the line of *dpp* expression (green), interrupts *dpp* expression autonomously. (G-I) A close up view of a wing pouch containing a *sgg* clone (arrow) in the middle of the stripe of *dpp* expression (green). Anterior is up and dorsal is to the right in these panels. (G) A double stain showing myc expression (red) and *dpp* expression (green) and a *sgg* clone (arrow). (H) The location of the *sgg* clone (arrow) is revealed by absence of staining when only the myc channel is shown. (I) This panel presents only the *dpp* staining showing that a *sgg* clone in the wing continues to express *dpp*.

Fig. 5. Loss of *sgg* or *dsh* affects *wg* and *dpp* expression in the eye-antennal disc. Anterior is to the left and dorsal is up in all panels. (A,B) *wg-lacZ* expression is monitored by β -galactosidase activity. (A) A *sgg* clone in the developing eye field (arrow) shows ectopic patterning and a low level of ectopic *wg* expression. (B) A *sgg* clone in the ventral antenna also exhibits ectopic growth and *wg* expression (arrowhead). (C) A *sgg* clone in the eye field (asterisk) is indicated by the absence of myc staining (red). Expression of *dpp* is indicated by green. The *sgg* clone sharply interrupts *dpp* expression in the morphogenetic furrow. (D) A *dsh* clone (asterisk), marked by absence of myc staining (red), is located in the region of the eye disc that normally makes the ptinulum. In normal discs, two discrete domains of *wg* expression occur, one in the region that will make the ptinulum and one in the area that will make dorsal head cuticle (see arrow 1 and arrow 2 in B). In this disc, the more anterior domain of *wg* expression (green) is split into two regions indicated with arrows 1a and 1b. The more posterior region of *wg* expression is indicated with arrow 2. Note the sharp boundaries between the edge of the mutant clone and the reporter expression in both C and D.

ate secreted signal. We believe the data argue against a strictly paracrine/autocrine mechanism, but rather, for an intracellular (autonomous) mechanism in a number of important ways.

Changes in gene expression at the clone boundaries and the behavior of clones as a function of location, provide important clues to distinguish between autocrine/paracrine mechanisms and cell autonomous, cytokine-independent mechanisms of regulating gene expression. The abrupt transition from expression to non-expression of both *wg* and *dpp* at the boundary of *sgg* clones (Figs 4B,D,F, 5C) shows that the changes in expression are mediated by cytokine-independent effects of SGG and not by paracrine signaling. If activation of *wg* in *sgg* clones were due to removal of *dpp* transcription, endogenous DPP nearby should still repress *wg* transcription at the clone edge; it doesn't. The fact that all cells in *sgg* clones express *wg* and that activation of *wg* coincides precisely with the clone boundary indicates that SGG activity affects some intracellular effector of *wg* expression. The fact that only those clones near the A/P border express *wg* indicates HH is also required to overcome the general repression of *wg* expression in the anterior compartment (Phillips et al., 1990; Basler and Struhl, 1994; Capdevila et al., 1994; Jiang and Struhl, 1995;

Fig. 6. *dsh* clones in leg imaginal discs. In all panels, anterior is to the left and dorsal is up, red indicates myc expression, absence of red indicates a clone, and green indicates *dpp* expression. A DNA dye (blue) to indicate the presence of tissue is included in B. (A) A ventral *dsh* clone near the A/P border (arrow) shows some ectopic growth and is filled with ectopic *dpp* expression. Autonomous activation of *dpp* in *dsh* clones near the A/P border is also described by (Jiang and Struhl, 1996). (B) A ventral posterior *dsh* clone (asterisk) shows no change in morphology and no expression of *dpp*. (C,D) Two focal planes of the same leg disc containing a very proximal *dsh* clone, (asterisk), that causes ectopic growth and extensive ectopic *dpp* expression. (C) This focal plane shows that some mutant cells (lack of red) do not express *dpp* indicating that expression of *dpp* in anterior clones distant from the A/P border is not autonomous. (D) In this focal plane, some neighboring normal cells (red) also express ectopic *dpp* which is indicated by yellow (arrow) indicating a paracrine effect of the *dpp*-producing clone on neighboring cells in this clone with extensive growth and patterning.

