Requirements for *hedgehog*, a Segmental Polarity Gene, in Patterning Larval and Adult Cuticle of *Drosophila*

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ABSTRACT

Mutations of the *hedgehog* gene are generally embryonic lethal, resulting in a lawn of denticles on the ventral surface. In strong alleles, no segmentation is obvious and the anteroposterior polarity of ventral denticles is lost. Temperature shift analysis of a temperature-sensitive allele indicates an embryonic activity period for *hedgehog* between 2.5 and 6 hr of embryonic development (at 25°) and a larval/pupal period from 4 to 7 days of development (at 25°). Mosaic analysis of *hedgehog* mutations in the adult cuticle indicates a series of defined defects associated with the failure of appropriate *hedgehog* expression. In particular, defects in the distal portions of the legs and antenna occur in association with homozygous *hedgehog* clones in the posterior compartment of those structures. Because the defects are associated with homozygous clones, but are not co-extensive, a type of "domineering" nonautonomy is proposed for the activity of the *hedgehog* gene.

THE development of a highly structured organism from a comparatively unstructured egg is an awe-inspiring process (ARISTOTLE 335 BC). The initial classical and subsequent molecular genetic analysis of segmentation during embryogenesis of *Drosophila melanogaster* has provided considerable insight into how the spatial pattern of a developing organism is regulated (for a review, see AKAM 1987). The zygotically active segmentation genes fall into three classes, gap, pair-rule and segment polarity, defined initially by cuticular defects associated with mutations in those genes (NÜSSLEIN-VOLHARD and WIESCHAUS 1980). The gap genes are active in contiguous domains along the anteroposterior axis of the blastoderm embryo (JÄCKLE *et al.* 1986) and regulate segmentation within those domains presumably via their action on the pair-rule genes (CARROLL and SCOTT 1986; FRASCH and LEVINE 1987). The pair-rule genes are expressed in stripes along the length of the blastoderm embryo with a periodicity corresponding to every other segment (see review by AKAM 1987) and appear to define the segmentation parameters via their regulatory effects on the segment polarity genes (DINARDO and O'FARRELL 1987). The segment polarity genes regulate the spatial pattern within each segment (NÜSSLEIN-VOLHARD and WIESCHAUS 1980; BAKER 1988; DINARDO *et al.* 1988) and the differential expression of many of the segment polarity genes (*e.g.*, *en*—KORNBERG *et al.* 1985; *wg*—BAKER 1987; and *gsb*—BAUMGARTNER *et al.* 1987) along the anteroposterior axis is thought to give rise to the periodic segmentation pattern of the *Drosophila* larva. The mechanisms by which the segment polarity genes regulate the

spatial pattern within the segment are still unknown.

The patterning of structures of the adult cuticle of *Drosophila* has also been intensively studied. Studies of regeneration following ablation of imaginal discs suggest an underlying "positional information" that directs particular cell fates and is regulated according to particular rules (FRENCH, BRYANT and BRYANT 1976). Studies of the effects of homeotic genes on the developmental potential of cells within various imaginal discs suggest that this "positional information" is similar among discs and that each disc has a unique program for interpreting that information (POSTLETHWAIT and SCHNIEDERMAN 1971; LEWIS 1978). The nature of this "positional information" remains a mystery, so it is relevant to determine the extent that the genes involved in patterning within the embryonic segment are involved in patterning the adult cuticle.

This study characterizes the mutational effects of one of the segment polarity genes, *hedgehog*, on both the larval and adult cuticular patterns. This study also analyzes the temporal requirements for *hedgehog* gene activity through development, via analysis of temperature sensitive periods of a temperature sensitive allele, and investigates the spatial requirements for *hedgehog* in imaginal cuticle by mosaic analysis.

MATERIALS AND METHODS

***hedgehog* mutations used in this study:** This study used primarily the eight EMS-induced alleles of *hedgehog* identified by JÜRGENS, WIESCHAUS and NÜSSLEIN-VOLHARD (1984); *hh^U*, *hh^{IK}*, *hh^{IO}*, *hh^{6N}*, *hh^{9K}*, *hh^{10B}*, *hh^{13E}* and *hh^{13C}* (mutations with allele names beginning with a Roman numeral were isolated on a third chromosome from a *cn bw* stock, those beginning with an even Arabic numeral from *st*

e, and those beginning with an odd Arabic numeral from *rucuca*). An additional eleven alleles, identified in an F1 screen for their inability to complement *bar-3*, were generated for this study by either γ -irradiation (4000 R), hybrid dysgenesis, or treatment with triethylenemelamine [according to the protocols of KIDWELL (1986) and GRIGLIATTI (1986), except that the triethylenemelamine concentration was reduced from 150 μ M to 10 μ M due to complete sterility at that dose]. For these new alleles, the first letter of the allele name indicates the mutagen used (G = γ -irradiation, H = hybrid dysgenesis, T = triethylenemelamine) and the second letter indicates the strain in which they were induced (S = *st e*, R = *rucuca*, W = *w*). Other strains used in this study can be found in LINDSLEY and GRELL (1968).

Larval cuticle preparations: For photomicrographs, larval cuticle preparations of artificially hatched homozygous *hedgehog* embryos raised at 25° were prepared according to the protocol of WIESCHAUS and NÜSSEIN-VOLHARD (1986) and mounted in Hoyer's mountant. For determination of larval phenotypes after temperature shifts, cuticle preparations of unhatched larvae in the vitelline membrane were used (WIESCHAUS and NÜSSEIN-VOLHARD 1986).

Temperature shift analysis of *hh^{SK}*: For the analysis of the embryonic temperature sensitive period of single dose individuals, embryos were collected on apple-juice agar plates for thirteen hourly intervals at both 18° and 25° from each of the two reciprocal crosses of: *hh^{SK}/+ × hh^{13C}/+*. The heterozygous parents were obtained from an outcross of the *hh/TM3* laboratory stock with Oregon-R to decrease viability effects. Sets of hourly collections were shifted from the initial to the final temperature *en masse* at 13 hr from the beginning of the first hourly collection. Embryos were allowed to proceed through embryogenesis, the number of hatched and unhatched embryos were counted, and the hatched and unhatched larvae were mounted to determine embryonic phenotype. Results from the two reciprocal crosses were pooled.

