Proximal–distal pattern formation in *Drosophila*: cell autonomous requirement for *Distal-less* gene activity in limb development

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Communicated by H. Jäckle

Limb development in the *Drosophila* embryo requires a pattern-forming system to organize positional information along the proximal–distal axis of the limb. This system must function in the context of the well characterized anterior–posterior and dorsal–ventral pattern-forming systems that are required to organize the body plan of the embryo. By genetic criteria the *Distal-less* gene appears to play a central role in limb development. Lack-of-function *Distal-less* mutations cause the deletion of a specific subset of embryonic peripheral sense organs that represent the evolutionary remnants of larval limbs. *Distal-less* activity is also required in the imaginal discs for the development of adult limbs. This requirement is cell autonomous and region specific within the developing limb primordium. Production of genetically mosaic imaginal discs, in which clones of cells lack *Distal-less* activity, indicates the existence of an organized proximal–distal positional information in very young imaginal disc primordia. We suggest that this graded positional information may depend on the activity of the *Distal-less* gene.

Key words: *Drosophila*/limb development/*Distal-less* gene

Introduction

Genetic analysis of pattern formation in *Drosophila* has identified many of the genes required for the organization of axial polarity in the embryo. The embryonic anterior–posterior (AP) and dorsal–ventral (DV) axes are established by independent sets of maternally acting genes (reviewed by Nüsslein-Volhard *et al.*, 1987; Anderson, 1987; Ingham, 1988) and are elaborated through the action of the maternal genes on subordinate zygotic genes (e.g. Schröder *et al.*, 1988; Driever and Nüsslein-Volhard, 1989). The AP and DV systems are required early in embryogenesis and the pattern that they produce is molecularly detectable in the cellular blastoderm (reviewed by Ingham, 1988). The blastoderm is a single layered epithelium and so is formally a two-dimensional sheet requiring only two axes of polarity. This represents the developmental situation of primitive annelid-like organisms which are segmented but do not develop limbs. Development of the more advanced organisms, like insects, requires in addition to AP and DV pattern-forming systems a third zygotic pattern-forming system to provide proximal–distal positional information for limb development.

The expression of proximal–distal pattern formation is not morphologically obvious in the embryos of higher dipteran insects like *Drosophila* since the larvae have only rudimentary limbs. Consequently, the need for this pattern-forming system in the embryo has been overlooked. Since the larvae of simpler insects do possess well developed limbs (Kellogg, 1902; Anderson, 1972a,b) we can take advantage of the information provided by comparative morphological studies to identify the evolutionary homologues of these structures in *Drosophila*. For example, the Keilin's organs of the thoracic segments correspond to the distal sensory apparatus of the legs of lower insects (Keilin, 1915–16). In this context it is clear that the basic pattern-forming processes required for limb organization must function in the *Drosophila* embryo.

The segmented appendages of insects are believed to have evolved from the simple unsegmented structures found in their annelid-like ancestors. Segmented appendages are considered to be composed of two evolutionarily distinct domains: a proximal domain, homologous to the unsegmented appendages of annelids, and a distal domain which is considered to be the true limb (Snodgrass, 1935). The distal domain is further subdivided into segments in a variety of ways to allow the development of functionally distinct articulated appendages such as legs, mouth parts and antennae (see Kellogg, 1902). Consequently, the true limb may be considered as an evolutionary specialization super-added to the more primitive annelid-type appendage. If we take the view that acquisition of a new developmental quality during evolution requires the functions of new genes that must act within the context of pre-existing systems, we expect the need for a gene that promotes the development of limb structures above a developmental ground state. We report here that by genetic and morphological criteria the *Distal-less* gene of *Drosophila* appears to fulfill this function.

Results

In order to determine the consequences of completely eliminating the activity of the gene we must identify complete lack-of-function (amorphic) alleles. A number of *Distal-less* (*Dll*) alleles are homozygous lethal (Table I). Some features of several of these alleles have been described previously under the name *Brista* (Sato, 1984; Sunkel and Whittle, 1987). Many of the lethal alleles are associated with cytologically visible chromosomal aberrations and share a common breakpoint in the cytogenetic locus 60E5,6. The allele MP is a cytologically visible deficiency that deletes the *Dll* transcription unit and is therefore an amorphic allele (Cohen *et al.*, 1989). In order to be certain that the MP phenotype is not due to the absence of additional genes contained within the deficiency, we have examined the phenotypes of other *Dll* mutants (Table I). All of the other lethal alleles including both X-ray-induced (e.g. SA1) and ethylmethane sulphonate-induced apparent point mutants

(e.g. 5) produce the same embryonic phenotype as MP, whether as homozygotes or as transheterozygotes. These results indicate that the cuticular phenotype caused by the deficiency MP (shown in Figure 1) reflects the consequences of removing activity at only the *Dll* locus.

