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Common and diverged functions of the *Drosophila* gene pair *D-Sp1* and *buttonhead*

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Abstract

The *Drosophila* gene *buttonhead* (*btd*) is required for the formation of the mandibular, the intercalary and the antennal head segments of the embryo. The *btd* protein (BTD) is functionally and structurally related to the human C_2H_2 zinc finger transcription factor Sp1. A second Sp1-like *Drosophila* gene, termed *Drosophila Sp1* (*D-Sp1*), had been identified on the basis of a partial sequence showing that the gene encodes a characteristic zinc finger domain, composed of three finger motifs similar to both *Sp1* and *btd*. *D-Sp1* is located in the same cytological location as *btd* in chromosome band 9A on the X-chromosome. It had been proposed that *D-Sp1* and *btd* are likely to act as a gene pair and function in a at least partially redundant manner. Here we report the molecular analysis of *D-Sp1* and its expression pattern during embryonic and larval development. We show that *D-Sp1* acts as a transcriptional regulator. Lack-of-function analysis combined with rescue and gain-of-function studies indicates that *btd* and *D-Sp1* play essential and redundant roles for mechanosensory organ development. However, *D-Sp1* lacks the specific features of BTD required for embryonic intercalary and antennal segment formation.

Keywords: D-Sp1; BTD; Zinc finger; Peripheral nervous system; Chordotonal organ

1. Introduction

Drosophila head development involves the activity of the gene *buttonhead* (*btd*). *btd* is expressed and functionally required in the blastoderm anlagen of the mandibular, intercalary and antennal segments (Wimmer et al., 1993) which fail to develop in *btd* mutant embryos (Cohen and Jürgens, 1990). It encodes a transcription factor similar to the human transcription factor Sp1 (Wimmer et al., 1993). The *btd* protein (BTD) has an Sp1-like modular design with separable DNA-binding and activation domains, represented by C_2H_2 zinc fingers (Klug and Schwabe, 1995) and glutamine- and serine/threonine-rich domains, respectively. BTD is able to activate transcription through the GC-rich DNA target site of Sp1 (Wimmer et al., 1993) and supports in vitro transcription via the same components of the basal transcription machinery as Sp1 (Schöck et al., 1999). Despite these similarities and the identical biochemical features of the two proteins, transgene-dependent expression of Sp1 in place of BTD rescued only mandibular segment structures of *btd* mutant embryos, indicating that Sp1 cannot replace all aspects of BTD required for head development.

Recently, a second gene which encodes a BTD/Sp1-like zinc finger domain had been identified and termed *D-Sp1* (Wimmer et al., 1996). Partial sequence analysis of the gene encompassing the putative zinc finger domain suggested that it is a member of the Sp1 family of transcription factors, including BTD and the vertebrate proteins Sp1, Sp2, Sp3 and Sp4 (Kadonaga et al., 1987; Hagen et al., 1992; Kingsley and Winoto, 1992; Wimmer et al., 1993). These proteins share the critical three amino acids in each zinc finger loop which are known to contact DNA and ensure target specificity of human Sp1 (Narayan et al., 1997). *btd* and *D-Sp1* were shown to be located in chromosome band 9A on the X-chromosome. Additionally, the two genes were found to be expressed in similar spatial and temporal patterns during postblastodermal embryogenesis (Wimmer et al., 1996). The common expression domains include a restricted pattern in the developing central nervous system and embryonic brain, as well as in the leg anlagen, where the

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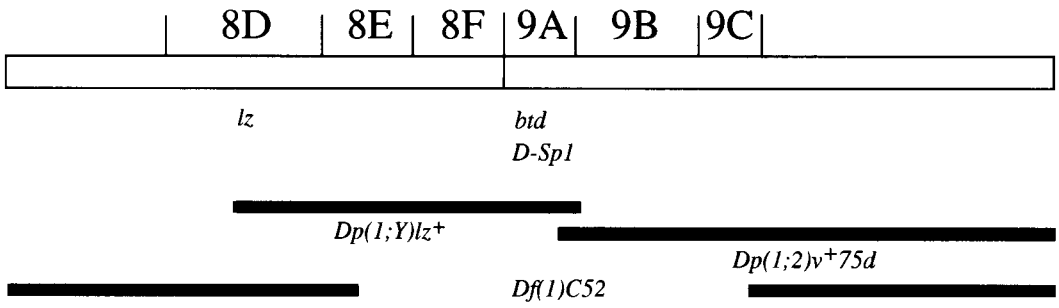

