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The novel homeodomain gene *buttonless* specifies differentiation and axonal guidance functions of *Drosophila* dorsal median cells

Chin Chiang[1], Nipam H. Patel[2], Keith E. Young[1], Philip A. Beachy[1,*]

[1]Howard Hughes Medical Institute, Department of Molecular Biology and Genetics, The Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA
[2]Department of Embryology, Carnegie Institution of Washington, 115 West University Parkway, Baltimore, MD 21210, USA

*Author for correspondence

SUMMARY

We have identified a novel homeodomain gene, *buttonless* (*btn*), that is specifically expressed in 20 cells of a single type during *Drosophila* embryonic development. These cells, the dorsal median (DM) cells, are arranged as a single pair within each segment along the dorsal midline of the CNS. Distinctive features of the DM cells include a large cell body and a long thick process extending laterally to the muscle attachment site. In the absence of *btn* gene function the initial commitment to the DM cell fate is made but differentiation fails to occur and the DM cells are lost. The *btn* mutation thus specifically eliminates the DM cells, and this genetic ablation in turn reveals a requirement for DM cells as cellular cues for axonal guidance during transverse nerve outgrowth and bifurcation of the median nerve.

Key words: axon guidance, *buttonless*, *Drosophila*, homeodomain, midline, *shibire*

INTRODUCTION

The homeodomain DNA-binding motif, initially discovered in *Drosophila*, is now understood to play a major role in the development of most multicellular eukaryotes. The first homeodomain genes identified in *Drosophila* were associated with mutant phenotypes ranging from homeotic transformations of segment identity to repetitive segmentation defects to gross defects in embryonic polarity. Extensive genetic and molecular studies indicate that these homeodomain genes contribute to the establishment and maintenance of a detailed positional reference system; homeodomain genes operating at this level appear to influence the development of a variety of cell types in multiple germ layers.

In contrast, several more recently identified homeodomain genes appear to function in a more restricted range of cell types. For example, the *Drosophila* homeodomain genes *H*2.0, *msh*-2, *tinman*, and *bagpipe* are expressed primarily in mesoderm (Barad et al., 1988; Bodmer et al., 1990; Azpiazu and Frasch, 1993), expression of the *bsh* homeodomain gene is restricted to a subset of neurons in the *Drosophila* brain (Jones et al., 1993) and the *rough* gene functions in photoreceptor cell differentiation in the developing retina (Saint et al., 1988; Tomlinson et al., 1988). In other organisms, the homeodomain genes *mox-1* and *mox-2* are expressed only in vertebrate mesoderm and its derivatives (Candia et al., 1992), *Csx* expression is restricted to the developing vertebrate heart (Komuro and Izumo, 1993), and *mec-3* functions in a particular set of sensory neurons of the nematode (Way and Chalfie, 1988). Homeodomain genes in this second class thus appear to be more strongly associated with the commitment to or implementation of specific differentiation pathways as opposed to specification of positional information.

We report here the characterization of *buttonless* (*btn*), a novel *Drosophila* homeodomain gene which falls into the second group. Its expression is exquisitely specific, being restricted to a single pair of cells per segment in the trunk of the embryo. These cells, the dorsal median (DM) cells, are closely associated with the central nervous system and appear biochemically and morphologically similar to the *Drosophila* cultured cell line from which the *btn* gene was isolated. DM cell bodies are tightly paired at the dorsal midline of the ventral cord while each cell's single large process projects laterally to the muscle attachment sites in the body wall. In embryos lacking *btn* gene function the DM cells fail to form and, as a consequence, axons that normally project laterally along the DM cell processes to form the transverse nerve instead appear to wander along the surface of the ventral cord. These observations indicate that the *btn* gene function is critical for the specification of DM cell fate and for transverse nerve pathfinding.