For temperature pulse studies of homozygous *hh^{SK}* embryos, embryos were collected for 12 hourly intervals at 25° from heterozygous *hh^{SK}* animals outcrossed to Oregon-R. This set of hourly collections was given a 90 min 18° pulse 12 hr from the beginning of the first collection. Embryos were allowed to complete embryogenesis, and the hatch rate was counted.

For temperature shift analysis of larval and pupal stages, embryos were collected from heterozygous *hh^{SK}* parents for 19 daily intervals at 18° and allowed to develop in standard milk bottles. For up-shift experiments, this set of daily collections was shifted to 25° 19 days from the beginning of the first collection. For down-shift experiments, each collection was shifted to 25° after three days from the beginning of the collection period and then the set of daily collections was shifted back to 18° 11 days after the beginning of the first collection of the set. For each set, abnormal adults were characterized and the proportion of homozygous *hh^{SK}* individuals was calculated and normalized against proportions in parallel, untreated 18° cultures. The relative developmental rate between 18° and 25° was calculated as the ratio of the time of first enclosed pupae at 18° and 25°.

Mosaic analysis of *hedgehog* mutations: Two series of irradiations were conducted. In the Princeton series, for each of five mutations (*hh¹⁰*, *hh^{6N}*, *hh^{10B}*, *hh^{13E}* and *hh^{13C}*) embryos were collected at daily intervals from the cross *hh/TM3 × Ki Sb M(3)^{w¹²⁴}/TM2*. Each collection was allowed to develop either 24 or 48 hr, when it was irradiated with 1000 R. Adults of the genotype *hh/Ki Sb M(3)^{w¹²⁴}* were quickly scored while anesthetized for cuticular defects and *Ki⁺ Sb⁺*

patches. For *hh^{13C}* adults, 81 of 161 were either mosaic or defective; whereas for *hh^{13E}*, 114 of 200 were mosaic or defective. These mosaic and defective animals were stored in 1:3 glycerol:ethanol. These were later prepared for observation by leaching soft tissue with 10% NaOH for 10 min at 50° and mounting in Faure's mountant. In the Barnard series, for each of five mutations (*hh^{SK}*, *hh^{10B}*, *hh^{13C}*, *hh^{13E}* and *hh^{GS1}*) embryos of the same cross were collected at 12-hr intervals and were irradiated with 1000 R either 24, 36, 48 or 60 hr later. The adults were again quickly scored for cuticular defects and *Ki⁺ Sb⁺* patches and were stored in 1:3 glycerol:ethanol until mounting. All *hh^{13C}* and *hh^{13E}* animals were mounted in 2:1 mixture of methyl salicylate and a commercial preparation of Canada Balsam in xylene. Among the 124 *hh^{13E}* individuals in this series judged to be normal by casual inspection, 25 were found to possess clones or defects; among the 118 "normal" *hh^{13C}* individuals, 32 had clones or defects. This suggests the frequency calculations for the Princeton series underestimates the frequency of clones and defects by about 10% and that the frequency estimates from pooling both series (Table 2) may be underestimates by about 5%. Because the experimental frequencies exceed those of the control in most cases, no important errors should arise from this underestimation.

RESULTS

Phenotypes of *hedgehog* alleles

The *hedgehog* gene was initially identified following the production of mutations affecting embryonic cuticular pattern (NÜSSEIN-VOLHARD and WIESCHAUS 1980). In the mutational screen of JÜRGENS, WIESCHAUS and NÜSSEIN-VOLHARD (1984), a total of eight alleles of *hedgehog* were generated by EMS mutagenesis. Several other *hedgehog* alleles have been isolated by γ -irradiation, hybrid dysgenesis, and TEM (triethylenemelamine) mutagenesis. These alleles are listed in Table 1.

The original EMS-induced *hedgehog* mutations exhibit a range of embryonic cuticular defects. Animals homozygous for strong alleles (such as *hh^{13C}*, *hh^{6N}*) are approximately 40% the length of a wild-type larva and exhibit a ventral lawn of denticles associated with the loss of naked cuticle from the ventral surface. In mutants of strong alleles, the denticle pattern reflects a complete lack of segmentation; lateral denticles point toward the ventral midline rather than posteriorly as in wild-type and ventral denticles are arranged in a number of whorls of variable number (Figure 1f). Although no segmentation is obvious, denticles at the anterior end of the larva exhibit a thin, thoracic character, whereas denticles in the middle and posterior regions of the larva show a thicker, abdominal character, indicating a differentiation into segmental type independent of segmentation. This phenotype is similar to that described for amorphic alleles of *eve* (NÜSSEIN-VOLHARD, KLUDING and JÜRGENS 1985). All the chromosome rearrangement mutations (e.g., *hh^{GS1}*, *hh^{HL1}*) produce the strong embryonic phenotype, suggesting that this strong phenotype reflects the null phenotype for the *hedgehog* locus. For

TABLE 1
hedgehog mutations

Allele	Rank		Viability 18° allele/hh ^{9K}	Cytology
	18°	25°		
9K	0	1	0.66	
10B	1	2	0.18	
13E	2	3	0.27	
11K	2	3.5	0.09	
IJ	3	4	0.02	
GS2	NT	4	0.04	
110	3.5	4.5	0.02	
GR1	NT	4.5	0.01	In(3R)81F; 94D10-E5
GS1	NT	4.5	0.01	In(3R)92B4-11; 94D10-E5
HL2	NT	4.5	0.03	
GW1	NT	4.5	NT	
GW2	NT	4.5	NT	
TW1	NT	4.5	NT	
6N	1.5	5	0.01	
HL1	NT	5	0.02	Tp(3R)85D; 94D10-E5; 97E7-8
HL3	NT	5	0.01	
13C	4.5	5	0.01	
GR2	NT	5	NT	Df(3R)93F11-14; 94E2-5

NT = not tested.

purposes of comparison among alleles, this strong phenotype has been assigned an arbitrary rank value of 5.