Fig. 1. Genetic and physical organisation of the *D-Sp1* locus. (A) Genomic organisation and conceptual translation of the *D-Sp1* cDNA. The genomic sequence is shown in italics; the putative TATA box and initiator sequence upstream of the transcription start site are underlined. The cDNA sequence which corresponds to the genomic sequence apart from the poly(A)$^+$ tail is shown in plain letters; the putative translation initiation site (Cavener, 1987) is underlined, non-matching nucleotides are indicated in lower case letters. Conceptual translation of the *D-Sp1* open reading frame is depicted below the DNA sequence; *D-Sp1* consists of an alanine-, a serine/threonine- and a glutamine-rich region and three zinc fingers in between (underlined). The sequence has been deposited into the EMBL nucleotide sequence database (accession number AJ131022). Note that the single-exon structure of the gene was verified by translation of in vitro transcribed RNA derived from a subcloned genomic DNA fragment containing the open reading frame. Only a single protein of the expected 67 kD size was obtained (not shown). (B) Localization of *btd* and *D-Sp1* at position 9A on the X-chromosome. Note that both genes are contained within the duplication *Dp(1;Y)lz*$^+$ and deleted from the deficiency *Df(1)C52*. Centromere is to the right. Black bars denote either duplicated parts of the chromosome or parts not deleted.

pattern is similar to the gene *Distal-less* (*Dll*) (Wimmer et al., 1996). However, only *btd* is expressed in a circumferential ring covering the anlagen of the antennal, intercalary and mandibular segments at blastoderm stage (Wimmer et al., 1993). In contrast to *btd*, transcripts of *D-Sp1* were found in low amounts throughout the early embryo but not at blastoderm stage, suggesting that the gene is expressed maternally (Wimmer et al., 1996). Based on these few observations, it had been proposed that *btd* and *D-Sp1* represent a gene pair with possibly redundant functions after the blastoderm stage (Wimmer et al., 1996).

Here we describe a molecular characterization and functional analysis of *D-Sp1*. Lack-of-function analysis combined with rescue experiments show that the development of chordotonal organs, a segmentally repeated mechanosensory organ of the peripheral nervous system, requires the combined *btd* and *D-Sp1* activities. Furthermore, each of the two genes is sufficient to induce sensory hair development when ectopically expressed in cell clusters expressing proneural genes in wing imaginal discs. The data suggest that the two genes represent a gene pair with overlapping neural functions. However, we also show that *D-Sp1* can only rescue mandibular development, but not intercalary and antennal segment formation, when expressed in *btd* mutant embryos. *D-Sp1* can therefore act as a transcriptional regulator but lacks the specific features of BTD to support the formation of pregnathal segments. We propose that *btd* has acquired head segmentation function through novel *cis*-acting control elements and by gaining protein domains that mediate intercalary and antennal in addition to mandibular segment development.

2. Results

2.1. Molecular characterization of D-Sp1

The zinc finger region of *D-Sp1* was previously cloned by a polymerase chain reaction (PCR)-based approach to isolate *btd*-homologous sequences from *Drosophila* genomic DNA (Wimmer et al., 1996). We used these sequences to isolate corresponding genomic and cDNA clones from various libraries and performed $5'$ rapid amplification of cDNA ends (RACE) to determine the transcription start site (see Experimental procedures, Section 4.2). Sequence analysis of the cDNA clones and corresponding genomic DNA revealed an unspliced, single transcript of 2270 bp in length (Fig. 1A; EMBL nucleotide sequence database accession number AJ131022). The genomic sequence upstream of the transcribed region includes a putative TATA box and an initiator sequence (Purnell et al., 1994), but no conventional polyadenylation signal was found upstream of the poly(A)$^+$ tail of the cDNA. It is therefore possible that the $3'$ untranslated region continues beyond the sequences shown in Fig. 1A.

Conceptual translation of the open reading frame shows that *D-Sp1* codes for a 523 amino acid polypeptide including the Sp1-like zinc finger domain described earlier (Wimmer et al., 1996). The deduced protein *D-Sp1* contains serine/threonine-and glutamine-rich regions as has been observed for both Sp1 and BTD (Wimmer et al., 1993). Furthermore, *D-Sp1* contains a long stretch rich in glycine and serine residues between the zinc finger domain and the glutamine-rich region and a C-terminal histidine-rich region. In addition, *D-Sp1* contains a stretch of alanines and the 'BTD-box' which is conserved in a number of Sp1 family members (Wimmer et al., 1993). Other diagnostic protein motifs or sequence similarities to other transcription factors were not found within D-Sp1.