MATERIALS AND METHODS

Isolation and characterization of *btn* cDNA and genomic clones

Total RNA was prepared according to standard procedures (Sambrook et al., 1989) from the *shibire* cell line (Simcox, 1981) and from an isolate of this parent line stably transfected with a construct

carrying the open reading from for the *Ultrabithorax* open reading frame under control of the *Drosophila* metallothionein promoter. Poly(A)$^+$ RNA was isolated using oligo(dT)-cellulose (Boehringer Mannheim) and a cDNA library (2×10^6 independent clones) was prepared from the induced stably transfected line using the Lambda Zap cDNA cloning kit (Stratagene). The probe used to screen this library was an end-labelled 1024-fold degenerate oligonucleotide corresponding to a widely conserved region of the third helix of the homeodomain (Burglin et al., 1989). Hybridization to filters proceeded in 6× SSC, 5× Denhardt's solution, 0.05% sodium pyrophosphate, 1% SDS and 100 μg/ml salmon sperm DNA at 42°C; washes were in 6× SSC, 0.05% sodium pyrophosphate and 0.2% SDS at room temperature, then at 50°C. A *btn* cDNA clone isolated from this library was used as a probe to screen a genomic library (Maniatis et al., 1978) and a 4-8 hour embryonic cDNA library (Brown and Kafatos, 1988).

Sequence determination utilized the dideoxy termination method with Sequenase (US Biochemical). For the largest embryonic *btn* cDNA (650 bp) flanking vector primers and synthetic primers were used. For genomic sequence, nested exonuclease III deletions spanning a 2.4 kb *Hin*dIII/*Eco*RI genomic fragment were generated using the Erase-a-Base kit (Promega). The deduced amino acid sequence of *btn* protein was used to search the NBRF and SWISS-PROT data bases using the FASTA algorithm.

Mapping and mutagenesis of the *btn* gene

Hybridization to polytene chromosome squashes was as described (Langer-Safer et al., 1982) using the alkaline phosphatase-based DNA detection system (GIBCO/BRL). A probe was generated from the 6.5 kb *Eco*RI *btn* genomic fragment (from 0 to 6.5 in Fig. 1) using the random hexamer priming kit (Boehringer Mannheim) in the presence of biotinylated dUTP (ENZO Biochemicals). The *btn* sequences hybridized near polytene division 94B, which is the location of P-element insert in P[rib(94B)] described by Karpen et al. (1988).

Southern hybridization with the 6.5 kb *Eco*RI *btn* genomic fragment as a probe established that the P element was inserted near *btn* coding sequences and its precise location was determined by isolation of sequences flanking the P[rib(94B)] insert using inverse PCR (Ochman et al., 1990). Briefly, two primers, GTATACTTCG-GTAAGCTTCGGCTAT and CGAAATGCGTCGTTTAGAGC-AGCAG, which hybridize to the 5′ and 3′ portions of a region near the 5′ end of the P element, were used as primers in a PCR reaction with 1.5 μg of DNA from the homozygous P[rib7(94B)] line. Prior to PCR, this DNA was digested with *Sau*3A, ligated overnight in a 300 μl reaction at 4°C, and digested with *Ase*I.

The P-element insert in P[rib(94B)] was mobilized by exposure to transposase from the P[Δ*2-3 ry*$^+$] *Sb* chromosome. Excision events were identified by loss of the *ry*$^+$ marker present in P[rib(94B)]. One of the chromosomes recovered was homozygous lethal and carried a deletion of 3.3 kb that removed the entire *btn* transcription unit (see Fig. 1). The extent of this deletion was inferred by Southern hybridization to DNA digested with various restriction endonucleases from heterozygous *btn* individuals using the *btn* cDNA and a 6.5 kb *Eco*RI fragment (from 0 to 6.5 kb in Fig. 1) as probes.

Analysis of *btn* RNA expression

For northern analysis, 5 μg of poly(A)$^+$ RNA derived from different developmental stage embryos or tissue culture cells was electrophoresed on 1.5% formaldehyde gels and transferred to nitrocellulose membranes (S&S or Costa). Filters were hybridized with radiolabeled *btn* cDNA and washed according to standard procedures (Sambrook et al., 1989).

In situ hybridization to whole embryos was performed as described by Tautz and Pfeifle (1989). Probes were generated by random hexamer priming using digoxigenin-dUTP and detected with the Genius Kit (Boehringer Mannheim). Stained embryos were prepared for sectioning by embedding and freezing in OCT solution (Tissue Tec), and 10 μm sections were prepared with the Microm 500 OM-Cryostat.