Canonical alleles of *hedgehog*, such as *hh*^{13E}, as described by NÜSSEIN-VOLHARD and WIESCHAUS (1980), show a more intermediate phenotype (Figure 1d). In mutants of these alleles, naked cuticle is also missing from the ventral region. However, the denticle lawn is arranged in segmental arrays with mirror-image symmetry: denticles in the anterior portion of each segmental array point posteriorly, whereas denticles in the posterior portion of each segmental array point anteriorly. The canonical intermediate phenotype of *hedgehog* has been assigned an arbitrary rank value of 3.

The weakest embryonic phenotype of *hedgehog* mutations results in a number of denticle belt fusions at numerous points along the anteroposterior axis (*hh*^{9K}, Figure 1b). These fusions appear to delete most of the naked cuticle between denticle belts and substitute a small length of ectopic denticles. The fusions occur most frequently between abdominal segments 6, 7 and 8 and between abdominal segments 1 and 2. This weak phenotype has been assigned an arbitrary rank value of 1.

Among the EMS-induced alleles, all exhibit a stronger phenotype at 25° than at 18° (Table 1), implying a differential temperature requirement for *hedgehog* locus activity between high and low temperatures, rather than a thermolability of any given allele. The weakest allele, *hh*^{9K}, is embryonic lethal at 25° and viable at 18°. The adult viability of homozygous *hh*^{9K} individuals at 18° is approximately 65% the

viability of heterozygous *hh*^{9K}/TM3 siblings. Homozygous *hh*^{9K} adults raised at 18° are visibly mutant and have a reduced number of eye facets and a "bobbed" scutellum, in which the posterior portion of the scutellum is lost and the posterior scutellar bristles are displaced or missing. A similar reduction of eye facets is the predominant phenotype of *bar-3*, an adult visible mutation mapping at 3-79.3 (LINDSLEY and GRELL 1968) near the previously reported position of *hh* (3-81) (JÜRGENS, WIESCHAUS and NÜSSEIN-VOLHARD 1984). All *hedgehog* alleles fail to complement the eye phenotype of *bar-3*, indicating that *bar-3* is a weak, adult viable allele of *hedgehog*.

The implication that the *hedgehog* alleles fall into a hypomorphic series of increasing gene activity, as suggested by the arbitrary ranking of phenotypes, is strengthened by a number of observations. First, the phenotype of individuals heterozygous for two *hedgehog* alleles invariably is intermediate on this scale to the phenotype of the homozygotes (at least for all the EMS alleles, data not shown), implying an additivity of gene activity among these alleles. Second, the viability at 18° of individuals heterozygous for a particular *hedgehog* allele and *hh*^{9K} is proportional to the strength of the allele as judged by its embryonic phenotype (Table 1). Because no embryonic inviability of individuals heterozygous for *hh*^{9K} and any other allele is apparent at 18°, the reduction in the adult viability of these heterozygotes probably reflects the degree of gene activity in post-embryonic development. A similar relationship is also observed between the degree of the eye facet reduction in heterozygotes of lethal *hedgehog* alleles and *bar-3* and the rank of the embryonic phenotype of the homozygous allele (data not shown). Thus, the strength of any particular *hedgehog* allele as judged by its embryonic phenotype is indicative of its gene activity in establishing post-embryonic pattern as judged by its partial complementation of *hh*^{9K} and *bar-3*.

Temperature sensitive periods of *hh*^{9K}

Embryonic temperature sensitive period: Because animals homozygous for *hh*^{9K} are viable and phenotypically normal at 18° but mutant at 25°, it is possible to use this allele in temperature shift analyses to determine times when the mutant allele provides insufficient function at the restrictive temperature for viability and, probably, times at which the *hedgehog* gene is most likely to be active. Unfortunately, animals homozygous for *hh*^{9K}, with two doses of the *hh*^{9K} allele, possess only a marginally mutant embryonic phenotype (rank 1) and have a substantial embryonic viability (about 20% hatch as phenotypically normal larvae). Animals with only one dose of *hh*^{9K}, i.e., heterozygotes for *hh*^{9K} and a strong allele, such as *hh*^{GS1}, have complete lethality at 25° and possess a stronger, rank 2 phenotype, but are phenotypically normal and viable

FIGURE 1.—Larval cuticular phenotypes of *hedgehog* mutations at 25° (120X). a, Wild-type larva (rank 0); b, *hh*^{9K} (rank 1); c, *hh*^{10B} (rank 2); d, *hh*^{13E} (rank 3); e, *hh*¹¹⁰ (rank 4); f, *hh*^{6X} (rank 5).

FIGURE 2.—Embryonic temperature shift analysis of temperature sensitive allele, *hh*^{9K}. a, Fraction of embryos from the cross (*hh*^{9K}/+ × *hh*^{19C}/+) phenotypic mutant as a function of the time of temperature shift. Open circles are the results from up-shifts (18° to 25°), closed circles the results from down-shifts (25° to 18°). Vertical bars are ± SE. b, Fraction of inviable embryos from heterozygous *hh*^{9K} parents raised at 25° and pulsed at 18° for 90 min as a function of the time of the pulse. Horizontal broad bars indicate the duration of the pulse, vertical bars are ± SE.

at 18° through embryogenesis. These one dose animals were used for the determination of the temperature sensitive period by temperature shifts because of the greater differential between the phenotypes at restrictive and permissive temperatures.