2.2. D-Sp1 expression patterns

In contrast to *btd*, *D-Sp1* transcripts were observed in low amounts throughout the early embryo up to the preblastoderm stage (Wimmer et al., 1996). Thus, *D-Sp1* was expected to be expressed maternally. Fig. 2A shows that *D-Sp1* transcripts accumulate during oogenesis in the nurse cells and are transported into the growing oocyte. This observation explains the presence of maternal transcripts in eggs. The transcripts are likely to have a relatively

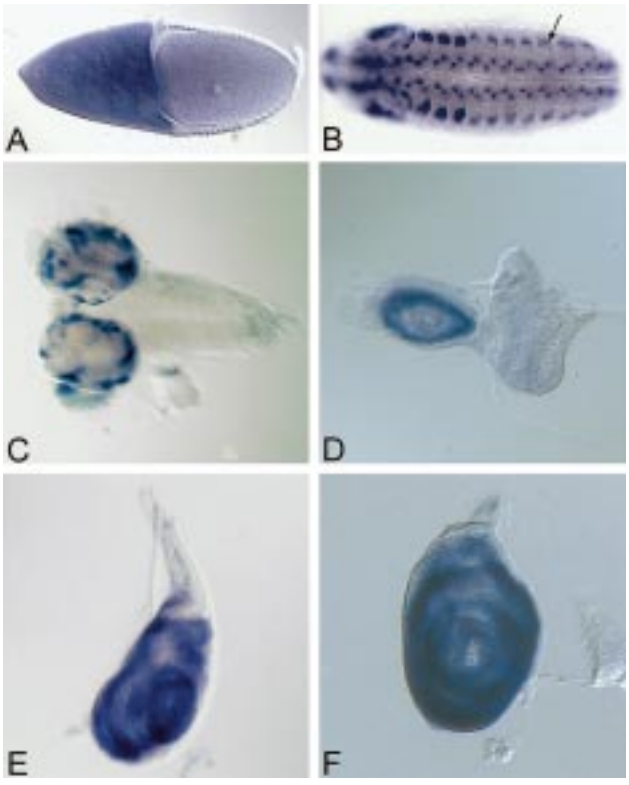

Fig. 2. *D-Sp1* expression. Whole-mount preparations were in situ hybridized with antisense *D-Sp1* RNA probe visualizing ovarial *D-Sp1* expression (A) and the expression of DSp1 in lateral chordotonal organ precursors in a stage-13 embryo (indicated by an arrow) (B), in distinct subsets of the larval brain and the ventral nerve cord (dorsal view) (C), and in concentric rings in the antennal (D), T2 leg (E) and T3 leg (F) imaginal discs of third instar larvae.

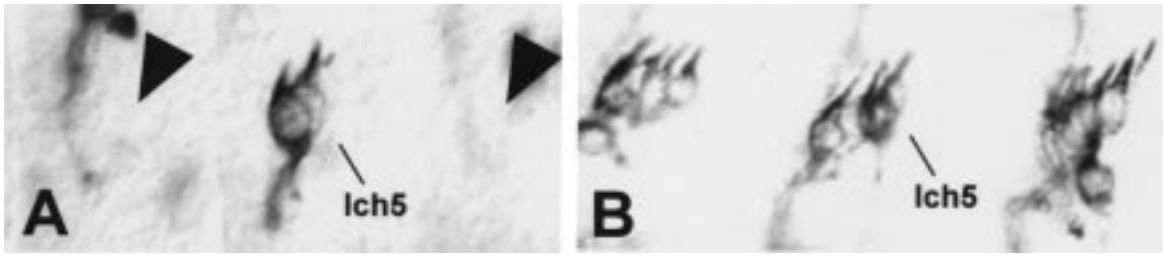

Fig. 3. *btd-D-Sp1* rescues chordotonal organ development of *btd* mutant embryos. (A) Enlarged lateral region of a hemizygous *Df(1)C52* mutant embryo showing a reduced lateral pentascolopidial chordotonal organ (lch5) in only one out of the three abdominal segments shown. Note that the lch5 is composed of two instead of normally five scolopidia (for wild-type see Figure 4a in Wimmer et al., 1996) and that two segments fail to develop the lch5 organs (arrowheads). (B) Transgene-dependent *D-Sp1* rescues lch5 development. Note the formation of 4 or 5 scolopidia in each segment. lch5 organs were visualized in stage 15 embryos (for staging see Campos-Ortega and Hartenstein, 1997) by mAb22C10 staining.

