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Mechanisms of Development 59 (1996) 53–62

buttonhead and *D-Sp1*: a novel *Drosophila* gene pair

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Received 23 May 1996; revision received 24 June 1996; accepted 24 June 1996

Abstract

The *Drosophila* gene *buttonhead* (*btd*) is a gap-like head segmentation gene which encodes a triple zinc finger protein structurally and functionally related to the human transcription factor Sp1. Here we report the pattern of *btd* expression during embryogenesis. *btd* is not only expressed and required in the blastoderm anlagen of the antennal, intercalary and mandibular segments as reported previously, but both expression and requirement extend into the anlage of the maxillary segment. From gastrulation onwards, *btd* is expressed in distinct spatial and temporal patterns, suggesting that *btd* might be required for a number of developmental processes beyond head segmentation. In fact, analysis of *btd* mutant embryos revealed that *btd* participates in the formation of the peripheral nervous system. However, no other morphologically apparent phenotype was observed. We identified a *btd*-related gene, termed *D-Sp1*, which is expressed in temporal and spatial patterns similar to *btd* during postblastodermal development. No localized expression domains of *D-Sp1*, which is located in the same X-chromosomal band as *btd*, were seen during the blastoderm stage. The results suggest that *D-Sp1* and *btd* represent a novel gene pair with partially redundant functions after the blastoderm stage.

Keywords: Chordotonal organs; Gene pair; Head development; Peripheral nervous system; Zinc finger proteins

1. Introduction

Embryonic pattern formation is based on the elaboration and interpretation of maternally deposited morphogens that define spatially restricted expression of zygotic genes (reviewed in St Johnston and Nüsslein-Volhard, 1992). The three genes *orthodenticle* (*otd*), *empty spiracles* (*ems*) and *buttonhead* (*btd*) act as gap-like segmentation genes mediating the function of the morphogen bicoid (bcd) in the anterior head region (Cohen and Jürgens, 1990). The blastodermal expression of the three genes is regulated by bcd in a concentration-dependent manner (Dalton et al., 1989; Finkelstein and Perrimon, 1990; Walldorf and Gehring, 1992; Wimmer et al., 1995), and each of these genes is required in a contiguous block of two or three head segments (Cohen and Jürgens, 1990). Their domains of action overlap, but are out of phase by one segment at their posterior margins, suggesting that a combinatorial input of these genes directly specifies head segmentation (Cohen and Jürgens, 1990).

otd and *ems* code for homeodomain proteins, whose embryonic expression and requirement is not restricted to the blastoderm stage. In the extended germband stage, *otd* is expressed in epidermal cells along the ventral midline, and *otd* mutants show defects in the pattern of the ventral medial denticles and abnormalities in the specification of medial cells in the central nervous system (Finkelstein et al., 1990; Wieschaus et al., 1992). In the same stage, *ems* is expressed in a metameric pattern surrounding the tracheal pits and *ems* mutations result in posterior spiracles devoid of Filzkörper (Dalton et al., 1989; Walldorf and Gehring, 1992). *btd* (Wieschaus et al., 1984) encodes a zinc finger protein with sequence and functional similarity to the human transcription factor Sp1. Initial *btd* expression at blastoderm was previously shown to be restricted to a head stripe and an anterior dorsal spot (Wimmer et al., 1993). Here we show that *btd* is also expressed in multiple spatio-temporally restricted patterns during postblastodermal development. The lack of these aspects of *btd* activity affects only the development of

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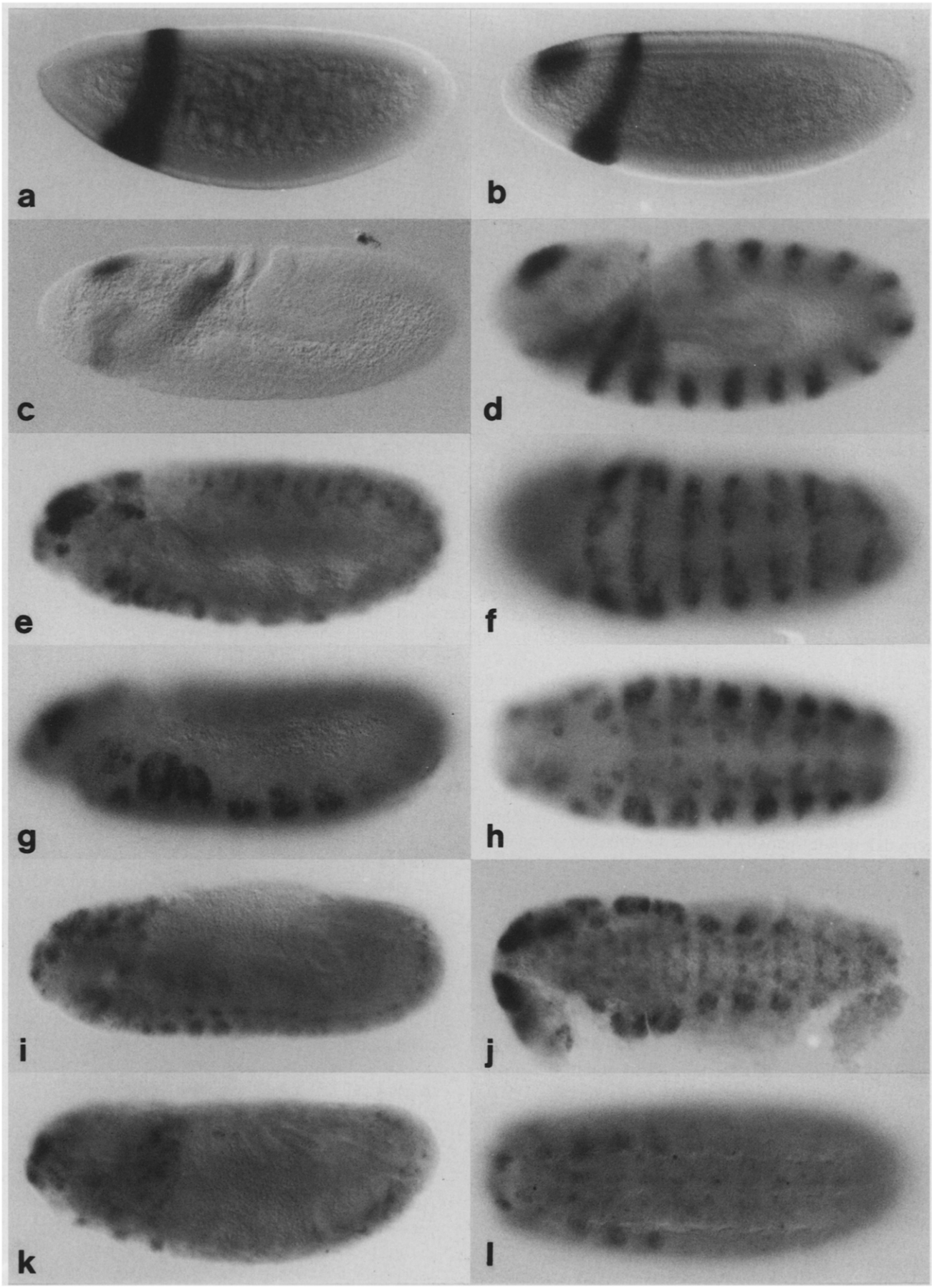
PII S0925-4773(96)00575-8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polyscolopidial chordotonal organs, indicating that *btd* functions in the formation of the peripheral nervous system but does not seem to be required in regions corresponding to the other expression domains. We identified a novel *btd*-related gene, termed *D-Sp1*, which is expressed in patterns covering most of the postblastodermal *btd* expression domains. Functional redundancy between the two genes might explain why *btd* mutants develop no morphologically apparent mutant phenotype in the regions of overlapping *btd* and *D-Sp1* expression.