Immunohistochemisry

Embryos and larvae were fixed and immunostained as described (Patel, 1994). Embryos were viewed on a Zeiss Axioplan microscope and photographed on Ektachrome 64 slide film or captured electronically using a Sony DXC-760MD camera and a Nuvista Plus capture board. Slides were digitized using a Kodak RFS 3500 scanner. Figures were arranged using Adobe Photoshop and Aldus Pagemaker and printed using a Kodak XL7700 dye-sublimation printer.

Drosophila strains and cell culture

A 1.85 kb *Eco*RI-*Pst*1 genomic fragment, which contains 5′ flanking sequences and part of of the *btn* ORF (Fig. 2), was first subcloned into the *Eco*RI-*Pst*I site of Bluescript (Stratagene). The *Eco*RI-*Bam*HI fragment of the resulting construct was then subcloned into the *Eco*RI-*Bam*HI site of the pCasperβgal vector (Thummel et al., 1988) to create an in-frame fusion to a *lacZ* reporter gene. This construct, p*btn*1.85/*lacZ*, was coinjected with p25.7wc (Karess and Rubin, 1984) into W^{1118} embryos at a DNA concentration of 500 μg/ml and 100 μg/ml, respectively. Germ-line transformation was performed essentially as described (Rubin and Spradling, 1982).

For cell fate analysis in the *btn* mutant background, embryos were collected from a stable line P[*btn*1.85-*lacZ*]/P[*btn*1.85-*lacZ*]; btn^{r3}/*TM3*, P[*ftz-lacZ*]. *btn* mutant embryos from this strain can be identified by the absence of the *ftz* expression pattern.

Mutant *btn* embryos were collected from *btn*/P144 parents. The P144 enhancer-detector line carries a *lacZ* gene with strong expression in the central and peripheral nervous systems of late stage embryos (unpublished observations), and homozygous *btn* embryos therefore can be unambiguosly identified by the absence of *lacZ* antibody staining.

The *shibire* cultured cell line (Simcox, 1981) was maintained in Schneider's medium (Gibco/BRL) supplemented with 10% fetal calf

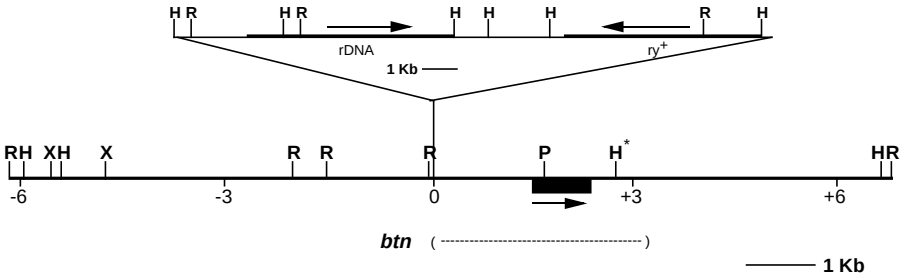

Fig. 1. Molecular organization of the *btn* locus. Genomic DNA at the *btn* locus is represented by the thin line with the 0 coordinate (in kilobases) corresponding to the site of insertion for the P[rib7(94B)] element. The *btn* transcription unit is represented by a filled box, with the direction of transcription indicated by an arrow. The structure of the P[rib7(94B)] element is diagrammed above the genomic map, with the locations of the rDNA and *ry*$^+$ transcription units denoted by arrows. The 3.3 kb genomic region deleted by imprecise excision of the P element in the *btn* mutant allele is indicated by the dashed line within parentheses. The *Hin*dIII restriction site at +2.8 (asterisk) is polymorphic (present in Canton-S but not in the ry^{506} chromososome). Abbreviations: H, *Hin*dIII; R, *Eco*RI; X, *Xho*I.

serum (Gibco/BRL) and an antibiotic/antimycotic mixture (Gibco/BRL).