Johnson et al., 1995; Lepage et al., 1995; Li et al., 1995; Dominguez et al., 1996).

Loss of *dpp* expression in *sgg* clones also appears to be cytokine independent (Figs 4F, 5C). It is unlikely that repression of *dpp* is due to antagonism by ectopic *wg* both because the repression of *dpp* is abrupt at the clone border (Figs 4F, 5C) and because WG is not capable of fully repressing *dpp* expression in discs (Theisen et al., 1996; Jiang and Struhl, 1996; Brook and Cohen, 1996). Thus, the data suggest a cell autonomous requirement for SGG activity to stimulate *dpp* expression.

The repression of *dpp* and activation of *wg* provide a mechanism to explain why *sgg* mutant cells produce structures characteristic of *wg*-expressing cells such as extreme ventral bristles in legs (Fig. 2C,D,G,H), while ectopic expression of *wg* never produces these ventral-most cell fates (Struhl and Basler, 1993; Wilder and Perrimon, 1995). Since WG and DPP exert antagonistic influences on cell fate (Theisen et al., 1996; Brook and Cohen, 1996), the effect of ectopic expression of *wg* will be diminished by antagonism of endogenous DPP, but, in *sgg* clones, DPP is absent leaving WG uninhibited influence over cell fate.

Fig. 7. A model for the regulatory interactions in the leg and eye-antennal discs. A schematic of a leg disc is shown with the anterior compartment divided into dorsal and ventral territories. Expression of *sgg* and *dsh* is uniform throughout the disc. In this model, SGG activity promotes *dpp* expression and inhibits *wg* expression. DPP signaling and SGG activity together (indicated by the + sign) inhibit *wg* expression; either alone is much less effective. WG does not directly inhibit *dpp* but removes an activator (SGG) via a DSH-mediated inhibition of SGG activity. The WG-mediated inhibition of SGG is not 100% as indicated by the fact that weak *dpp* expression is seen in the *wg* domain of normal leg discs. This model provides a molecular mechanism to account for the antagonistic interactions between WG and DPP in leg and eye-antennal discs using experimentally demonstrated effects. It incorporates lateral inhibition and autoactivation which are two elements that are formally essential for the generation of stable pattern (Meinhardt, 1982). The diagram shows the regulatory interactions occurring in the dorsal and ventral regions of the disc, respectively. In the ventral region where WG predominates, WG acts through DSH to inhibit SGG activity thus removing the activator of *dpp* (SGG) and promotes its own expression by removing the combinatorial inhibition of SGG and DPP. In the dorsal region, DPP signaling and SGG together inhibit *wg* expression and the consequent lack of inhibition of SGG promotes further *dpp* expression. The asymmetry that initially biases *wg* to the ventral region could follow from the initial asymmetry of *wg* and *dpp* in the disc anlage of the embryo or from other positive inputs on *wg* expression. No other activators would be formally needed although they likely exist. General inhibition of *dpp* and *wg* expression in the anterior compartment, which is relieved by HH signaling from the posterior compartment, normally restricts these interactions to the region near the A/P boundary. Both *wg* and *dpp* are repressed by EN in the posterior compartment. In general, inhibition is dominant over activation of transcription unless relieved.

Clones of *dsh* near the ventral A/P compartment boundary of the leg disc cause ectopic activation of *dpp* in all mutant cells of the clone (Fig. 6A). On the contrary, *wg* expression is repressed throughout the clone (Fig. 5D). Again, the sharp demarcation between cells of the clone expressing only *dpp* and the surrounding normal cells expressing only *wg* argues that the changes in gene expression are not due to secreted factors repressing transcription of other genes. Rather, the sharp borders suggest that changes in DSH activity lead to changes in intracellular components that affect expression of both *wg* and *dpp*.