2. Results and discussion

2.1. Embryonic expression pattern of buttonhead

The early stripe domain of *btd* expression at the blastoderm stage covers the anlagen of the head segments affected in *btd* mutants and vanishes during germ band extension (Fig. 1a–c) (for details see Wimmer et al., 1993). During cellularization of the blastoderm a dorsal head spot appears in the proneural region anterior to the head stripe (Fig. 1b). The head spot expression continues (Fig. 1c–e) and splits up during germ band retraction into several smaller spots which become integrated into the developing brain, marking different brain areas (Fig. 1i). During the early phase of germ band extension *btd* starts to be expressed in a metameric pattern (Fig. 1d,f). This pattern of expression decays at the fully extended germ band stage leaving single *btd*-expressing cells (Fig. 1e,h,j). The *btd*-expressing cells represent subgroups of neuroblasts, which finally end up in the ventral nerve cord (Fig. 1i,l).

During germ band extension, a second metameric expression pattern of *btd* can be observed. It is restricted to the lateral region of the embryo (Figs. 1e,f,h and 2a), corresponding to the area of the proneural clusters from which the peripheral nervous system originates (Bodmer et al., 1989). This aspect of *btd* expression is transient and vanishes during the late phase of germ band extension (Fig. 1g,j), when *btd* is expressed in the leg anlagen located in the thoracic segments and in several restricted areas of the developing head (Fig. 1g,j). At this stage, the pattern of *btd* expression resembles *Distal-less* (*Dll*) expression (Cohen, 1990). However, *btd* expression is delayed compared to *Dll* expression. Furthermore, *btd* is expressed in the mandibular but not in the labral segments, whereas *Dll* is expressed in the labral, but not in the mandibular segments. As observed with *Dll*, *btd* expression remains in the leg anlagen until the end of embryogenesis, when they form small epidermal sacs representing the early imaginal discs of the first instar larva at the ventral side of the thoracic segments (Fig. 1k,l).

2.2. Lack of buttonhead activity affects the anterior portion of the maxillary segment

The *btd* head stripe expression in the syncytial blastoderm embryo extends from 65% to 77% egg length (100% represents the anterior pole; Wimmer et al., 1993). This region of the blastoderm contains the anlagen of the antennal, intercalary and mandibular segments as well as the anlage of the maxillary segment, which maps around 70% egg length (Jürgens et al., 1986). A first phenotypical analysis of *btd* mutants, undertaken before the cloning of the gene (Cohen and Jürgens, 1990), was limited to an examination of the larval cuticle markers and the expression of segment polarity genes during gastrulation. These analyses revealed that *btd* mutant embryos fail to develop antennal, intercalary and mandibular segments (Cohen and Jürgens, 1990). However, since the most anterior part of the maxillary segment does not give rise to a morphologically distinct cuticular structure, and since the expression of the marker genes *Dll*, *wingless* (*wg*) and *engrailed* (*en*) is limited to the central and posterior part of the segment, disturbances affecting the anterior portion of the maxillary segment would have escaped previous examinations.

In order to see whether the maxillary segment is affected in *btd* mutant embryos, we examined the development of wild type and *btd* mutant embryos by scanning electron microscopy. The results shown in Fig. 3 confirm that *btd* mutant embryos lack the antennal, intercalary and mandibular lobes. In addition, those embryos lack the anterior border of the maxillary lobe. This finding is consistent with an earlier study on head organization by ectopic expression of the homeotic selector gene *Ultrabithorax*, suggesting that the most anterior part of the maxillary segment is deleted in *btd* mutants (González-Reyes and Morata, 1991). The deletion pattern of head segments caused by *btd* mutations is therefore neither in segmental register as previously suggested (Cohen and Jürgens, 1990), nor in parasegmental register, since the respective *wg* and *en* stripes are affected together (Cohen and Jürgens, 1990).