RESULTS

Isolation of *buttonless* clones and mutations

The *buttonless* (*btn*) gene was first isolated in cDNA form using as hybridization probe a degenerate 23-base oligonucleotide corresponding to a widely conserved region from within the third helix of the homeodomain (Burglin et al., 1989). The library screened with this probe derived from a cultured *Drosophila* cell line called *shibire* (originally established from *shibire* mutant embryos; Simcox, 1981) in which expression of the *Ultrabithorax* homeotic protein had been experimentally induced (see Materials and Methods). From an initial screen of 150,000 clones, five of eight positives contained genuine homeodomain coding sequences, of which one corresponded to *Ubx*, one to another homeotic gene, *Abdominal-B*, and one to the previously described mesoderm-specific homeodomain gene, H2.0 (Barad et al., 1988). The remaining two homeodomain clones represented the gene *buttonless* (*btn*), named for its expression pattern and mutant phenotype (see below). Further cDNA and genomic clones were isolated using the initial *btn* cDNA clone from the *shibire* cell line as a probe; only three embryonic cDNA clones were recovered from screening of a million colonies from an embryonic cDNA library, consistent with a low overall abundance of *btn* message within the embryo (see below).

A restriction map of the *btn* genomic region is shown in Fig.

A

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EcoRI                                   ∇
GAATTCTCGACGATGTCGTAGATGA**CATGTG**GCGGTCCGGTGGTTCCGAGTAGCGAAAACCCGAAACCGGAATGCTGCTCGCTGCGCCGC 90
ACGACCACTTTCAGTTCCTCGCTCATTTTGGGTCTTCGGCGTCCTGCTGCCGATCGGTTCACACTCTTTTCACCGAGTTTTCGTTTGGTT 180
GGCGGTGTGGGTGGGTAACCCCTGGGCGTGATTAATCCTTTGGGCGACGGCCACTTTTCTTAGTCGCTTTAATTAATTCTCGCTGTTTTG 270
GGCTTGGCCAACTTCATTGCACACTCTCATTTTGTTTTCACTCTTTCCACTTCACAACATGCACACAAACACACACGCTCACAAACACTC 360
ATACACGCACTCACGGATGACGATTTGAAAAAAAGAATGTTTCTCCTTGGCGAATTTGTTTAAACATTAAACATTATTGCATTTCGTGCG 450
CTGCTTCTTCTTCTACTTCGTTGGCTTTCTTTCCACGCAATTCTCGGTTCCAGCGAAACGAAACTTGTTTAATTCGCCCGTCTGCTTCTC 540
GCTTCGTTTCTTTATGTCGCTTTTGTCCGACTGTTTCGCCGTCCTTTGCGGTTCGCGGGGGGTTTTAGCCGTCACATATTTTGGCGAATG 630
TGGATGCCCAGATATCGCAGATTGTACTAAATGATTGTAAATTGTAAATTGTACTATTGCACTGGGGATGGGAAGCATAAAAGGTATTAA 720
TATATCGATAAGATATCGGAAAGTATTTCTACCGTTGCTTCATTTCTATCGATAGATATATTTAAACTTCTTGTATACTTTTTCTAGATT 810
AAATGAGATTTATTAGCTGACAAAGGAAAATTAAAAGAAATATATGAATAAATCTAGCCAAACAATAACTACACAGCGAAGGACAACAGA 900
TATCACTAATGCCCTTTGGAATGTCATAGTCTGGCAA**CACTTG**AAATGTGC**CATATG**CCTTAACCAAAAAAGATTACCCAACACTTATTC 990
AGTTTGAAAATAAATGCGGATAATTTAAAACCGGAACCGGTTAATTTTCATATACCGGTATATTATTTAATAAAAACTCAAATAAAAAAG 1080
GGTATACCTAAGCCCCAATAAAAATAAG**CAGTTG**CTAGAATTTACGTTTTTGCTGTGTCCATTTTATAATTGGTTTCATAATAGGATATC 1170
AAAATTTTTTGATTTCTTTTCTAATTTCTAAAACCCCCGTAAGACTTATTTTATTGAACTATTTTATCCCTTAACCATTTCTGTGACACG 1260
CACTTATTTCCCCATTAATTATTATATAAAACTCCACTTTGTCATGGATTATCTAGGTATATCACTCAATGGTCACTCGTAATCCGCCGC 1350
ATCGACTTTAAAAGCGCGTG**CAATTG**CGACTTTCAGATAAATCTCCGACTGGGTCTTACGACTCGCCGACGGTTTCTATTAAAATTTTAT 1440
ACGTCCG**CAGTTG**GTGGCACAAAG**CACTTG**ACTCATGCGTCCACTTCCGTTTCTGACCGGACGCCTGGGAAGGGGCTGGGAATCGGATTC 1530
GGGTTTGTTGTGACAACAGTTCGTAGGCAAAGACAACAAGCCGATCTGCGGGTGTCATAACCACTCATACCCGAGGTC**CAGATG**TTGCGC 1620
ACAATTAT**CATATG**AGCAGCACAT**CATTTG**CGACTAAGTGAAAAAGCAGATTGGCACACTCTGCGCCTTGTTTAAAAAGCACGTCCGCCG 1710
          ↓
AATCTGAGATTTCAGTAATAGTTTAGACCTAGAAGCAAAGACATCGGCAATGAAGAACTCCCACGCGAACGTCTACCAAGAATCCTATTG 1800
                                              M  K  N  S  H  A  N  V  Y  Q  E  S  Y  C  14
                                                 PstI
CTACGAGGATATTACGAAATCGTGGCTGAACCAAACGGAGGGCTATACGGACTGCAGCTATGTATATGACCACAGTGCGTCTTCTTCCTA 1890
 Y  E  D  I  T  K  S  W  L  N  Q  T  E  G  Y  T  D  C  S  Y  V  Y  D  H  S  A  S  S  S  Y  44