Embryos from each of the two reciprocal F₁ crosses (*hh*^{9K}/+ × *hh*^{GS1}/+) were collected at hourly intervals at either 18° or 25° and shifted at various times to the other temperature. Embryos from this F₁ cross collected at 25° and shifted to 18° (down-shift) prior to 4 hr of embryonic development were phenotypically normal and survived at high frequencies (Figure 2a). Full expression of the mutant phenotype (rank 2) was consistently observed in embryos down-shifted after 6 hr, whereas mutant embryos shifted between 5 and 6 hr of embryonic development were equally divided among rank 1 and rank 2 phenotypic classes. Embryos from this F₁ cross collected at 18° and shifted to 25° (up-shift) after 7 hr of embryonic development, corresponding to 4 hr of embryonic development at 25°, survived embryogenesis at high frequencies and were phenotypically normal. Embryos

up-shifted prior to 5 hr (equivalent to about 2.5 hr of development at 25°) consistently displayed full expression of the mutant phenotype (rank 2), whereas those down-shifted between 5 and 7 hr of embryonic development at 18° (2.5–4 hr at 25°) were divided between rank 1 and rank 2 phenotypic classes.

Because temperature shifts to the permissive temperature as late as 6 hr of embryonic development at 25° and temperature shifts from the permissive temperature as early as a time corresponding to 2.5 hr of embryonic development at 25° can ameliorate the mutant effects of *hh*^{9K}, these results indicate an activity period of *hedgehog* for segmental cuticle pattern between 2.5 and 6 hr of embryonic development at 25°, corresponding roughly to a period of embryonic development from the onset of gastrulation (stage 6 of CAMPOS-ORTEGA and HARTENSTEIN 1985) to the time when segmentation becomes visible (stage 10). Growth at the permissive temperature for either the first or last half of this period is sufficient for viability of single dose *hh*^{9K} individuals, suggesting that the period required for proper cuticular patterning is the same as the period of expression required for viability. However, because the thermosensitivity for *hh* alleles appears to reflect a differential temperature requirement for *hedgehog* activity, rather than a thermolability of a protein product, this temperature sensitive period reflects the timing of the thermosensitive requirement for *hedgehog* function. Although it seems likely that the thermosensitive process that requires *hedgehog* activity should be coincident with *hedgehog* expression, the thermosensitive period may either precede or succeed actual *hedgehog* gene activity.

To better define the thermosensitive period for *hedgehog* activity as required for viability, a more sensitive regime of temperature shifts were employed. Because embryos homozygous for *hh*^{9K} are marginally sub-threshold for viability at 25°, brief pulses of increased *hedgehog* activity should substantially increase viability of these embryos. Such increases in *hedgehog* activity may be obtained by growth for brief times at permissive temperatures during periods of *hedgehog* gene activity. As shown in Figure 2b, 1.5 hr 18° pulses delivered between 2.5 and 5.5 hr of embryonic development resulted in high viability of homozygous *hh*^{9K} embryos raised otherwise continuously at 25°, confirming this period as the main activity period affected by *hh*^{9K}. Pulses delivered after 6 hr of embryonic development also caused a slight increase on embryonic viability. Two alternative explanations for this late effect seem plausible: this may reflect a second thermosensitive period for *hedgehog* that is less sensitive to the *hh*^{9K} mutation or this may reflect a stochastic ability of residual *hedgehog* gene product to complete its normal function after a stage when that process would normally be completed. In light of the

FIGURE 3.—Post-embryonic temperature shift analysis of temperature sensitive allele, *hh*^{9K}. Relative viability (compared to 18° controls) of viable homozygous *hh*^{9K} adults as a function of time of temperature shift. Closed circles are results from up-shifts (18° to 25°), open circles are the results from down-shifts (25° to 18°). Vertical bars are $\pm$ SE.

fact that no evidence was found for ameliorative effects of permissive temperature on a single dose of *hh*^{9K} after 6 hr of embryogenesis, on either cuticular phenotype or viability, the second explanation seems more likely.

Postembryonic temperature sensitive period: To determine whether there was an activity period for *hedgehog* after embryogenesis, during which growth of *hh*^{9K} at permissive temperature was required, temperature shifts during larval and pupal development were employed. Embryos were collected at daily intervals at 18° and either shifted to 25° at various times during larval and pupal stages (up-shift) or were shifted to 25° after three days of development at 18° (to avoid the embryonic temperature sensitive period) and subsequently shifted back to 18° at various times during post-embryonic development. As shown in Figure 3, the last time animals could be left at the restrictive temperature and retain high viability corresponds to a stage at approximately 4 days of development at 25°, roughly the middle of the third larval instar. The earliest time animals could be shifted to 25° and retain high viability was 13 days of development at 18° (equivalent to 7 days at 25°, at early to mid-pupal stages). Because *hh*^{9K} is a weak allele, with substantial residual activity at 25°, the period from 4 to 7 days of development (at 25°) is a minimal estimate of the postembryonic period in which *hedgehog* gene activity is required. The weak activity of *hh*^{9K} at 25° may provide sufficient activity for some developmental process that employs *hedgehog* gene function or, if the process has a long duration, 18° *hh*^{9K} allele expression within this 4–7-day window may provide a sufficient boost in *hedgehog* gene activity to override insufficient expression at other times.

Surviving flies that were exposed to the restrictive temperature between 3 and 5 days of age (25° developmental time) possessed a number of defined defects in adult cuticular morphology; more extreme defects were associated with longer treatments at 25°. Many of these flies were missing antennae, legs and portions of the eye and scutellum. Defects in the eye were associated with duplications of surrounding bristles (Figure 4a); duplicated bristles were also observed in the humerus and male genitalia. Twelve-hour exposures to 25° administered during this period to animals otherwise raised continuously at 18° also caused weak versions of these defects, although these pulses had no obvious effect on viability when delivered during larval or pupal development.