Fig. 1. *btd* expression during embryogenesis. In situ hybridization with *btd* cDNA 5-2 (Wimmer et al., 1993) to embryo whole mount preparations. (a) Syncytial blastoderm: *btd* expressed in a head stripe. (b) Cellular blastoderm: head stripe plus anterior dorsal head spot. (c) Gastrulation: head stripe expression vanishes. (d,f) Early phase of germ band elongation: expression in metameric pattern. (e,h) Germ band elongated embryos: first metameric expression decays leaving single cells expressing *btd*. Lateral metameric pattern becomes visible. (g,j) Late phase of germ band elongation: *btd* expressed in *Dll*-like pattern. (i) Germ band retracting embryo: *btd* head spot expression splits into several patches in the brain. (i,l) Single cells in the ventral nerve cord express *btd*. (k,l) Embryos after dorsal closure: *btd* expressed in central nervous system and in *Dll*-like expression pattern. Small epidermal sacs corresponding to the early leg discs of first instar larva show *btd* expression. (a–e,g,i,k) Lateral view, dorsal up. (f,h,j,l) Ventral view. Anterior to the left.

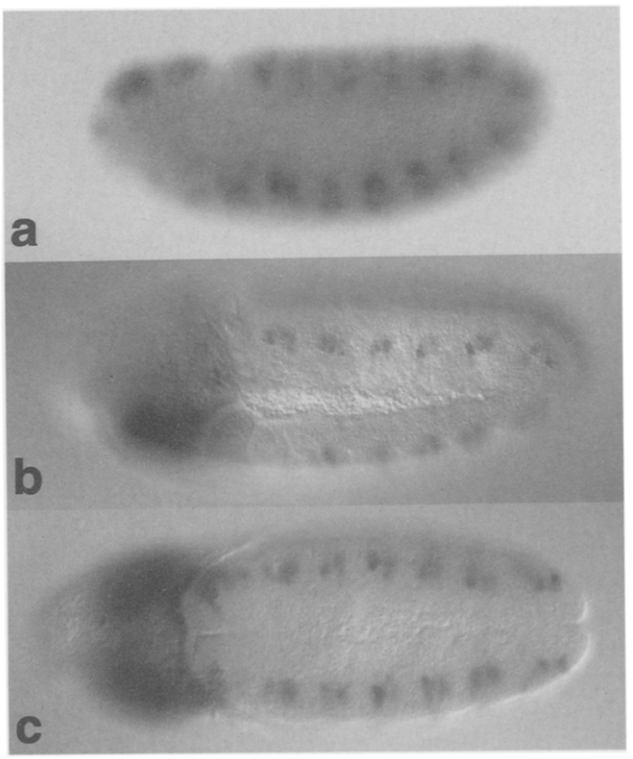

Fig. 2. Expression pattern of *btd* reporter gene construct containing the 5.2 kb upstream region of a *btd* transgene. (a) In situ hybridization with *btd* cDNA 5-2: germ band extended embryo showing the lateral metameric expression pattern of *btd* in the peripheral nervous system anlage. (b,c) Antibody stainings with anti-β-galactosidase antibodies: germ band extended embryos carrying the reporter gene construct *btd* RV-2ndB (Wimmer et al., 1995) which mediates staining in the peripheral nervous system anlage resembling *btd* expression. (b) Lateral, (c) dorsal view.

2.3. *buttonhead participates in the formation of the peripheral nervous system*

btd is expressed in the proneural clusters of the peripheral nervous system (see above; Fig. 2a). In order to identify possible morphological disturbances in the peripheral nervous system, we analyzed *btd* mutant embryos stained with the neuronal marker mab22C10 (Fujita et al., 1982). *btd* mutants show reduced numbers of scolopidia in thoracic and abdominal polyscolopidial chordotonal organs (Fig. 4a,b), while the other structures of the peripheral nervous system appear normal. Chordotonal organs are internal organs for the reception of stretching and vibration, and each chordotonal organ consists of a defined number of aligned scolopidia (McIver, 1985).

For a more detailed analysis of the *btd* phenotype in the peripheral nervous system, we focused on the lateral pentascolopidial chordotonal organs (lch5) in the abdominal segments of the embryo. In wild type, these organs consist of five scolopidia each, while the lch5 of *btd* mutants contain between two and five scolopidia. The expressivity of the *btd* phenotype varies from segment to segment and from embryo to embryo. We scored several hundred lch5 of *btd* mutant embryos of the btd^{XO}, btd^{XA} and btd^{XG} genotypes (Cohen and Jürgens, 1990). In btd^{XO} and btd^{XG}, the lch5 display an average of three scolopidia, whereas in btd^{XA} the average number of scolopidia is between three and four (Table 1). This result is consistent with the previous observation that btd^{XA} produces a weaker head phenotype than btd^{XO} and btd^{XG} (Cohen and Jürgens, 1990).

btd expression in the peripheral nervous system anlage is mediated by the *btd* 5.2 kb upstream region which is contained in a *btd* transgene shown to rescue *btd* mutants to viability (Wimmer et al., 1993), since embryos expressing a reporter gene under the control of the *btd* 5.2 kb upstream region (construct *btd* RV-2ndB; Wimmer et al., 1995) show expression in the proneural clusters resembling *btd* expression (Fig. 2). This expression is, besides the *btd* head stripe, the only other pattern mediated by the *btd* 5.2 kb upstream region. None of the other reporter gene constructs used in the analysis of *btd* cis-regulatory elements (Wimmer et al., 1995) mediate *btd*-like expression in the anlagen of the peripheral nervous system, suggesting complex cis-regulatory requirements which can only be found in the intact 5.2 kb upstream region.