A model for the mutual repression of *wg* and *dpp* expression

The observations presented here, suggest a model for the regulatory interactions of WG, DPP, DSH and SGG in leg and eye-

antennal discs (Fig. 7). We make the following postulates. SGG activity activates *dpp* expression (Fig. 4F, 5C). DPP signaling (Theisen et al., 1996; Penton and Hoffmann, 1996; Jiang and Struhl, 1996; Brook and Cohen, 1996; Morimura et al., 1996) and SGG activity in combination (Fig. 4D), inhibit *wg* expression (either alone is much less effective or not effective at all). DSH inhibits SGG activity upon WG signaling as suggested by the double mutant clones (Fig. 2E,K; Peifer et al., 1994; Siegfried et al., 1994). General repression of *wg* and *dpp* expression in the anterior compartment (Phillips et al., 1990; Capdevila et al., 1994; Jiang and Struhl, 1995; Johnson et al., 1995; Lepage et al., 1995; Li et al., 1995) dominates over activation of *dpp* by SGG unless the repression is relieved by HH (Basler and Struhl, 1994). In this model, a dorsal cell that lost *sgg* would autonomously stop expressing *dpp* (Figs 4F, 5A) and lose an inhibitor of *wg* expression thus leading to activation of *wg* (Fig. 4B,D). Adjacent cells expressing DPP would not suppress *wg* expression at the boundary because the combinatorial action of SGG plus DPP is lost. On the contrary, a cell that lost *dsh* would have elevated SGG, which, if the clone is near the ventral A/P border, autonomously activates *dpp* (Fig. 6C,D; Jiang and Struhl, 1996) and represses *wg* (Fig. 5D). The different behavior of *sgg* clones in proximal anterior parts of the leg discs (i.e. far from the A/P boundary) with respect to ectopic transcription of *wg* is accounted for by the lack of HH available to relieve the inhibition to *wg* expression in those locations (Basler and Struhl, 1994). Since *dsh* and *sgg* are ubiquitously expressed in discs (Theisen et al., 1994; unpublished results), we assume that transcriptional regulation of *dsh* and *sgg* do not play a significant role.

It has been argued on theoretical grounds that mutually repressing interactions must be accompanied by mutually stimulatory activities to maintain a stable state (Meinhardt, 1982). This concept is incorporated here since in our model, WG and DPP mutually repress each other's transcription and promote their own. The patterning of the leg discs may be the first example of this type of mutual repression, selfactivation other than the hydra head activator (Meinhardt, 1982). By this model, SGG stands at the center of the regulatory interactions whereby both DPP and WG activities repress the other's expression and stimulate their own expression.

Regulatory interactions in wing discs are different

The requirement for SGG function to support *dpp* transcription does not hold in wing discs (Fig. 4G-I). Ectopic *wg* expression does not inhibit *dpp* expression in wing discs (Theisen et al., 1996). In normal wing discs, a band of *dpp* expression crosses several bands of *wg* expression as it extends the entire length of the A/P compartment boundary from dorsal to ventral but it does not repress *wg* at any of these intersections nor does WG repress *dpp*. Further, *dsh* clones in wing discs are not associated with ectopic *dpp* expression (data not shown). Thus, the elements necessary for repression of *dpp* expression by WG signaling are absent or different in wing discs than they are in leg and eye-antennal discs (see also Rulifson and Blair, 1995; Theisen et al., 1996; Brook and Cohen, 1996; Morimura et al., 1996).

Local cell interactions also play a role in the response to *sgg* and *dsh* clones

Interestingly, clones located far from the A/P compartment border behave differently than clones adjacent to the border. Interactions, other than simple changes in expression of *wg* and *dpp*, may play

a role in the patterning response of clones far from the boundary. When *dsh* clones occur far from the A/P border, the domain of ectopic activation of *dpp* includes some, but not all, of the mutant cells as well as some of the cells neighboring the clone (Fig. 6C,D). Moreover, the boundary between *dpp*-expressing and non-expressing cells is not sharp. These observations are consistent with local cell interactions playing a role in the patterning and growth response and ultimately in the activation of *dpp* in these clones. Similarly, *sgg* clones that fall far from the A/P compartment boundary in either the dorsal anterior or dorsal posterior compartment can cause ectopic growths and yet *wg* expression is absent from these clones. No clone was ever observed with ectopic *wg* or *dpp* expression that had not already experienced growth and patterning. Conversely, clones associated with growth and patterning were observed with either none, little or extensive ectopic expression (e.g. Fig. 3) suggesting that locally driven pattern regulation may precede ectopic activation of *wg* or *dpp*. Alternatively, other cytokines not yet identified may play a role in the responses of these clones distant from the A/P border.

