The role of *buttonhead* and *Sp1* in the development of the ventral imaginal discs of *Drosophila*

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Summary

The related genes *buttonhead* (*btd*) and *Drosophila Sp1* (the *Drosophila* homologue of the human *SP1* gene) encode zinc-finger transcription factors known to play a developmental role in the formation of the *Drosophila* head segments and the mechanosensory larval organs. We report a novel function of *btd* and *Sp1*: they induce the formation and are required for the growth of the ventral imaginal discs. They act as activators of the *headcase* (*hdc*) and *Distal-less* (*Dll*) genes, which allocate the cells of the disc primordia. The requirement for *btd* and *Sp1* persists during the development of ventral discs: inactivation by RNA interference results in a strong reduction of the size of legs and antennae. Ectopic expression of *btd* in the dorsal imaginal discs (eyes, wings and halteres) results in the formation of the corresponding ventral structures (antennae and legs). However, these structures are not patterned by the morphogenetic signals present in the dorsal discs; the cells expressing *btd* generate their own signalling system, including the establishment of a sharp boundary of *engrailed* expression, and the local activation of the *wingless* and *decapentaplegic* genes. Thus, the Btd product has the capacity to induce the activity of the entire genetic network necessary for ventral imaginal discs development. We propose that this property is a reflection of the initial function of the *btd/Sp1* genes that consists of establishing the fate of the ventral disc primordia and determining their pattern and growth.

Key words: *Drosophila*, Imaginal discs, *btd*, *Sp1*, Zinc-finger transcription factors

Introduction

One principal question in developmental biology is how the body of multicellular organisms is genetically subdivided. In *Drosophila* there is a great deal of information about the subdivision along the anteroposterior axis (AP) body axis: the successive activities of maternal and segmentation genes (reviewed by Lawrence and Morata, 1994) generate a number of equivalent body units that form the parasegmental trunk. The various Hox genes become active in specific positions of the parasegmental trunk and confer different identities, thus generating the morphological diversity in the AP axis.

Within the domains defined by the activity of the Hox genes there are other genes that discriminate the identity of more restricted parts. For example, *pannier* (*pnr*) appears to be responsible for distinguishing between medial and lateral regions of all thoracic and abdominal segments (Calleja et al., 2000; Herranz and Morata, 2001). The gene *apterous* (*ap*) distinguishes between the dorsal and ventral regions of the wing appendages (Díaz-Benjumea and Cohen, 1993), and *Distal-less* (*Dll*) specifies the growth and identity of ventral appendages (Cohen and Jurgens, 1989; Gorfinkiel et al., 1997).

A major morphological distinction in the embryonic, larval and adult body is that between dorsal and ventral regions. There is evidence that the dorsal and ventral adult structures of the thorax share a common lineage in early development (Steiner, 1976; Wieschaus and Gehring, 1976; Lawrence and Morata, 1977), but by late embryogenesis the dorsal and ventral primordia have different lineages. It is not clear whether this restriction corresponds to a genuine compartment segregation or whether it is the result of the physical separation of the two primordia, which can be observed during embryogenesis (Goto and Hayashi, 1997; Kubota et al., 2000). Nonetheless there is a clear difference between dorsal and ventral patterns, which is also reflected in the activity of different developmental genes. There are identity conferring genes which are restricted to either the dorsal or the ventral regions, such as *pnr*, *Dll* or *vestigial* (*vg*) (reviewed by Mann and Morata, 2000).

We have developed a method (Calleja et al., 1996) which allows the visualisation of gene expression patterns in living flies. It is a modification of the Gal4/UAS procedure of Brand and Perrimon (Brand and Perrimon, 1993): mobilisation of the pGalw element yields a collection of insertion lines, which are tested using an *UAS-y* construct. Individual adult flies showing dark (y^+) patches of interest were used to establish new Gal4 lines. We have used this method for a systematic screen of genes showing restricted expression in adult flies. Among those, the line MD808 was found to confer y^+ expression in ventral adult derivatives, proboscis, antennae, legs and genitalia, suggesting the gene driving its expression may have a general function in the formation of these structures.

We report a functional characterisation of the gene driving MD808 expression. It turned out to be *buttonhead* (*btd*), which encodes a zinc-finger transcription factor (Wimmer et al.,

1993) and is known to be required for the formation of the intercalary, antennal and mandibular head segments (Cohen and Jürgens, 1990). A related adjacent gene, termed *Sp1* (previously known as *D-Sp1*), has been previously characterised (Wimmer et al., 1996), the function of which is partially redundant with that of *btd* (Schock et al., 1999). We find that in addition to their role in head development *btd* and *Sp1* are involved in the development of the ventral imaginal discs. Their expression is under the control of the Wg and Dpp signals and is also regulated by other factors such as the bithorax complex genes. Once activated, they induce the function of genes such as *Dll* and *headcase* (*hdc*), which are involved in the specification of adult ventral structures. Our results also show that the Btd product is able to trigger the entire process necessary for leg and antennal development, including the activation of the *wg* and *dpp* signalling genes.

Materials and methods

Molecular localisation of MD808

Using inverse PCR (http://www.fruitfly.org/about/methods/inverse.pcr.html), we cloned and sequenced the flanking sequence 3′ of the pGalw element (Brand and Perrimon, 1993). The insert is located at 55,584 bp within the scaffold AE 003448 (FlyBase), 753 bp upstream of *btd* transcription start. *Sp1* is located immediately adjacent 60 kb downstream of *btd*.

Fly stocks

The btd^{XG81} is a strong allele of *btd* with the same phenotype as the deletion of the gene (Cohen and Jurgens, 1990). The *Df(1)C52* is a deletion of the 8E4-9C region, deficient for *btd*, *Sp1* and about 60 other genes (Cohen and Jurgens, 1990) (FlyBase). The $Dp(1;Y)lz^{+}$ covers the 8B-9A region (Schock et al., 1999) and also rescues the *btd* and *Sp1* larval phenotypes, indicating the genes responsible for the latter are within the 8E4-9A interval. This interval contains about 44 genes (FlyBase). The Ubx^{1} allele has been described by Kerridge and Morata (Kerridge and Morata, 1982) and Dll^{SA1} by Cohen and Jürgens (Cohen and Jürgens, 1989). The reporter genes used were *dpp-lacZ* (Blackman, 1991), *wg-lacZ* (Ingham, 1991), *Dll-lacZ* (Dll^{01092}) (Spradling et al., 1999), *esg-lacZ* (Whiteley et al., 1992) and *hdc-lacZ* (Weaver and White, 1995)

Production of mutant *btd* clones

Heat shocks were given to larvae of different stages of genotype btd^{XG81} f^{36a} *FRT18A/y w FRT18A; hsFLP/+*. Mitotic recombination in the first chromosome would produce clones of mutant btd^{XG81} cells marked with f^{36a} and twin btd^{+} control clones marked *yellow*.

RNA interference

The GAL4-inducible constructs for RNA interference were made as follows: for *btd*, a 400 bp fragment was amplified by PCR using a 5′-gaaggatccgccgccaccgccgccgct-3′ upper primer and a 5′-cggggtaccgtaactggctgttcccgcacc-3′ lower primer. For *Sp1*, a 813 bp fragment was amplified using a 5′-gccggatcctggctggatatggg-3′ upper primer and a 5′-gccggtaccggccccgcccgtctgtggg-3′ lower primer. Each PCR product was independently cloned as a *Bam*HI-*Kpn*I fragment in the pHIBS vector (Nagel et al., 2002), to make the pHIBS-*btd* or pHIBS-*Sp1* constructs. The *Bam*HI-*Sac*I fragments from pHIBS-*btd* or pHIBS-*Sp1* were subcloned in the Bluescript vector, generating the BS-*btd and* BS-*Sp1*, respectively. *Sal*I and *Kpn*I fragments (containing the intron and the gene fragment) from pHIBS-*btd* and pHIBS-*Sp1* constructs were cloned in the opposite direction in the BS-*btd* and BS-*Sp1* vector, thus forming the final RNAi constructs BS-*btd* RNAi and BS-*Sp1* RNAi. The RNAi constructs were cloned in the pUAS *Kpn*I site and injected in y w^{1118} embryos by standard procedures. They are referred to as *UAS-btdi* and *UAS-Sp1i* respectively.

RT-PCR

We used larvae of genotype *btd-Gal4>UAS-btdi* and *btd-Gal4>UAS-Sp1i* to measure the transcripts levels in comparison to *btd*-Gal4 larvae used as control.

To isolate RNA for RT-PCR, anterior halves of larvae were lysed in Trizol (Invitrogen), and extracted RNA was dissolved in water. Synthesis of first strand cDNA was primed by oligo (dT) and random hexamers. RT-PCR was performed using published primers to amplify the ribosomal protein 49 (RP49) mRNA (Foley et al., 1993), which serves as internal control, and two pair of primers outside the region cloned in the UAS RNAi to amplify *btd* and *Sp1* mRNA.