The frequency of the transgene-rescued *btd* mutant adults was low (Wimmer et al., 1993). In order to determine the portion of *btd* mutant embryos which were rescued by transgene-dependent *btd* activity, we marked the X-chromosome carrying btd^{XG} with the mutation *shaven baby* (*svb*) (Wieschaus et al., 1984). The *svb* mutation causes a reduction of the larval denticles, which serves as a morphological marker for the *btd* mutation-bearing chromosome (Fig. 5). The head phenotype of embryos containing the double mutant chromosome is rescued with about 100% frequency by the *btd* transgene (Fig. 5d). In *btd* mutant embryos carrying the *btd* transgene the number of scolopidia in lch5 is still slightly reduced, varying between two and five. However, the average number of scolopidia is increased by more than one as compared to the *btd* mutants lacking the transgene (Table 1, Fig. 4b,c). This indicates that *btd* activity participates in the formation of lch5.

Chordotonal organs serve a multitude of proprioceptive, tactile and auditory functions during the life cycle of a fly, and they are involved in various behaviors like the larval withdrawal from touch and coordinated movement of adult flies (McIver, 1985). The function of these organs has been shown not to be required for larval and pupal survival if raised in a non-competitive environment. However, the adult flies with chordotonal defects are disabled, showing uncoordinated or sedentary behavior (Kernan et al., 1994). Such a behavior is also seen with transgene-rescued *btd* mutant males (Wimmer et al., 1993), i.e. they never fly nor mate, they rarely move and show very uncoordinated footwork when moving. Part, but not all of these defects may be explained by leg malformations, which vary in penetrance and expressivity (Wimmer, 1995). The involvement of *btd* in the forma-

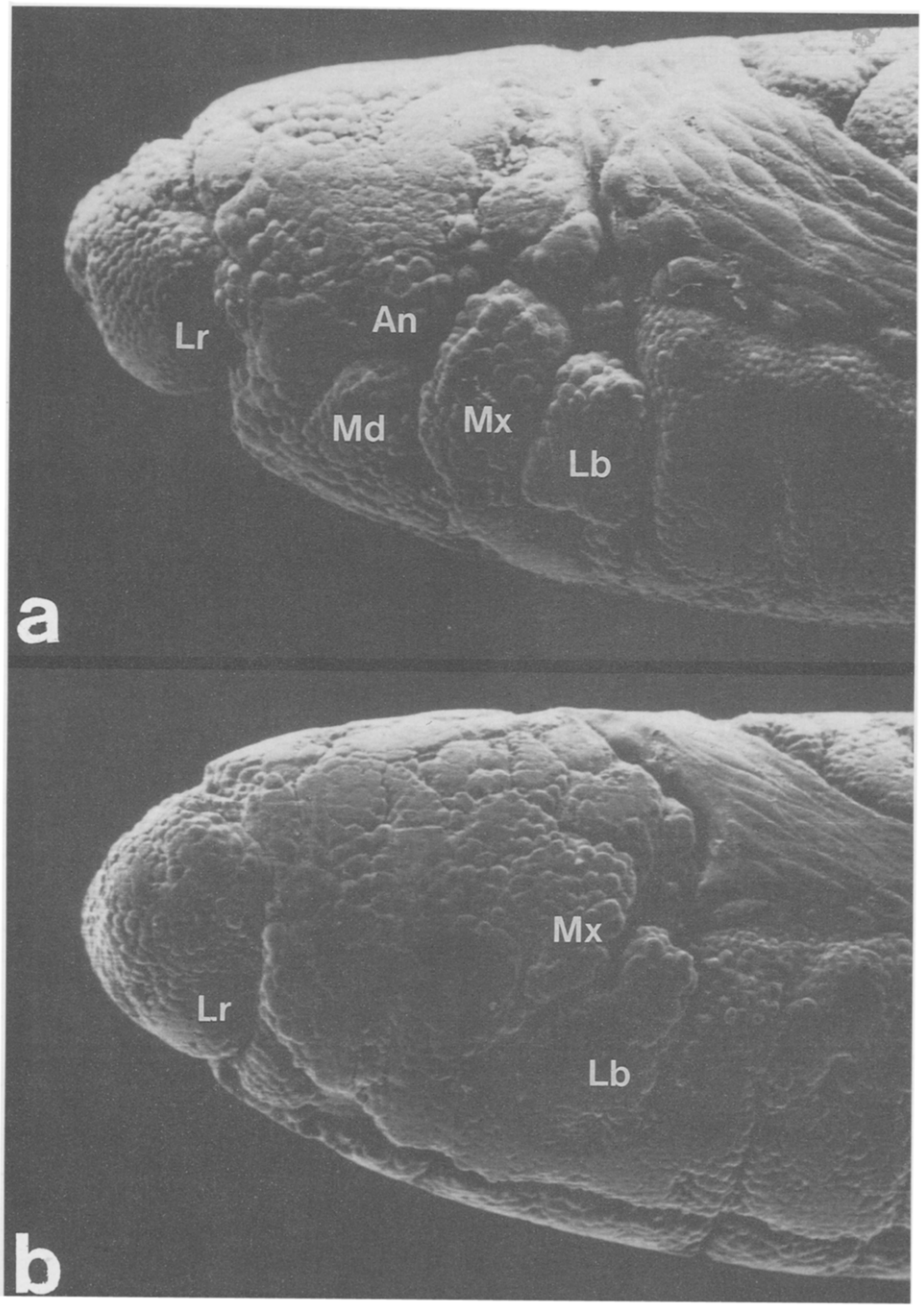

Fig. 3. *btd* head phenotype. Scanning electron microscopy of wildtype and *btd* mutant embryos. Depicted are the head regions of embryos in extended germ band stage (anterior to the left and dorsal up). (a) Wildtype embryo. (b) btd^{XG} mutant embryo: labral (Lr) and labial (Lb) segments as well as the posterior part of the maxillary (Mx) segment can be identified, whereas the anterior border of the maxillary segment plus the mandibular (Md) and the antennal (An) segments are missing.

tion of embryonic chordotonal organs and the participation of chordotonal organs in coordinate behavior allow, however, the speculation that *btd* is involved in the formation or function of chordotonal organs in adult flies.