CGCGGATTACAATAAACTAGAAACTAATTGGTGCAACGAAGCGAACGATCAATGGTTACAAAATGAGGCACCTACTGGTCAAGAACTTCC 1970
 A  D  Y  N  K  L  E  T  N  W  C  N  E  A  N  D  Q  W  L  Q  N  E  A  P  T  G  Q  E  L  P  74
                                                 c
AAGCCAAAGGTCCAAACTCCGGGCAATCAGCTCGAATCGCAAGGAACGCACAGCATTTTCAAAAACGCAGCTCAAGCAGCTGGAAGCGGA 2060
 S  Q  R  S  K  L  R  A  I  S  S  N  R  K  E  R  T  A  F  S  K  T  Q  L  K  Q  L  E  A  E  104
                      c           g
GTTCTGCTATTCCAACTATTTGACTCGCTTGCGTCGCTACGAAATCGCAGTGGCGCTGGAGCTGACCGAACGCCAGGTGAAGGTGTGGTT 2150
 F  C  Y  S  N  Y  L  T  R  L  R  R  Y  E  I  A  V  A  L  E  L  T  E  R  Q  V  K  V  W  F  134

CCAAAATCGACGCATGAAGTGCAAGCGCATCAAACTGGAGGAGCAGCAAGGCAGCGCGGCCAAAACGCCATTTTAGTTTGTACAAGGACA 2240
 Q  N  R  R  M  K  C  K  R  I  K  L  E  E  Q  Q  G  S  A  A  K  T  P  F  *                 158

TATTTTAACACAATTCATCTTACAAAATGTTTTCGCTTACTTAAAGAAAGTATAGTAAATGTATTTTAAGCT                  2312
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B

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                                HELIX I         HELIX II        HELIX III
        btn             NRKERTAFSKTQLKQLEAEFCYSNYLTRLRRYEIAVALELTERQVKVWFQNRRMKCKRIK