Mosaic analysis of adult cuticle

To further identify requirements for *hedgehog* in patterning of the adult cuticle, mosaic clones homozygous for various *hedgehog* alleles were generated by X-ray induced somatic recombination of heterozygotes during early larval stages. Mutant clones were identified by the absence of the *Ki* and *Sb* markers present on the homologous chromosome (*e* and *e'* markers present in many *hedgehog* mutant strains proved unreliable in scoring mosaic clones). Results for mosaic analysis of a strong allele (*hh*^{13C}, rank 5) and an intermediate allele (*hh*^{13E}, rank 3) are shown in Table 2. Results for other alleles are similar, except that for *hh*^{6N} and *hh*^{GS1} very few clones were recovered, possibly due to the presence of other mutations on the mutant chromosomes.

Homozygous *hedgehog* clones were identified in all portions of the adult fly, where the phenotype of the macrochaetae could be scored: in the head capsule, antenna, legs, mesonotum and abdominal tergites (Table 2). In all tissues, homozygous *hedgehog* clones were generated at a frequency similar to or higher than the generation of homozygous clones from control (*st e*) flies. In general, these results suggest that *hedgehog* mutations are cell viable in most imaginal disc tissue. However, because the large macrochaetae affected by the markers *Ki* and *Sb* are present in only limited areas of the adult cuticle, substantial proportions of adult cuticle may be in fact affected by these clones without a significant effect on the numbers of such clones observed. In particular, clones in the proboscis (except for the palp), posterior second and third legs and genitalia would not be observed.

Irradiation of flies heterozygous for all *hedgehog* alleles resulted in a set of defined defects in the adult cuticle in particular portions of the animal. Among the various alleles screened, no association was detected between the strength of the allele and the frequency, severity or type of adult defects. These defects include a reduction in the size of the eye, elimination of ocelli and associated bristles, loss of

FIGURE 4.—Imaginal defects arising from misexpression of *hedgehog*. a, Defective head structures from homozygous *hh*^{9K} animal raised at restrictive temperature (25°) for 4 days during larval stages. Note missing eye and duplicated bristles on animal's right, missing left antenna (A) and greatly reduced left eye (E). b–d, Defects arising from irradiation of heterozygous *hedgehog* larvae. b, Shrunk left antenna with missing arista. Note *Ki*⁺, *Sb*⁺ bristles (at arrow) indicating a homozygous *hh* clone. (*hh*^{13C}, irradiated 48–60 hr.) c, Shrunk first leg with sex comb, transverse bristle rows (out of focal plane) and single distal claw (arrow). (*hh*^{13E}, irradiated 48–72 hr.) d, Missing ocellar bristles and ocellus (arrow) associated with adjacent homozygous *hh* clone. (*hh*¹¹⁰, irradiated 24–48 hr.)

distal portions of the antenna and legs, “bobbing” of the scutellum and loss of structures in the male and female genitalia (Table 2). Homozygous *hedgehog* clones were frequently recovered in tissue adjacent to defects in the head and antenna, but only rarely with defects in the legs and scutellum. This implies that the defects in the head and antenna are caused by the existence of a clone of mutant tissue. Although the defects often appear to be associated with mutant clones, it cannot be determined whether the missing tissue was originally mutant. The failure of the defects to respect the compartment boundary in the antennae and legs (see below) suggests that at least some non-mutant tissue contributes to the defects. The lack of clones in association with leg, scutellar and genital defects may be due either to a different etiology for those defects (e.g. synergistic effects of radiation damage and a haplo-insufficiency for *hedgehog*) or by undetected *hedgehog* clones (either because the clone is confined to the defective tissue or due to the paucity of marker bristles).

The similar defects found in the antennae and legs

of irradiated *hedgehog* heterozygotes suggest a requirement for *hedgehog* in the same portion of the posterior compartment for the structural integrity of the entire appendage. In both the legs and antennae, the principal defect is the loss of distal structures and shrinkage of more proximal ones. In the antenna, the most prevalent and most severe defect is the loss of the aristae and segment 4 of the antenna and a severe reduction in the size of antennal segment 3 (Figure 4b). Weaker defects are missing part or all of the arista, associated with lesser reductions of antennal segment 3. These distal defects were frequently found to be associated with homozygous *hedgehog* clones in the posterior compartment of second antennal segment (Table 3). Approximately 80% of the defective antennae were associated with posterior compartment clones and approximately 80% of posterior compartment clones were in antennae with distal defects. No defects were found in the 36 antennae with homozygous *hedgehog* (*hh*^{13C} and *hh*^{13E}) clones that appeared to be restricted to the anterior compartment. In the 20 defective antennae resulting from irradiation be-

TABLE 2
Clone/defect frequency following induced mitotic recombination (24–72 hr)

	Head								
	Eye	OCE	ANT	Leg 1	Leg 2	Leg 3	MS	MG	FG
<i>st e</i>									
Clones	2.1	3.8	1.3	1.9	0.8	1.9	—	—	—
Defects	0	0	0.2	0	0.2	0.2	0	0.8	0
C+D/D	—	—	0/1	—	0/1	0/1	—	—	—
Total				526				125	139
<i>hh^{13E}</i>									
Clones	4.2	2.7	3.8	5.8	1.5	1.8	—	—	—
Defects	1.9	0.7	1.2	1.5	6.9	0.4	0.3	2.2	2.7
D+C/D	0/14	5/5	8/9	0/11	4/51	0/3	0/2	—	—
Total				734				182	185
<i>hh^{13C}</i>									
Clones	4.0	4.9	2.7	4.1	2.1	2.3	—	—	—
Defects	1.0	0.3	1.6	1.6	2.8	1.6	0.5	22.3	8.6
D+C/D	5/7	2/2	8/11	1/11	2/20	1/12	0/2	—	—
Total				710				157	198

Frequency of clones and defects (expressed in percent) after irradiation of *st e*, *hh^{13E}* and *hh^{13C}* heterozygotes in various structures: Head = head capsule, OCE = ocellus, ANT = antenna, MS = mesonotum, MG = male genitalia, FG = female genitalia. D+C/D = number of defects associated with adjacent clones over total number of defects. Total represents the number of animal sides (for bilaterally symmetric structures), or male/female genitalia scored. (— = not detectable.)