Gal4/UAS experiments

ey-Gal4 was provided by Walter Gehring and directs expression identical to the *eyeless* gene. The *nub*-Gal4, *omb*-Gal4 and *Ubx*-Gal4 lines were isolated in our laboratory and drive expression essentially in the domains defined by the genes in which the pGalw element is inserted. *ptc*-Gal4 is described elsewhere (Wilder and Perrimon, 1995). The MD743 line directs expression in the hinge and the pleural region of the wing (M.C., unpublished). The 444-Gal4 line gives an overall uniform embryonic expression (Azpiazu and Morata, 1998). The *mata*-Gal4VP16 Vp15 lines (a gift from Daniel St Johnston) yield maternal Gal4 expression. The *UAS-btd* stock (Schock et al., 1999) was provided by Herbert Jackle.

To induce marked clones of cells ectopically expressing *btd* we used three different chromosomes as convenient: *y w hs FLP122* f^{36a}; *abx<*f^{+}*<Gal4-lac-Z* (de Celis and Bray, 1997), *hsFLP act<CD2<Gal4* (Pignoni and Zipursky, 1997) and *y w hsFLP122; act<*y^{+}*<Gal4 UAS-GFP* (Ito et al., 1997). They were crossed to *UAS-btd* flies and the F1 larvae were given heat shocks at different stages.

Histochemical methods

Embryos were stained using standard procedures for confocal microscopy (Gonzalez-Crespo et al., 1998); secondary antibodies were coupled to Red-X and FITC fluorochroms (Jackson Inmunoresearch) and were analysed under a laser-scan BioRad microscope. For double fluorescent in situ/antibody label we followed the method as described by (Knirr et al., 1999). For the in situ label we used a btd RNA probe reported previously (Wimmer et al., 1996) and provided by Herbert Jackle. As probe for *Sp1* we used an RNA transcribed from a cloned 2 kb DNA fragment obtained by PCR using primers from the 5′ and 3′ UTR.

Imaginal discs were dissected in PBS and fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature. They were blocked in PBS, 1%BSA, 0.3% Triton for 1 hour, incubated with the primary antibody over night at 4°C, washed four times in blocking buffer, and incubated with the appropriate fluorescent secondary antibody for 1 hour at room temperature in the dark. They were then washed and mounted in Vectashield (Vector Laboratories).

The primary antibodies used were: rabbit and mouse anti-β-gal (Capel and Promega), mouse anti-CD2 (Serotec), rabbit anti-vestigial (Williams et al., 1991), mouse anti-dachshund (Mardon et al., 1994), guinea pig anti-homothorax (a gift of Natalia Azpiazu), rabbit anti-Distal-less (a gift of Grace Panganiban), mouse anti-Distal-less (Duncan et al., 1998), mouse anti-engrailed (a gift of Peter Lawrence) and mouse anti-wingless (Hybridoma Center).

Preparation of larval and adult cuticles

Adult flies were by the standard methods for microscopic inspection. Soft parts were digested with 10% KOH, washed with ethanol and mounted in Euparal. Embryos were collected overnight and aged an additional 12 hours. First instar larvae were dechorionated in commercial bleach for 3 minutes and the vitelline membrane removed using heptano-methanol 1:1. Then, after washing with methanol and

0.1% Triton X-100, larvae were mounted in Hoyer's lactic acid 1:1 and allowed to clear at 65°C for at least 24 hours.

Results

The MD808 Gal4 line is an insertion at the *buttonhead* gene

In our search for genes with restricted expression in the adult cuticle, the MD808 Gal4 line was found to direct expression in the ventral derivatives of the adult body; proboscis, antennae, legs and genitalia. In the abdomen and analia we could not discern a clear expression. We also noticed that the insertion was located in the first chromosome and associated with a lethal mutation. The mutant larvae showed a head phenotype resembling that described for mutants at the *btd* gene: loss of antennal organ and the ventral arms of the cephalopharyngeal skeleton (Cohen and Jürgens, 1990), and complementation analysis indicated that the chromosome carrying the insert contained a mutation at *btd*. The expression pattern found in *MD808/UAS-lacZ* embryos was also similar to that reported for *btd* (see below), suggesting that the Gal4 insertion was located at this gene. In addition, the imaginal expression of MD808 and of *btd* was largely coincident.

Further to the genetic analysis and the expression data we cloned DNA fragments at the insertion site to map the position of the P-element on the genome (see Material and methods). It is located 753 bp 5′ of the *btd* gene (FlyBase). We note that the related gene *Sp1*, the *Drosophila* homologue of the human *SP1* (Wimmer et al., 1993; Wimmer et al., 1996) is immediately adjacent (FlyBase). It is likely that *btd* and *Sp1* have originated by a tandem duplication of a primordial *btd*-like gene.

btd and *Sp1* expression domains in the thoracic segments

The expression patterns of *btd* and of *Sp1* during embryogenesis have already been described (Wimmer et al., 1993; Wimmer et al., 1996). In early embryos *btd* is expressed in the head region, but by the extended germ band stage the expression domain has expanded to the ventral region of cephalic, thoracic and abdominal segments. During germ band retraction most of the abdominal and thoracic expression is lost, except in derivatives of the peripheral nervous system and the primordia of the imaginal discs. *Sp1* is not expressed in early embryos, but from stage 11 onwards it shows the same pattern as *btd* (Wimmer et al., 1996).

We paid special attention to the btd/Sp1 expression domain in the thoracic imaginal discs primordia, as it may suggest a novel function related to imaginal development. Double labelling with Dll and btd probes (Fig. 1A-C) indicates that *btd* precedes *Dll* expression, but by stage 12 the two genes are co-expressed in a group of thoracic cells. However, the Dll domain is smaller and is included within the btd/Sp1 domain: there are cells expressing *btd* that do not show *Dll* activity, although all the cells expressing *Dll* express *btd* (Fig. 1B,C).

The ventral disc primordia include not only cells expressing *Dll* but also other cells containing expression of *escargot* (*esg*) and *hdc*, markers of the diploid cells that form the imaginal primordia (Whiteley et al., 1992; Hayashi et al., 1993; Weaver and White, 1995). In late embryonic stages, *esg* is expressed in a ring domain surrounding the *Dll*-expressing cells

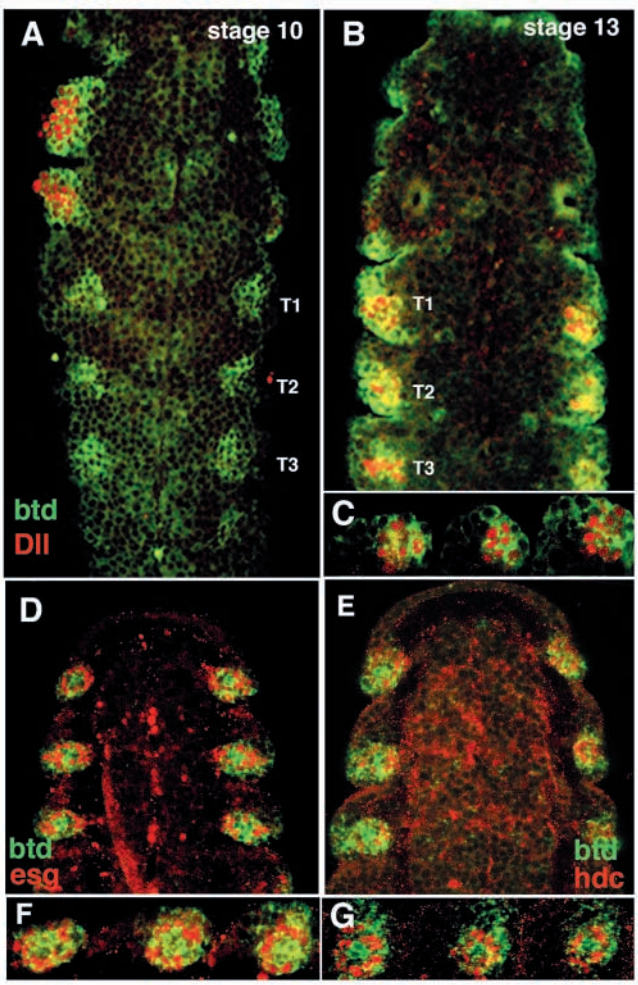


Fig. 1. Some aspects of embryonic expression of *btd* in relation to that of *Dll*, *esg* and *hdc*. Double immunofluorescent staining of in situ hybridisation for *btd* and anti-β-gal antibodies for *Dll*, *esg* and *hdc*. (A) Stage 10 embryo showing *btd* expression (green) in two cephalic segments (maxillary and labial) and in three thoracic discs primordia (T1-T3). Note that at this stage *Dll* (red) is not active in the thoracic primordia. (B) Stage 13 embryos equally stained for *btd* and *Dll* to show that at this stage *Dll* is co-expressed with *btd* in the thorax. (C) Higher magnification of the thoracic disc primordia of a stage 13 embryo. Note that the btd domain is bigger than that of Dll. (D) Double staining for *btd* and *esg* in a stage 14 embryo. Higher magnification is shown in F. (E) Stage 14 embryo stained for *btd* and *hdc*. Higher magnification is shown in G.