2.4. Compensation for the lack of buttonhead function by other gene activities?

The lack of scorable phenotypic effects in the absence of *btd* activity in the dorsal spot, the central nervous system and in the regions corresponding to the *Dll*-like expression domain might be compensated for by the activity of other genes. In a search for such genes, we used a PCR-based approach to isolate *btd*-homologous sequences from *Drosophila* genomic DNA. For this, we used a pair of degenerate primers directed to sequences corresponding to the Sp1-like zinc finger domain of the *btd* protein. With the obtained fragment, we succeeded in isolating a partial cDNA clone encoding a triple zinc finger domain similar to *btd* and related members of the

Table 1
Number of scolopidia in lch5 of different *btd* mutants and transgene rescued *btd* mutants

	Mutant	Rescued
btd^{XO}	3.2 (140)	ND
btd^{XG}	2.9 (329)	4.3 (168)
btd^{XA}	3.6 (196)	4.7 (91)

The average number of scolopidia per lch5 is shown, with the number of scored lch5 indicated in parentheses. Counting the scolopidia in lch5 of wildtype embryos resulted in an average number of 4.9 (70), probably due to the difficulty in identifying all scolopidia, which are sometimes located behind each other. The mutant embryos carrying the *btd* transgene have been identified by showing head sensory organs, which are missing in *btd* mutants (Schmidt-Ott et al., 1994). Mutant embryos not carrying the *btd* transgene (missing head sensory organs) of the same cross show the average number of scolopidia derived for mutant embryos from the corresponding stock. ND, not determined.

vertebrate Sp1-like gene family. The degree of sequence identity of the zinc finger domain between the newly identified gene, *btd* and the different members of the vertebrate Sp1 gene family indicates that its DNA-binding domain is more similar to Sp1 than to *btd* (Fig. 6a). We refer to it as *Drosophila Sp1* (*D-Sp1*).

Cytogenetic analysis by in situ hybridization of the *D-Sp1* cDNA to polytene chromosomes revealed that *D-Sp1* maps to the same cytogenetic location as *btd*, i.e. band 9A of the X-chromosome (Fig. 6b). Thus, *btd* and *D-Sp1* may represent a pair of genes with partially overlapping functions as has been observed for a number of *Drosophila* genes such as *knirps* and *knirps-related* (González-Gaitán et al., 1994), *en* and *invected* (Simmonds et al., 1995), *gooseberry* and *gooseberry-neuro* (Li and Noll, 1994), *sloppy-paired 1* and *sloppy-paired 2* (Cadigan et al., 1994) as well as the forkhead-domain genes FD3 and FD4 (Häcker et al., 1992). In fact, in situ hybridization of the *D-Sp1* cDNA to whole mount embryos shows that *D-Sp1* is expressed in *btd*-like patterns during post-blastodermal stages of embryogenesis (Fig. 7). However, the early expression patterns of the two genes are different (compare Figs, 1 and 7). Low levels of *D-Sp1* transcripts are found evenly distributed throughout the pre-blastoderm embryo (Fig. 7a), suggesting that *D-Sp1* is maternally expressed. At blastoderm, when *btd* is expressed in the head stripe domain and the dorsal spot, no corresponding *D-Sp1* transcripts are observed (Fig. 7b). First zygotic expression of *D-Sp1* occurs in the proneural region of the head during early gastrulation and expression continues in the corresponding domain until the germ band is fully extended (Fig. 7c,d). From then on, the expression patterns of the two genes are very similar (Fig. 7e–j) except that the metameric pattern is much weaker than observed with *btd* (compare Figs. 7c and 1d,f). These results, the highly conserved DNA-binding domain and the lack of a *btd* phenotype in the regions corresponding to the overlapping expression domains suggest that the two genes carry redundant functions in these regions of the embryo. The question of whether the lack of the combined *D-Sp1* and *btd* activities cause developmental defects in their common expression domains remains to be elucidated.

