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Localized enhancement and repression of the activity of the TGF-β family member, *decapentaplegic*, is necessary for dorsal-ventral pattern formation in the *Drosophila* embryo

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Summary

Seven zygotically active genes are required for normal patterning of the dorsal 40% of the *Drosophila* embryo. Among these genes, *decapentaplegic* (*dpp*) has the strongest mutant phenotype: in the absence of *dpp*, all cells in the dorsal and dorsolateral regions of the embryo adopt fates characteristic of more ventrally derived cells (Irish and Gelbart (1987) *Genes Dev.* 1, 868-879). Here we describe the phenotypes caused by alleles of another of this set of genes, *tolloid*, and show that *tolloid* is required for dorsal, but not dorsolateral, pattern. Extragenic suppressors of *tolloid* mutations were isolated that proved to be mutations that elevate *dpp* activity. We studied the relationship between *tolloid* and *dpp* by analyzing the phenotypes of *tolloid* embryos with elevated numbers of the *dpp* gene and found that doubling the dpp^+ gene dosage completely suppressed weak *tolloid* mutants and partially suppressed the phenotypes of *tolloid* null mutants. We conclude that the function of *tolloid* is to increase *dpp* activity. We also examined the effect of doubling dpp^+ gene dosage on the phenotypes caused by other mutations affecting dorsal development. Like *tolloid*, the phenotypes of mutant embryos lacking *shrew* gene function were suppressed by elevated *dpp*, indicating that *shrew* also acts upstream of *dpp* to increase *dpp* activity. In contrast, increasing the number of copies of the *dpp* gene enhanced the *short gastrulation* (*sog*) mutant phenotype, causing ventrolateral cells to adopt dorsal fates. This indicates that *sog* gene product normally blocks *dpp* activity ventrally. We propose that the *tolloid*, *shrew* and *sog* genes are required to generate a gradient of *dpp* activity, which directly specifies the pattern of the dorsal 40% of the embryo.

Key words: *decapentaplegic*, *tolloid*, TGF-β, dorsal-ventral pattern formation, *Drosophila*.

Introduction

The result of the cascade of maternal gene action that patterns the dorsal-ventral axis of the *Drosophila* embryo is a gradient of nuclear localization of the product of the *dorsal* gene (Rushlow et al., 1989; Roth et al., 1989; Steward, 1989). High levels of the *dorsal* protein are present in ventral nuclei, while the *dorsal* protein is excluded from nuclei on the dorsal side of the embryo. The protein product of the *dorsal* gene is homologous to the transcription factor NF-κB and the *rel* oncogene (Steward, 1987; Kieran et al., 1990; Ghosh et al., 1990) and appears to specify pattern by the differential transcriptional activation or repression of specific downstream zygotic genes (Ip et al., 1991; Thisse et al., 1991).

While the mechanisms of action of the maternal genes are known in some detail, much less is known about how the early acting zygotic genes fix and elaborate embryonic dorsal-ventral pattern. Seven zygotically active genes, *decapentaplegic* (*dpp*), *tolloid*, *screw*, *shrew*, *zerknüllt* (*zen*), *twisted gastrulation* and *short gastrulation* (*sog*), are required early in embryogenesis for the generation of normal pattern in the dorsal 40% of the embryo (Nüsslein-Volhard et al., 1984; Wieschaus et al., 1984; Jürgens et al., 1984; Wakimoto et al., 1984; Zusman and Wieschaus, 1985; Irish and Gelbart, 1987). At the syncytial blastoderm stage, the *dpp*, *tolloid* and *zen* genes are transcribed with apparent uniform intensity in the dorsal 40-50% of the embryo (St. Johnston and Gelbart, 1987; Shimell et al., 1991; Doyle et al., 1986; 1989), in the region where no *dorsal* nuclear protein is detectable. For *zen*, it has been demonstrated that dorsal-specific expression is caused by repression of *zen* transcription by the high levels of *dorsal* protein present in ventral nuclei (Rushlow et al., 1987; Ip et al., 1991). These results suggest that the gradient of *dorsal* protein may define the dorsal-ventral extent of the expression of the zygotic genes required for dorsal development, but may not

affect the level of transcription within the region where they are expressed. Thus, if the gradient of *dorsal* protein only specifies the spatial extent of the transcription of the zygotic genes required for dorsal development, the pattern within the dorsal region of the embryo must be specified by interactions among these zygotic genes, rather than directly by *dorsal*.

Embryos that lack the *dpp, tolloid, screw, shrew, zen* or *twisted gastrulation* genes undergo abnormal morphogenetic movements at the time of gastrulation, including failure of normal germ band extension, and subsequently differentiate larval cuticles that are missing characteristic subsets of dorsally and dorsolaterally derived cuticular structures. The gastrulation and cuticular defects of these mutants are similar to those observed in the ventralized embryos caused by a number of maternal effect mutations that ventralize the embryonic fate map (for example, see Rushlow and Levine, 1990; Rushlow and Arora, 1991). Since the early phenotypes of these mutations also appear to shift the fate map of the blastoderm embryo, we refer to them as zygotic ventralizing mutations.

The phenotypic and genetic analysis of the *sog* gene suggests that its mechanism of action is different from the six genes identified by zygotic ventralizing mutations. Although *sog* mutants also fail to undergo normal germ band extension, the dorsal-most pattern elements are not completely deleted in *sog* mutant embryos (Zusman et al., 1988), suggesting that *sog* mutations affect the blastoderm fate map differently than the zygotic ventralizing mutations. In addition, genetic mosaic studies showed that the *sog* gene is required in the ventral embryonic cells to allow normal dorsal development (Zusman et al., 1988), indicating that the *sog* product acts cell non-autonomously to influence the fates of dorsally derived cells.

In this paper, we describe genetic studies on mutations in the zygotic genes required for normal dorsal development that allow us to begin to order the function of these genes. By studying the phenotypes of *tolloid* mutant embryos and genetic interactions between *tolloid* and *dpp*, we conclude that the function of the *tolloid* gene product is to increase the activity of *dpp*. Studies on the relationship of the other zygotically active genes to *dpp* lead us to conclude that *dpp* plays the central role in organizing the pattern of the dorsal region of the *Drosophila* embryo and that the activity of the *dpp* gene product is differentially regulated over the dorsal-ventral axis of the embryo.

The *dpp* gene encodes a member of the TGF-β family of proteins (Padgett et al., 1987). Other TGF-β family members are believed to be involved in an array of developmental events in vertebrate embryos (Melton, 1991). Our results suggest that during vertebrate development the activity of TGF-β family members could be regulated by post-translational interactions with other gene products to organize the fates of fields of cells.

Materials and methods

Stocks and alleles

Fifteen *tolloid* alleles were isolated in the third chromosomal screen for zygotic pattern mutants (Jürgens et al., 1984; Tearle and Nüsslein-Volhard, 1986). Seven *tolloid* alleles, tld^{B1}, tld^{B2}, tld^{B3}, tld^{B4}, tld^{B6}, tld^{B7}, tld^{B8}, were isolated in various screens as zygotic lethal ventralizing mutations (K. V. A. and E. L. F., unpublished, P. Hecht and D. Morisato, personal communication). $tld^{68\text{-}62}$ is a P element-induced allele of *tolloid* (Shimell et al., 1991) that is a small deficiency that deletes the DNA encoding the *tolloid* transcript. *Df(3R)XTA1*, obtained from G. Jürgens, deletes bands 94C1-5 to 96B6-7. The *tolloid* alleles can be arranged in an allelic series from weak to strong as judged both by complementation behavior and by the progressive deletion of dorsally derived cuticular structures of the head and tail (see Fig. 3A): very weak, tld^{5H}, tld^{7M}; weak, incompletely penetrant, tld^{6B}, tld^{9K}; weak, completely penetrant, tld^{7O}, tld^{B8}, tld^{9D} at 29°C, tld^{9Q1}; moderate alleles, tld^{B3}, tld^{9Q7}, tld^{8L}, tld^{B1}, tld^{9B}, tld^{B7}, tld^{6P1}, tld^{6P4}, tld^{B2}; and apparent null alleles, tld^{10F}, tld^{7H}, tld^{B6}, $tld^{68\text{-}62}$, tld^{10E}, tld^{B4}. A P element that contained a 14 kb insert of genomic DNA surrounding the *tolloid* gene and that was integrated on the X chromosome was used to increase $tolloid^{+}$ gene dose (Shimell et al., 1991).

The recessive partial loss-of-function alleles of *dpp*, $dpp^{hin\text{-}r27}$ and $dpp^{hin\text{-}r4}$, the haploinsufficient null allele of *dpp*, dpp^{Hin48}, the deficiency of *dpp, Df(2L) DTD2*, and the dpp^{+} duplication, *Dp(2;2) DTD48*, are described either in Spencer et al. (1982) or in Irish and Gelbart (1987). *Dp(2;2) DTD48* is the smallest chromosomal duplication of the *dpp* region, and it contains the $dpp^{d\text{-}ho}$ mutation, a distal 3′ regulatory mutation that affects *dpp* expression in the wing disk. The dpp^{+} duplication, *In (2LR) CyO P20*, P[dpp^{Hin+}], is a P-element construct that has a 20 kb insertion including the *dpp* transcription unit and surrounding regulatory sequences and is inserted into the second chromosome balancer, CyO (R. Padgett, personal communication).

The two putative null alleles of shrew, srw^{B4} and srw^{B5}, isolated as zygotic lethal ventralizing mutations (P. Hecht and D. Morisato, personal communication) allelic to srw^{10K} (Jürgens et al., 1984), cause identical phenotypes that are stronger than any of the other four *shrew* alleles (data not shown). The phenotypes of these alleles are not enhanced in *trans* to a deficiency of the *shrew* gene, *Df (3L)* e^{13} (obtained from M. Simon), suggesting that these mutations completely eliminate *shrew* gene activity. However, since embryos homozygous for the two known *shrew* deficiencies do not differentiate identifiable cuticular structures, we cannot prove that the srw^{B4} and srw^{B5} mutations cause the complete absence of *shrew* activity.