Embryonic lack-of-function phenotype

The *Drosophila* larva has rudimentary limbs, although these are not so morphologically well developed as the limbs in simpler insects (e.g. caterpillar legs, see Figure 4C and N). In the absence of *Dll* gene activity these vestigial larval limbs are deleted. The anatomical and evolutionary relationships among these structures are clearest in the thoracic segments. The Keilin's organs of the *Drosophila* larva are homologous to the distal sensory apparatus of the larval appendages of simpler insects and have been shown to be directly associated with the developing leg imaginal disc primordia (Keilin, 1915–16; see Figure 4C). These sense organs therefore are the rudimentary legs of the *Drosophila* embryo. In *Dll*[−] embryos the three sensory hairs of the Keilin's organs are deleted (Figure 1E,F). Note that the position at which the sense organ should develop is still visible in the embryonic cuticle, which suggests that the mechanism used by the embryo to position the developing limbs with respect to the other body axes is still active in the mutant. The capacity of the mutant embryo to activate the development of the larval limb in response to this signal appears to be impaired.

The sense organs are segmentally repeated structures which acquire unique identities through the action of the homeotic selector genes (reviewed in Akam, 1987). The antennal sense organ is developmentally homologous to the Keilin's organs of the thoracic segments and represents the vestigial larval antenna. Similarly, the maxillary sense organ represents the maxillary appendage of the larval mouth parts. The antennal and maxillary sense organs are deleted in mutant embryos (Figure 1A,B). The labial and labral sense organs represent the labial and labral appendages of the larval mouth parts and are also deleted in the mutant embryos (Figure 1C,D and G,H). More detailed descriptions of these structures and their relationships are presented in the legend to Figure 1. In weaker, homozygous viable, mutant alleles of

Dll corresponding defects can be found in all of the adult limbs that are developmentally homologous to the larval limbs (Cohen and Jürgens, in preparation).

The larval limbs of *Drosophila* are rudimentary structures of which only the distal sensory apparatus remains (e.g. Keilin, 1915–16). The defect in *Dll*[−] embryos is specific for limb development and does not reflect a generalized defect in the development of the peripheral nervous system (PNS) since other peripheral sense organs are not affected in *Dll*[−] embryos (see Figure 1). Sense organs that are not associated with developing limbs are normal in the mutant embryos. Moreover staining of the nervous system with a monoclonal antibody (22C10, Zipursky *et al.*, 1985) shows no developmental abnormality in the organization of the PNS. All of the sensory nerve cells that innervate the affected sense organs are present in the mutant embryos [the ves cells for the Keilin's organs (Ghyssen *et al.*, 1986) and the dorsal and terminal organs for the antennal and maxillary sense organs (Campos-Ortega and Hartenstein, 1985)] which indicates that only the epithelial portions of the sense organs are affected (data not shown).

Localized requirement for *Dll* activity in adult limbs

The adult limbs of *Drosophila* are formed by imaginal discs which begin to develop in the embryo (Madhavan and Schneiderman, 1977). Since the *Dll*[−] amorphic condition is homozygous lethal early during larval development (Figure 1 legend) it is not possible to assess directly the developmental potential of an imaginal disc that completely lacks *Dll* activity. It is possible, however, to generate genetically mosaic imaginal disc primordia in which clones of homozygous *Dll*[−] cells propagate in a background of heterozygous cells. Genetically mosaic disc primordia were produced in first instar larvae by X-ray-induced mitotic recombination. The developmental potential of the genetically marked clones of cells can be assessed by analysing the distribution of their progeny in the resulting genetically mosaic adult structure. Based on this analysis we conclude that *Dll* activity is not required in the abdomen, the dorsal thorax or the genitalia since *Dll*[−] clones are as frequent as wild-type control clones and differentiate