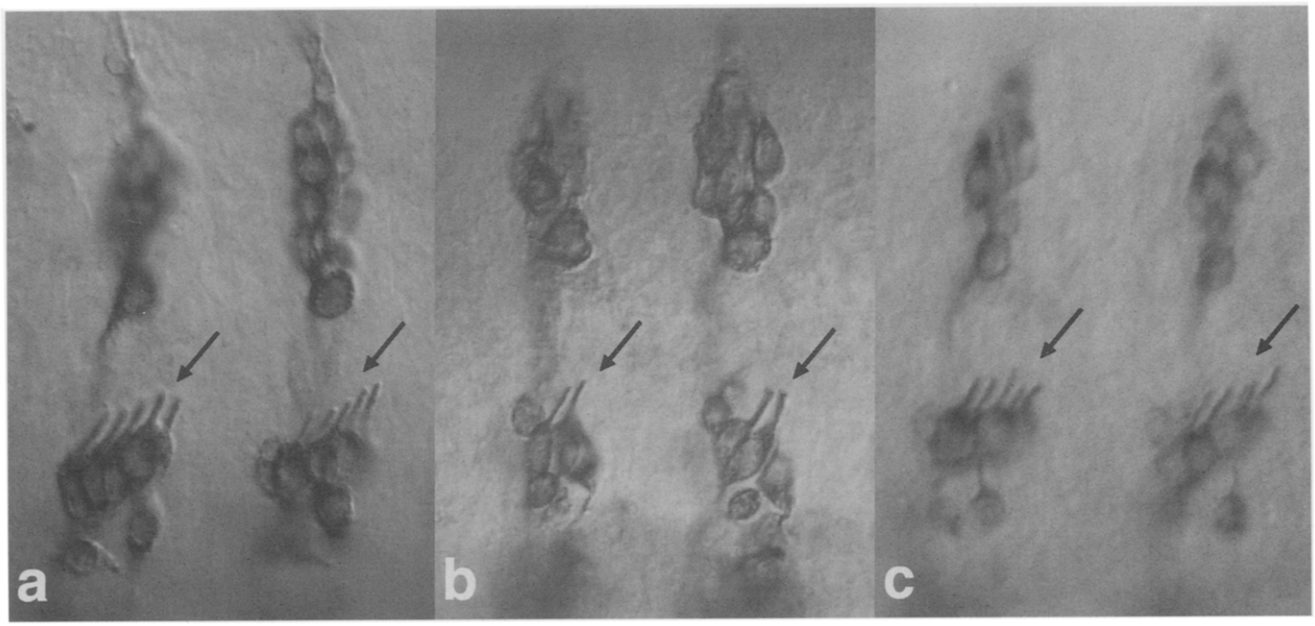

Fig. 4. *btd* phenotype in the peripheral nervous system. Antibody stainings with the neural marker mab22C10 (Fujita et al., 1982). Depicted are lateral parts of the peripheral nervous system in abdominal segments of embryos at the stage of dorsal closure (lateral views, anterior to the left and dorsal up). (a) Wildtype embryo: lch5 (arrows) consist of five scolopidia. (b) btd^{XG} mutant embryo: lch5 (arrows) are reduced to two scolopidia. (c) btd^{XG} mutant embryo carrying *btd* transgene: rescued lch5 (arrows) consist of five scolopidia.

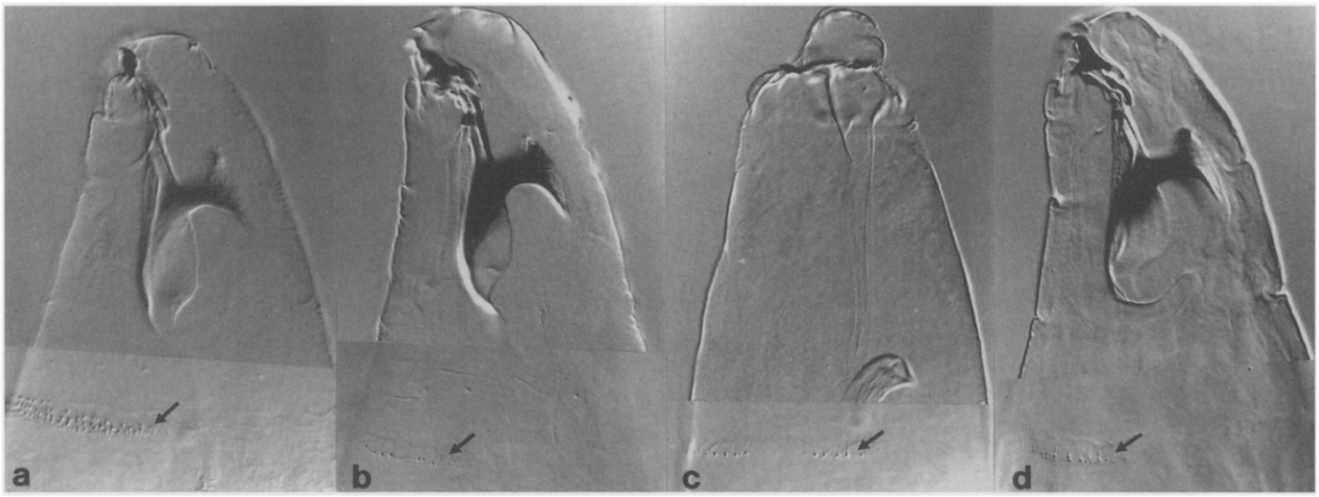

Fig. 5. *btd* transgene rescues *btd* embryonic head phenotype. Head structures of cuticular preparations (anterior up, ventral to the left). Arrows point to the first abdominal denticle belt (denticle belts and head structures of the same embryo have been photographed using different focal planes). (a) Wildtype embryo. (b) Hemizygous *svb* embryo: head structures not affected, but denticle belt reduced. (c) Hemizygous *svb*, *btd* embryo: head shows *btd* phenotype, denticle belt reduced. (d) Hemizygous *svb*, *btd* embryo, carrying a *btd* transgene (Wimmer et al., 1993). The reduced denticle belt indicates the presence of the mutant *svb*, *btd* chromosome, whereas the normal head structures show that the *btd* transgene can fulfill *btd* function.

2.5. Conclusions

We present evidence for a novel *Drosophila* gene pair, *btd* and *D-Sp1*. If one accepts sequence similarity as an argument for relationship, it appears that *D-Sp1* represents the closer relative to the vertebrate Sp1 gene family (Hagen et al., 1992; Kingsley and Winoto, 1992). An interesting link between the vertebrate and *Drosophila* members of the Sp1 gene family can be established on the basis of their expression in the brain. Recently, the other gap-like genes *otd* and *ems* have been shown to be expressed in the developing embryonic brain and specific roles for them in brain formation have been suggested (Hirth et al., 1995). Since initial *btd* expression in the dorsal spot is continuous with expression in different regions of the embryonic brain, and since both *btd* and *D-Sp1* expression continue during brain development, a more detailed analysis concerning the potential role of *btd*, *D-Sp1* or both of them for embryonic brain formation will be important. Moreover, based on the expression