(Gonzalez-Crespo et al., 1998; Goto and Hayashi, 1999) (reviewed by Morata, 2001) and *hdc* is expressed in a similar pattern (C.E., G.R., M.C. and G.M., unpublished; Fig. 1E,G). We have carried out double label experiments with *btd*, *hdc* and *esg* probes and found (Fig. 1D-G) that the expression of the two latter genes overlaps with that of *btd* (and with *Sp1*, not shown) in the thoracic disc primordia.

The overlap of the *btd* and of *esg* domains indicates that *btd* is also expressed in the hth domain, which is coincident with that of *esg* (Goto and Hayashi, 1999). As the hth/esg domain marks the precursor cells of the proximal region of the adult leg (reviewed by Morata, 2001) the embryonic expression data indicate that *btd* and *Sp1* are active in the entire primordia of

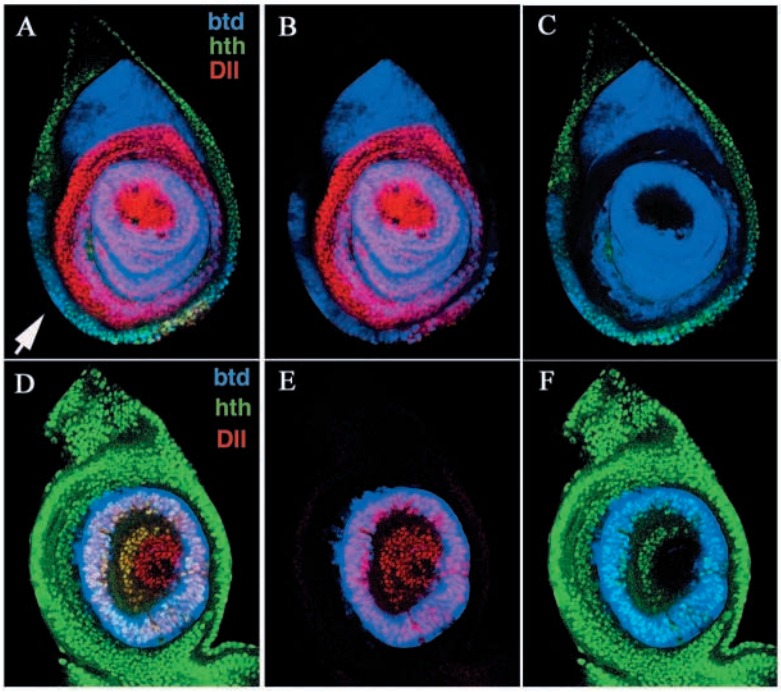
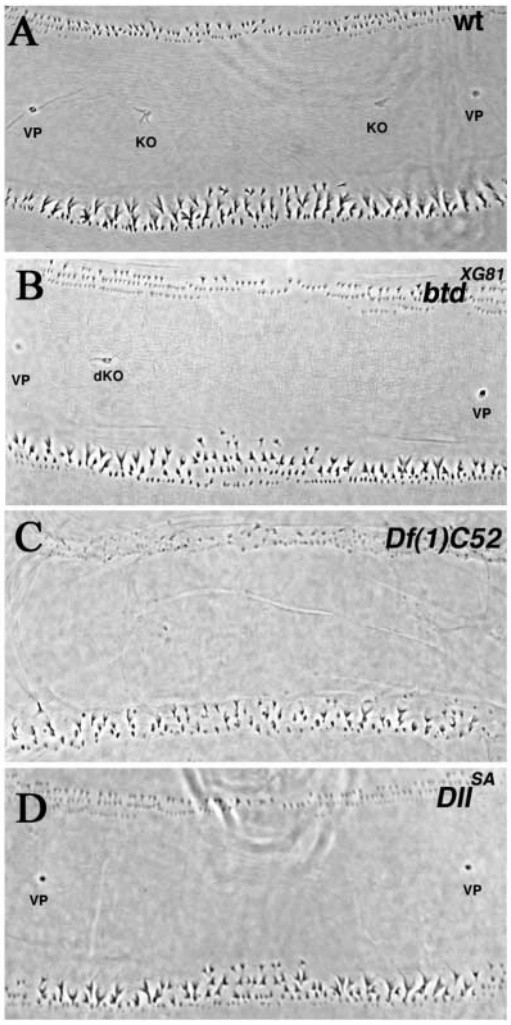


Fig. 2. Expression of *btd* (blue), *hth* (green) and *Dll* (red) in leg (A-C) and antennal (D-F) imaginal discs. (A) Triple staining for three genes. (B) *btd* and *Dll*. (C) *btd* and *hth*. Notice that the *btd* domain overlaps partially with those of *Dll* and *hth*, indicating that it is expressed both in the proximal and the distal leg domain. The arrow indicates the ventral region where *btd* and *hth* overlap. (D) Triple label. (E) *btd* and *Dll*. (F) *btd* and *hth*.

the ventral adult structures, including the distal and the proximal parts.

The different expression patterns in third instar leg and antennal imaginal discs are illustrated in Fig. 2. In the mature antennal disc, *btd* expression is restricted mostly to the region corresponding to the second antennal segment, where it co-localises with both *Dll* and *hth*. In the leg disc *btd* also overlaps in part with *Dll* and with *hth* (Fig. 2A-C). The latter result is significant, for the expression of *Dll* and *hth* define two major genetic domains, which are kept apart by antagonistic interactions (Gonzalez-Crespo et al., 1998; Abu-Shaar and Mann, 1998). The fact that *btd* is expressed in the two domains suggests that its regulation and function is independent from the interactions between the two domains. This observation is consistent with the results obtained in embryos (Fig. 1) and suggests that the btd domain includes the precursors of the whole ventral thoracic structures regions from the beginning of development.



btd and *Sp1* are required for the formation of the ventral disc primordia

The observation that the original btd/Sp1 domain includes the Dll, hdc and esg domains suggested that *btd* and *Sp1* might be necessary for the activation of these genes and hence for the formation of the primordia. Therefore, we studied whether the loss of *btd* and *Sp1* function affect *Dll*, *esg* and *hdc* expression. We also checked for the effect on the formation of Keilin's organs (KO), larval structures which share the same lineage of the leg discs and known to require *Dll* activity (Cohen and Jürgens, 1989; Cohen, 1990).

Larvae mutant for the strong btd^{XG81} allele are defective in the formation of KOs (Fig. 3); in 45% of the larvae the KO are absent and in the rest the KO are defective. The incomplete effect of the btd^{XG81} mutation in suppressing KOs suggested that its function might be redundant and shared by another gene, the obvious candidate being *Sp1*. As no individual *Sp1* mutation is available, we tested the contribution of *Sp1* to KO formation by examining first instar larvae hemizygous for *Df(1)C52* (Schock et al., 1999), which lacks both *btd* and *Sp1*

Fig. 3. Effect of the loss of *btd*, *Sp1*and *Dll* on the formation of Keilin's organs (KO) and ventral pits (VP). The third thoracic and first abdominal denticle belts are shown. (A) Wild-type segment displaying the normal set of KOs and VPs, a pair of each per segment. (B) btd^{XG81} larva. Only one KO is present and it is defective. The two ventral pits are present in this case, but they are frequently lacking in the mutant larvae (see main text). (C) *Df(1)C52* larva showing complete absence of KOs and ventral pits. (D) Dll^{SA1} larva. The KOs are always missing but the ventral pits are present.