Fig. 1. Complete lack-of-function embryonic phenotype. The amorphic condition is typified by the allele MP shown here in panels B, D, F and H. Wild-type structures are shown in panels A, C, E and G. A,B: the antennal (AntSO) and maxillary (MxSO) sense organs are deleted in the mutant, except for the dorsolateral (DLP) and dorsomedial (DMP) papillae. The DMP and DLP become physically associated with the MxSO but are of different developmental origin (Jürgens *et al.*, 1986). C,D: the labial sense organ (LiSO) and the bridge of the H piece (H, also derived from the labial segment) are deleted in the mutant (arrowhead). The lateral arms of the head skeleton (LG) are not directly affected but are distorted because of the partial collapse of the head skeleton as a result of the missing labial and labral structures. E,F: thoracic segments. The three hairs of the Keilin's organ (KO) are deleted but the position at which the structure would form is marked in the cuticle of the mutant (arrowhead). Scanning electron micrographs confirm that the sensory hairs have been deleted and that the position of the Keilin's organ in the cuticle is marked by a depression (not shown). Other thoracic sense organs like the ventral black dots (vbd) are unaffected. G,H: the sclerotic structures of the head skeleton derived from the labral segment, the labrum (lr) and epistomal sclerite are much reduced or missing in the mutant. The labral sense organ is also deleted (not visible in G or H). Deletion of the labial (LiSO) sense organ and the H piece, as well as the labral sense organ, the labrum (lr) and the epistomal sclerite can cause partial collapse of the head skeleton (C,D and G,H). The extent of the structural abnormality of the head skeleton is somewhat variable and probably accounts for the failure of some homozygous embryos to hatch. In addition to the impairment of larval sensory functions due to loss of sense organs homozygous larvae probably have difficulty feeding since the pharynx and oesophagus could be compressed by the collapsed head skeleton. These aspects of the phenotype probably account for the disperse embryonic/larval lethality observed. The identification of the larval Keilin's organ as the vestigial larval leg has been made on comparative morphological grounds since the two structures are in close physical proximity in *Drosophila* as in other more primitive insects where the larval limbs are better developed (Keilin, 1915–16). Genetic evidence for the correctness of this interpretation lies in the observation that both the larval and adult legs are affected identically by certain mutations in the Bithorax-complex genes (Struhl, 1984). The identification of the other affected sense organs as limb structures rests in part on the developmental homology between these structures and the legs (see Figure 4A–C and Jürgens *et al.*, 1986; Akam, 1987) and also on comparative morphology. For example, the relationship between the larval AntSO and the developing imaginal antenna has been shown in other holometabolous insects, e.g. the moth *Pieris* (Eassa, 1953; Figure 4P). Note that although the sense organs and the disc primordia are closely linked developmentally they need not remain in physical proximity during subsequent morphogenetic movements, particularly in the region of the head where major reorganizations occur during head involution in *Drosophila*. The locations of the affected sense organs and the corresponding imaginal discs on the fate maps of the blastoderm, the extended germ band embryo and in the mature larva are schematized in Figure 4A–C. The development of the homologous larval appendages in the moth are shown in Figure 4M,N.

Table I. *Distal-less* alleles

Allele	Mutagen	Cytology	<i>Dll</i> /+ ^a	<i>Dll</i> /+/+ ^b	<i>Dll</i> / <i>Dll</i> /+ ^c	Source ^d
<i>Dll</i> ^J	X-ray	T(2;3) 60E5,6; 64B12,13-C12	D	+	L	1
<i>Dll</i> ^B	X-ray	In(2R) 48E,F; 60E5,6	D	D	L	2
<i>Dll</i> ^{MP}	X-ray	Df(2R) 60E3,4-5,6	D	+	D	2
<i>Dll</i> ^W	spontaneous	T(1;2) 3C; 60E5,6	D	+	L	2
<i>Dll</i> ^I	X-ray	normal	D	+	LD	3
<i>Dll</i> ^S	EMS	normal	D	+	D	3
<i>Dll</i> ^{G48i}	?	Tp(2;3)52E-60E; 3R centric heterochromatin	D	nd	nd	4
<i>Dll</i> ^{En(Arp)}	dysgenesis	normal	D	nd	L	5
<i>Dll</i> ^{SA1}	X-ray	normal	D	nd	nd	1

Genetic properties of homozygous lethal *Distal-less* alleles. Many of the alleles involve cytologically detectable rearrangements that share a common breakpoint in the cytogenetic position 60E5,6 on the right arm of chromosome 2. Our cytological determinations do not agree with previously published cytological localization of some alleles (under the name *Brista*, Sunkel and Whittle, 1987). 'normal' indicates that no visible cytological abnormality was detected. 'nd' indicates not done.

^a*Dll*/+: the phenotype of the heterozygote between mutant and wild-type alleles. 'D' indicates the dominant transformation of antenna to leg (not shown, see Sunkel and Whittle, 1987).

^bPhenotype of the dominant alleles in the presence of an additional wild-type copy of the locus as a Y-borne duplication [Dp(2;Y)L9 60AB-F, *sp*⁺, *Dll*⁺, *gsb*⁺, *Kr*⁺ kindly provided by C.Sunkel]. The dominant phenotype is fully suppressed in male flies of the genotype *Dll*/+/+ in comparison to their female *Dll*/+ siblings (indicated as +). These alleles are haplo-insufficient. The dominant phenotype (D) of B is only partially suppressed under these conditions. B is not a simple loss-of-function allele but is probably an abnormal gain-of-function or neomorphic allele.

^cThe column *Dll*/*Dll*/+ indicates the phenotype of male progeny homozygous for the mutant allele on the 2nd chromosome, but carrying the *Dll*⁺ duplication. Male flies of the genotype MP/MP/+ and 5/5/+ are indistinguishable from their MP/+ and 5/+ female siblings indicating that these are simple loss-of-function alleles. MP is associated with a cytologically visible deficiency that deletes the entire *Distal-less* transcription unit (Cohen *et al.*, 1989); this allele therefore defines the complete loss-of-function, or amorphic, phenotype. 1/1/+ flies exhibit a leg defect (LD) indicating that this allele is antimorphic (the mutant copy is worse than no copy as in MP/MP/+, which suggests that the mutant copy interferes with the activity of the normal copy) as described by Sunkel and Whittle (1987). It was not possible to isolate flies of this genotypic class for the alleles J, B, W, En(Arp) indicating the presence of additional lethal mutations on these chromosomes that lie outside the region covered by the duplication (indicated as L).