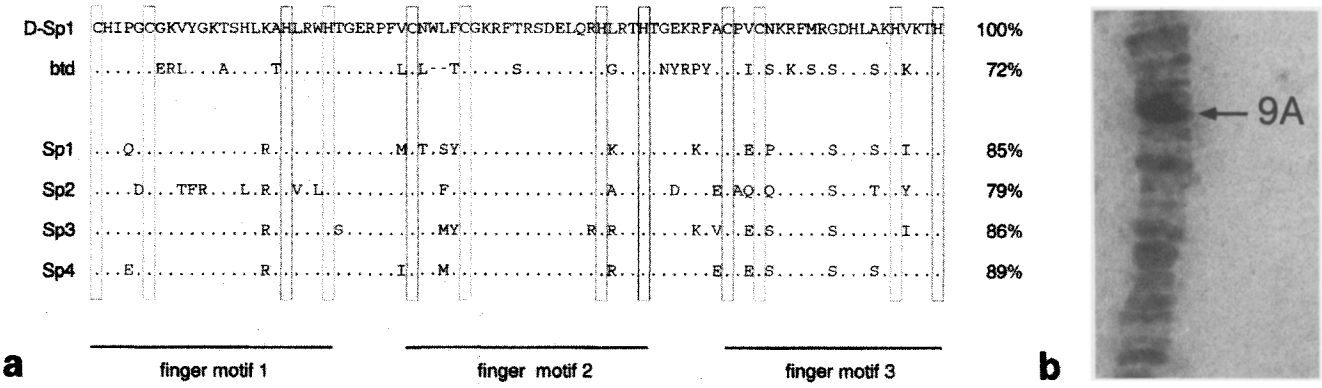

Fig. 6. Sequence comparison of the zinc finger domain of *D-Sp1* with other members of the Sp1 family and chromosomal localization of the transcription unit. (a) Amino acid sequence of the newly identified zinc finger domain encoded by *D-Sp1* and the corresponding DNA-binding motifs encoded by *btd* (sequence taken from Wimmer et al., 1993) and by the known members of the murine Sp1 gene family (sequences taken from Hagen et al., 1992; Kingsley and Winoto, 1992; note that Sp3 is identical to SPR-2 and Sp4 has previously been called SPR-1). The *D-Sp1* DNA-binding motif is more closely related to the murine DNA-binding motifs of Sp1-Sp4 than to the one encoded by *btd* (% values to the right of the sequence indicate % identity). Since the latter binds to the same DNA target site as murine Sp1 (Wimmer et al, 1993), the similar sequences suggest that the *D-Sp1* protein can bind to the same target sites as *btd* protein and Sp1. Bars highlight the cysteines and histidines of the zinc finger motifs 1–3. (b) Section 9 of the polytene X-chromosome of *Drosophila melanogaster* showing an in situ hybridization signal with the *D-Sp1* cDNA in chromosomal band 9A (arrow). Note that *btd* is localized in the same chromosomal band (Wimmer et al., 1993).