short half life since no *D-Sp1* transcripts are detected at blastoderm stage (Wimmer et al., 1996). A further undescribed aspect of the *D-Sp1* expression is its distinct pattern in the developing peripheral nervous system, covering the lateral region of the embryo which corresponds to the cluster of cells expressing proneural genes (Jarman et al., 1993) (Fig. 2B). In addition, *D-Sp1* expression is found in the larval brain (Fig. 2C) and in ventral imaginal discs of the larva, i.e. in the antenna but not the eye imaginal disc (Fig. 2D), and in the leg (Fig. 2E,F) but not the wing imaginal discs (data not shown). In the antenna disc, the pattern of *D-Sp1* expression is similar to *atonal* (*ato*) expression which is necessary for sensory organ development (Jarman et al., 1993, 1995). In the leg imaginal disc, the *D-Sp1* expression pattern is similar to *Distal-less* (*Dll*) (Diaz-Benjumea et al., 1994). Both *ato* and *Dll* code for transcription factors (Jarman et al., 1993; Cohen et al., 1989). Thus it is possible that *D-Sp1* is required for the expression of these genes or conversely, *ato* and *Dll* are necessary to express *D-Sp1*. *D-Sp1* and *ato* as well as *D-Sp1* and *Dll* could be part of a common genetic pathway, a proposal to be addressed by future experiments.

2.3. D-Sp1 and BTD carry redundant functions for chordotonal organ development

btd participates in chordotonal organ development (Wimmer et al., 1996). Chordotonal organs serve a multitude of proprioreceptive, tactile and auditory functions during the life cycle of the fly. They are involved in various behaviors such as the larval withdrawal from touch and the coordinated movements of larvae and adults (McIver, 1985). Chordotonal organs are required for the reception of stretching and vibration, and each organ consists of a defined number of scolopidia. Here we focus on the lateral pentascolopidial chordotonal organ (lch5), which is segmentally repeated in the abdominal segments of the embryo. It is composed of five scolopidia which are easily identified by morphological means due to their characteristic size and shape (McIver, 1985).

In embryos carrying a *btd* point mutation, the number of scolopida in the lch5 varies between two and five. The variability in the phenotype suggested that *btd* participates in chordotonal development, but that the development of the remaining scolopidia is likely due to the activity of other genes that potentially compensate for the loss of *btd* activity in the developing scolopidia (Wimmer et al., 1996). In view of the common expression domains of *btd* and *D-Sp1* in the developing peripheral nervous system, we reasoned that *D-Sp1* represents a candidate for carrying a *btd*-like function for lch5 development. This implies, that the absence of both genes, in deficiency mutant embryos, should enhance the lch5 phenotype.

In order to obtain such double mutant embryos, we examined chromosomal deficiencies and duplications uncovering the 9A region of the X-chromosome by in situ hybridization with antisense RNA probes for the two genes, asking whether the chromosomes carry or have lost one or both genes. Fig. 1B summarizes the results indicating that deficiency *Df(1)C52* lacks both genes. Embryos which are hemizygous for the deficiency *Df(1)C52* allow therefore to examine the contribution of *D-Sp1* to chordotonal organ development. As shown in Fig. 3, such embryos develop indeed a stronger lch5 phenotype than observed with *btd* mutants. Whereas *btd* mutants develop at least two, and usually in average three scolopidia, the deficiency mutants lacking both *btd* and *D-Sp1* develop no scolopidia in most cases and up to a maximum of two scolopidia in few chordotonal organs per embryo (Fig. 3A). Thus, *D-Sp1* or another gene within the deficiency is necessary for scolopidia development in addition to *btd*. Furthermore, since some scolopidia were found in *btd/ D-Sp1* -deficient embryos, their development must be promoted by the activity of at least a third gene outside the deficient region.

A 5.2-kb *btd cis*-acting element drives expression in the precursors of the peripheral nervous system (Wimmer et al., 1996). This feature of the *btd* enhancer allowed us to assess whether *D-Sp1* in addition to *btd* participates in lch5 development. Transgene-dependent expression of *D-Sp1* in *btd* mutant embryos showed at least four and frequently five scolopidia per lch5 (Fig. 3B) and is therefore similar to the rescue obtained with *btd* (Wimmer et al., 1996). The results show that *btd* and *D-Sp1* carry overlapping or even redundant functions for scolopidia development, but also