^dSources of the alleles: 1, this work, see Materials and methods. 2, James Kennison, alleles isolated on the basis of their dominant phenotypes. MP by J.Moscoso del Prado, also called Art². W, by J.Williams, also called Art¹. B, by J.Botas, also called Art³. 3, Claudio Sunkel (Sunkel and Whittle, 1987); we retain their allele designations where possible but use the name *Dll* rather than *Ba*. 4, William Gelbart. 5, isolated by Paul Adler as a dominant enhancer of *Aristapedioid*. En(Arp).

normally in these structures (Table II). The eye-antenna disc produces the eye, the antenna, the maxilla and the majority of the head capsule. Of the eye-antenna disc derivatives only the antenna and maxilla require *Dll* activity. All portions of the head capsule surrounding the eye do not. Although the eye was not directly scored in this analysis, it is not affected in the most severely affected viable mutant alleles, so we conclude that the eye also develops independently of *Dll* activity.

In order to interpret the consequences of removing *Dll* activity in mutant clones in the adult limbs it is important to bear in mind that imaginal discs are folded sheets of epithelial cells in which distal structures derive from the centre and proximal structures derive from the periphery (reviewed in Bryant, 1978; see also Figure 4). Since clones grow as radial sectors of the imaginal discs the progeny of a single genetically marked cell become distributed in stripes along the longitudinal (proximal–distal) axis of the adult appendage following eversion of the disc to form the limb. In all cases described below the structural defects associated with the *Dll*[−] clones are not observed in control clones which are *Dll*⁺. Control clones in the antenna and leg are shown in Figures 2A,C,D and 3A,B respectively.

Three classes of clones were found in the antenna (Figure 2): *Dll*[−] clones of the first class occupy the entire antenna. There is no genetically wild-type tissue in these antennae. In this situation the first antennal segment (AI) develops and is genetically *Dll*[−], but the second and third antennal segments (AII, AIII) and the arista are deleted. This result indicates that there are two developmentally distinct domains in the antenna. The proximal domain (the AI segment in the case of the antenna) develops independently of *Dll* activity,

Table II. Mitotic recombination clones

Adult derivatives of:	Clone frequency (%)	
	<i>stw</i>	<i>stw Dll</i>
eye-antenna disc	6.4	7.5
leg discs	3.4	3.5
dorsal thoracic discs	1.6	1.2
genital disc	1.6	1.3
number of flies scored	157	372

Mitotic recombination clones were induced in imaginal discs by X-ray irradiation during the first instar of *lt stw*/M(2)c33a larvae (control clones, wild-type for *Dll*) and *lt stw Dll*^{SA1}/M(2)c33a larvae (experimental clones, SA1 is an amorphic allele). Results are presented as clone frequency per imaginal disc derivative scored. Leg discs include 1st, 2nd and 3rd thoracic legs. Dorsal thoracic discs include humerus (T1), notum and wing (T2). Haltere (T3) could not be scored with the markers used. The derivatives of the genital disc that can be scored using the *stw* marker include the anal plates, claspers, lateral plate and genital arch in the male and the anal plates, thorn bristles and long bristle of the female. All of these structures develop normally in the absence of *Dll* activity. Note that the absolute frequencies/disc differ. This reflects both the number of target cells at the time of irradiation and the ease of identification of clones in particular discs and depends on factors like the number of potentially scoreable bristles.

while the distal domain (the second and third antennal segments and the arista) strictly requires *Dll* activity. 11% of *Dll*[−] antennal clones are of this type.

The second class of clones occupy only a part of the antenna and cause deletions of structures in the distal domain. Adjacent *Dll*⁺ cells that lie outside the clone develop normal structures according to their position in the distal

domain (AII*, Figure 2F). This situation indicates that cells in the antenna behave autonomously with respect to *Dll* activity. The gene product is required in individual cells to promote the development of distal structures. Absence of the product in one cell cannot be compensated for by its presence in adjacent cells. Cells lacking the gene are therefore constrained to forming proximal structures, by default. In this situation the remaining wild-type tissue does not regenerate all missing distal pattern elements. 28% of *Dll*⁻ antennal clones are of this type.

The third class of clones also occupy only a portion of the antenna but are not associated with structural abnormalities in the distal domain. In such clones the entire proximal domain is *Dll*⁻ and the distal domain is structurally normal and composed entirely of *Dll*⁺ cells (not shown). The majority of *Dll*⁻ antennal clones, 61%, fall into this third class. Since clones are normally arranged as longitudinal strips in the mature antenna (see Bryant, 1978) this pattern is unlikely to occur by chance and is not observed in the control clones. All of the control clones in the antenna contribute to the formation of at least some distal structures. When pattern abnormalities are associated with *Dll*⁻ clones in the antenna the most distal elements are always deleted (i.e. the class 1 and 2 clones). No clones are observed in which interstitial deficiencies occur that do not also delete the most distal structure (i.e. arista).