        mox-2           A-------T-E-IRE-----AHH-------------N-D----------------W--V-
        mox-1           A-------T-E-IRE-----AHH-------------N-D-S--------------W--V-
        gax             P-------T-E-IRE-----AHH-------------N-D----------------W--V-
        Xelmox2         P-------T-E-IRE-----AHH-------------N-D----------------W--V-
        hoxb-2          S-RL---YTN---LE--K--HFNK--C-P--V---AL-D----------------H--QT
        pb              P-RL---YTN---LE--K--HFNK--C-P--I---AS-D----------------H--QT
        Antp            PKRQ---YTRH-ILR--K--HYNR----R--I---HT-V-S---I-I--------W-KDN
        Abd-B           V--K-KPY--F-TLE--K--LFNA-VSKQK-W-L-RN-Q-------I--------N-KNS
```

Fig. 2. Sequence of the *btn* gene and its deduced protein product. (A) The longest embryonic cDNA isolated begins at position 1722 (marked by an arrow) and extends to the end of the sequence displayed. Sequence extending upstream was derived from genomic clones and the insertion site of the P[rib7(94B)] element is indicated by an inverted triangle. The 158 amino acid sequence is encoded by the largest open reading frame within the *btn* cDNA that is initiated by a methionine codon; lower case letters above the line represent silent differences in base sequence between cDNA and genomic sequences. Homeodomain residues within the open reading frame are underlined. Consensus DNA-binding sites for the *twist* protein are indicated by bold lettering (CTNNAG; Ip et al., 1992; see text). The *Eco*RI and *Pst*I sites delimit the fragment used in the *btn*1.85-*lacZ* reporter construct. (B) The amino acid sequence of the *btn* homeodomain is aligned with those of the murine *mox-1* and *mox-2* genes (Candia et al., 1992), the rat *gax* gene (Gorski et al., 1993), the *Xenopus Xelmox2* gene (Candia et al., 1992), murine *hoxb-2* (ref), and the *Drosophila pb* (Cribbs et al., 1992) and *Antp* (Laughon and Scott, 1984) genes. A dash indicates residues identical to those in *btn*. Three regions corresponding to the α-helical regions from the crystallographically derived structure of the *engrailed* homeodomain are underlined (Kissinger et al., 1990).

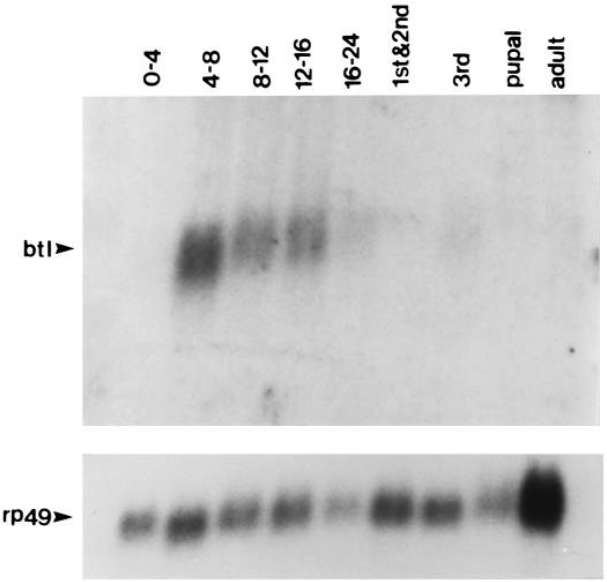


Fig. 3. Temporal profile of the *btn* transcript. Each lane contains 5 μg of poly(A)$^+$ RNA derived from several embryonic stages (time given in hours), in two larval stages, and in the pupal and adult stages, as indicated. Following a 7 day autoradiographic exposure with the entire 608 bp *btn* cDNA used as a hybridization probe, no transcript was detected in 0-4 hour embryos, but a sharp peak of expression was observed in 4-8 hour embryos as a single 0.7 kb band. Lower level expression is detectable in all subsequent embryonic stages, and in larval, pupal and adult stages. The lower panel shows the same blot subsequently hybridized with the *Drosophila rp49 gene* and exposed for 8 hours.

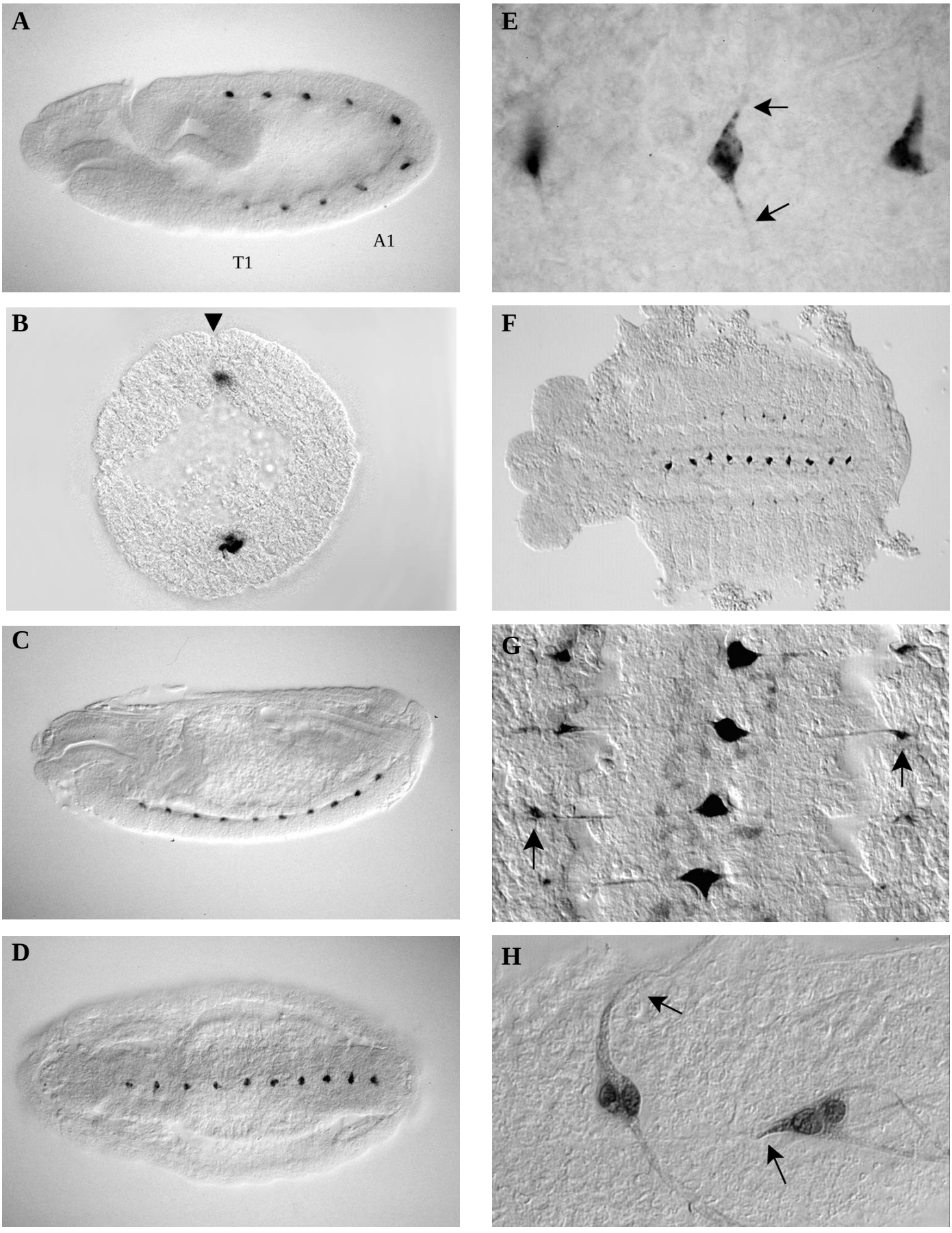