The zen^{w36} allele appears to be null by phenotypic and molecular criteria (Wakimoto et al., 1984; Rushlow et al., 1987). Other alleles, zen^{MAS1}, zen^{f75} and zen^{f62} (M. Seeger, personal communication; Wakimoto et al., 1984), are partial loss-of-function alleles, as assayed by cuticular phenotype (Table 3).

The embryonic lethal alleles of *short gastrulation* are described in Wieschaus et al., 1984 and Zusman et al., 1988. Since *sog* maps on the X chromosome, allelism between these seven mutations had previously been assumed based on similarities of phenotype and map position. The weak, homozygous viable allele of *sog* that was isolated as a suppressor of the tld^{5H}/tld^{10E} phenotype (see Results) failed to complement all seven lethal *sog* mutations, confirming their allelism.

The *twisted gastrulation* alleles, tsg^{XB86} and tsg^{YN97}, are

described in Wieschaus et al. (1984) and the *screw* allele, scw^{1G76}, is described in Nüsslein-Volhard et al. (1984).

Phenotypic analysis

Cuticle preparations were done as described (Wieschaus and Nüsslein-Volhard, 1986). In addition to the criteria of general morphology and ventral denticle band width, the severity of *dpp*, *tolloid*, *shrew*, *screw*, *twisted gastrulation* and *zen* mutant phenotypes was determined by scoring mutant cuticles for the presence and/or morphology of a number of dorsal and dorsolateral cuticular structures of the head and tail derived from known locations in the blastoderm fate map, as shown in Fig. 3A (based on Jürgens et al., 1986 and Jürgens, 1987). To examine the ventral sides of the normally tail-up *sog* embryos, a 23 gauge needle was used to tear the dorsal cuticle in the mid abdominal region and each embryo was oriented in the Hoyer's - lactic acid mix to give a ventral view of its cuticle. So that the weight of the coverslip would not flatten the torn cuticles excessively, Sephadex G-50 beads were added to the mix.

tolloid *germ-line clones*

Germ-line clones for a *tolloid* null mutation were induced by mitotic recombination in the developing germ lines of females heterozygous for the *tolloid* mutation tld^{10Er1}. Females of genotype Tl^{10b}, *mwh e*/tld^{10Er1}, *ru h th st sr bar-3* were irradiated with 1000R in an Astrophysics Research Corporation (Long Beach, CA) X-ray source at the end of the first larval stage. Upon eclosion, these females were individually mated to males of genotype tld^{B4}, *th st cp in ri* p^{p}/*TM3, Sb* in egg-laying blocks, and the phenotypes of their eggs were examined. Tl^{10b} is a dominant maternal-effect mutation that causes an extreme ventralization of the embryonic pattern such that all cells in embryos derived from females heterozygous for the Tl^{10b} mutation adopt the fate of the ventralmost cells in the wild type and form mesoderm (Hudson, 1989). Because mesodermal cells do not secrete cuticle, in general no cuticular structures are visible in embryos derived from females carrying the Tl^{10b} mutation. If, however, a mitotic recombination event is induced in a cell within the developing germ line of a female heterozygous for the Tl^{10b} mutation, some descendants of that cell will be homozygous for the chromosome in *trans* to the Tl^{10b} mutation and can be recognized by their ability to produce cuticle.

Approximately 600 individual irradiated females were examined. Fourteen females produced some embryos (ranging from 2-7) with cuticular structures, which were the result of a recombination event in the female germ line. Of 54 embryos with cuticular structures, 25 (of presumptive genotype tld^{10Er1}/*TM3*) hatched, and 29 had a phenotype indistinguishable from tld^{10Er1}/tld^{B4} embryos derived from heterozygous parents, indicating the absence of a detectable maternal contribution to the *tolloid* phenotype.

Temperature-sensitive period of tld^{9D}

The onset of gastrulation, as evidenced by the formation of the ventral furrow, was used to precisely stage *tolloid* mutant embryos. To shift embryos prior to gastrulation, homozygous tld^{9D} flies were allowed to lay eggs on apple juice agar plates for two hours at either the permissive (18°C) or restrictive (30°C) temperature. The eggs were then transferred to a second plate at the opposing temperature, and all embryos that gastrulated within set intervals of time (every 15 minutes at 30°C and 30 minutes at 18°C) were put on separate plates. The developmental stage of the embryos at the time of the temperature-shift was calculated based on the amount of time from the temperature-shift until the onset of gastrulation. To shift embryos after the onset of gastrulation, tld^{9D} embryos were collected at 18°C for four hours. The embryos were observed at 18°C and those embryos that gastrulated during each 30 minute period were put on a separate plate. All embryos were then simultaneously transferred to the restrictive temperature. The developmental stage of the embryos at the time of the temperature-shift was calculated based on the amount of time from the onset of gastrulation until the temperature shift.

Reversion of tld^{10E} *and isolation of dominant extragenic suppressors of the* $tld^{5H/10E}$ *phenotype*

Mutagenized males of genotype tld^{10E} opa^{9C}, *h th st cu sr/TM1, kni Me* p^{p} were mated to virgin females of genotype tld^{5H} kni^{1ID}, *h sr e/TM1, opa Me* p^{p}. Balancers with zygotic lethal mutations *kni* and *opa* were isolated by K. V. A. All four progeny genotypes from this cross normally die as embryos. Rare adult survivors should carry a second site mutation in *tolloid* that eliminates the antimorphic activity of the tld^{10E} mutation, a dominant extragenic suppressor of the $tld^{5H/10E}$ mutant phenotype, or a suppressor of *odd paired* (*opa*) or *knirps* (*kni*). EMS mutagenesis was performed according to the protocol of Lewis and Bacher (1968), using 40 mM EMS. For X-ray mutagenesis, males were irradiated with 4000 R. For P element mutagenesis, a P-cytotype stock of genotype $\pi 2$; *ru h th st cu sr e Pr ca/TM1, kni* was constructed. Males from this stock were mated to females from the M-cytotype strain tld^{10E} opa^{9C}, *h th st cu sr/TM1*. The dysgenic F_1 progeny males of genotype tld^{10E} opa^{9C}, *h th st cu sr/TM1 kni* were mated to the females described above. Approximately 25,000 EMS treated F_1 zygotes, 50,000 X-ray treated F_1 zygotes and 150,000 hybrid dysgenic zygotes were tested. Zygotes that survived to adulthood were scored for visible markers and only the $tld^{5H/10E}$ flies (*h sr*) were analyzed further. Adult flies of other genotypes appeared to be rare homozygous *opa* escapers or flies in which mutations on the original chromosomes had recombined with the *TM1* balancer chromosome.

From 554 candidates, 16 lines were recovered in which more than 10% of the zygotes of genotype $tld^{5H/10E}$ survived to adulthood. By segregation analysis, six lines carried a third chromosomal suppressor, nine lines carried a second chromosomal suppressor, and one line carried an X chromosome suppressor. All third chromosomal suppressors mapped within 2% of the *tolloid* locus and therefore were most likely second-site mutations within *tolloid* that eliminated the antimorphic activity of the tld^{10E} allele. Based on 42 recombinants between *vermilion* (*v*) and *forked* (*f*), the X chromosomal suppressor mapped at 53.3 map units.

The nine second chromosomal dominant extragenic suppressors of the $tld^{5H/10E}$ mutant phenotype (*12D, 26A, 26B, 28A, 35A, 39A, 39D, 44F, 89D*) appear to be mutations in *dpp* that elevate the activity of the gene or duplications of a region of the chromosome including the *dpp* transcript. All nine second chromosomal suppressors suppressed the haplolethality of a *dpp* null allele. Based on the suppression of *dpp* haplolethality, seven of the suppressors (*26A, 26B, 28A, 35A, 39A, 39D, 44F, 89D*) were mapped between *al* (0.0) and *dp* (13.0) (*dpp*=4.0). Two suppressor chromosomes (*12D* and *28A*) had chromosomal breaks in 22F, near *dpp* (22F2-3). Three suppressor strains had imaginal phenotypes like those associated with known alleles of *dpp* (Spencer et al., 1982; St. Johnston et al., 1990). The wings of *12D*/dpp^{Hin48} flies (dpp^{Hin48} is a haploinsufficient allele of *dpp*; Irish and Gelbart, 1987) were heldout. *39D*/dpp^{Hin48} flies often had a central gap in the cuticle covering their notum (cleft notum) a defect associated with certain mutations in the 3′ regulatory