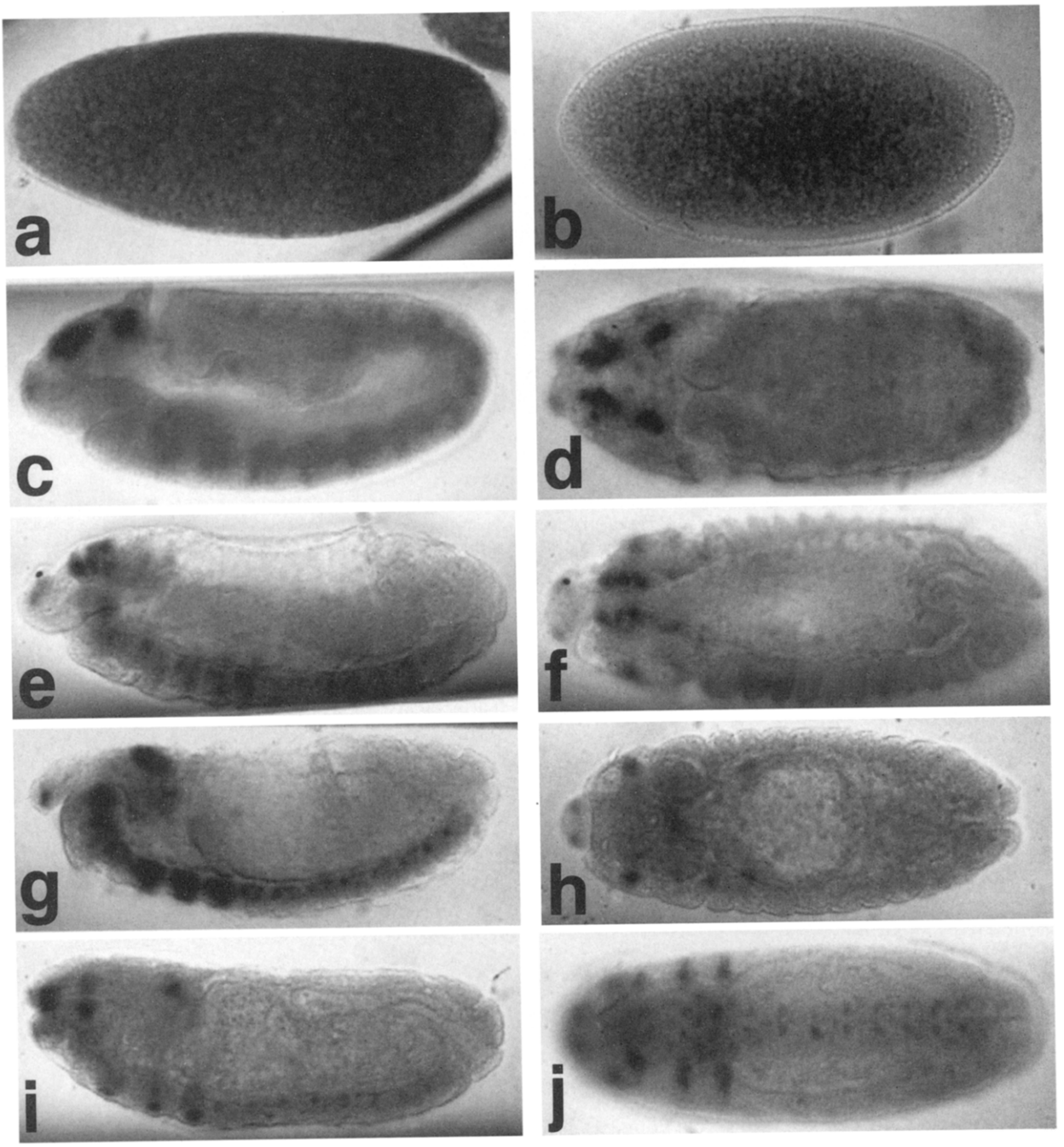

Fig. 7. *D-Sp1* expression during embryogenesis. In situ hybridization with *D-Sp1* cDNA to embryo whole mount preparations. (a) Early cleavage stage containing a low level of maternally expressed *D-Sp1* transcripts. (b) Lack of expression during blastoderm when *btd* is expressed in a head stripe and an anterior head spot (compare Fig. 1b). (c,d) Germ band elongated embryos: weak metameric pattern and head spot equivalent become visible (compare Fig. 1e). (e,f) Germ band retracting embryo. (g,h) Embryos after germ band retraction. (i,j) Embryos after dorsal closure: *D-Sp1* expressed in central nervous system and in *Dll*-like expression pattern. (a–c,e,g,i) Lateral view, dorsal up; (d,f) dorsal and (h,j) ventral views. Anterior to the left.

patterns of vertebrate homologs of *otd* and *ems*, an evolutionarily conserved program for brain development has been proposed (Thor, 1995). In this context it is interesting to note that the closest related vertebrate homolog of *D-Sp1* is *Sp4* (Fig. 6a) (previously named *SPR-1*; Hagen et al., 1992, 1995), which is specifically expressed in mouse brain tissue (Hagen et al., 1992). The embryonic expression analysis and functional study of the vertebrate transcription factor Sp4 and the analysis of *Drosophila* brains lacking the activity of *btd* and/or *D-Sp1* may elucidate a common role for members of the Sp1 family in brain development as previously found for the two other head gap-like genes *otd* and *ems* (reviewed in Boncinelli et al., 1995).

3. Materials and methods

3.1. Drosophila fly stocks

In order to generate a double-mutant X-chromosome btd^{XG}, svb^{YP17b}, we crossed very rare male escapers of a svb^{YP17b}/FM7 stock to virgins of the stock btd^{XG}/FM7. Virgin female progeny not carrying the balancer chromosome were crossed to FM7 males, and single virgin females from this cross were then again mated with FM7 males. To score for meiotic recombination events linking the *btd* and *svb* alleles together, we analyzed cuticle preparations of the independently set-up lines, and one of the lines showing both the *btd* and *svb* phenotypes was propagated. To analyze the ability of the *btd* transgene to rescue the *btd* mutant phenotype in the peripheral nervous system, we used male flies carrying the FM7, *ftz-lacZ* balancer chromosome (Struhl et al., 1993) and a *btd* transgene on the third chromosome. These males were crossed to virgin females of *btd* mutant stocks balanced over the marked FM7, *ftz-lacZ* chromosome. Male embryos carrying a *btd* mutation in hemizygous condition coming from this cross could be recognized since they are the only embryos not showing β-galactosidase expression.

3.2. Embryo analysis

In situ hybridization to whole mount preparations of embryos was performed as described by Tautz and Pfeifle (1989). Antibody double stainings with anti-β-galactosidase antibodies (Cappel) and mab22C10 antibodies (Fujita et al., 1982) to whole mount embryos were carried out as described by Macdonald and Struhl (1986) using the Vectastain ABC Elite horseradish peroxidase system for detecting mouse antibodies and an alkaline phosphatase-conjugated antibody to detect rabbit antibodies (Jackson ImmunoResearch Laboratories). For cuticle preparations, embryos were dechorionated, devitellinized, incubated in glycerol/glacial acetic acid (1:4) at 65°C, mounted in Hoyer's medium/lactic acid (1:1; Roberts, 1986) and incubated at 65°C. For scanning electron microscopy, embryos were dechorionated, fixed with glutaraldehyde (2.5%), devitellinized and fixed with osmium tetroxide (1%). Fixed embryos were dehydrated in an ethanol series, dried by the critical-point method, mounted on double-stick tape, gold-coated in an argon atmosphere and examined with a Cambridge Stereoscan 150 microscope.

3.3. PCR amplification and cDNA cloning

D-Sp1 DNA was amplified by PCR using 100 ng of genomic *Drosophila* DNA. Degenerate primers corresponding to the amino acid sequence of parts of the first and third zinc finger motif of *btd* protein were used. The sequences of the primers were 5′-CGCCTAGGTG(AG)TC(AG)CTNC(GT)CAT(GA)AANC-3′ (5′; open reading frame left to right) and 5′-GCTTAAGAA(GA)CA(GA)CA(TA)ATNTG(TC)CA(TC)AT-3′ (3′; opposite strand), respectively. The reaction mixture was denatured at 94°C (330 s) followed by 40 cycles at 94°C (60 s), 50°C (60 s) and 72°C (30 s) and a single termination step at 72°C (10 min). PCR, cloning and handling of the DNA fragments were as described by Frommer et al. (1996). A corresponding cDNA clone was isolated by screening a lambda ZAP library (Stratagene).

Acknowledgements

We thank U. Schmidt-Ott for providing initial preparations of mab22C10-stainings, I. Gajewski for preparing photographs, D. Schmucker for mab22C10 antibody, P. Gergen for the *svb*-allele, J. Winkler and M. Kahlweit for the use of the scanning electron microscope (SEM), G. Brönner, W.-D. Kötting and D. Capco for help with the SEM-preparations and microscopy, and especially M. González-Gaitán and S.M. Cohen for stimulating discussions. This work was supported by the Max-Planck Society (GF and HJ) and the Deutsche Forschungsgemeinschaft (HJ). EAW was a fellow of the Boehringer Ingelheim Fonds; BAP receives an Alexander von Humboldt fellowship.

Note added in proof

Supp et al. (Dev. Biol. 176, 284–299) showed that *sp4* is highly expressed in the developing central nervous system of mouse embryos and is required for normal murine growth, viability and male fertility.

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