The different classes of antennal clones can be explained if we propose the existence of a graded positional information in the early imaginal disc primordium; in other words, if cells in the immature primordium have discrete identities with respect to the developing proximal-distal pattern of the adult limb. In this view when cells committed to forming the distal-most structures are eliminated in a clone, the damage to the primordium cannot be repaired and a deletion of distal structures will result. However, since clones do not always include the most distal structures (e.g. Figure 2D) one would expect subterminal deletions to occur in some mutant clones. These are not observed, but appear to be replaced by the class 3 clones in which genetically wild-type and morphologically normal AII, AIII and arista lie adjacent to genetically mutant AI structures. The absence of internal deletions when more distal structures are present suggests that the remaining wild-type cells can compensate for the deleted pattern elements by intercalary regeneration, but only when cells having more distal positional values exist in the disc. In other words, intermediate values can be regenerated in the presence of the extreme distal values but the extreme values cannot be generated *de novo* when they have been deleted. Therefore if a *Dll*⁻ clone were to be induced in an antenna disc in which cells of distal-most positional value were not affected, we would expect the *Dll*⁻ cells to be restricted to the proximal domain in an otherwise structurally normal antenna. This is what has been observed.

All of the clones generated in the leg are of the third class (Figure 3). Genetically *Dll*⁻ clones in the leg are not associated with distal structural deletions, but, as above, are developmentally restricted to forming proximal leg structures (coxa = AI, Figure 3C–E). The adjacent portions of the affected legs are structurally normal and genetically *Dll*⁺ as in the class III clones of the antenna. If the foregoing interpretation of the antennal clones is correct, the fact that we find only class III clones in the leg suggests that cells expressing distal-most positional values cannot be eliminated

from the leg disc primordium, as they can from the antenna. One significant difference between the antenna and leg imaginal disc primordia is that from the earliest testable stage the leg disc primordium is subdivided into two distinct cell populations occupying adjacent territories, called compartments (Steiner, 1976; Wieschaus and Gehring, 1976a,b; see Figure 4F). Distal pattern elements can only be deleted from a single compartment of the leg in any given clone (except in rare cases where two clones might be induced in one disc). The distal-most positional values would therefore remain in the other compartment to provide the information necessary to allow distal intercalation of the entire leg from the remaining *Dll*⁺ tissue. Cells lacking *Dll* activity would then be restricted to forming coxa in an otherwise normal leg. This explanation requires that cells from the unaffected compartment be able to interact with *Dll*⁺ cells from the affected compartment (or possibly to contribute directly to that compartment) and in fact the AP compartment boundary has been demonstrated to reversibly disappear during regeneration of the wing disc (Szabad *et al.*, 1979). The eye-antenna disc does not have an anterior-posterior compartment boundary during the first larval instar when the clones were induced (Morata and Lawrence, 1979) therefore there are no restrictions on the distributions of *Dll*⁻ clones in this disc (see Figure 4D–F). A single clone in the antenna, but not in the leg, can eliminate all cells committed to form distal structures. Consequently *Dll*⁻ clones in the antenna can be associated with deletions of distal structures while *Dll*⁻ clones in the leg cannot.

The frequency with which clones are recovered in either the antenna or the leg, relative to wing, does not depend on their genotype (Table II). This indicates that *Dll*⁻ clones in the leg and antenna are not being preferentially lost, but rather suggests that such clones are restricted to forming proximal structures. The cells in both the control and experimental clones are genetically provided with a growth advantage which shortens their cell cycle (Morata and Ripoll, 1975; see Materials and methods) and thereby allows them to contribute to disproportionately large regions of the adult structure (see Figures 2A,C,D and 3A,B). However, since the developmental fate of the *Dll*⁻ cells of the experimental clones is restricted, these cells cannot contribute to distal structures. Consequently, the relatively large number of cells produced by these clones must either be incorporated into proximal structures, or eliminated. This suggestion is consistent with the observation that the first antennal segment is expanded at the expense of distal structures in clones which cover the entire antenna (see Figure 1E). Similarly *Dll*⁻ clones which affect the entire anterior compartment of the coxa of the leg have significantly more bristles than do control clones (Table III and compare Figure 3A with D and E). The observed expansion of the proximal domains is likely to be a consequence of the superimposition of a developmental restriction on the population of rapidly dividing *Dll*⁻ cells. It is also possible that the expansion of the proximal domains reflects abnormal growth of the proximal stump in an abortive attempt to compensate for the missing distal positional values. This explanation seems less likely since no such compensatory growth is seen in less severely affected clones of the class II type.