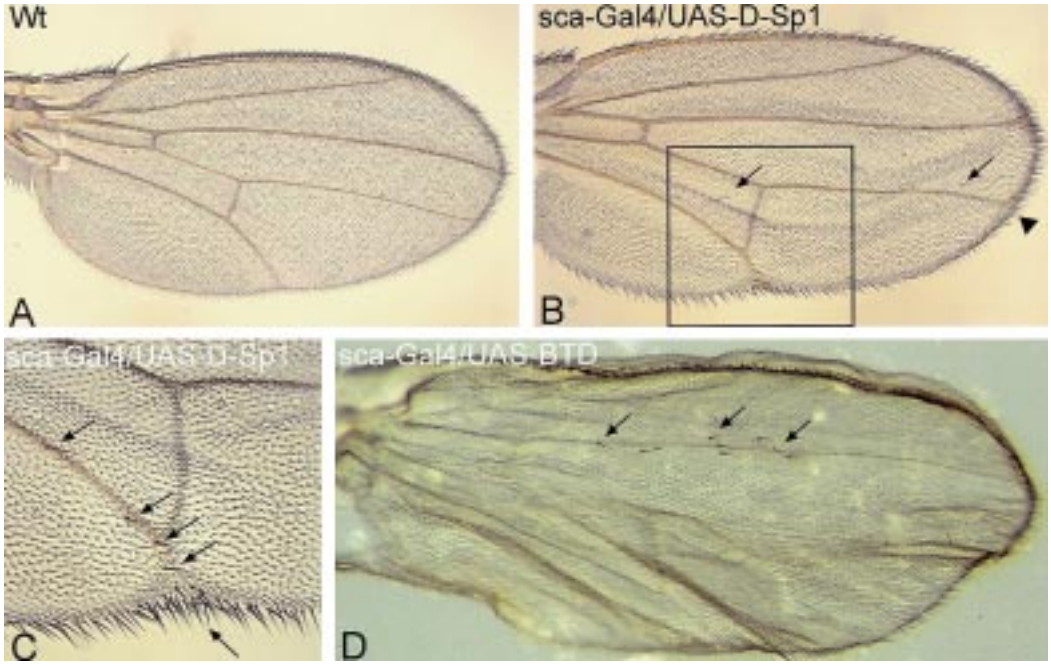

Fig. 4. Ectopic expression of *D-Sp1* in proneural clusters with the scabrous-Gal4 driver line results in ectopic delta and mechanosensory stout bristle formation. (A) Wild-type wing. (B) sca-Gal4/UAS-D-Sp1 wing at 25°C. Ectopic stout mechanosensory bristles form along the posterior wing margin, on veins and sometimes in the intervein region (arrows). Deltas can often be observed (triangle). Campaniform sensilla on L3 are absent or transformed to stout bristles. Expression of the driver line in a double row along the anterior wing margin probably causes the messed-up appearance of the anterior wing margin with less stout bristles in the middle row and ectopic bristles in rows 1 and 3. For wing bristle nomenclature please refer to Jan and Jan (1993). (C) Enlarged view of (B). (D) UAS-BTD crossed to sca-Gal4 shows ectopic stout bristles on the L3 vein (arrows).

indicate that *D-Sp1* is more efficient than BTD in rescuing scolopidia development.

2.4. D-Sp1 and BTD induce stout bristles upon ectopic expression in wing imaginal discs

Expression of *D-Sp1* and *btd* in the developing nervous system and the rescue of the lch5 suggested that *D-Sp1* and BTD act in the same neurogenic or neural pathway. In order to see whether the two genes can generate neuronal cell fates in tissues that normally do not express the genes we employed the Gal4/UAS system (Brand and Perrimon, 1993). To drive ectopic expression of *D-Sp1* or *btd* in proneural clusters of the wing imaginal disc we used a *scabrous*-Gal4 driver line (Hinz et al., 1994). Ectopic expression of *D-Sp1* caused development of mechanosensory stout bristles in ectopic places of the wing, including the posterior margin, veins and intervein regions (Fig. 4A–C). In addition, and probably due to *scabrous*-Gal4 driving *D-Sp1* expression in a double row of cells in the anterior wing margin, ectopic stout bristles develop in row 1 and 3 (for nomenclature on wing bristles see Jan and Jan, 1993). Ectopic expression of BTD caused a similar but somewhat weaker phenotype as *D-Sp1* (Fig. 4D). Since scolopidia and stout bristles are both mechanosensory cells, the results suggest that *D-Sp1* and *btd* are redundant or functionally overlapping components of a genetic circuit which specifies the mechanosensory fate of cells.