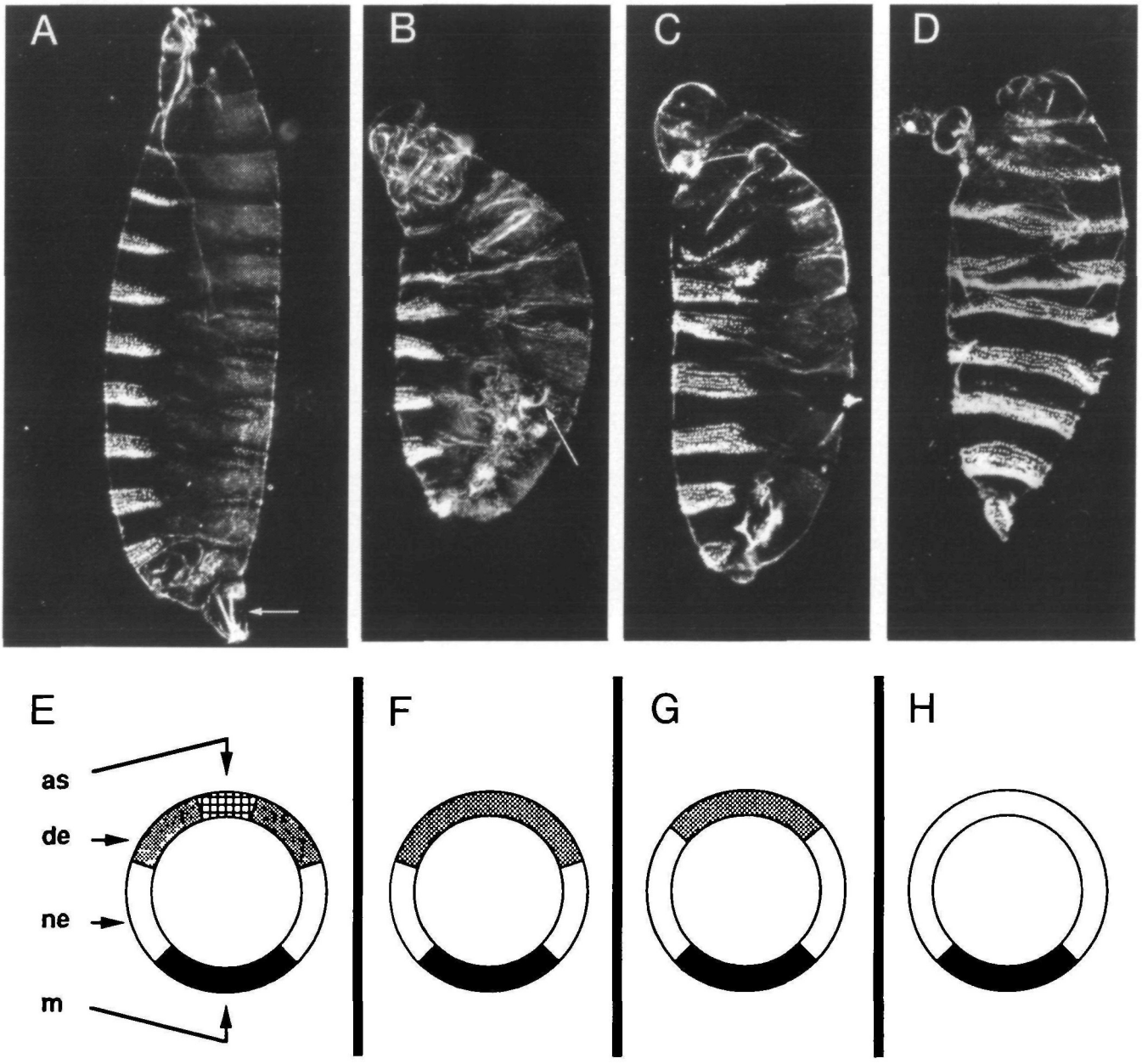
region of *dpp*. Flies homozygous for the suppressor mutation *39A* often had a terminal gap at the end of the L4 longitudinal wing vein (shortvein), a defect characteristic of mutations in the 5′ regulatory region of *dpp* (Segal and Gelbart, 1985). By Southern analysis (Maniatis et al., 1982), four suppressor lines (*12D, 35A, 39A, 39D*) had rearrangements in the 48 kb of the *dpp* gene surrounding the *dpp* transcript (+67kb to +115 kb on the molecular map of *dpp*; St. Johnston et al., 1990; kindly provided by M. Hoffmann). In three of the four lines, a single extra band was observed when probed with DNA from the 5′ (*12D, 35A, 39A*) or 3′ (*35A, 39A*) regulatory regions, which would be consistent with a duplication of the *dpp* coding region and part of the surrounding regulatory region of the gene. We hypothesize that those suppressor strains that displayed imaginal disk phenotypes characteristic of *dpp* alleles contained chromosomal rearrangements that both duplicated the coding region of *dpp* and disrupted the *cis*-regulatory regions of the gene.

Results

The phenotypes of zygotic ventralizing mutations

Zygotic ventralizing mutations cause the loss of a subset of structures normally derived from the cells in the dorsal 40% of the blastoderm embryo (Fig. 1). In the wild-type embryo, the dorsal-most blastoderm cells give rise to the extraembryonic amnioserosa, which does not contribute to the final cuticular structures of the larva, but is necessary for proper morphogenetic movements immediately after gastrulation (Lohs-Schardin et al., 1979). Most embryonic cells from 90 to 60% egg circumference (where 100% egg circumference is the dorsal midline) form the dorsal epidermis, characterized in cuticle preparations by a lawn of fine dorsal hairs.

Null mutations in six genes, *dpp, tolloid, screw, shrew, zen* and *twisted gastrulation*, delete different



subsets of dorsal structures. The absence of *dpp* causes the most severe phenotype: these embryos lack the amnioserosa and the entire dorsal epidermis, which are replaced in the larval cuticle by ventral denticle belts that encircle the embryo (Fig. 1D, H; Irish and Gelbart, 1987). In embryos lacking *tolloid* or *screw* activity, the amnioserosa and some, but not all, of the dorsal epidermal structures are deleted (Fig. 1C, G; Arora and Nüsslein-Volhard, 1991). The partial loss of dorsal structures in *tolloid* and *screw* embryos is accompanied by an expansion of the neurogenic ectoderm, as evidenced by an increase in the dorsal extent of each denticle band. Mutant embryos that lack *shrew, twisted gastrulation* or *zen* activity are missing the amnioserosa (Fig. 1 B, F; Zusman and Wieschaus, 1985; Wakimoto et al., 1984). Partial loss-of-function mutations in *dpp* fall into an allelic series in which weak alleles delete the amnioserosa, like null alleles of *shrew, twisted gastrulation* or *zen*, while stronger alleles delete the same set of structures as null alleles of *tolloid* and *screw* (Fig. 1 legend; K. Wharton, R. Ray and W. Gelbart, personal communication).

In contrast to *zen*, *shrew* and *twisted gastrulation* mutations, which affect only the amnioserosa, *tolloid* mutations delete both the amnioserosa and part of the dorsal epidermis, suggesting that the *tolloid* gene product does not control differentiation of a particular cell type, but rather must be involved in organizing the overall pattern within the dorsal region of the embryo. We therefore chose to analyze the *tolloid* mutant phenotypes in detail. These studies led us to investigate interactions between alleles of *tolloid* and ventralizing mutations in other genes.

Characterization of tolloid *gene action*

The temperature-sensitive period (TSP) of a temperature-sensitive *tolloid* allele, tld^{9D}, corresponds to the one hour immediately preceding gastrulation (Fig. 2). This suggests that wild-type *tolloid* gene activity is required for normal patterning only during the cellularization of the blastoderm. The *tolloid* mutant phenotype is visible within 15 minutes after the end of the TSP: at that time in *tolloid* embryos the normally lateral head fold begins to appear on the dorsal side, suggesting that the dorsal cells of *tolloid* embryos are behaving like the lateral cells of the wild-type embryo. The same shift in head fold position has been seen in *dpp* and *zen* mutants (Irish and Gelbart, 1987; Rushlow and Levine, 1990). Subsequently, *tolloid* embryos do not extend their germ bands fully, as the dorsal-most cells of the embryo fail to form the squamous epithelial sheet characteristic of the amnioserosa.

Fig. 1. Darkfield cuticle patterns and fate maps of wild-type embryos (A, E) and of embryos homozygous for apparent null alleles of the zygotic ventralizing genes *shrew* (B, F), *tolloid* (C, G) and *decapentaplegic* (D, H). (A) Wild-type embryo cuticle. Anterior up and dorsal to the right. In the thoracic and abdominal regions of the embryo, the ventral epidermis is characterized by a repeating pattern of denticle bands. The dorsal epidermis is characterized by a lawn of fine dorsal hairs. Cuticular structures in the head and tail of the wild-type larva that are derived from specific dorsal-ventral regions of the blastoderm fate map are particularly useful in defining the regions deleted in the zygotic ventralizing mutants (Fig. 3A). The arrow points to one such structure, the dorsolaterally derived filzkörper of the tail. (B) Cuticle of a srw^{B5}/srw^{B4} embryo. Embryos mutant for *shrew* lack amnioserosa tissue. Because of this defect, the germ band moves into the interior of the embryo, thereby causing the abnormal position and morphology of the filzkörper (arrow). *shrew* embryos lack all components of the head skeleton except the vertical arm, which remains external due to a failure in head involution. *shrew* embryos retain all external cuticular structures of the head, the cirri, mouthhooks, antennal sense organ and, in a majority of embryos, the maxillary sense organs. Embryos that are homozygous for null alleles of *zen* or *twisted gastrulation* or embryos that are heterozygous for a null mutation of *dpp* have similar phenotypes (Wakimoto et al., 1984; Zusman and Wieschaus, 1985; Irish and Gelbart, 1987; data not shown). (C) A $tld^{B4}/tld^{68\text{-}62}$ cuticle. Null *tolloid* embryos lack both the amnioserosa and some, but not all, of dorsal epidermal structures. There is an increase in the left-right extent of the ventral denticle bands, indicating an expansion in the ventral epidermal anlage. *tolloid* embryos lack all dorsally and some dorsolaterally derived cuticular specializations of the head, including all structures of the head skeleton, the maxillary sense organs, the antennal sense organs, the cirri and, in about 50% of the embryos, the mouthhooks. In the posterior, approximately two-thirds of *tolloid* null embryos lack the dorsolaterally derived filzkörper. Embryos homozygous for a strong *screw* allele or the partial loss-of-function allele of *dpp*, $dpp^{hin\text{-}r27}$, have cuticular phenotypes similar to embryos that lack *tolloid* activity (Arora and Nüsslein-Volhard, 1992; data not shown). (D) The cuticle of an embryo lacking *dpp* activity, $dpp^{Hin48}/Df\ (2L)\ DTD2$. In the absence of *dpp*, the cells that would normally generate the amnioserosa and dorsal epidermis instead give rise to structures characteristic of the more ventral neurogenic ectoderm (Irish and Gelbart 1987), so that bands of ventral denticles encircle the embryo. *dpp* null embryos also lack all dorsally and dorsolaterally derived cuticular specializations of the head and tail. (E-H) Blastoderm fate maps of the thoracic and abdominal regions of wild-type and mutant embryos. The changes in the fate map of mutant embryos were deduced from a combination of their cuticular phenotypes, their patterns of gastrulation and the altered expression of region specific markers. In *tolloid* and *shrew* mutant embryos, the dorsal-most cells of the embryo, which would normally form the amnioserosa, express a marker characteristic of the dorsal epidermis, supporting the hypothesis that the dorsal-most embryonic cells adopt dorsal epidermal fates in these embryos (data not shown). (E) Wild-type embryo. At the blastoderm stage, the embryo is subdivided into four discrete regions: the amnioserosa, from 100 to 90% of egg circumference (hatched); dorsal epidermis, from 90 to 60% egg circumference (stippled); neurogenic ectoderm, from 60 to 25% egg circumference (white); and mesoderm, from 25 to 0% egg circumference (black). (100% egg circumference defines the dorsal midline of the embryo.) (F) Fate map of embryos lacking *shrew* activity. (G) Fate map of embryos lacking *tolloid* activity. (H) Fate map of embryos lacking *dpp* activity.