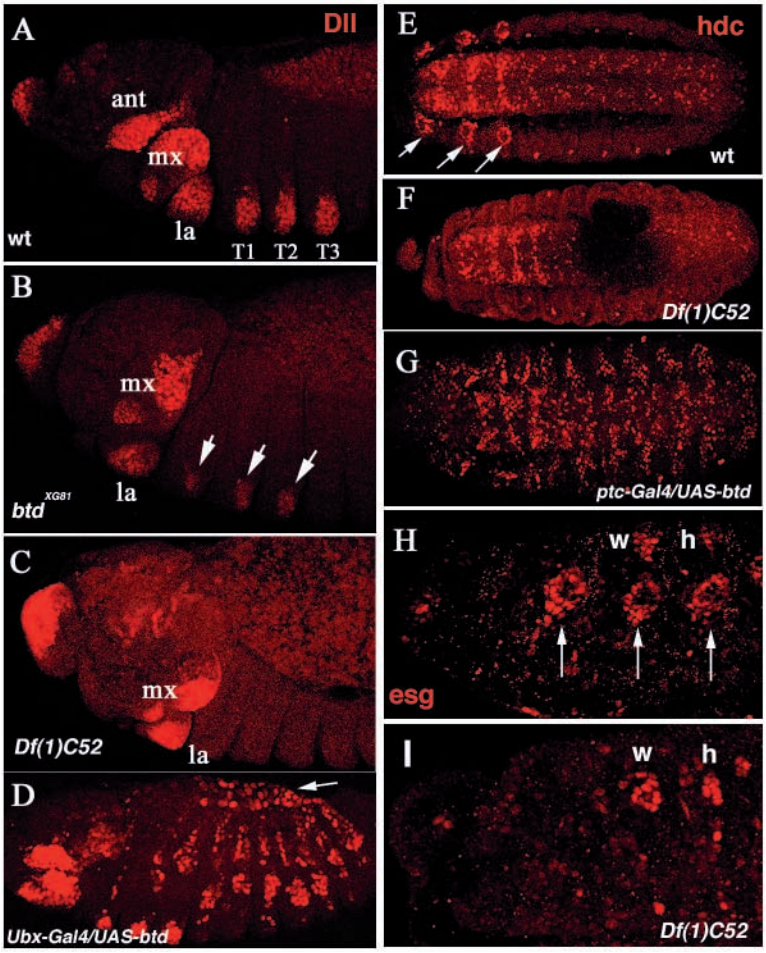


Fig. 4. Alteration of embryonic expression of *Dll*, *hdc* and *esg* in the absence of *btd* and *Sp1* function. (A) Wild-type *Dll* expression. The antennal (ant), maxillary (mx) and labial (la) head segments contain *Dll* activity, as well as the three thoracic (T1, T2, T3) primordia. (B) btd^{XG81} embryo. The expression in the antennal segment is lost and there is a marked reduction of *Dll* activity in the three thoracic primordia (arrows). (C) *Df(1)C52* embryo showing complete loss of *btd* activity in the thoracic primordia. The expression in the maxillary and labial segments is not altered. (D) *Ubx-Gal4/UAS-btd* embryo exhibiting ectopic *Dll* expression in the abdominal segments, which do not normally contain *Dll* activity. Note the expression in the amnioserosa cells (arrow). (E) Ventral view of a wild-type embryo showing *hdc* expression restricted to the anterior region of the ventral cord and the thoracic discs primordia (arrows). (F) *Df(1)C52* embryo with loss of *hdc* activity in the thoracic primordia. (G) Ventral view of *ptc-Gal4/UAS-btd* embryo showing high levels of *hdc* activity (compare with E). (H) Wild-type expression of esg in the thoracic discs primordia. Wing (w) and haltere (h) clusters are indicated. The three arrows point to the three leg primordia. (I) *esg* expression in a *Df(1)C52* embryo. Expression in the wing and haltere primordia remain but no activity is seen in the region corresponding to the leg primordia.

(see Materials and methods). *Df(1)C52* larvae show *btd* mutant phenotype in the head as reported previously (Cohen and Jürgens, 1990), but in addition we find that no KO are present (Fig. 3). Both the *btd* phenotype and the lack of KOs of *Df(1)C52* larvae are covered by the duplication *Dp(1;Y)* lz^{+}, indicating that the responsible genes are located in the interval 8E-9A, which contains *btd* and *Sp1* and about 40 other genes (FlyBase). These results suggest that both *btd* and *Sp1* play a role in the formation of the disc primordia and that their functions are partially redundant, but we cannot rule out the possibility that some other gene(s) located in the 8E-9C interval may also be involved.

We tried to test the specific contribution of *btd* and *Sp1* to the mutant phenotype reducing their transcript levels by means of RNA interference. *UAS-btdi* and *UAS-Sp1i* genes were synthesised as described in Materials and methods and were found to reduce significantly the transcripts levels of *btd* and *Sp1* (see Fig. 6). Various Gal4 lines (*Ubx-Gal4*, *ptc-Gal4*, *444-Gal4*, *mata-Gal4*) were used to test the effect of the two constructs either separately or together. However, in contrast with the results obtained for adult patterns (see below), we failed to see a clear phenotypic effect. We suspect that although the RNAi experiments significantly reduce *btd* and *Sp1* activity, the remaining function is sufficient for the formation of ventral larval patterns.

The loss of KOs in *Df(1)C52* larvae activity already suggested an alteration in *Dll* function, known to be essential for the formation of KOs (Cohen and Jürgens, 1989). *Dll* expression in btd^{XG81} and in *Df(1)C52* embryos is shown in Fig. 4: in btd^{XG81} embryos there is a strong reduction in *Dll* activity in the thoracic primordia when compared with the wild-type, although the majority still show some label (Fig. 4B). In *Df(1)C52* embryos there is no *Dll* activity in the same primordia (Fig. 4C), although in a few cases we observe some hints of expression, which might be due to the maternal component of *Sp1* (Schock et al., 1999). In the two mutant genotypes, there is normal *Dll* expression in the labial and maxillary segments, where it does not depend on *btd/Sp1*.

The lack of *btd* and *Sp1* not only suppresses *Dll*, but it also affects the expression of *hdc* and *esg* in the imaginal discs primordia. In *Df(1)C52* there is no *hdc* activity in the ventral discs (Fig. 4E,F). There was some expression in the dorsal discs, especially in the prothoracic one, but the groups of cells corresponding to the wing and haltere discs could not be discerned clearly. The expression of *esg* is also affected (Fig. 4H,I); it is abolished in the leg discs, but not in the wing and haltere discs. Other markers of dorsal discs such as *vestigial* (not shown) are also unaffected. As *hdc* and *esg* are considered as markers of the imaginal primordia (Hayashi et al., 1993; Whiteley et al., 1992; Weaver and White, 1995), their lack in the ventral region of *Df(1)C52* embryos suggests that ventral discs are not formed in the absence of *btd/Sp1*.

btd acts upstream of *Dll* and *hdc* in the formation of the disc primordia and appears to mediate the control by Wingless and the BX-C genes

The results above demonstrate that *btd* and *Sp1* are necessary for normal *Dll*, *hdc* and *esg* expression. Moreover, *btd* expression precedes that of *Dll*, *esg* and *hdc*, which suggests it might act as an early activator of these genes. We have tested this possibility by forcing *btd* activity using the Gal4/UAS method (Brand and Perrimon, 1993) and examining the effect

on *Dll* and *hdc* expression. In *Ubx-Gal4>UAS-btd* embryos, *btd* activity extends from the second thoracic segment to the seventh abdominal one. In this region, the presence of the Btd product induces ectopic *Dll* activity in many places, although not in all of the zones where *btd* is expressed (Fig. 4D). Those embryos also show gain of *hdc* expression. In *ptc-Gal4/UAS-btd* embryos there is a general gain of *hdc* activity in the ventral region (Fig. 4G). In the case of *Dll*, we checked whether there is a mutual requirement for *Dll* and *btd/Sp1* genes by looking at *btd* and *Sp1* expression in *Dll* mutant embryos. The result was that their expression is normal, supporting the idea that *btd* and *Sp1* act upstream of *Dll*.

The activator role of *btd* (and presumably of *Sp1*) on *Dll* suggests that *btd* and *Sp1* may play a role in mediating some of the factors known to be involved in *Dll* regulation during embryogenesis. Previous work has identified some of these regulators, e.g. *wingless* (*wg*), *decapentaplegic* (*dpp*) and the bithorax complex genes (Cohen, 1990; Vachon et al., 1992; Goto and Hayashi, 1997) and we therefore tested whether their role is mediated by an effect on *btd* and *Sp1*.

The secreted molecule Wg is necessary for the early activation of *Dll* in the cephalic and thoracic primordia (Cohen, 1990; Goto and Hayashi, 1997). We find that in the absence of *wg* function *btd*/*Sp1* expression in the disc primordia is abolished, although it remains in the central nervous system (Fig. 5B). In addition, early *Dll* expression is repressed by *dpp*; in embryos deficient for *dpp* function *Dll* expression is expanded (Goto and Hayashi, 1997). We observe a similar situation with early *btd* expression, which extends to the dorsal side in *dpp* mutant embryos (not shown). The BX-C genes act as repressors of *Dll* in the abdominal region (Cohen, 1990; Vachon et al., 1992). In *Ultrabithorax* (*Ubx*) mutations the first abdominal segment is transformed into a thoracic one and correspondingly there is an additional domain of *Dll*. We find that *Ubx* mutant embryos also exhibit an additional btd/Sp1 domain (Fig. 5C) in the first abdominal segment. These results suggest that *btd* mediates the regulation of *Dll* exerted by *wg* and *Ubx*.

There are two major points emerging from the preceding results. The first is the delimitation of *btd/Sp1* activity to a particular group of cells in the mid-ventral region of the embryonic thorax. This is achieved by the regulatory activities of *wg*, *dpp*, the BX-C genes and perhaps of other factors. The second point is that once activated, *btd/Sp1* induces the activity of genes like *Dll* and *hdc*, which are necessary for ventral disc development.

Requirement for *btd* and *Sp1* during imaginal development

As shown above, *btd* and *Sp1* are necessary for the initial formation of the disc primordia, but are also expressed in mature ventral imaginal discs (Fig. 2), suggesting that they may be required during imaginal disc development. To test the imaginal function we first induced a large number of marked btd^{XG81} clones (see Materials and methods) during larval period in all body structures, including legs and antennae. All these clones developed and differentiated normally according to their position, and behaved like the twin btd^{+} control clones. The specific function of *Sp1* could not be tested because there is no individual mutation at this gene. We tried to recover clones homozygous for the *Df(1)C52*, in which both *btd* and *Sp1* are deleted, but as expected (they are deficient for over 60 genes included in the interval 8E4-9C), these clones did not survive.