TABLE 3

Antennal defects vs. anterior-posterior compartment clones

Allele	No defect				Distal defect			
	—	A	P	A+P	—	A	P	A+P
<i>st e</i>	506	12	8	0	0	0	0	0
<i>hh^{13E}</i>	711	12	1	1	1	0	7	1
<i>hh^{13C}</i>	668	26	2	1	3	0	8	0

— = no clone, A = anterior clone, P = posterior clone, A+P = overlapping clone.

tween 24 and 72 hr, 16 contained clones in the posterior compartment, only one of which overlapped into the anterior compartment. Although these clones were probably induced prior to the establishment of the anterior-posterior restriction boundary in heterozygous *M(3)w¹²⁴* flies (it is established at about 72 hr in heterozygous *M(2)o^{5p}* flies) (MORATA and LAWRENCE 1979), the high correspondence between posterior compartment clones and defects and the extremely low correspondence between anterior compartment clone suggests that *hedgehog* is required in some portion of the posterior compartment of the antenna for the proper patterning of the distal portion of the antenna. Because the arista is formed entirely from the anterior compartment of the antenna (MORATA and LAWRENCE 1978), these results suggest that the action of *hedgehog* in patterning the distal antenna is nonautonomous: mutant posterior compartment

cells disrupt the differentiation of presumably non-mutant anterior compartment cells.

In the leg, the strongest and most frequent defect consisted of the slight reduction in the size accompanied by a slight twist of the femur, a substantial reduction in the size of the tibia, capped by a "knob" resulting from the fusion of remnants of more distal tarsal segments. Shrunk forelegs invariably retained the transverse rows of bristles in the tarsus and, in males, the sexcombs, both of which are located in the anterior compartment of the foreleg (STEINER 1976). Legs with slightly weaker defects, such as that seen in Figure 4c, retain a single distal claw (normal legs have two distal claws, one in the anterior and one in the posterior compartment) and are missing large portions of the tarsus. Thus, irradiation of heterozygous *hedgehog* individuals appears to affect preferentially distal structures of the posterior compartment and secondarily, anterior distal structures, indicating that in both the leg and the antenna, structures distal to a common homologous segment (AIII in the antenna and the femur in the leg, POSTLETHWAIT and SCHNEIDERMAN 1971) are reduced or lost in irradiated *hedgehog* heterozygotes. While these defects are not found among irradiated *st e* individuals or among non-irradiated *hedgehog* heterozygotes, homozygous clones are associated with these defects at a frequency only slightly elevated from the frequency of clones in general (Table 2). While these might be due to causes other than the induction of mutant clones, the homologies with the antennal defects and clones suggest a similar association of defects with undetected posterior compartment *hedgehog* clones. Because only the posterior macrochaetae of the femur of the first leg, which are lost in severe defects, could be used to identify posterior compartment clones of the legs, detection of posterior compartment clones in association with defects may be frequently impossible. Significantly, both cases of coincident clones and defects in the first legs of *hh^{13C}* heterozygotes were among the six clones identified in the posterior compartment and involved weak defects that did not extend into the femur (close scrutiny of the other four legs with posterior compartment clones revealed two with very weak defects, missing solely a distal claw, that would not have been detected or scored as defects). This may suggest that for the leg, like the antenna, *hedgehog* is required in the same portion of the posterior compartment to provide the structural integrity of the distal leg.

Defects in the ocellar region (consisting of absent ocelli and ocellar and post-vertical bristles) were invariably associated with clones present in adjacent orbital or vertical bristles (Figure 4d). Only one homozygous *hedgehog* clone in the ocellar region (affecting a single ocellar bristle) was detected in the 1444

irradiated *hh*^{13C} and *hh*^{13E} heterozygous flies. These results suggest that the loss of structures caused by homozygous *hedgehog* clones in the ocellar region is at least region autonomous, and possibly cell-autonomous (assuming the single bristle clone was the result of a somatic recombination event resulting in the loss of the *Ki* and *Sb* markers, but not *hh*⁺).

Defects in the genitalia differed considerably according to sex. Many male irradiated *hedgehog* heterozygotes were missing all external structures derived from the genital disc, both the male genital and male analia structures. In contrast, many female irradiated heterozygotes had defects in or were totally missing the female analia, but in no case was the female external genitalia affected. Gyandromorph studies by NÖTHIGER, DÜBENDORFER and EPPER (1977) have indicated that the genital disc structures were derived from three separate primordia: one for the male genital structures, one for the female genital structures and one for the analia. Thus, irradiation of homozygous mutant *hedgehog* appears to affect the products of two of these primordia, the analia and male genitalia, but to have no effect on the primordia for the female external genitalia. Because of the inability to score for *Ki*⁺ *Sb*⁺ clones in the genitalia, it is not possible to determine whether these defects were clonally derived.

DISCUSSION

Relationship between embryonic and imaginal requirements for *hedgehog*: The results suggest that *hedgehog* is required during two phases of *Drosophila* development: during early embryogenesis (3 to 6 hours of embryonic development at 25°) and during late larval and early pupal stages (4–7 days of development at 25°). The *hedgehog* gene is required during early embryogenesis to establish the normal segmentation pattern of the larva. The *hedgehog* gene is also required during larval stages for the correct patterning of various imaginal cuticular structures in the adult.

The strong, presumed null phenotype caused by *hedgehog* mutations is consistent with the idea that all the cells of the ventral hypoderm adopt the same cell fate, namely that of the cells that produce the third or fourth denticle row of each segment. The fact that a large proportion of these denticles point toward the ventral midline indicates that these cells no longer sense the normal anteroposterior polarity of the animal. It seems likely, therefore, that the normal polarity of these cells is not defined by the global polarity of the animal, but rather derives from the comparison of "positional information" among adjacent cells. Because all cells of the segment possess the same "positional information" in a null *hedgehog* mutant, the

sense of an anteroposterior polarity at the cellular level is lost.