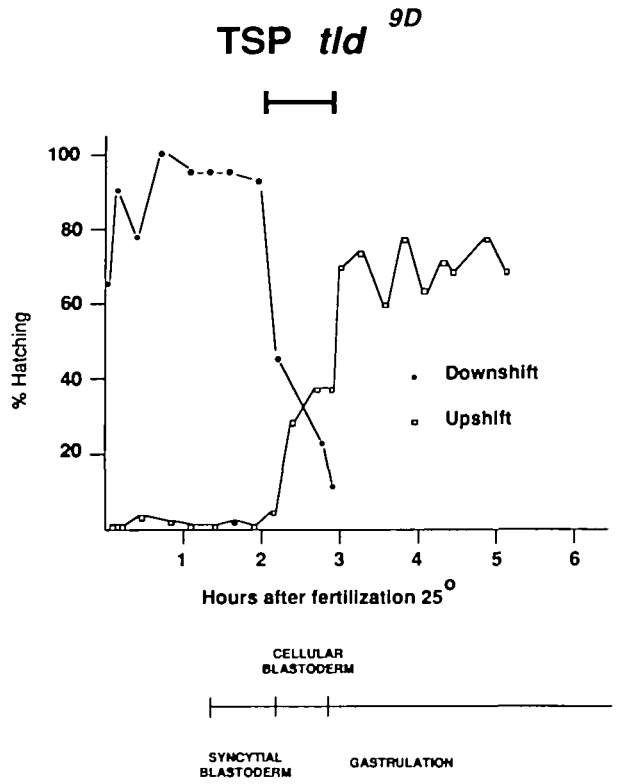


Fig. 2. The temperature-sensitive period of tld^{9D}. For each time point, from 20 to 60 embryos were hand-shifted as described in Materials and Methods from either the permissive to the restrictive temperature (open squares) or from the restrictive to the permissive temperature (filled circles). All developmental times were normalized to 25°C. The temperature-sensitive period begins at two hours after fertilization, the first time at which embryos shifted from the restrictive to the permissive temperature displayed a more severe phenotype than embryos raised at the permissive temperature. Similarly, the temperature-sensitive period ends at three hours after fertilization, the last time embryos shifted from the permissive to the restrictive temperature displayed a more severe phenotype than embryos raised at the permissive temperature. This period of time precisely coincides with the time of cellularization of the blastoderm.

The larval cuticular patterns of differentiated *tolloid* embryos lack a portion of the dorsal epidermis, as seen by the increased width of the ventral denticle bands. We wished to determine the exact regions within the dorsal epidermis that were affected by loss of *tolloid* activity; however, the thoracic and abdominal regions of the dorsal epidermis do not contain scorable cuticular markers that define different dorsolateral positions. Instead, we determined the severity of the phenotypes of *tolloid* mutants by scoring the specialized cuticular structures of the head and tail derived from stereotyped dorsal-ventral positions on the blastoderm fate map (Jürgens et al., 1986; Jürgens, 1987) (Fig. 3A). By these criteria, six of the 23 alleles examined resulted in as strong a mutant phenotype as does a deficiency of the *tolloid* locus and thus are likely to encode mutant polypeptides with little or no activity (Fig. 3A, Table 1, data not shown).

The remaining 17 *tolloid* mutations could be ordered in an allelic series (Table 1 and data not shown) in which weaker alleles (Fig. 3B) deleted only the most dorsally derived structures, while stronger alleles deleted dorsally and dorsolaterally derived structures (compare the dorsal-ventral extents of the top and bottom lines in Fig. 3A, which represent the structures deleted in weak and strong *tolloid* mutants, respectively). Thus, like *dpp*, *tolloid* is a component of a process that is more active in the dorsal-most regions of the embryo than in the dorsolateral regions.

Because lack of *tolloid* activity in the zygote deletes only a portion of the dorsal epidermis, it was possible that the maternal component of $tolloid^{+}$ gene activity partially rescues the zygotic mutant phenotype. To test this hypothesis, female germ cells that lacked *tolloid* activity were produced by inducing mitotic recombination in the developing germ line of females heterozygous for a *tolloid* null mutation (Materials and Methods). The lack of *tolloid* activity in the maternal germ line did not enhance the severity of the *tolloid* mutant phenotype (compare Fig. 3C to Fig. 1C), indicating that there is no detectable maternal contribution to *tolloid* gene function in the developing embryo.

The *tolloid* gene is unusual among the zygotic lethal pattern genes because of a complex pattern of complementation among alleles (Jürgens et al., 1984). We repeated the complementation matrix and found that part of the apparent complexity of this pattern was caused by the incomplete penetrance of the four weakest *tolloid* alleles: a percentage of embryos carrying each of these alleles in *trans* to a *tolloid* deficiency survived to adulthood (Table 2 and data not shown).

The remaining complexity of the complementation pattern could be accounted for by the observation that three alleles, tld^{9Q1}, tld^{6P4} and tld^{10E}, behaved more strongly than a deficiency of the *tolloid* locus. For instance, fewer than 2% of the zygotes carrying any of these three alleles in *trans* to the weak alleles tld^{5H} or tld^{7M} survived to adulthood, while 20-35% of the zygotes carrying tld^{5H} or tld^{7M} in *trans* to a *tolloid* deficiency survived (Table 2). These three *tolloid* alleles also failed to complement a recessive, partial loss-of-function *dpp* allele: embryos carrying one copy of both mutations died with a partially ventralized phenotype (Table 2 and data not shown). In contrast, embryos carrying a deficiency of the *tolloid* locus and the same *dpp* allele were fully viable, again indicating that the three *tolloid* alleles behaved more severely than a *tolloid* deficiency. The antimorphic (Muller, 1932) behavior of the tld^{9Q1}, tld^{6P4} and tld^{10E} mutations could be explained if their products block the remaining activity of the other allele, either by binding to the product of the other allele or by competing with that allele for association with the product of another gene required for the same process, possibly *dpp*.

To test whether the tld^{10E} allele did in fact encode an

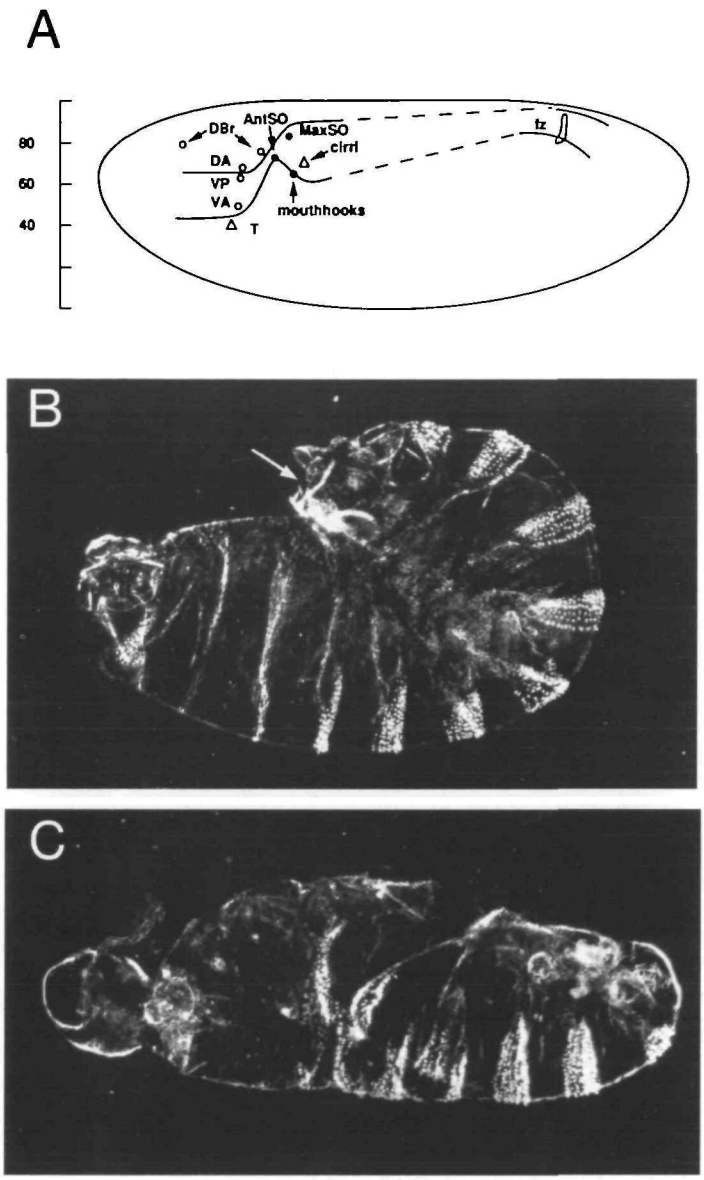


Fig. 3. Phenotypes of *tolloid* mutant embryos. (A) Fate map of the blastoderm embryo (adopted from Jürgens et al., 1986; Jürgens, 1987) showing the positions of some dorsally and dorsolaterally derived cuticular structures affected by *tolloid* mutations. All structures above the bottom line are deleted in embryos lacking *tolloid* activity, while only those structures above the top line are deleted in embryos homozygous for a weak *tolloid* mutation, tld^{6B}. Because there are no obvious cuticular markers that define specific dorsal-ventral domains within the thoracic and abdominal regions of the dorsal epidermis, the effects of *tolloid* mutations on these regions of the embryo could not be determined precisely and are represented by dotted lines. Internal head skeleton: VA, vertical arm; VP, ventral plate; DA, dorsal arm and DBr, dorsal bridge. External cuticular structures of the head: MaxSO, maxillary sense organ; AntSO, antennal sense organ; Mh, mouthhooks; and Ci, cirri. Tail structure: fk, filzkörper. (B) Cuticle of an embryo homozygous for a weak *tolloid* mutation, tld^{6B}. In this embryo, the head is abnormal due to the loss of some components of the internal pharyngeal skeleton and a failure of head involution during late embryogenesis. The filzkörper (arrow) are of normal morphology, but are mispositioned due to a failure of germ band retraction. (C) Cuticle of an embryo lacking *tolloid* activity of genotype tld^{10Er1}/tld^{B4} derived from a female germ cell homozygous for the tld^{10Er1} mutation (see Materials and Methods for experimental details). The cuticular phenotype of this embryo is identical to that of a *tolloid* embryo of similar genotype derived from female germ cells heterozygous for another *tld* null mutation (Fig. 1C), indicating that there is no detectable maternal rescue of the *tolloid* phenotype.