2.5. D-Sp1 cannot replace btd activity required for intercalary and antennal segment formation

The largely identical expression domains and the close relationship with respect to sequences encompassing the zinc finger region (72% identity at the amino acid level) suggested that *D-Sp1* and *btd* constitute a gene pair. Due to their partially redundant function in the peripheral nervous system, *D-Sp1* and *btd* were expected to carry similar or even redundant functions for head development. We tested this proposal by asking whether *D-Sp1* can rescue head segmentation in *btd* mutant embryos. We expressed *D-Sp1* cDNA under the control of a 5.2-kb *btd* enhancer fragment. This *cis*-acting region conducts gene expression in the *btd* blastodermal expression domain and rescues all head defects when driving expression of a *btd* cDNA in *btd* mutants (Wimmer et al., 1993, 1995, 1996, 1997). In *btd* mutant embryos, transgene-derived *D-Sp1* expression rescued only mandibular structures as shown by mandibular *engrailed* (*en*) expression and the presence of mandibular head structures. Antennal or intercalary structures were not rescued (Fig. 5A–D). Mandibular rescue and the failure to rescue the pregnathal segments lacking in *btd* mutant embryos was independent of the number of *D-Sp1* transgene copies (data not shown). Thus, as has been observed with human Sp1, *D-Sp1* lacks the specific features of BTD necessary for pregnathal segment development. The results allow the conclusion that *D-Sp1* acts as a transcriptional regulator

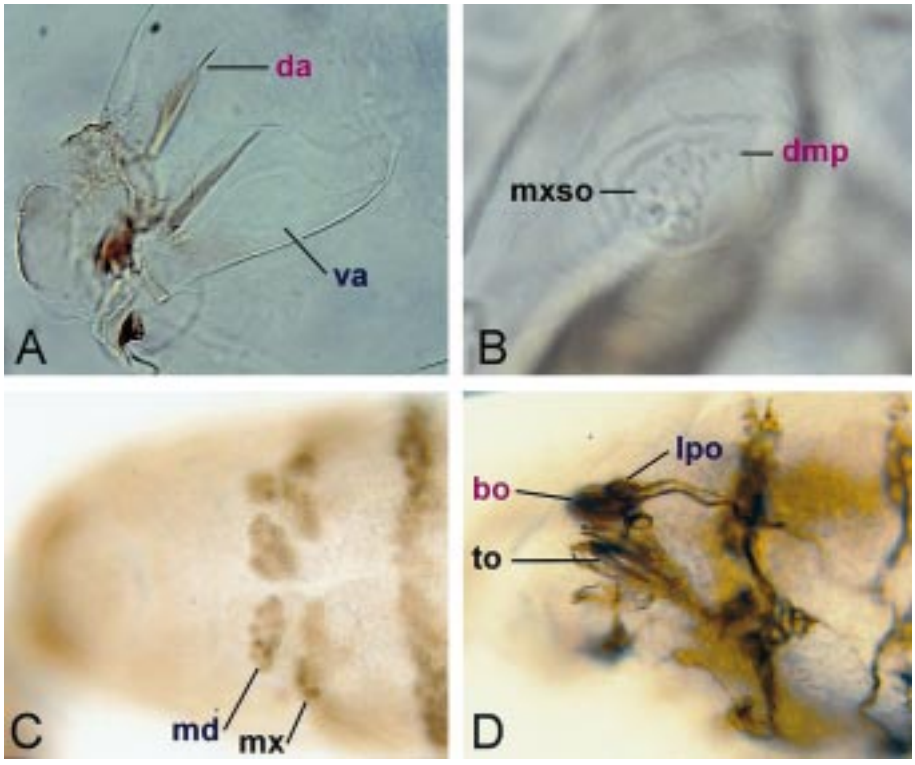

Fig. 5. *btd-D-Sp1* partially rescues the *btd* mutant embryonic head phenotype. (A–D) *D-Sp1* expressing *btd* mutant embryos showing a head skeleton with rescued ventral arms of mandibular origin (A), an antennomaxillary complex with no sign for antennal or intercalary rescue (B), the prospective head region of an embryo (ventral view) showing rescue of the mandibular *engrailed* expression domain (C) and mAb22C10 staining at a representative focal plane (lateral view of the head region) showing that a sensory organ of mandibular origin (lpo) is rescued whereas antennal and intercalary organs are absent (D). Anterior is to the left. Anti-Engrailed-antibody-stained embryos are at stage 11, mAb22C10-stained embryos are at stage 15, cuticles were prepared from unhatched larvae (stage 17; stages according to Campos-Ortega and Hartenstein, 1997). Abbreviations : amc, antennomaxillary complex. Cuticle structures or head sensory organs of ocular (oc) origin: bo, Bolwig organ; da, dorsal arms; dmp, dorsomedial papilla. Of mandibular (md) origin: lpo, lateropharyngeal organ; va, ventral arms. Of maxillary (mx) origin: mxso, maxillary sense organ; to, terminal organ. For details on the wild-type head structures and identity of the head sensory organs see Schmidt-Ott et al. (1994); Wimmer et al. (1993).

which is able to control the BTD target genes necessary for mandibular development. However, BTD must contain additional protein domains that are missing in Sp1 as well as in *D-Sp1* and are necessary for the promotion of pregnathal segments.