1, with the location and structure of the *btn* transcription unit as determined by comparison of genomic and *btn* cDNA sequences (Figs 1 and 2). The *btn* transcript, ~600-700 bases in length (Fig. 3), is difficult to detect and appears to derive from a single exon. The homeodomain coding sequences lie within an open reading frame (ORF) capable of encoding a 158 residue protein; this is the largest ORF within a fully sequenced 607 bp cDNA, and is preceded by two in frame chain termination codons just upstream of the initiating methionine codon. A stretch of 12 adenines at the 3′ end of the cDNA is preceded by several imperfect matches to the polyadenylation signal AAUAAA. We can not distinguish between the possibilities that this stretch of adenines reflects a genuine polyadenylation event or a site of internal priming by oligo(dT) during cDNA synthesis; neither possibility affects the extent of the ORF, which ends 86 bases upstream.

Outside the homeodomain, the predicted *btn* protein shares no significant homologies with other proteins in the databases (see Materials and Methods). The *btn* homeodomain itself is most related to a divergent family of vertebrate homeobox genes (Fig. 3B), sharing amino acid identities of 77-78% with the *Mox-1* and *Mox*-2 genes in the mouse (Candia et al., 1992), the *Gax* gene in the rat (Gorski et al., 1993) and the *Xelmox*2 gene in *Xenopus* (Candia et al., 1992). All of these homeodomains, including that of *btn*, contain a lysine residue at position 3 (K3). The majority of other homeodomains carry an arginine residue (R3) at this position, which is known to make a specific contact with the second of four bases within the core of the homeodomain recognition site (TAAT). The *Abd-B* homeodomain of *Drosophila*, which also carries a lysine residue at position 3, has been shown to prefer a T at the second position within the core (TTAT), but this difference in specificity also involves other residues (Ekker et al., 1994). The most closely related *Drosophila* homeodomain (63% identity) is that encoded by the homeotic gene *proboscipedia* (Cribbs et al., 1992); the *Antennapedia* homedomain is 53% identical. To our knowledge, the *btn* mRNA encodes the smallest reported homeodomain protein.

Embryonic expression of *btn*

Nothern blot hybridization indicates that the *btn* transcript is expressed throughout the embryonic period beginning sometime after 4 hours of development, but is only weakly detectable by blotting during the larval, pupal and adult periods (Fig. 3). Expression is first detectable by in situ hybridization in a group of two to four midline cells in each of the three thoracic and in the first seven abdominal segments at stage 11 (the extended germ band at $5\frac{1}{2}$ to 6 hours of development; Fig. 4A). As determined by simultaneous detection of the *fushi tarazu* and *btn* mRNAs (not shown), these cells are located between