antimorphic product that interfered with normal dorsal development, we carried out a genetic selection to recover revertants that eliminated antimorphic activity of the tld^{10E} allele. If a second mutation were introduced into the tld^{10E} allele and eliminated all activity of the tld^{10E} gene product, the resulting double mutant allele should behave less severely in genetic crosses than the tld^{10E} gene product. For example, although only 0.25% of the embryos of genotype tld^{5H}/tld^{10E} survive, 20-35% of tld^{5H}/Df embryos survive to adulthood. Thus, if a second mutation that eliminated all *tolloid* activity were introduced into the tld^{10E} mutant allele, it should allow survival in *trans* to tld^{5H}. Using this as the basis of a selection scheme (Materials and Methods), we recovered 16 lines from 225,000 mutagenized F_1 zygotes in which more than 10% of the $tld^{5H}/tld^{10E(*)}$ zygotes survived. One of the revertant lines contained a large deletion encompassing the *tolloid* locus, indicating that the elimination of the antimorphic *tolloid* product leads to a less severe phenotype. The newly induced mutations in five of the other lines were inseparable from the original tld^{10E} mutation (see Materials and Methods) and most likely are second site mutations within *tolloid* that inactivate the gene. In all six cases, the second site mutation eliminated the antimorphic activity of the tld^{10E} mutation both in interactions with weak *tolloid* alleles (the basis on which these mutations were selected) and in interactions with the *dpp* allele (Table 2 and data not shown).

Extragenic suppressors of tolloid *are mutations in* dpp *and* short gastrulation

The selection for intragenic revertants of the tld^{10E} mutation relied upon the fact that reversion of the antimorphic activity of the tld^{10E} mutation resulted in the survival of the $tld^{5H}/tld^{10E(*)}$ flies to adulthood. However, this selection scheme could also be used to identify dominant extragenic suppressors of the tld^{5H}/tld^{10E} mutant phenotype. In fact, in ten suppressor lines the mutations responsible for the survival of the tld^{5H}/tld^{10E} embryos were not linked to the *tolloid* locus and therefore defined dominant, extragenic suppressors of *tolloid*. Nine of these suppressors mapped to the second chromosome, and one suppressor mapped to the X chromosome. Because extragenic suppressors of a *tolloid* mutant could define genes that also affected dorsal development, we analyzed these mutations further.

In the course of characterizing the second chromo-

Table 1. *Phenotypes of* tolloid *mutants used in this study*

tolloid allele	Penetrance of mutant phenotype	Number counted	Expressivity of mutant phenotype: % mutant embryos with						Number of embryos
			Pharyngeal skeleton		Sense organs		Filzkörper		
			VP	VA	MaxSO	AntSO	present	normal morphology	
5H	63	1079	48	97	67	85	100	85	54
7M	65	875	42	85	66	98	100	92	59
6B	98	529	44	94	67	85	100	82	51
7O	100	577	3	32	35	67	100	56	63
9D at 29°	100	628	0	13	26	65	100	15	46
9Q1	100	449	0	2	20	56	90	2	60
6P4	100	483	0	0	2	31	77	0	49
10F	100	496	0	0	0	23	60	0	43
10E	100	489	0	0	0	33	59	0	63
B4	100	499	0	0	0	31	37	0	154
68-62	100	377	0	0	0	38	31	0	64
Df(XTA1)	–	–	0	0	0	31	41	0	54

The penetrance of the tld^{5H} and the tld^{7M} mutations was calculated as the ratio of the number of embryos that displayed a mutant phenotype to the number of embryos homozygous for the *tolloid* mutation, which was calculated as one quarter of the total number of embryos counted. For the remaining alleles, the penetrance of the mutation was determined as the percentage survival to adulthood of zygotes carrying the *tolloid* allele in *trans* to a deficiency of the locus, compared to balancer siblings. The expressivity of the mutant phenotype indicates the percentage of phenotypically mutant embryos in each strain that differentiated the dorsally or dorsolaterally derived cuticular structure indicated in the Table. Thus, a low expressivity of dorsally derived structures in *tolloid* mutant embryos is indicative of a stong mutant phenotype. Abbreviations are as in Fig. 3A.

Filzkörper present: percentage of embryos that differentiated any filzkörper material.

Filzkörper normal morphology: percentage of embryos that had two filzkörper of normal morphology. Filzkörper that did not have a tube-like morphology or that did not connect to the exterior of the embryo were not counted as normal.

Table 2. *Percentage survival to adulthood of zygotes carrying selected* tolloid *alleles either in* trans *to weak* tolloid *alleles,* tld^{5H} *and* tld^{7M}*, or heterozygous with a recessive, partial loss-of-function allele of* dpp, $dpp^{hin\text{-}r27}$*, compared to balancer siblings*

gene	allele	tolloid allele							
		antimorphic			null			revertants	
		9Q1	6P4	10E	10F	Df(68-62)	Df(XTA1)	10Er1	10Er2
tolloid	5H	1	1	0.3	27	19	34	22	31
	7M	2	0	0	26	28	20	18	21
decapentaplegic	hin-r27	14	8	0	108	91	100	108	99

Antimorphic alleles of *tolloid* behaved more severely than a deficiency of the *tolloid* locus in these crosses. Null alleles caused the complete absence of *tolloid* gene activity by phenotypic (tld^{10F}), molecular ($tld^{68\text{-}62}$), or cytological (*Df(3R) XTA1*) criteria. Revertants of tld^{10E} were recovered by selecting for survival in *trans* to tld^{5H} (see Materials and methods).

somal suppressors, we found that they could all suppress the haplolethality of null alleles of *dpp* (Irish and Gelbart, 1987), suggesting that they act either by elevating *dpp* activity or by partially bypassing the requirement for *dpp*. Although we have not characterized these suppressors in great detail, a variety of criteria suggested that most, if not all, of these mutations elevated *dpp* activity, possibly by duplicating the region of the *dpp* gene that included the open reading frame (see Materials and Methods).

To determine whether an elevation in *dpp* gene dosage was sufficient to suppress the tld^{5H}/tld^{10E} mutant phenotype, we constructed *tolloid* mutant strains carrying either of two known duplications of the dpp^+ gene, *Dp(2;2) DTD48*, which duplicates dpp^+ and 8 known complementation groups (W. Gelbart, personal communication) or *In (2LR) CyO P20*, P[dpp^{Hin+}], a P element construct with a 20 kb insert that includes the *dpp* transcription unit and surrounding regulatory sequences (R. Padgett, personal communication). Increasing the number of copies of the *dpp* transcription unit from two to three by introduction of either duplication resulted in a degree of suppression of the tld^{5H}/tld^{10E} mutant phenotype that was similar to the degree of suppression that we observed with the chromosome two suppressors (data not shown). Thus, we consider it likely that some or all of the chromosome two suppressors contain a duplication of the coding region of the *dpp* gene.

The results of other experiments indicated that an elevation of dpp^+ gene dosage could suppress a variety of weak *tolloid* alleles. We constructed *tolloid* mutant strains that were homozygous for *Dp(2;2) DTD48* and observed that doubling the dosage of the dpp^+ gene

completely suppressed the phenotypes of three weak *tolloid* alleles, tld^{5H}, tld^{6B} and tld^{7O}. In each case, fewer than 2% of the homozygous *tolloid* embryos with four copies of dpp^+ had a ventralized phenotype, compared to 60-100% ventralized embryos with two copies of dpp^+. In addition, the degree of suppression was proportional to the dosage of the dpp^+ gene: for several weak, completely penetrant *tolloid* allelic combinations, three copies of dpp^+ allowed some *tolloid* zygotes to survive to adulthood, while four copies of dpp^+ promoted greater survival (data not shown). Thus, the higher the gene dosage of dpp^+, the less *tolloid* activity was required.

The suppression of weak *tolloid* mutations by duplications of dpp^+ indicated that the two gene products are required for the same process, but did not order their activities relative to one another. To try to order the activities of *dpp* and *tolloid*, we tested whether an elevation in the dosage of dpp^+ could suppress the phenotype resulting from complete absence of *tolloid* gene activity. We reasoned that if *dpp* were required for the activity of *tolloid* or if *dpp* and *tolloid* were required independently for dorsal development, an elevation in dpp^+ activity would not suppress the phenotype of a *tolloid* null embryo. Conversely, if *tolloid* functioned to increase *dpp* activity, an elevation in dpp^+ gene dosage could compensate for the loss of all *tolloid* activity. For two different *tolloid* deficiencies, *Df (3R) XTA1* and $tld^{68\text{-}62}$, and one phenotypically null allele, tld^{B4}, the fraction of *tolloid* embryos that differentiated cuticular structures of the head and tail derived from dorsolateral positions on the blastoderm fate map increased with a doubling of the dpp^+ dosage. For example, the percentage of *tolloid* null embryos in which some filzkörper material was observed in the tail of the embryo went from 31-41% of the embryos with two copies of dpp^+ to 90-94% with filzkörper with 4 copies of dpp^+ (at least 50 embryos of each genotype scored). Similarly, the fraction of *tolloid* null embryos differentiating the antennal sense organs of the head more than doubled with a doubling of the dosage of dpp^+. We then asked whether an increase in $tolloid^+$ gene dosage could suppress a *dpp* mutant phenotype by constructing strains containing a *dpp* mutation and a P element insertion with *tolloid* genomic DNA (Shimell et al., 1991). We found that while doubling of the dose of $tolloid^+$ could suppress a very weak *dpp* mutant phenotype, a doubling of $tolloid^+$ gene dosage did not modify the phenotype caused by $dpp^{hin\text{-}r4}$, a partial loss-of-function allele of *dpp* that resulted in a phenotype less severe than null alleles of *tolloid* (data not shown). Because an increase in the dosage of the dpp^+ gene can partially bypass the requirement for *tolloid*, but an increase in *tolloid* gene dosage does not suppress a *dpp* mutant phenotype, we conclude that a normal action of *tolloid* is to elevate the activity of *dpp*.