3. Discussion

We present evidence that *D-Sp1* and *btd* represent a gene pair with overlapping or redundant functions in the peripheral nervous system. Both genes are expressed in the proneural clusters and participate in the formation of the lateral chordotonal organ. In addition, the ectopic expression of either *D-Sp1* or *btd* in proneural clusters of the wing imaginal discs, where both genes are normally not expressed, caused the formation of stout mechanoreceptory bristles. *D-Sp1/btd* expression in the proneural cluster corresponding to the lateral chordotonal organ, and *D-Sp1*expression in a ring of cells within the antenna imaginal disc corresponding to the future second antennal segment, coincide or at least overlap with the expression domains of *ato*. This proneural gene is necessary for the specification of chordotonal organs and several adult sensory organs including the Johnstons organ of the second antennal segment (Jarman et al., 1993, 1995). Thus, *ato* represents a prime candidate for being a direct or indirect target of *D-Sp1* and *btd* activity, or vice versa.

In contrast to the common neural function, *D-Sp1* and *btd* have clearly diverged functions which are reflected in non-overlapping spatial and temporal expression patterns during oogenesis and early embryonic development. *D-Sp1* is expressed maternally and its transcripts are also observed, although in low amounts, in the early embryo up to the preblastoderm stage. In contrast, *btd* is first expressed at blastoderm stage in a stripe covering the region of the three head segments that fail to develop in *btd* mutant embryos (Wimmer et al., 1993). *D-Sp1* cannot compensate for the lack of *btd* activity, but is able to cause mandibular development when expressed in place of BTD. This observation shows that the two genes differ not only with respect to different cis-acting control regions but also hold different potentials in generating head segments. The intercalary and antennal segments require besides *btd* also the activity of the genes *empty spiracles* and *empty spiracles* plus *orthodenticle*, respectively (Cohen and Jürgens, 1990). Exactly these

segments are not rescued in response to D-Sp1. Thus, *D-Sp1* can replace BTD in a manner similar to human Sp1 (Wimmer et al., 1993), although the rescue of mandibular structures was more complete in response to *D-Sp1* as compared to Sp1. Since Sp1 has been shown to act as a transcriptional activator, these results establish by biological means that *D-Sp1* is a transcriptional regulator likely to function as an Sp1-like activator.

The molecular conservation, the close chromosomal localization and the overlapping expression patterns and functions of BTD and *D-Sp1* in neural development argue in favor of a gene pair derived from a common ancestor with neural function. In fact, by molecular means, the closest relative of *D-Sp1* is the vertebrate factor Sp4 (Wimmer et al., 1996) which is specifically expressed in the nervous system. *Sp4* knock-out mice do not show embryonal lethality but show rather ill-defined defects during adult life (Supp et al., 1996). Due to the lack of a *D-Sp1* point mutation, we do not know the exact *D-Sp1* mutant phenotype. However, the extremely variable leg malformation phenotype observed in adult *btd*-transgene rescued *btd* mutants (Wimmer et al., 1996) and the fact that the *btd*-transgene cannot rescue *Df(1)C52* mutants to adulthood (data not shown) argue for a gene that functions partially redundant to *btd* in larval and pupal stages, which is likely to be *D-Sp1*.

Studies in yeast, vertebrates and *Drosophila* indicate that the lack of certain specific genes can have remarkably little effect on the phenotype of cells or embryos (González-Gaitán et al., 1994, and references therein), since the function of such genes is buffered by the activity of other genes involved in the same or a parallel genetic pathway. This phenomenon is attributed to gene duplications during evolution. Evidence for such duplications has been observed with a number of *Drosophila* segmentation genes, including *engrailed*, *knirps*, *gooseberry* or *sloppypaired*, which are structurally related genes expressed in similar or identical temporal and spatial patterns. In addition, the genes of each pair are located in close chromosomal proximity (González-Gaitán et al., 1994, and references therein). We propose that BTD and *D-Sp1* derived from a common ancestor with neural function. This proposal is also consistent with the observation that *D-Sp1* is more closely related to the *Sp4* gene of mouse which is expressed in neural tissue exclusively (Supp et al., 1996; Wimmer et al., 1996). This implies that *btd* has acquired novel functions in head segmentation. In this case, *btd* had to gain new *cis*-acting control regions for the expression in the head anlagen at blastoderm stage. Furthermore, BTD can promote intercalary and antennal segment formation, a feature not associated with D-Sp1. The common rescue of mandibular development by both D-Sp1 and Sp1, and the additional segments generated in response to BTD, show that BTD might also have gained protein domains to promote development of pregnathal head segments, a phenomenon not yet seen with other known gene pairs. Alternatively, the common ancestor of *btd* and *D-Sp1* may have been involved in establishing anterior structures, and *D-Sp1* has lost this feature, the required control and the corresponding domains of the protein. A distinction between these possibilities has to await further analysis of the genes and their functions in other species.