The excess growth potential of these, by default, proximal cells also emphasizes the developmental restrictions placed on *Dll*⁻ cells. The boundary between regions that do

Fig. 3. Developmental restrictions exist in clones of cells lacking *Dll* activity in the legs. The approximate boundaries of the clones are indicated by dashed lines. **A** shows a control clone in the male foreleg which begins in the coxa, includes a part of the trochanter and femur and covers the entire face of the tibia and metatarsus visible in this view (compare with the unaffected control leg). Minute⁺ control clones run as long strips and can cover the entire leg from coxa to claw, although the clone shown here ends in the tarsal segments and does not reach the claw. **B** shows another control clone running through the sex comb but not affecting all teeth. Wild-type sex comb teeth are indicated with arrowheads. **C** shows a *Dll*⁻ clone in the male foreleg which covers a portion of the coxa. The clone does not extend to more distal segments. **D** and **E** show other mutant foreleg clones which are also restricted to coxa. The single unaffected bristle in the coxa in **E** is indicated by an arrowhead. In cases where the clones cover most of the anterior compartment the number of bristles in the coxa is increased (compare **D** and **E** with **A**).

Fig. 2. Developmental restrictions exist in clones of cells lacking *Dll* activity in the head. The approximate boundaries of the clones are indicated by dashed lines in the higher magnification panels. **A**, **C** and **D** show control clones in the antenna. The clone in **A** includes most of the right side of the head capsule (as oriented in the photograph). The region of the antenna is shown enlarged in **C**. The A1 and most of AII are included in the clone. The markers are not scorable in AIII and the arista. **D** shows a wild-type clone covering a different region of AII (arrowhead). **B**, **E**, **F** show *Dll*⁻ clones. The clone in **B** (enlarged in **E**) is similar in extent and distribution to the control clone in **A**. Note that the head capsule and A1 segment develop normally in the absence of *Dll* activity. AII, AIII and the arista are completely deleted. Note also that A1 is somewhat expanded (A1*). **F** shows another *Dll*⁻ clone in the antenna. This clone does not include the entire structure and leaves a sector of AII which is genotypically *Dll*⁺ (AII*). The maxillary palp (MP) is the distal domain of the maxillary appendage (as AII, AIII and arista are for the antenna). The maxillary palp is deleted in a *Dll*⁻ clone, while the proximal maxillary structure (e.g. lacinia, La) are present (**B** arrowhead, see Figure 4D,I). Note the presence of a large ectopic bristle on the base of the affected maxilla which resembles a bristle normally found on the coxa. Control clones in the maxilla are normal (not shown).

and regions that do not require the activity of the gene corresponds to the morphologically defined boundary between the evolutionarily distinct proximal and distal domains of the appendages, called coxopodite and telopodite respectively (Snodgrass, 1935). The coxopodite domain has been described as an outgrowth of the body wall upon which the true limb, or telopodite, develops. Our results indicate that the first antennal segment or the coxa of the leg behave genetically like parts of the body wall (the head capsule or ventral thorax respectively) in that they do not require the activity of *Dll*. In contrast the distal segments of the antenna or leg strictly require *Dll* activity and therefore correspond to the limb proper. The boundaries between the proximal and distal domains of each of the appendages is indicated in Figure 4 (D–F for the imaginal disc fate maps and G–K for the adult structures). For example, the coxa of the leg corresponds to the coxopodite of the hypothetical primitive arthropod leg, while the distal segments—trochanter, femur, tibia and tarsi—correspond to the telopodite (Figure 4K,L). Our definition of this boundary by genetic means in the antenna and maxilla is consistent with both morphological descriptions, and with the known homologies between antenna and leg defined through the study of the homeotic mutant *Antennapedia* (Postlethwait and Schneiderman, 1971). We therefore conclude that the activity of the *Distal-less* gene is required in a cell-autonomous manner to promote the development of limb structures above the evolutionary ground state of non-limb, or body wall.

Discussion

The analysis of pattern formation in the *Drosophila* embryo has been principally concerned with the anterior–posterior (AP) and dorsal–ventral (DV) axes. Mutations that disrupt the organization of these axes are readily detectable as defects in the segmentation and gastrulation of the embryo (reviewed in Nüsslein-Volhard *et al.*, 1987; Anderson, 1987). The need for an embryonic proximal–distal pattern-forming system

Table III. Bristle density in the anterior compartment of the proximal domain of legs (coxa) containing mitotic recombination clones (see Table II)

	<i>stw</i>	(n)	<i>stw Dll</i>	(n)	ratio
T1 leg	13 ± 2	(55)	23 ± 6.0	(11)	1.7
T2 leg	11 ± 1	(33)	16 ± 3.5	(8)	1.5
T3 leg	8.1 ± 0.7	(38)	11 ± 2.3	(4)	1.4

Results are presented as average number of bristles per coxa ± standard deviation. (n) indicates number scored. The effect is only detectable in cases where most of the anterior compartment is contained in the clone (compare Figure 3C with D and E). Coxae in which >90% of bristles were included in the clone were scored.

has been largely overlooked because the expression of this activity in the *Drosophila* embryo is morphologically subtle. This system, along with the AP and DV systems, is required for pattern formation in the larval appendages. In *Drosophila* the development of the larval limbs depends on the activity of the *Distal-less* gene.