The remaining extragenic suppressor of *tolloid* mapped to the X chromosome. The X-linked suppressor did not suppress the haplolethality of a *dpp* null allele, and therefore was not a transposition of a wild-type copy of the *dpp* gene to the X chromosome. The suppressor mapped to within 0.1 cM of the gene *short gastrulation* (*sog*) (Wieschaus et al., 1984; Zusman and Wieschaus, 1985). The suppressor mutation, which itself was homozygous viable, failed to complement all seven lethal *sog* alleles: only 1.5 - 10% of flies carrying the suppressor in *trans* to any of the lethal *sog* alleles survived to adulthood. Thus, by the criteria of mapping and failure of complementation, the suppressor is a partial loss-of-function allele of *sog*. In addition, we found that a lethal *sog* allele acted as a dominant suppressor of the *tolloid* heteroallelic combination used in the reversion selection: 12% of the zygotes of genotype tld^{5H}/tld^{10E}; sog^{YS06}/+ survived to adulthood. Other weaker, but still completely penetrant, *tolloid* heteroallelic combinations were more strongly suppressed by one copy of sog^{YS06} (data not shown). Thus the *sog* gene, while it has a different mutant phenotype than the zygotic ventralizing mutations (Zusman and Wieschaus, 1985), appears to be a component of the same pathway promoting dorsal development as *tolloid*.

shrew, zen *and* screw *act in the same pathway as* dpp

The suppression of the phenotype of *tolloid* mutants by an increase in the dosage of dpp^+ suggested that we could use increased *dpp* gene dosage to order the action of other zygotic ventralizing genes relative to *dpp*. Apparent null alleles of *shrew* (see Materials and Methods) cause completely penetrant phenotypes similar to those caused by weak alleles of *tolloid*. In *shrew* mutant embryos, the dorsally derived structures of the head skeleton are absent, and the dorsolaterally derived filzkörper of the tail are present, but are abnormal in morphology (Table 3). In *shrew* mutant embryos with four copies of the dpp^+ gene, there was a restoration of the dorsally derived components of the head skeleton and the filzkörper were of normal morphology (Table 3). Since a doubling of the dose of dpp^+ suppressed the phenotypes of putative null alleles of *shrew*, we conclude that *shrew*, like *tolloid*, functions upstream of *dpp* to increase *dpp* activity.

The homeobox-containing *zen* gene is required for the production of the amnioserosa cells. After the initial broad dorsal expression of *zen* defined by *dorsal*, *zen* expression becomes confined at the cellular blastoderm stage to the presumptive amnioserosa cells (Doyle et al., 1986; 1989). One of *dpp*'s functions in patterning the dorsal region of the embryo appears to be to activate *zen* transcription at this later stage (Rushlow and Levine, 1990) We found that doubling the dosage of dpp^+ had no effect on the phenotype caused by a strong, apparently null allele of *zen* (Table 3), which is consistent with *zen* acting downstream of *dpp*. In contrast, doubling the dosage of dpp^+ partially or completely suppressed the phenotypes caused by three weak alleles of *zen* (Table 3). This suppression of weak *zen* alleles suggests that the components of the *dpp* signal transduction pathway can transmit a doubling of the dosage of the dpp^+ gene to increase the activity of a downstream target gene.

The two remaining zygotic ventralizing genes, *screw* and *twisted gastrulation*, could not be ordered unam-

Table 3. *Suppression of the ventralizing phenotypes of* shrew *and* zerknüllt *mutants by increasing* dpp$^+$ *gene dosage*

Gene	Allele (strength)	Number of copies of dpp$^+$	Penetrance of mutant phenotype	Number of embryos	Expressivity of mutant phenotype: % mutant embryos with pharyngeal skeleton: VP	MaxSO	filzkörper: normal morphology	Number of embryos
shrew	B4 (null)	2	104	549	4	76	31	55
		4	101	509	82	98	91	44
	B5 (null)	2	95	565	4	72	23	53
		4	96	567	78	90	88	58
zerknüllt	MAS1 (weak)	2	29	492	97	97	91	32
		4	4	457	–	–	–	–
	F75 (moderate)	2	81	703	39	89	91	44
		4	56	380	96	100	100	56
	F62 (moderate)	2	101	398	82	75	80	44
		4	57	395	100	92	100	64
	W36 (null)	2	98	1079	0	45	4	60
		4	100	414	0	46	12	57

The penetrance of each mutant genotype was calculated as the ratio of the number of embryos that displayed a mutant phenotype to the number of embryos homozygous for the ventralizing mutation, which was inferred as one quarter of the total number of embryos counted. The expressivity of the mutant phenotype indicates the percentage of phenotypically mutant embryos in each strain that differentiated the dorsally or dorsolaterally derived cuticular structure indicated in the Table. An increase in the expressivity of dorsally derived structures is indicative of a suppression of the mutant phenotype by elevation of *dpp*$^+$ gene dose. Phenotypic characterizations as in Table 1. Greater than 95% of the embryos listed in this Table differentiated antennal sense organs, the vertical arm of the pharyngeal skeleton and some filzkörper material.

biguously relative to *dpp* with this test. The *screw* gene was originally defined by a single partial loss-of-function allele, *scw*^{IG76} (Nüsslein-Volhard et al., 1984), that produces a weak ventralization of the embryonic pattern. The *scw*^{IG76} phenotype was completely suppressed by two extra copies of *dpp*$^+$ (data not shown), indicating that *screw* is also likely to function in the same process as *dpp*. However, since we did not determine whether null alleles of *screw* were suppressed by duplications of *dpp*$^+$, we cannot conclude whether *screw* acts upstream or downstream of *dpp*. Two putatively null mutants of *twisted gastrulation*, which have a phenotype similar to *shrew* mutants, were not suppressed by extra copies of the *dpp*$^+$ gene (data not shown), suggesting that *twisted gastrulation* acts downstream of, or in parallel with, *dpp*.

Increasing dpp *in a* sog *background affects patterning in the neurogenic ectoderm*

While doubling the gene dosage of *dpp*$^+$ suppressed the phenotype of zygotic ventralizing mutations in four different genes, extra copies of the *dpp*$^+$ gene enhanced the phenotype of *sog* mutants, causing profound alterations in the cuticular pattern of the neurogenic ectoderm. We observed that in most *sog* mutant embryos with four copies of *dpp*$^+$ the ventral denticle bands were completely absent. Since doubling the dose of the *dpp*$^+$ gene in *sog*$^+$ embryos does not cause alterations in the embryonic cuticle, this phenotype indicated that the *sog* gene is required to inhibit *dpp* activity in the ventral regions of the embryo, and that ectopic *dpp* activity interferes with normal patterning in the neurogenic ectoderm.

To characterize the loss of ventral cuticular structures, we quantitated the left-right extent of the first and second abdominal denticle bands in wild-type embryos and in *sog* mutant embryos with two or three copies of the *dpp*$^+$ gene (Table 4). We found that, compared to wild-type embryos (Fig. 4A), there was a reduction in the width of the denticle bands in *sog* mutants (Fig. 4B), and that an additional copy of the *dpp*$^+$ gene in *sog* mutant embryos led to a further reduction in this width (Fig. 4C). This narrowing of the neurogenic ectoderm was accompanied by an expansion of the dorsal epidermis, as measured by a decrease in the left-to-right distance of the ventral-most dorsal hairs (data not shown), indicating that cells that would normally contribute to the ventral ectoderm were transformed to dorsal ectodermal fates.

We also observed cuticular defects within the neurogenic ectoderm in *sog* mutant strains. The Keilin's organs are paired sensory structures located at stereotyped positions within the neurogenic ectoderm of the three thoracic segments. We found that the distance between the Keilin's organs was reduced in *sog* embryos compared to the wild type and that increasing *dpp* dosage in *sog* embryos caused a further reduction in this distance (Table 4 and Fig. 5). These results suggest that in a *sog* mutant background, elevating *dpp*$^+$ gene dose causes a ventral shift in the dorsal-ventral position of cuticular pattern elements within the neurogenic ectoderm and that the extent of this shift is correlated with the number of copies of the *dpp*$^+$ gene.

The *sog* embryos with extra copies of dpp^+ also appeared to have another patterning defect within the neurogenic ectoderm. For example, many *sog* embryos with four copies of the dpp^+ gene had Keilin's organs but completely lacked the second and third thoracic denticle bands (Fig. 5B), suggesting that only ventral ectodermal structures at a particular anterior-posterior position within each segment were completely deleted. These phenotypes suggest that, in addition to *dpp*'s effect on dorsal-ventral patterning in these embryos,

Table 4. *Phenotypic effects of increasing* dpp^+ *gene dosage in embryos mutant for the* sog *gene*

Genotype	Number of copies of dpp^+	Distance between pairs of Keilin's organs in segments		Number of denticles in the fifth row of the denticle band in segments		Number of embryos
		T2	T3	A1	A2	
wild type	2	22 ± 1	32 ± 1	53 ± 3	52 ± 4	21
sog^{YS06}	2	19 ± 2	23 ± 2	40 ± 4	38 ± 4	17
	3	16 ± 3	17 ± 3	22 ± 7	23 ± 2	18
	4	9 ± 4	10 ± 3	ND	ND	19
sog^{XM42}	2	20 ± 3	25 ± 3	38 ± 9	42 ± 6	33
	4	11 ± 3	9 ± 3	ND	ND	20
sog^{YL26}	2	21 ± 4	30 ± 3	50 ± 5	44 ± 6	33
	3	16 ± 3	22 ± 3	33 ± 7	29 ± 4	20
	4	11 ± 4	13 ± 5	17 ± 7	14 ± 6	29

An eyepiece micrometer marked in arbitrary units was used to measure the left-right distance between the pairs of Keilin's organs in the second (T2) and third (T3) thoracic segment. Embryos lacking any of the Keilin's organs in T2 and T3 were not counted. The left-right extent of the denticle bands in the first (A1) and second (A2) abdominal segments was measured by counting the number of denticles in the fifth row in each band. N.D., not determined because most embryos lacked any denticle bands in the abdominal segments, possibly as a result of a segment polarity defect caused by the ectopic *dpp* activity within the neurogenic ectoderm. In embryos of genotype sog^{YL26}; *Dp(2;2) DTD48*, over half of the embryos had denticle bands in the first and second abdominal segments and were therefore included here.