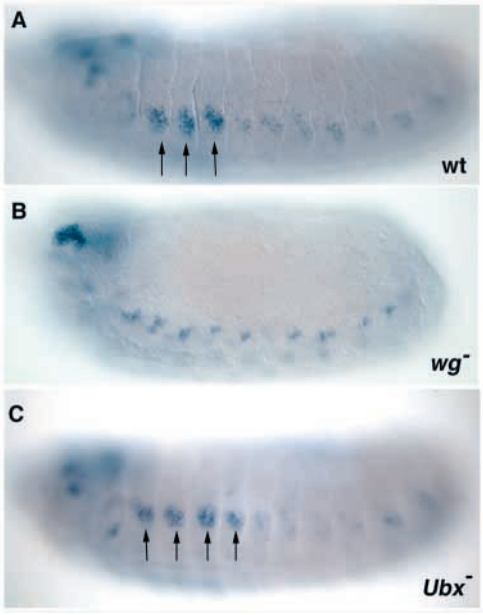


Fig. 5. *btd* expression in disc primordia requires *wg* activity and is downregulated by *Ubx* in the first abdominal segment. (A) *btd* expression in a wild-type embryo. Arrows indicate the three thoracic imaginal primordia. (B) wg^{-} embryo lacking *btd* expression in the thoracic imaginal primordia. Dots of expression observed probably correspond to the precursors of the CNS. (C) In an Ubx^{1} mutant embryo, there is an additional imaginal primordium in the first abdominal segment, which also expresses *btd* (arrows).

The negative result obtained with btd^{XG81} clones was not conclusive because there was the possibility that the loss of *btd* function could be rescued by that of *Sp1*, which is expressed in the same cells. Besides, there is evidence that *btd* and *Sp1* have partially redundant roles (Schock et al., 1999). We then tested the imaginal requirements for *btd* and *Sp1* by RNA interference using *UAS-btdi* and *UAS-Sp1i* constructs (see Materials and methods). As shown in Fig. 6, these are able to reduce strongly *btd* and *Sp1* transcripts levels. As a rule the *btd*-Gal4 driver was used to check on the morphological effects on legs and antennae. This driver was chosen because it directs expression specifically in the btd/Sp1 domain. Moreover, it is mutant for *btd*, thus diminishing the amount of wild-type function in the combinations. The general result is that the inactivation of either *btd* or *Sp1* alone (*btd-Gal4>btdi* or *btd-Gal4>UAS-Sp1i*) fails to affect legs and antennae; at most some reduction of the tarsus was observed in some *btd-Gal4>UAS-Sp1i* legs. However, when the two genes are inactivated, as in *btd-Gal4>UAS-btdi UAS-Sp1i* flies grown at 29°C, these are unable to hatch and the antennae and legs are affected in all cases, though to a different extent. The morphological effects are illustrated in Fig. 6A-C. There is no change of identity, but the growth is severely impaired. In the antennae the stronger effect is in the second segment, which is very reduced or absent. The first ant third segments are also affected in size, but the arista is normal. In the legs there is a strong reduction in size, which appears to affect all segments, which often fuse. We also observed similar but weaker effects with the *Dll*-Gal4 line (not shown).

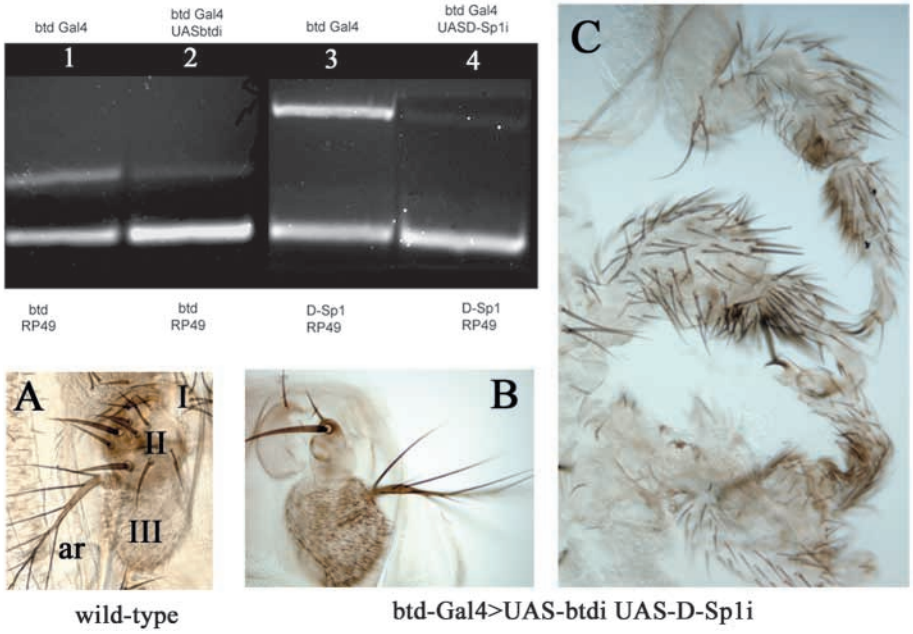


Fig. 6. RNA interference experiments. The upper left panel shows the transcript levels for RP49, as an internal control, and of *btd* and *Sp1*. Lanes 1 and 3 show normal levels of *btd* and *Sp1* in control *btd-Gal4* larvae. Lane 2 shows decreased *btd* levels in a *btd-Gal4>UAS-btdi* larva (compare with lane 1). Lane 4 shows the very low *Sp1* transcript levels in *btd-Gal4>UAS-Sp1i* in comparison with control (lane 3). (A) Wild-type antenna. Antennal segments I, II and III, and the arista (ar) are indicated. (B) Antenna of a fly of genotype *btd-Gal4>UAS-btdi UAS-Sp1i*. Note the reduction in size of the segments I and II. (C) Legs of a fly of the same genotype. All leg segments are reduced in size and often fuse.

The pattern abnormalities observed in the double RNAi experiment reflects a requirement during imaginal disc development. Taking advantage that the phenotype of *btd-Gal4>UAS-btdi UAS-Sp1i* flies is temperature dependent (at 29°C there is strong effect, but at 17°C there is practically no alteration) we performed temperature shifts to ascertain whether *btd* and *Sp1* are required during the larval period. Shifting the temperature from 17 to 29°C during the second or during the third larval period produced morphological alterations in the adult flies that were very similar to those of flies grown entirely at 29°C, indicating a requirement during the late phases of imaginal disc development.

The effects of RNA interference fit very well with the expression pattern in the antennal and leg discs (Fig. 2), and indicate that *btd* and *Sp1* have a imaginal function necessary for the growth of antennae and legs. This function appears to be redundant.

Ectopic *btd* activity transforms dorsal disc derivatives into ventral ones.

The developmental potential of the Btd protein was tested by inducing ectopic *btd* activity in different adult regions. In some experiments, we used Gal4 lines to direct activity in specific body regions whereas in others induced flip out clones of *btd*-expressing cells all over the body (see Materials and methods). The principal result is that ectopic *btd* activity is able to transform dorsal cephalic and thoracic adult structures into the corresponding ventral ones according to the segment.

The effect in the eye was studied with the *eyeless*-Gal4 line, which confers expression in the eye cells, and also inducing marked clones of *btd*-expressing cells. In *ey-Gal4/UAS-btd* flies the eye disappears and is replaced by an additional antenna which often fuses with the normal one (Fig. 7A). The same transformation was observed in clones of *btd*-expressing cells: clones located in the eye differentiate antennal structures (Fig. 7B).

In the thoracic segments we used the *nub*-Gal4, MD743 and *omb*-Gal4 lines (see Materials and methods). The *nub*-Gal4 line confers high expression levels in the wing pouch and also in the corresponding region in the haltere, reflecting accurately the domain of activity of the *nubbin* gene (Ng et al., 1995). The result of expressing *btd* in these regions is illustrated in Fig. 7C,D: the wings and halteres differentiate leg tissue. The leg tissue in these flies is not arranged in segments as normal legs are, but it is possible to recognise pattern elements corresponding to most leg segments; distal markers such as claws are found as well as tarsal and tibia-like bristles. The amount of leg tissue produced in each of these appendages is greater than the corresponding individual legs, a phenomenon for which we do not have a clear explanation. In favourable cases it is possible to find morphological markers that indicate the segmental identity of the leg tissue; in the wing segment the leg tissue often contains the ‘edge’ bristle that indicates second leg identity.

We note that *btd* is able to induce a complete set of leg structures even if its activity is induced in different regions of the wing disc. For example, the MD743 and the *omb*-Gal4 lines drive expression in different and non-overlapping regions, the hinge/pleura and the center of the wing respectively. Yet *MD743/UAS-btd* and *omb-Gal4/UAS-btd* flies develop similar sets of leg structures in the wing disc derivatives, suggesting that *btd* has a capacity to induce leg development independently of the local context.