The expression of proximal–distal pattern is specifically required for appendage development. Since appendages arose later in evolution than segmentation it is reasonable to suppose that the proximal–distal pattern-forming system will function downstream of those required for the organization of the embryonic AP and DV axes. In this context, Meinhardt (1983) has proposed a theoretical model to account for the spatial organization of the imaginal disc. In this model AP and DV boundaries intersect at unique positions in the embryonic epidermis and initiate the formation of proximal–distal axis. The intersection of two lines of embryonic positional information provides an elegant mechanism to accurately position the appendages with respect to the other body axes. The identities of these proposed AP and DV boundaries are a matter of speculation at present although it seems likely that the AP boundary will be the parasegment boundary (e.g. Struhl, 1984; Lawrence

Fig. 4. Summary diagram of proximal–distal pattern formation in the limbs. A–K are *Drosophila* and M–S are *Pieris*, a moth (after Eastham, 1930; Eassa, 1953; Kim, 1959). (A) Blastoderm fate map of the anterior half of the embryo showing the segment boundaries and the primordia of both imaginal discs and sense organs of the vestigial larval appendages. Abbreviations: Ac, acron; Ic, Li, Lr, Md, Mx, T1, primordia of the intercalary, labial, labral, mandibular, maxillary and first thoracic segments; antso, liso, lrso, mxso, Ko are sense organs of the corresponding segments; cl, ea, li, fl, imaginal primordia of the clypeolabrum, eye-antenna, labium and foreleg discs. Note that the eye-antenna primordium straddles the boundary between the non-segmental acron (eye), the antennal and maxillary segments. (B and M) Extended germ band stage. B shows the fate map of the primordia relative to morphological boundaries. In the moth the larval appendages are morphologically apparent at this stage. New abbreviations: Cl, clypeolabrum; Hy, hypopharyngeal lobe; Pl, procephalic lobe; St, stomodeum. (C and N) First larval instar. C shows the imaginal disc primordia and the larval sense organs. N shows the larval appendages in the moth. Note that the larval leg is segmented. Abbreviations: an, antenna; cp, clypeus; cps, head skeleton; cx, coxa; eps, epistomal sclerite; fe, femur; lip, labial palp; lr, labrum; mxp, maxillary palp; ta, tarsus; ti, tibia. (D–F) Fate maps of mature third instar imaginal discs. The boundaries between distal and proximal domains of the appendage primordia are indicated by dashes. D shows the eye-antenna disc which includes the antennal and the maxillary limb primordia. E shows the labial disc which gives rise to the labellar cap and prementum of the proboscis. F shows the foreleg disc. The AP compartment boundary is indicated by a dashed line in E and F (after Struhl, 1981 for the labial disc; Steiner, 1976 for the leg disc). Abbreviations: A, P, anterior and posterior compartments; AI,II,III, segments of the antenna; ar, arista; eye indicates the eye and head capsule; eh, edge hairs; mxb, base of the maxilla; mxp, maxillary palp; lc, labellar cap; pm, prementum; ps, pseudotracheae; c, claw; cx, coxa; tr, trochanter; ta, tarsus. (O–S) show comparable stages of limb development in the moth. Stippled regions indicate the imaginal epithelium which is continuous with the larval epidermis. The imaginal epithelium is generated by localized growth of the larval epidermis which invaginates. O shows the clypeolabrum (there is no fate map of the equivalent disc in *Drosophila*). P shows the antenna. Q shows the maxilla. R shows the labium. S shows the foreleg. New abbreviation: so, sense organ. (G–K) show the adult appendages of *Drosophila*. The boundaries between proximal and distal domains are indicated by dashes. The demarcation of the boundaries in G and J is based on data derived from the phenotypes of adult viable hypomorphic alleles (Cohen and Jürgens, manuscript in preparation). These conclusions are only presented for comparison with those in the leg and antenna. G shows the left half of the clypeolabrum. H shows the antenna. Note that AI is proximal. I shows the maxilla. Note that the lacinia is a lobe of the maxillary base (the proximal domain). J shows the labial derivatives in the proboscis. The posterior view is on the left and anterior is on the right. Note that the entire labellar cap is distal and the prementum is proximal. K shows the male foreleg. Coxa is proximal. Compare with L which shows the basic subdivision of the arthropod limb into coxopodite and telopodite domains according to Snodgrass (1935). Abbreviations: ac, pc anterior and posterior cibarial plates; cxp, coxopodite; ft, fish trap bristles; la, lacinia; mt, metatarsus, sxc, sex comb; transverse rows of bristles are stippled; tlp, telopodite.

et al., 1987; A.Simcox and A.Sheran, personal communication).

In all but the evolutionarily most advanced insects there is clear morphological evidence that development of the adult appendages makes use of the pre-existing organization of the larval structures (Miall and Hammond, 1892; Kellogg, 1902; Eassa, 1953; Anderson, 1972a,b). In these insects the imaginal appendages develop within, or at least in physical contact with, the appendages of the earlier developmental stage (for a more detailed description of these structures in the moth *Pieris*, see Figure 4M–S, compared with *Drosophila*, Figure 4A–J). This mode of appendage development is also found in lower dipteran species like the black fly (Kellogg, 1902). Consequently we expect that the embryonic pattern-forming systems which organize the larval appendages are also used to organize the adult appendages in higher dipteran insects like *Drosophila*. In this context it is not surprising that the *Distal-less* gene also plays a key role in the development of the adult limb. By genetic criteria we have shown that the development of the entire adult limb depends critically on the activity of *Dll*, while the development of non-limb structures that make up part of the appendage do not.