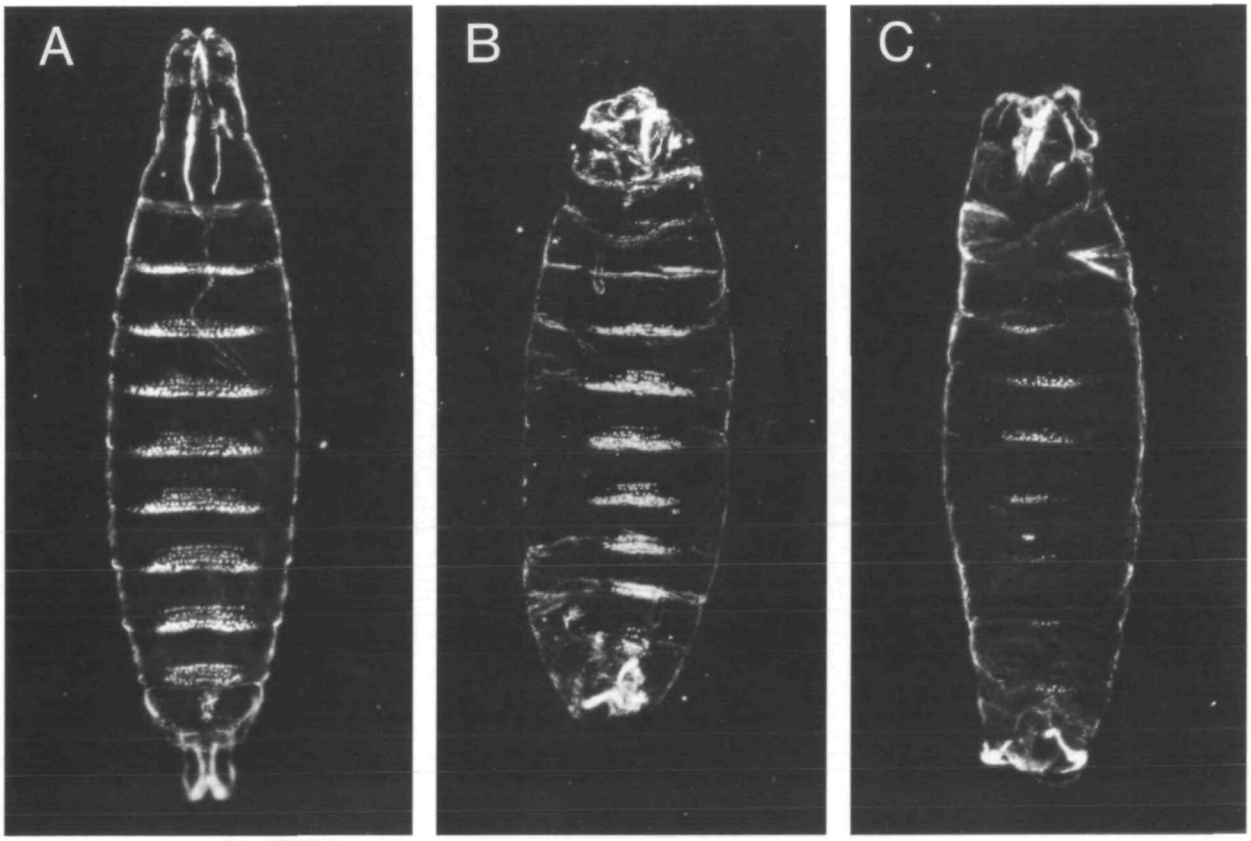


Fig. 4. Effects of increasing the dosage of the dpp^+ gene on the ventral denticle band morphology of *sog* mutant embryos. (A) A ventral view of a wild-type embryo, anterior up. (B) A ventral view of an embryo mutant for sog^{YL26}. (C) A ventral view of an embryo of genotype sog^{YL26}; *Dp(2;2) DTD48*/+. The extra copy of the dpp^+ gene causes a large reduction in the left-right extent of each denticle band, suggestive of a decrease in the size of the neurogenic ectoderm.

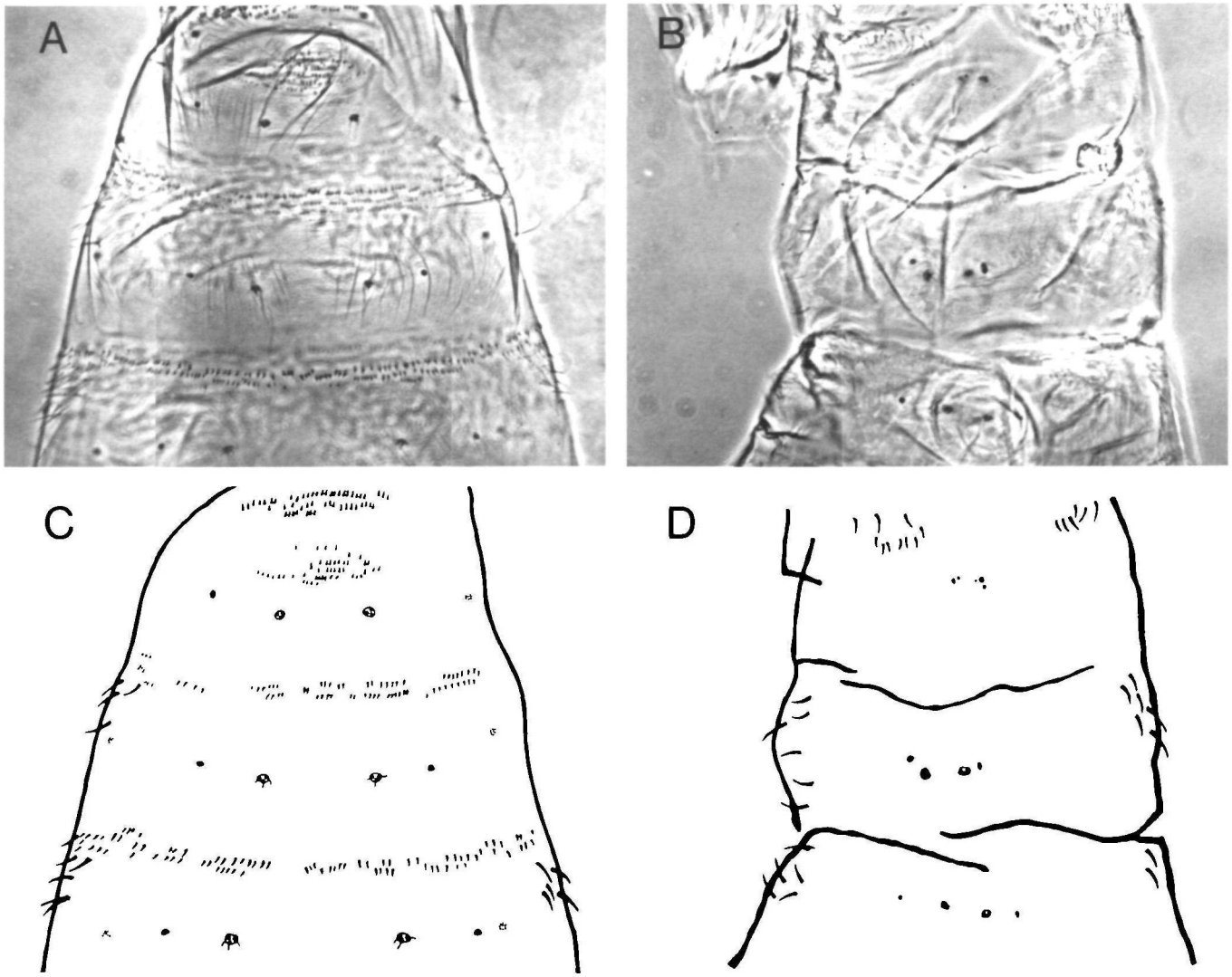


Fig. 5. Effect of increasing the dosage of the dpp^+ gene in a *sog* mutant background on patterning within the neurogenic ectoderm, as shown by the positioning of the Keilin's organs and the ventral black dots. (A and C) A phase contrast photomicrograph (A) and drawing (C) of a ventral view of a wild-type cuticle showing the Keilin's organs and ventral black dots in the three thoracic segments. In each thoracic segment, the two Keilin's organs, which are each composed of three hairs, are located at stereotyped positions on either side of the ventral midline. The ventral black dots are positioned more laterally than are the Keilin's organs. (The stippled dots in the drawing represent the lateral black dots, which are out of the plane of focus on the opposite side of the embryo.) (B and D) A photomicrograph (B) and drawing (D) of a ventral view of a cuticle from an embryo homozygous for sog^{Ys06} and for the dpp^+ duplication *Dp(2;2) DTD48*. The distance between the two Keilin's organs in each of the three thoracic segments is greatly reduced compared to the wild-type distance, indicating a loss of ventral-most pattern elements of the neurogenic ectoderm. In the embryo in the photograph, there are no ventral denticle bands in the second and third thoracic segments. We propose that the lack of denticle bands in *sog* mutant embryos with four copies of the dpp^+ gene reflects a segment polarity defect, in which the region within each segment that produces the denticle bands is transformed to produce naked cuticle.

ectopic *dpp* activity in the ventral epidermis causes a segment polarity defect such that the cells that normally produce the denticle bands within each segment are transformed to produce naked cuticle.