4. Experimental procedures

4.1. Fly work

The following fly strains were used in this study: *Df(1)C52*, *Dp(1;Y)lz*$^+$, *Dp(1;2)v*$^+$*75d* (obtained by the Bloomington stock center, USA), *btd*^{XG}, *btd*^{XG} *svb* and transgenic lines carrying a genomic *btd* rescue fragment (P_{btd}; Wimmer et al., 1993), *D-Sp1* driven by the *btd cis*-acting element (btd-D-Sp1, this work), UAS-BTD (this work), UAS-D-Sp1 (this work) and sca-Gal4 (Hinz et al., 1994). To determine the lethal stage of P_{btd} and wild type crossed to *Df(1)C52/+*, 200 embryos were collected, aligned and 24 h later the fraction of unhatched embryos was calculated. btd-D-Sp1 was constructed by cloning a 3.4-kb genomic *Xba*I fragment containing the intronless *D-Sp1* gene into the *Xba*I-digested P-element vector pCbtdRV–2ndΔXba (Wimmer et al., 1997). UAS-D-Sp1 was generated by cloning a 2.3-kb *Xba*I/*Xho*I genomic *D-Sp1* fragment into the respective sites of pUAST (Brand and Perrimon, 1993). UAS-BTD was made by cloning a 2.6-kb *Bam*HI/*Not*I fragment of pKSbtd (Wimmer et al., 1997) into pUAST digested with *Bgl*II and *Not*I. To generate transgenic flies, the constructs were injected in *w* embryos using pΔ2-3 (Laski et al., 1986) as helper (Rubin and Spradling, 1982). Transgenic progeny was balanced over CyO or TM3. At least two independent transgenic lines were analysed. For the Gal4/UAS misexpression experiment, we analysed adults of the crosses sca-Gal4 to UAS-D-Sp1 or UAS-BTD at 18, 25 and/or 29°C.

4.2. Genetic and molecular characterization of the D-Sp1 locus

Df(1)C52 alone or crosses of *Df(1)C52* with *Dp(1;Y)lz*$^+$ and *Dp(1;2)v*$^+$*75d* were analysed for the presence of *D-Sp1* by RNA in situ hybridization using *btd* and *D-Sp1* antisense RNA probes. Genomic and cDNA clones were obtained by screening a genomic λFixII phage library (strain Canton S, Stratagene) or a 0–18-h embryonic plasmid cDNA library (strain Canton S, Clontech), respectively. 5′-RACE was performed with a cDNA library (a kind gift of Ralf Pflanz) made from embryonic poly(A)$^+$ RNA with the Marathon cDNA Amplification Kit (Clontech). Nested PCR (primer pair AP11: 5′-CCATCCTAATACGACTCACTATAGGGCT-3′ and dSp2: 5′-CGCCCACACCAACGCCAACTCC-3′; nested primer pair AP2: 5′-ACTCACTATAGGGCTCGAGCGGC-3′ and dSp1: 5′-CGATCCCAACAGCGAATGACTC-3′) resulted in a single 420-bp fragment. All clones were sequenced using an ABI373A sequencer (Perkin-Elmer). [^{35}S]methionine-labelled in vitro translated D-Sp1

was generated using a coupled transcription/translation system (TNT, Promega).

4.3. In situ hybridization and immunocytochemistry

RNA in situ hybridization of whole-mount embryos was carried out with digoxigenin-labelled antisense RNA probes (Tautz and Pfeifle, 1989). Immunological stainings of whole-mount embryos were performed as described (González-Gaitán and Jäckle, 1997) using as primary antibodies anti-β-galactosidase (Cappel), anti-EN (4D9) (Patel et al., 1989) and mAb22C10 (Zipursky et al., 1984) together with the Vectastain ABC Elite Kit (Boehringer Ingelheim). Hemizygous *btd* mutant embryos were identified using blue balancers (González-Gaitán and Jäckle, 1997). Stained embryos and cuticle preparations (Wimmer et al., 1996) were analysed and photographed with a Zeiss Axiophot microscope.

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