Analysis of the developmental potential of genetically mosaic imaginal discs suggests that eliminating *Dll* activity not only alters the developmental fates of individual cells in the primordium of the adult limb by restricting them to forming proximal structures, but also affects the range of structures that can be formed. As outlined above, the production of different classes of *Dll*[−] clones in the antenna can be satisfactorily explained if we assume that cells already possess different proximal–distal identities in the first instar imaginal disc. The early disc primordium therefore apparently contains a set of graded positional values that in some way determine cell identity along the future proximal–distal axis of the limb. The important question to be answered at this point is whether the existence of the proximal–distal positional information in the limb primordium is a direct consequence of *Dll* gene activity. At present we have no direct way to address this issue; however, an independent line of evidence indicates that there actually is a graded requirement for the activity of the *Dll* gene during the development of the adult limbs. This conclusion is based on the phenotypes of partial loss-of-function *Dll* mutants which exhibit a graded series of structural defects in the adult legs as progressively more activity is removed in successively stronger mutant alleles (Sunkel and Whittle, 1987; Cohen *et al.*, 1989). Since there appears to be a graded requirement for the activity of the *Dll* gene during limb development it is tempting to speculate that the graded positional information revealed in the early imaginal disc primordium by the clonal analysis might depend directly on *Distal-less* activity. Molecular analysis of the *Distal-less* locus has shown that the gene encodes a homodomain-containing protein (Cohen *et al.*, 1989). It therefore seems likely that the *Dll* gene product exerts its activity by differential regulation of subordinate genes. It remains to be determined whether the graded requirement for *Distal-less* activity reflects a graded distribution of the gene product in the developing limb primordium.

Materials and methods

Stocks and mutant isolation

Fly stocks were cultured under standard conditions (Roberts, 1986). Marker stocks and balancers are described in Lindsley and Grell (1968) and Lindsley and Zimm (1985, 1986, 1987). Sources of *Distal-less* alleles are indicated in Table I. *Dll*^J was isolated as a second site suppressor of the dominant phenotype of Pc3. *Dll*^{SA1} was isolated as a second site suppressor of the dominant phenotype of the X2 allele of the gene *Abnormal leg pattern* (*Alp*) which specifically interacts with *Distal-less* (our unpublished data).

Cuticle preparations

Larval cuticle was prepared as discussed by Wieschaus and Nüsslein-Volhard (1986). Adult cuticle was prepared by cooking flies in 10% KOH until the eye pigment dissolved. The insoluble cuticle was rinsed well with water, dissected and mounted in Faure's medium (Wieschaus and Nüsslein-Volhard, 1986).

Induction of mitotic recombination clones

lt stw Dll^{SA1}/SM6a (experimental) or *lt stw*/SM6a (control) females were mated with *M(2)c33a*/SM5 males. Eggs were collected over 24-h periods and larvae were irradiated 36 h later. This corresponds to first instar due to the developmental delay caused by the elongated cell cycle of the *Minute* mutation (Morata and Ripoll, 1975). Cuticular preparations of adult flies of the genotypes *lt stw Dll*^{SA1}/*M(2)c33a* or *lt stw*/*M(2)c33a* were scored on the compound microscope for the presence of genetically marked clones. The genetic marker used for this analysis is *straw* (*stw*) which lightens the colour of bristles and is cell autonomous. *stw* is easily identified in the abdominal tergites and sternites, the leg, the head capsule, antenna and maxilla, the dorsal thorax and in most of the wing and in parts of the genitalia. In addition to the colour change, the size of the bristles in the clones is increased due to removal of the *Minute* mutation (Morata and Ripoll, 1975). Since the flies were scored as cuticle preparations the eye pigments were bleached out and the eye was not directly scored [*light* (*lt*) is an eye colour marker]. Homozygous *lt stw* or *lt stw Dll*[−] clones are *Minute*⁺ and so divide ~50% faster than the surrounding *Minute* tissue. Consequently the cells in these clones have a growth advantage and can occupy large regions of the final pattern. The large size of such clones helps to identify developmental restrictions on the fates of genetically mutant cells contained in the clones.

Acknowledgements

We are indebted to Herbert Jäckle for his support and encouragement during the course of this work, which was carried out in his laboratory. We thank Herbert Jäckle and Detlef Weigel for valuable suggestions which helped to improve the manuscript. We are grateful for financial support from a grant by the Deutsche Forschungsgemeinschaft (Leibniz program) to H.J. S.C. was supported by a Centennial Fellowship from the Medical Research Council of Canada.

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Received on March 3, 1989; revised on March 23, 1989