The results from mosaic studies of *hedgehog* mutations in adult antennae (and, to a lesser extent, legs) suggest that the *hedgehog* gene is required primarily in some portion of the distal posterior compartment and that the failure of *hedgehog* to properly act in the posterior compartment induces the additional loss of nonmutant distal structures of both the anterior and posterior compartments. In the head, no compartmental restriction boundary separates the ocellar region from the rest of the head (suggesting that the ocellar region is not in a separate posterior compartment, MORATA and LAWRENCE 1978, 1979), but BROWER (1986) found that the region of the imaginal disc fated to give rise to the ocellar region expressed *en*; *en* expression in thoracic discs corresponds to the limits of the posterior compartment in those discs (KORNBERG *et al.* 1985). Thus, it appears that the *hedgehog* requiring regions correspond roughly to posterior compartments (or *en* expressing domains) of several imaginal discs. It is tempting to generalize this conclusion to the embryonic segment to speculate that *hedgehog* is required primarily for the viability of cells in the posterior compartment (which corresponds to the posterior region of naked cuticle of each segment) and that the loss of either most or all of the cells of this compartment induces, respectively, either a mirror-image pattern or a uniform pattern of cell fates in the anterior region of each segment. The extreme reduction in the overall length of mutant *hedgehog* embryos is consistent with the inviability of a major portion of the cells of each segment.

The homology of the scutellar and genital defects to the leg and antennal defects is less obvious. The scutellum is divided by the anterior-posterior compartment boundary (like the distal leg and antenna), but lies in the proximal, not distal, portion of the disc (LAWRENCE and MORATA 1977). In the genitalia, a compartment boundary separates the genitalia from the analia (WIESCHAUS and NÖTHIGER 1982), but the homology of these compartments with the anterior-posterior compartments of the thorax and antenna is nebulous. Because no mutant clones were detected in association with either of these defects, it cannot be resolved whether these defects are the result of patches of mutant tissue or of radiation damage enhanced by a haplo-insufficiency for *hedgehog*. Therefore, while a posterior compartment requirement for the integrity of these structures is plausible, the lack of obvious homology to the leg and antenna defects lends no support for this idea.

Possible roles of *hedgehog* and related genes in development: Current models for the establishment of segmental pattern in *Drosophila* embryogenesis suggest that the blastoderm segment primordium is

subdivided into three or four domains, initially a single-cell wide, by the differential expression of segmental polarity genes (MEINHARDT 1986; DiNARDO *et al.* 1988). The alternating pattern of these domains thus defines the segmentation pattern of the larva. The establishment of this spatial expression pattern of segmental polarity genes is regulated by other segmentation genes (maternal, gap and pair-rule genes) and has been hypothesized to be directly defined by the combinatorial expression of particular pair-rule genes (DiNARDO and O'FARRELL 1987).

The expression patterns of three segmental polarity genes, *engrailed* (*en*), *wingless* (*wg*) and *gooseberry* (*gsb*) are consistent with this model. The *en* and the two *gsb* genes are expressed initially in single-cell wide stripes that are believed to be the precursors of the posterior compartments of imaginal structures (KORNBERG *et al.* 1985; BAUMGARTNER *et al.* 1987). The *wg* gene is also expressed in a single-cell wide stripe immediately anterior to the stripe of *en* expression (BAKER 1987). The interactions of the higher order gap and pair-rule genes to set up these segmental polarity domains is thought to rely, at least in part, upon diffusion among the transcriptional regulatory products of these genes between nuclei in the syncytial blastoderm.

After the initial spatial pattern of the various domains is established, errors in the initial establishment of the shape, size or orientation of the domains are believed to be regulated by interactions between cells of the various domains (the "editing" phase). Consistent with this hypothesis is the observation that the failure to express *wg* in its domain (as in *wg⁻* mutants) causes the premature decay of *en* expression in the adjacent domain (DiNARDO *et al.* 1988). Under this hypothesis, the segmental polarity genes should be subdivided into two classes: those which define the domain identity on a particular cell, and those which are involved in cell-cell communication of those identities. The *en* gene, because of the presence of a DNA binding, homeobox domain within the protein, is presumed to be a transcriptional regulator and fall into the former class (KORNBERG *et al.* 1985). The *wg* gene, because of its homology to the *int-1* oncogene of mouse expressed on the cell surface, is presumed to be in the latter class (RIJSEWIJK *et al.* 1987).

The regulative interactions among the segmental polarity genes seem likely to provide a general model for genetic regulation of patterning in cellular morphogenetic fields. Especially encouraging for this conclusion is the observation that many of the segmental polarity genes are utilized again for the patterning of the adult cuticle. Mutational effects in the adult cuticle have been observed for *fu* (LINDSLEY and GRELL 1968), *Cell* = *ci^P* (ORENIC, CHIDSEY and HOLMGREN 1987; LINDSLEY and GRELL 1968), *en* (KORNBERG

1981), *arm* (WIESCHAUS and NOELL 1986) and *wg* (BAKER 1988), as well as *hh* (see above). Remarkably, genes with a similar mutant embryonic phenotype often give a similar array of imaginal defects. For example, mutations in *hedgehog* and the majority of other segmental polarity genes (*fu*, *Cell*, *arm*, and *gsb*) are phenotypically similar, possessing deletions of naked cuticle in each segment and duplications of remaining denticle tissue in mirror-image arrays. Homozygous *fu* and heterozygous *Cell* animals are missing structures in the ocellar region and have a "bobbed" scutellum (LINDSLEY and GRELL 1968), defects that are seen in *hedgehog* and *armadillo* clones and heteroallelic *hedgehog* mutant animals (above; WIESCHAUS and NOELL 1986). Thus, mutations with a similar phenotype embryonically often have a similar phenotype with respect to adult cuticular defects, suggesting that they play a similar role in both embryonic and adult patterning.