The transformation of wings and halteres to leg was also observed in the flip out clones expressing *btd*. Both in the haltere and the wing *btd*-expressing clones differentiate leg tissue. These clones normally appear as either vesicles of tissue segregated from the wing or the haltere, or as protruding tissue (Fig 7E,F). They tend to form leg patterns resembling the normal ones, which include the formation of various leg segments, suggesting that the positional mechanism to form the proximodistal leg axis is being activated (see below). Moreover, these clones develop nearly complete leg patterns independently of the wing region where they localise. In the example shown in Fig. 7E,F, the clone is located in the posterior compartment but it forms a leg pattern that includes the ‘edge’ bristle, which corresponds to the inventory structures of the anterior leg compartment.

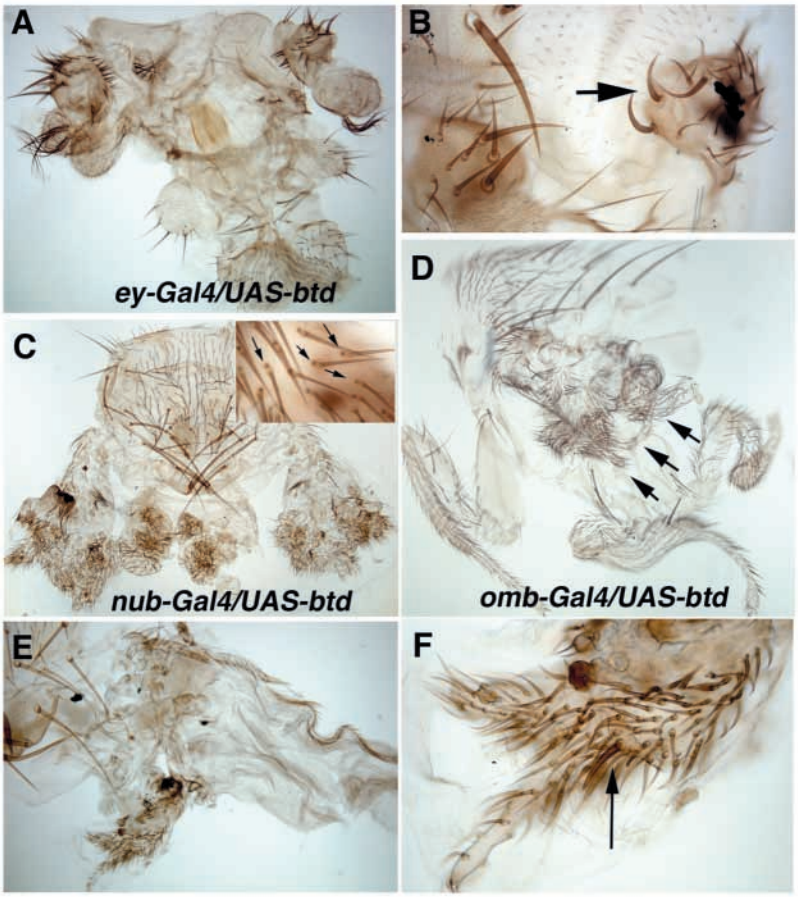


Fig. 7. Ectopic *btd* expression induces transformation of dorsal disc patterns into the corresponding ventral discs. (A) Head of an *ey-Gal4/UAS-btd* fly completely lacking eyes and showing duplication of antennae. The duplication is clear on the left side but not on the right because duplicated antennae tend to fuse. (B) Clone of *btd*-expressing cells marked with f^{36} (arrow) showing transformation towards antenna. Compare with the normal antenna towards the left. (C) Thorax of a *nub-Gal4/UAS-btd* fly. Wings and halteres are totally replaced by leg structures. Although overall leg patterns observed in this genotype are abnormal, the individual pattern elements of leg identity can be recognised. The inset shows a high magnification of a region of a transformed wing: all the bristles present an associated bract, a typical feature of leg bristles (arrows). Note that the bracts are similarly orientated with respect to the bristles in all cases, indicating that the ectopic leg patterns have acquired normal polarity. (D) Side view of an *omb-Gal4/UAS-btd* fly illustrating the transformation towards leg of most of the wing (halteres are also transformed although they are not visible). Although there are supernumerary leg structures, they often form local arrangements that reproduce normal leg patterns. Arrows indicate tibia-like and tarsus-like patterns. (E) A large clone of *btd*-expressing cells in the wing about to sort themselves from the wing cells. (F) Higher magnification of the clone in E showing the autonomously generated leg pattern and the presence of an edge bristle, which is characteristic of the midleg (arrow).

Ectopic *btd* recapitulates the formation of ventral discs primordia, including the activation of the Wg and Dpp signalling pathways

The transformation of the dorsal into ventral structures described in the preceding experiments can be visualised before differentiation takes place by the activation of specific ventral genes, accompanied by the repression of dorsal ones.

btd-expressing clones in the eye gain expression of *Dll* and *hth* (Fig. 8), the two selector-like genes that contribute to antennal identity (Casares and Mann, 1998; Dong et al., 2002). They also lose *eyeless* expression (not shown). These clones adopt a round shape, indicative of having acquired different cell affinities, probably owing to the change of identity. In the wing disc *btd*-expressing clones lose expression of *vestigial* (*vg*) (Fig. 8D,H), a gene that confers wing identity (Kim et al., 1996). In parallel these clones acquire *hth*, *dachshund* (*dac*) and *Dll* activity (Fig. 8E-K). The gain of *hth* expression is of interest, as it occurs in clones located in the center of the wing where there is no local *hth* activity. This suggests that *btd*-expressing clones tend to recapitulate the development of the entire leg disc, including the proximal region where *hth* is expressed (Rieckhof et al., 1997). This possibility was further explored by generating large clones in the first larval period. Their size facilitates the study of the expression of leg disc marker genes. These clones often show a spatially discriminated expression of *hth*, *dac* and *Dll* just like the normal leg disc; some are shown in Fig. 9. The activity of *hth* is always restricted to the periphery of the clone (Fig. 9A,B), whereas *dac* and *Dll* extend inside. Although the expression of *dac* and of *Dll* partially overlap, they occupy different parts of the clones (Fig. 9C-F). In some favourable cases (Fig. 9E,F), their domains define three different regions within the clone: a region containing only *dac* expression, a medial one containing *dac* and *Dll* and yet another only containing *Dll* activity. This arrangement resembles closely the normal distribution of these genes products along the PD axis of the leg disc (Lecuit and Cohen, 1997).

The same observation was made using Gal4 lines. For example, in *MD743/UAS-btd* flies only the pleural region is transformed towards leg and this transformation can be visualised by a local outgrowth in the wing disc (not shown). Although the size of the leg outgrowth is variable, *dac* and *Dll* are differentially expressed in all discs examined. Their domains are overlapping but not co-extensive.

The differential deployment of leg markers within the groups of cells expressing *btd* suggested that the Btd product triggers the signalling mechanism, involving the Hh, Dpp and Wg pathways, that normally patterns the leg disc (Basler and Struhl, 1994; Lecuit and Cohen, 1997). We have checked this by examining the expression of *engrailed* (*en*), *wg* and *dpp* in the *btd*-expressing clones.

The activity of *en* marks the distinction between anterior (A) and posterior (P) compartment, which is fixed during embryogenesis (Lawrence and Morata, 1977; Vincent and O'Farrell, 1992). Thus, all the *btd*-expressing clones, which are initiated during the larval period, are originally either of A (no cell expresses *en*) or P (all cells express *en*) provenance. We find that many of these clones exhibit *en* activity only in part