Loss of *sgg* function leads to accumulation of Armadillo protein (Peifer et al., 1994) which is a component of adherens junctions (Peifer, 1993; Peifer et al., 1993; Woods and Bryant, 1993). An increase in cell avidity due to increased ARM, may explain the round appearance of *sgg* mutant clones where the cells appear to adhere tightly together and avoid contact with their non-mutant neighbors (Figs 3A-C, 4A-D,F, 5C). Such a differential change in cell adhesion may constitute part of a putative contact-mediated interaction.

Surgically manipulated disc fragments that do not include a compartment boundary, and therefore lack *wg* and *dpp*-expressing cells (Baker, 1988; Masucci et al., 1990; Raftery et al., 1991), can respond by replacing both the missing boundary and the missing compartment (Schubiger, 1971; Abbot et al., 1981; Blair, 1995). This raises the possibility that cellular properties that affect local cell interactions, perhaps mediated by DSH and SGG, may participate in the regeneration of imaginal discs from these fragments.

***sgg* and *dsh* trigger opposing changes in positional identity**

Positional identity is operationally defined by two properties, the structures that a cell produces and the manner in which a cell interacts with its neighbors. Changing the activity of *dsh* or *sgg* causes cells to adopt characteristics of cells at specific locations in imaginal discs, including the property of interacting with neighboring non-mutant cells to organize new pattern (Fig. 2; Theisen et al., 1994; Klingensmith et al., 1994; Diaz-Benjumea and Cohen, 1994). Mutant cells in clones produce structures characteristic of a particular position and they are unable to participate in making structures of other positional identities even when involved in a major regulatory response. In both the leg and eye-antennal discs, the spatial requirements for *dsh* and *sgg* are complementary (Figs 1, 2G,H,J) as are the spatial requirements for *wg* and *dpp* (Held and Heup, 1996). Loss of *sgg* confers positional identities characteristic of *wg*-expressing cells while cells that have lost *dsh* adopt positional identities characteristic of cells from regions of *dpp* expression. For example, the adult structures produced by *dsh* clones are consistent with activation of *dpp* in *dsh* clones. Dorsal head clones of *dsh* cause ectopic ommatidia to form in place of dorsal head cuticle (Fig. 2J). This could be produced by a dorsal extension of the mor-

phogenetic furrow (i. e. *dpp* expression) into the clone but also must include the reprogramming of these cells to make eye tissue. It has been proposed that WG at the border of the eye restricts the extension of the morphogenetic furrow by inhibiting DPP (Ma and Moses, 1995; Treisman and Rubin, 1995). The extension of *dpp* expression into the clone is consistent with the loss of *wg* expression in these cells and hence loss of an inhibitory signal. Changing the activity of *dsh* or *sgg* changes two cellular properties, namely, the final differentiated structures that the mutant cells produce and the ability of those cells to organize non-mutant cells into ectopic structures thus, demonstrating that positional identity has been altered.

Parallels with vertebrates

Many of the molecules and regulatory interactions described in *Drosophila* appear in vertebrates. Posterior expression of *hh* during patterning of the A/P axis occurs in both groups (reviewed in Ingham, 1996). Similarly, as in *Drosophila*, adjacent territories of Wnt and BMP expression occur in vertebrate limbs including adjacent dorsal versus ventral or epidermal versus mesodermal territories of expression (reviewed in Tickle, 1995). The involvement of both *dsh* and *sgg* in these signaling pathways is conserved in vertebrates and indeed the insect and vertebrate homologues of both genes are functionally interchangeable (Ruel et al., 1993; Rothbacher et al., 1995). The regulatory interactions described here may play a role in establishing similar territories in vertebrates.

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