In this regard it is worth contrasting the activities of *hedgehog* and *wingless*. Null mutations in both genes have identical phenotypes: a nonsegmental ventral lawn of denticles, with a strong tendency for the denticles to point toward the mid-line, rather than anteriorly or posteriorly (above; BAKER 1988). However, the temperature sensitive period for a temperature-sensitive mutant of *wingless* (*wg^{ts}*) was found to be between 3 and 11 hr of embryonic development (at 25° rate), whereas the *hedgehog* temperature sensitive mutation described here had a relatively tight temperature sensitive period from 3 to 6 hr of embryonic development (at 25°). While this difference in temperature sensitive periods may be due to the use of a weak temperature sensitive mutation for *hedgehog*, causing some activity periods where less activity is required not to be detected, mutant *wingless* animals resulting from a late shift to the restrictive temperature had a phenotype that was never observed for *hedgehog*: the broadening of the denticle bands without a polarity reversal (BAKER 1988). In the adult cuticle, no defects were found associated with *wingless* clones, although temperature shifts of *wg^{ts}* during the second larval instar did result in a similar array of imaginal defects as with *hedgehog* (the primary *wg* defect, like the primary *fu* and *Cell* defects in the wing were not found to be associated with the *hedgehog* mutation, BAKER 1988). More surprising is the observation that two genes with such similar mutant phenotypes are apparently required in two separate sets of cells. If the results from the *hedgehog* mosaic antennae can be generalized to the blastoderm, *hedgehog* gene function is required in the posterior compartment (the *en* expressing cells) and *wingless* is only expressed in the anteriorly adjacent strip of cells (BAKER 1987). Thus *wingless* appears to act generally

in the patterning of a similar set of structures to *hedgehog*, but in a different manner.

WIESCHAUS and RIGGLEMAN (1987) investigated the question of autonomy of a number of segment polarity genes (*arm*, *gsb*, *hh* and *wg*) causing mirror-image duplication of denticle arrays as seen for *hedgehog* in the larval epidermis. They asked whether small homozygous patches of mutant tissue generated by somatic recombination in the cells fated to develop naked cuticle would produce ectopic denticles, for which only *arm* patches would. These results in mosaic studies of embryonic cuticle are consistent with observations here and by BAKER (1988) on *hedgehog* and *wingless* mosaics in adult cuticle. The *wingless* product appears to act nonautonomously to maintain proper expression of subsegmental identity in the naked cuticle; a small patch of *wg* tissue can be compensated for by neighboring tissue. The *hedgehog* product appears to act intrinsically to posterior compartment cells to maintain their viability; loss of a small patch of *hh* tissue would not induce ectopic denticles.

One likely role for the function of the *hedgehog* gene in embryonic patterning is to act within the cells of the posterior compartment to compare the internalized identity of the cell (conferred presumably by the activation of the *en* gene) with the external environment. *hedgehog* is not likely to be a member of the subclass of segment polarity genes that define the subsegmental domain identity on the cell, because it appears to be necessary only for a limited period in morphogenesis and because clones of mutant *hedgehog* tissue do not adopt another identity (as is the case with *engrailed*) (KORNBERG 1981). This suggests that *hedgehog* is more likely to be somehow involved in the regulation of aberrant spatial pattern (the "editing") of subsegmental domains. A properly positioned posterior cell should receive a signal from the anteriorly adjacent cell (mediated presumably by *wingless*) and a signal from the posteriorly adjacent cell. An improperly positioned posterior cell (expressing *en*) would fail to receive one or both of those signals, resulting in a mismatch between internalized identity and external cues, which should result in death of that cell (or an adjusted identity). If the role of the *hedgehog* gene is to perceive the match, nonexpression of *hedgehog* would result in the wholesale loss of cells from the posterior compartment. The wholesale loss of the posterior compartment would cause in turn internal mismatches in the adjacent cells (unable to receive a signal from nonexistent posterior cells). This would result in the loss of many of the adjacent cells to produce presumably the ventral lawn of uniform cell fate or, in less drastic cases, the mirror-image pattern characteristic of *hedgehog*. Failure to express the *wg* gene in the anteriorly adjacent cells would have a similar initial effect: a perceived mismatch of posi-

tional cue and internalized identity of posterior cells. A critical test of this hypothesis will be the identification of those cells in which *hedgehog* is transcribed and active (i.e., the *en* expressing domain?). It is to be hoped that the rearrangement mutations described here will aid in the molecular identification of the *hedgehog* locus.

On the nature of nonautonomy: Both *hedgehog* and *wingless* act nonautonomously in imaginal discs, yet the nonautonomy of each is distinct. Homozygous *wingless* clones were phenotypically normal and were present in all parts of the fly at wild-type frequencies (BAKER 1987) indicating that the nonautonomy for *wingless* is "passive": loss of function in a small mutant patch can be compensated for by some portion of the nonmutant tissue. In contrast, homozygous *hedgehog* clones and cuticular defects were adjacent, suggesting that the original clone and the missing region overlapped. This is consistent with a "domineering" nonautonomy: loss of function in a small mutant patch results in inappropriate behavior of the cells in that patch and secondarily in neighboring, nonmutant tissue. While both types of nonautonomy require some form of cell-cell interaction, these two types of nonautonomy are fundamentally distinct. "Passive" nonautonomy suggests that the gene is active in many cells but sufficient activity is supplied by only a portion of those cells. This is consistent with the production by the gene product of a diffusible substance, that can "cross-feed" adjacent cells, where the gene product itself acts "nonautonomously." "Domineering" nonautonomy involves a localized region of gene activity that is necessary for the entire tissue. This is consistent with either a cascade of failed processes radiating from the initial failure within the localized region (where the initial defect is intrinsic to the cell, and the gene product itself acts "autonomously") or the production from a point source of a diffusible substance necessary for the remaining tissue (e.g., a morphogen).

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