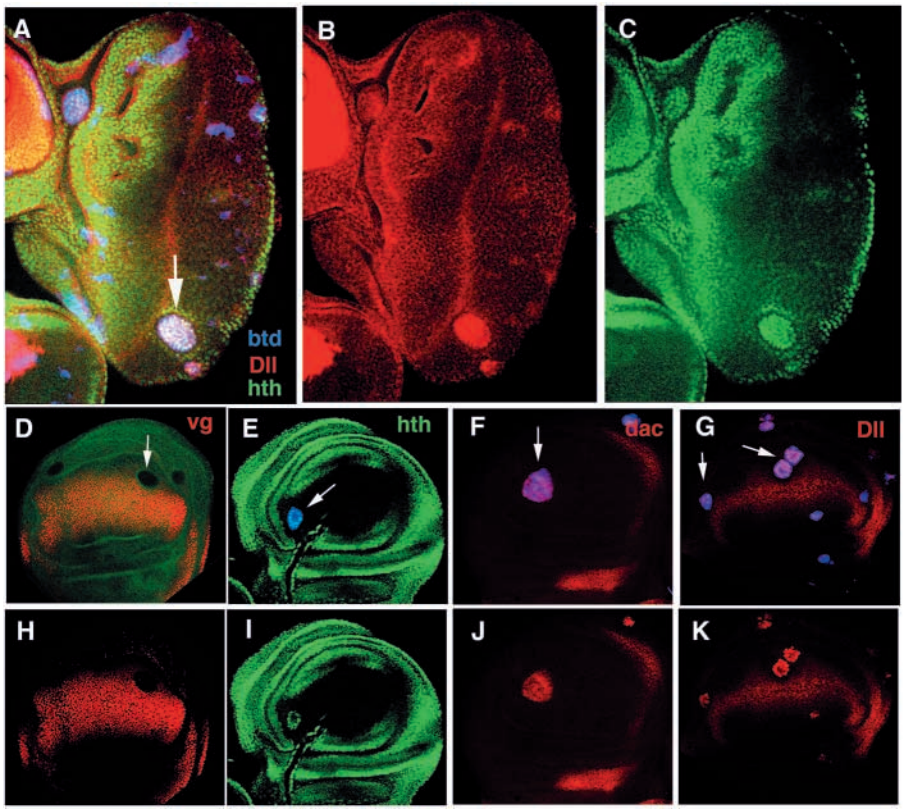


Fig. 8. Clones of *btd*-expressing cells in eye and wing discs induce activity of developmental markers of antenna and leg discs respectively. (A) The arrow indicates a clone of *btd*-expressing cells (blue) in the eye field, inducing *Dll* (red, B) and *hth* activity (green, C). Other clones scattered in the eye in the same picture also show similar effect. (D) Clone (arrow) of *btd*-expressing cells (marked by the loss of CD2, see Materials and methods) in the wing disc showing loss *vg* activity (red, H). (E) Another clone in the wing (arrow, blue) showing gain of *hth* function (green, I). (F) Clone inducing gain of dac activity (red, J) in the wing. (G) Several *btd*-expressing clones (some are indicated by arrows) inducing ectopic *Dll* activity (red, K).

of the clone (Fig. 10A-D). Thus, depending on their original assignment they either gain or lose *en* activity. Some are located within the P compartment and therefore lose *en*, whereas others are in the A compartment and gain *en*. The formation of this sharp border of *en* expression within the clones is expected to originate a zone of *hedgehog* (*hh*) activity, which in turn induces local activation of *wg* and *dpp* (Basler and Struhl, 1994). Indeed, we find that *wg* and *dpp* are activated in the *btd*-expressing clones. This is illustrated in Fig. 10E-H, showing that either gene is activated in part of *btd*-expressing clones. It is very likely that the appearance of the Wg and Dpp signals what induces the spatially discriminate expression of *dac* and *Dll* in the clones.

These results suggest that *btd* has the potential to induce the formation of the ventral imaginal discs, or at least the antennal and leg discs. The effect is not restricted to the appendage part, it also extends to the proximal region, defined by the activity of genes such as *hth* or *esg* (which is expressed in the same cells as *hth*).

Discussion

The *btd* gene performs several roles in development. Previous work has shown that it acts in combination with *orthodenticle* and *empty spiracles* to specify the development of mandibular, intercalary and antennal segments (Cohen and Jürgens, 1990; Wimmer et al., 1993). The related *Sp1* gene, located immediately adjacent to *btd* (FlyBase) performs partially redundant functions (Wimmer et al., 1996; Schock et al., 1999) in the peripheral nervous system and the head segments. Both genes are expressed in the primordia of the thoracic discs (Wimmer et al., 1996) (this report), but the functional significance of this expression domain was not known.

Our work demonstrates a novel and also redundant function of *btd* and *Sp1*: they are responsible for the formation of the ventral imaginal discs by activating the genetic network necessary for their development. Furthermore, we show that the Btd protein retains the capacity of inducing the entire ventral genetic network during the larval period. We propose that the activation of btd/Sp1 is the crucial event in the determination of the ventral structures of the adult fly.

btd and *Sp1* are responsible for the formation and required for the growth of the ventral discs

Our argument is based on the finding that *btd* and *Sp1* appear to mediate all events connected with the formation of the ventral discs. We center the discussion in the leg disc, but there is evidence that antennal primordium also requires *btd* (Cohen and Jürgens, 1990). Moreover, we have also observed that the genital primordium is lacking in *Df(1)C52* embryos (C.E. and G.M., unpublished), suggesting that this disc is also under the same control. Most of our experiments concern the function of *btd* but given the expression and functional similarities between the two genes (Wimmer et al., 1996; Schock et al., 1999) (this report), we assume that *Sp1* encodes the same or very similar role. Therefore, we will refer to *btd/Sp1* as a single function.

One crucial feature is that *btd* is an upstream activator of *Dll* and *hdc*, which are considered developmental markers of disc primordia (Cohen, 1993; Weaver and White, 1995): (1) *btd* expression precedes that of *Dll* and of *hdc*; (2) the btd expression domain includes those of *Dll* and *hdc*; (3) in *btd* mutants, *Dll* and *hdc* activity is much reduced, and completely absent in *Df(1)C52* embryos; (4) Ectopic *btd* function induces ectopic activation of *Dll* and *hdc*.

The role of *btd* in embryogenesis can be illustrated in the light of the models of *Dll* regulation (Cohen et al., 1993; Kubota et al., 2000). *Dll* is activated by *wg* and its expression modulated by the EGF *spitz* and by *dpp*, whereas it is repressed in the abdominal segments by the BX-C genes (Vachon et al., 1992).

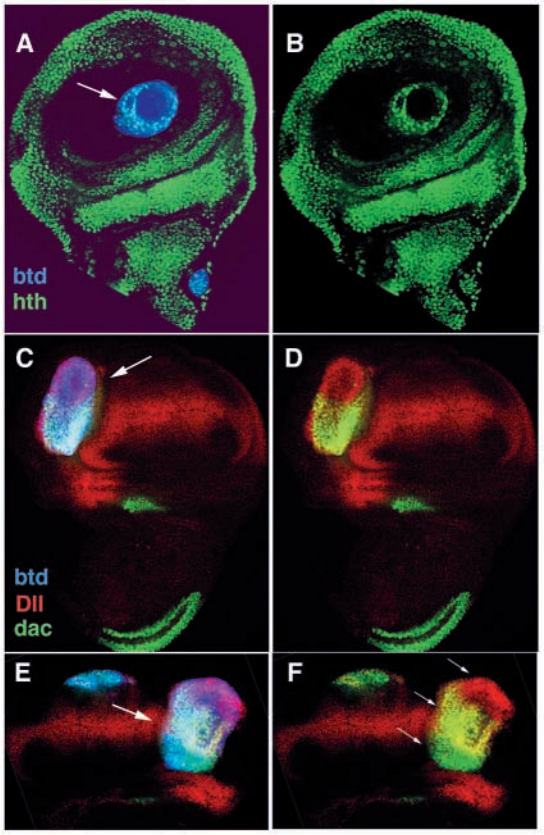


Fig. 9. Ectopic *btd* activity in wing induces spatially discriminated expression of leg developmental markers. (A) Large *btd*-expressing clone (blue) induces *hth* activity (green), but only on the periphery (B), resembling the disposition of the normal leg disc. (C) Another *btd*-expressing clone showing non-homogenous gain of Dll and dac activity: all cells express *Dll* (red) but only about half gain *dac* (green, D). (E) Another clone (arrow) showing a spatial deployment of *dac* and *Dll* resembling closely that along the PD axis in the normal leg (F): *dac* activity alone (green), *dac* and *Dll* (yellow), and *Dll* only (red). In the normal leg, these patterns of gene expression would correspond to the positions of the femur, tibia and tarsus, respectively.

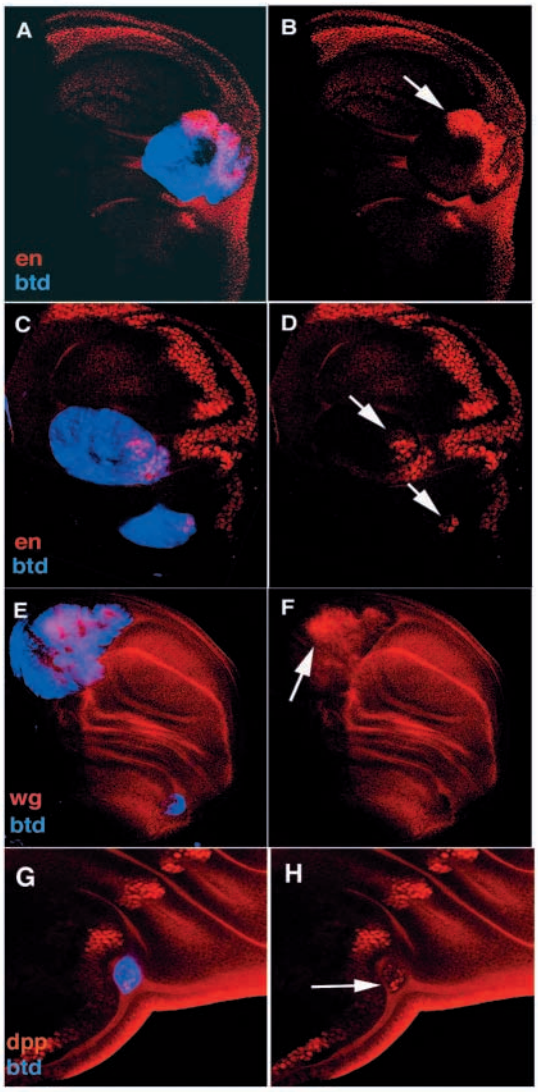


Fig. 10. Ectopic *btd* activity induces the production of morphogenetic signals in the wing and haltere discs. (A) Clone of *btd*-expressing cells in the wing disc showing *en* activity only in part of the clone (arrow, B). (C) Two clones in the haltere disc both showing *en* activity inside the clones but only in some cells (arrows, D). (E) Local gain of *wg* activity in a clone of *btd*-expressing cells. Only some cells show high *wg* levels (arrow, F). (G) Clone of *btd*-expressing cells in the hinge region of the posterior compartment showing gain of *dpp* activity in some cells (arrow, H).