Discussion

This analysis and the work of Irish and Gelbart (1987) indicate that *dpp*, a member of the TGF-β family of proteins, plays a central role in patterning the dorsal region of the *Drosophila* embryo. *dpp* is the only gene whose activity is required for the production of all dorsal structures. We have found that a two-fold increase in dpp^+ gene dosage has profound effects on dorsal-ventral patterning in various mutant backgrounds. From these observations, we conclude that at least three other genes required for dorsal development act by regulating *dpp* activity.

tolloid *and* shrew *potentiate* dpp *activity dorsally*

Two genes required for normal dorsal development,

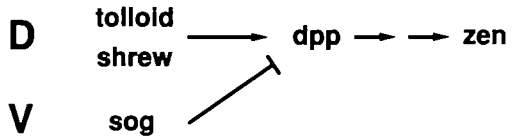


Fig. 6. Relationships among the genes required for specification of fate in the dorsal regions of the embryo. *tolloid* and *shrew* elevate *dpp* activity post-transcriptionally in the dorsal regions of the embryo. Both *tolloid* and *dpp* are expressed in the dorsal 40% of the embryo at the syncytial blastoderm stage (St. Johnston and Gelbart, 1987; Shimell et al., 1991); however, it is not clear whether *tolloid* activates *dpp* uniformly over the region in which they are co-expressed. The *sog* gene product lowers *dpp* activity within the ventral epidermis. Since reduction in *sog* activity also affects the fates of the dorsal and dorsolateral cells (Zusman et al., 1988; Rushlow and Levine, 1990), *sog*'s effect on *dpp* is not confined to the ventral regions of the embryo. We postulate that the interactions between the *tolloid*, *shrew*, *sog* and *dpp* gene products are necessary to generate a gradient of *dpp* activity, which provides positional information to pattern the dorsal regions of the embryo. In this model, high levels of *dpp* activity in the dorsal-most 10% of the embryo activate *zen* transcription at the cellular blastoderm stage. The activation of *zen* must be mediated by unidentified gene products that transduce the extracellular *dpp* signal to the nucleus.

tolloid and *shrew*, act by increasing the activity of *dpp* in the dorsal regions of the embryo (Fig. 6). Doubling the gene dosage of dpp^+ can partially suppress the phenotypes caused by null mutations in both *tolloid* and *shrew*. Since increased *dpp* gene dosage can compensate for the absence of *tolloid* or *shrew*, we conclude that *tolloid* and *shrew* function upstream of *dpp* to increase *dpp* activity. Our experiments do not allow us to conclude that the only functions of *tolloid* and *shrew* are to elevate *dpp* activity, since doubling the gene dosage of dpp^+ does not completely suppress null alleles of either gene, and it will be interesting to test whether further elevation in *dpp* activity completely bypasses the requirement for both genes.

The sequence of the *tolloid* gene (Shimell et al., 1991) indicates that *tolloid* is likely to elevate *dpp* activity at a post-translational level. The *tolloid* protein is 41% identical over 620 amino acid residues to BMP-1, one of a group of bone morphogenesis proteins (BMPs) isolated as components of a protein complex that can induce ectopic bone morphogenesis in rats (Wang et al., 1988; Wozney et al., 1988; Celeste et al., 1990). The remaining BMPs are TGF-β family members, and one BMP, BMP-2, is 75% identical in its active carboxy terminus to the *dpp* protein (Wozney et al., 1988). Since the mammalian homologues of the *tolloid* and *dpp* proteins appear to be present in a complex, we postulate that the *tolloid* and *dpp* proteins are physically associated. Furthermore, this result raises the possibility that *tolloid*-like proteins could be involved in the signal-generating pathways used by other TGF-β family members.

The sequence of the *tolloid* protein provides a possible explanation for the relatively frequent isolation of antimorphic alleles of the gene. The predicted product of the *tolloid* gene has an N-terminal domain similar to a zinc-binding metallo-protease and a C-terminal domain that contains EGF repeats and consequently may be involved in protein-protein interactions (Shimell et al., 1991). If *tolloid* acts as a protease, then the antimorphic alleles of *tolloid* could encode products that are able to form a complex with other proteins (perhaps including *dpp*), but render that complex inactive because they fail to cleave *tolloid*'s substrate.

sog *inhibits* dpp *activity ventrally*

In contrast to *tolloid* and *shrew*, the product of the *short gastrulation* (*sog*) gene appears to regulate *dpp* activity negatively (Fig. 6). While a twofold increase in the dosage of the dpp^+ gene has no effect on the dorsal-ventral pattern of sog^+ embryos, the addition of extra copies of the dpp^+ gene to *sog* mutant embryos causes multiple pattern abnormalities in the cuticular structures derived from the neurogenic ectoderm. These phenotypes indicate that one of the functions of the *sog* gene is to inhibit *dpp* activity in the ventral regions of the embryo. The ventral activity of *sog* observed in these experiments is consistent with the genetic mosaic data of Zusman et al. (1988) that the *sog* gene is required ventrally for embryonic survival.

Increasing the concentration of *dpp* activity in a *sog* mutant background appears to cause a greater number of cells to adopt dorsal fates. When we re-examined the cuticular defects of *sog* mutant embryos with two copies of the dpp^+ gene, we observed similar, although much less severe, defects in these mutant embryos, indicating that in the wild-type embryo sog^+ must inhibit *dpp* activity in the neurogenic ectoderm. Since the effects of *sog* mutations are visible at the time of germ band extension, we infer that *sog* inhibits *dpp* activity in the neurogenic ectoderm prior to or at the beginning of gastrulation. Because the initial transcription pattern of the *dpp* gene in the dorsal 40% of the embryo is not altered in *sog* mutants (Ray et al., 1991), the action of *dpp* on the cells of the neurogenic ectoderm of *sog* mutants must arise post-transcriptionally and be cell nonautonomous. We propose that in *sog* embryos the secreted *dpp* protein diffuses to ventral regions of the blastoderm embryo where it drives some ventrally located cells to differentiate along a dorsal epidermal pathway.

There are several possible mechanisms by which the *sog* gene product could inhibit *dpp* activity ventrally. The *sog* gene product could bind to and inactivate the *dpp* protein or it could inactivate any component of the *dpp* signal transduction pathway. Alternatively, the *sog* gene product could itself provide positional information in the ventral regions of the embryo, and the final fates of cells could reflect the local ratio of the activities of the *sog* and *dpp* gene products.

A dorsal-ventral gradient of dpp *activity*

Small increases or decreases in dpp^+ gene dosage can cause opposite shifts in the choice of cell fate along the

dorsal-ventral continuum of the embryo. A 50% decrease in *dpp* gene dose is sufficient to cause the most dorsally located cells to adopt a more ventral fate (Irish and Gelbart, 1987). Conversely, in a *sog* background, a 50% elevation in *dpp* gene dose is sufficient to cause laterally located cells to adopt a more dorsal fate. Thus, the level of *dpp* activity can act as a developmental switch to specify cell fate, with high levels of *dpp* activity necessary for dorsal development and low levels of *dpp* activity necessary for ventral development.

The phenotypic analysis of partial loss-of-function alleles of *dpp* indicate that *dpp* is a component of a graded patterning process that is more active in the dorsal-most regions of the embryo than in the dorsolateral regions (Irish and Gelbart, 1987; Fig. 1 legend; K. Wharton, R. Ray and W. Gelbart, personal communication). Because *dpp* plays the central role in this patterning process and because of the sensitivity of cells to incremental changes in dpp^+ gene dose, we propose that the graded requirement for *dpp* within the dorsal epidermis reflects an actual gradient of *dpp* activity. In this case, a high level of *dpp* activity in the dorsal region of the embryo specifies the amnioserosa, intermediate levels of *dpp* activity promote dorsal epidermal differentiation, and a lower level of *dpp* activity specifies the border between the dorsal epidermis and neurogenic ectoderm.

It is unclear what genes provide dorsal-ventral positional information within the neurogenic ectoderm (Ferguson and Anderson, 1991) and it is possible that a gradient of *dpp* activity could also contribute to positional information in that part of the embryo. In *sog* embryos with extra copies of the dpp^+ gene, the ventral shift in the border between the dorsal epidermis and neurogenic ectoderm is accompanied by the loss of the ventral-most pattern elements of the neurogenic ectoderm, as measured by a decrease in the left-right distance between the Keilin's organs. We conclude that positional information emanating from the dorsal region of the embryo influences, directly or indirectly, patterning within the neurogenic ectoderm. Thus it is possible that a gradient of *dpp* activity could provide positional information for patterning the entire dorsal 75% of the embryo.

If a gradient of *dpp* activity exists, it is likely to arise post-transcriptionally, since the *dpp* gene appears to be uniformly transcribed in the dorsal 40% of the syncytial blastoderm (St. Johnston and Gelbart, 1987). The data presented here suggest a gradient of *dpp* protein or activity could be produced by post-translational interactions between *dpp* and the products of other genes. We have shown that the *tolloid*, *shrew*, *sog* and possibly *screw* genes are all required for normal levels of *dpp* activity. Because we have found that *sog* inhibits *dpp* activity ventrally, we propose that the effect of *sog* mutations on dorsal and dorsolateral cell fates (Zusman et al., 1988; Rushlow and Levine, 1990) are also caused by *sog*'s effect on *dpp* activity. The global and nonautonomous effects of *sog* mutations make *sog* the most attractive candidate for a gene that functions in the formation of a gradient of *dpp* activity.

TGF-β family members and dorsal-ventral patterning in flies and frogs

Recently, the activins, TGF-β family members, have been shown to have dramatic effects on patterning the dorsal-ventral axis of both *Xenopus* (Thomsen et al., 1990; Smith et al., 1990; van den Eijnden-Van Raaij et al., 1990) and chick (Mitrani et al., 1990) embryos. Two groups have observed two different kinds of effect of the activins on early *Xenopus* embryos. Thomsen et al. (1990) found that exposure of regions of explanted animal cap to homogeneous concentrations of mammalian activin caused the production of embryoids with axial patterning, suggesting that an inherent polarity present in the animal cap is revealed upon induction by activin. In contrast, Green and Smith (1990) found that exposure of dissociated animal cap cells to activin caused these cells to adopt different fates according to the activin concentration, showing that activin can act as a dose-dependent morphogen. These two distinct interpretations of the role of activin in the patterning process could be reconciled if whole explants of animal cap tissue contain localized factors, possibly similar to the gene products that we have described here, that enhance or inhibit the activity of the applied activin to create a gradient of activin activity. We find it attractive to suppose that TGF-β family members act as graded morphogens to pattern the dorsal-ventral axis in both insects and vertebrates and that post-translational regulation is crucial in the establishment of activity gradients of TGF-β molecules.

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