Our experiments suggest that *Dll* regulation is mediated by *btd*: in *wg* mutants there is no *btd* expression and hence neither *Dll* nor *hdc* activity. In *dpp* mutant embryos, *btd* expands to the dorsal region resembling the effect on *Dll* (Goto and Hayashi, 1997). In *Ubx*$^{-}$ embryos there is an additional group of cells in the first abdominal segment expressing *btd*; the same cells that also express *Dll* in those embryos. The interpretation of the role of *btd* mediating *Dll* regulation by *Ubx* is complicated by previous observations (Vachon et al., 1992) showing direct repression of *Dll* by the Ubx protein. It is possible that *Ubx* regulates *Dll* both directly and by controlling *btd* activity.

We propose that the localisation of *btd/Sp1* activity to a group of ventral cells is a major event in the specification of adult structures. *btd* and *Sp1* are activated in response to spatial cues from Wg, Dpp, EGF and BX-C, and in turn their function induces the activity of the genes necessary for ventral imaginal development.

This hypothesis is strongly supported by the results obtained inducing ectopic *btd* activity in the dorsal discs, as just the presence of the Btd product alone is sufficient to bring about ventral disc development. In the wing and the haltere discs, Btd induces a transformation into leg, whereas in the eye it induces antennal development. This indicates that it specifies ventral disc identity jointly with other factors, e.g. the Hox genes, possibly through the activation of subsidiary genes such as *Dll*, known to contribute to ventral appendage identity in combination with Hox genes (Gorfinkiel et al., 1997).

The requirement for *btd* and *Sp1* activity appears to be restricted only to the ventral discs, even during the early phases of the thoracic disc primordia. In this context it is worth considering the observation that in *Df(1)C52* embryos there is *esg* expression in the wing and haltere disc primordia, even

though it is absent in the leg discs. Thus, the wing and haltere discs are formed in the absence of *btd* and *Sp1*. Because in these embryos there is a almost complete lack of *Dll* expression, this observation raises the question of the origin of the dorsal thoracic discs, which are currently considered to derive from the original ventral primordium, formed by cells expressing *Dll* (see Cohen, 1993; Kubota et al., 2000). Although some of the original group of ventral cells may contribute to the dorsal disc primordia, our data suggest that there may be cells recruited to form the dorsal discs that do not originate in the initial ventral primordium. Accordingly, it is worth considering that in the absence of *Dll* activity the leg and wing discs are formed (Cohen et al., 1993), although the leg only differentiates proximal disc derivatives. Thus, the activity of *Dll* cannot be considered a reliable marker for imaginal discs.

Our RNA interference experiments also indicate that both *btd* and *Sp1* are required for the growth of the antennal and leg discs. When the two gene functions are reduced simultaneously, leg segments fuse and there is an overall reduction in the size of antennae and legs. The reduction of growth affects the proximal and distal regions of the appendage (Fig. 6), and assigns a role to the expression observed in the imaginal discs. The two genes are able to perform this function on their own, for the inactivation of only one is not sufficient to impair growth. This conclusion is also supported by the observation that mutant *btd* clones do not have any effect, as they still possess *Sp1* activity, which is sufficient for normal development. At this point we do not know the mechanism by which *btd/Sp1* may affect growth.

btd activity induces the entire genetic network necessary for leg development

One particularly significant result about the mode of action of *btd* comes from the analysis of the ectopic leg patterns observed in the wing and halteres. The cells expressing *btd* tend to recapitulate the complete development of leg and antennal discs. For example, the whole genetic network necessary to make a leg appears to be activated. *btd* induces the activity of *hth*, *dac* and *Dll*, the domains of which account for the entire disc (Lecuit and Cohen, 1997; Abu-Shaar and Mann, 1998). Furthermore, *hth*, *dac* and *Dll* are activated in a spatially discriminated manner: in the clone shown in Fig. 9A,B *hth* is expressed only in the peripheral region, resembling the normal expression in the leg disc; in the clone in Fig. 9E,F the discriminate expressions of *dac* and *Dll* define three distinct regions. The formation of the dac and Dll domains is dependent on signalling from Wg and Dpp, although they require different signal thresholds (Lecuit and Cohen, 1997), but the hth domain is independent from Wg and Dpp (Abu-Shaar and Mann, 1998).

The generation of distinct hth, dac and Dll domains within the clones suggested that *btd*-expressing cells in the wing and haltere generate their own signalling process. Indeed, we find that within these clones there is local activation of *en*, the transcription factor that initiates Hh/Wg/Dpp signalling in imaginal discs (reviewed by Lawrence and Struhl, 1996). *btd*-expressing clones also acquire *wg* and *dpp* activity in subsets of cells (Fig. 10). It is probably in the boundary of *en*-expressing with non expressing cells where the Wg and Dpp signals are generated de novo; subsequently, their diffusion initiates the same patterning mechanism which operates during normal leg development. The result of this process is that the *hth*, *dac* and *Dll* genes are expressed in different domains contributing to form leg patterns containing DV and PD axes. One question for which do not have a clear answer is how the initial asymmetry is generated, so that a few cells within the group gain (or lose) *en* activity. We have observed that the cells expressing *en* within the clones are (Fig. 10A-D) those closer to the posterior compartment cells. It is conceivable that there might be an external signal, perhaps mediated by Hh, which triggers the initial asymmetry.

The ability of cells expressing *btd* to build their own patterning mechanism is also indicated by the observation that inducing *btd* activity in different parts of the wing disc results in the production of similar sets of leg pattern elements. For example, in *MD743/UAS-btd* and *omb-Gal4/UAS-btd* flies, *btd* is induced in different, non-overlapping wing regions, and yet all leg pattern elements are produced in both genotypes. Thus, the effect of *btd* is by and large independent of the position where it is induced, e.g. it does not depend on local positional signals.

A relevant issue is whether the ability of the Btd product to induce the formation of the full array of ventral structures has a functional significance in normal development. We believe that this property may be a faithful reflection of the original *btd/Sp1* function: the activation of the developmental program to build the ventral adult patterns. We envisage *btd/Sp1* function as follows. During the embryonic period, the conjunction of several regulatory factors (Wg, Dpp, EGF, Hox genes) allows activation of *btd/Sp1* in a group of cells in each thoracic segment (we assume a similar process takes place in the head). These cells become the precursors of the ventral imaginal discs and will eventually form the ventral thorax of the adult – which includes the trunk (the hth domain) and appendage (the Dll domain) regions. The activity of *btd/Sp1* is instrumental in segregating these ventral discs precursors from those of the larval epidermis and determining their imaginal fate. It is involved in specifying their segment identity (in collaboration with the Hox genes) and in establishing their pattern and growth. To achieve the latter role *btd/Sp1* induces the production of the growth signals Wg and Dpp, probably in response to localised activation of *en* and subsequent signalling by *hedgehog* (*hh*).

A problem with this model is that in normal development all the genes involved, *hth*, *en*, *hh*, *wg* and *dpp*, are expressed in embryos prior to *btd/Sp1*. Why should a new round of activation be necessary? Although we cannot offer a totally satisfactory answer, we note that clones of *btd*-expressing cells in wing or haltere lose their memory of *en* expression. Those that originated in the A compartment had no previous *en* expression, but gained it in some cells. Conversely, all cells in P compartment clones contained *en* activity but some lose it. The best explanation for this unexpected behaviour is that *btd* provokes a ‘naïve’ cell state in which the previous commitment for *en* activity is lost. Later, *en* activity is re-established. We believe this phenomenon may reflect the process that occurs in normal development. The initial btd/Sp1 domain may not inherit the previous developmental commitments and has to build a new developmental program. It is worth considering that the *btd/Sp1* function appears to determine ventral imaginal fate as different from larval fate. This is a major developmental decision, which may require de novo establishment of the

genetic system responsible for pattern and growth. A key aspect of this would be the localised activation of *en* in part of the btd/Sp1 domain. We speculate that there might be a short-range signal, perhaps Hh, emanating from nearby *en*-expressing embryonic cells, that acts as an inducer in the btd/Sp1 primordium. There is evidence that Hh can induce *en* activity (Guillen et al., 1995).

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