parasegment boundaries, approximately at the position of future segment boundaries. These cells are rounded in shape and are located along the dorsal midline of the mesodermal bridge that links the two halves of the mesoderm (Fig. 4B), thus indicating their mesodermal origin (Bate, 1993). A mesodermal origin for DM cells was also suggested by Beer et al. (1987) based on the fates of marked mesodermal cells transplanted into unmarked hosts. During germ-band retraction, the bodies of the *btn*-expresssing cells remain at the midline but begin to extend lateral processes until, by stage 14, the cells appear well differentiated and their processes extend beyond the ventral cord to the sites of muscle attachment (Fig. 4C-G). The number of cells expressing *btn* at this stage is reduced from four to two per segment by an as yet unresolved mechanism that could involve cell fusion or cell death. We shall refer to the mature, differentiated, *btn*-expressing cells as dorsal median (DM) cells. Note that in contrast to anterior DM cells, which display a characteristic oval shape with lateral processes, the cell bodies of posterior DM cells form a triangle

Fig. 4. Embryonic and larval patterns of *btn* gene expression. A-E derive from in situ hybridizations to whole embryos using digoxigenin-labeled *btn* cDNA as a probe. F-H display X-gal staining patterns of individuals carrying the *btn*1.85-*lacZ* reporter construct. (A) Lateral view of an extended germ-band embryo with the first thoracic (T1) and first abdominal (A1) segments indicated (stage 11). (B) Cross section of a stage 11 embryo showing expression in cells of the mesodermal bridge. The arrowheads mark the ventral furrow. (C) Lateral view of an embryo after germ-band retraction. The *btn*-expressing DM cells are situated just above the CNS (stage 14 embryo). (D,E) Low and high magnification ventral views of a stage 14 embryo. At the higher magnification (E) the DM cell processes are clearly visible (arrows). (F,G) Low and high magnification views of a stage 14 embryo dissected to expose the CNS. The arrows indicate the points of insertion of DM cell processes at the muscle attachment sites. (H) First instar larva dissected to expose the CNS and the DM cells. Note the anteriorly directed process (see arrow; also observed in embryos), which lends a triangular shape to the posterior-most pairs of DM cells. Except for B and G anterior is to the left; in G, anterior is down.


A
A1
T1
B
C
D
E
F
G
H

with processes that extend anteriorly and laterally from the vertices (Fig. 4E,H).

The distinctive morphological characteristics of the DM cells have been noted previously (Caudy et al., 1986). These cells have also been shown to express neuroglian (Bieber et al., 1989) and extracellular matrix proteins such as laminin, glutactin and collagen IV (Olson et al., 1990), molecules that appear to function in cell-cell interactions and adhesion.

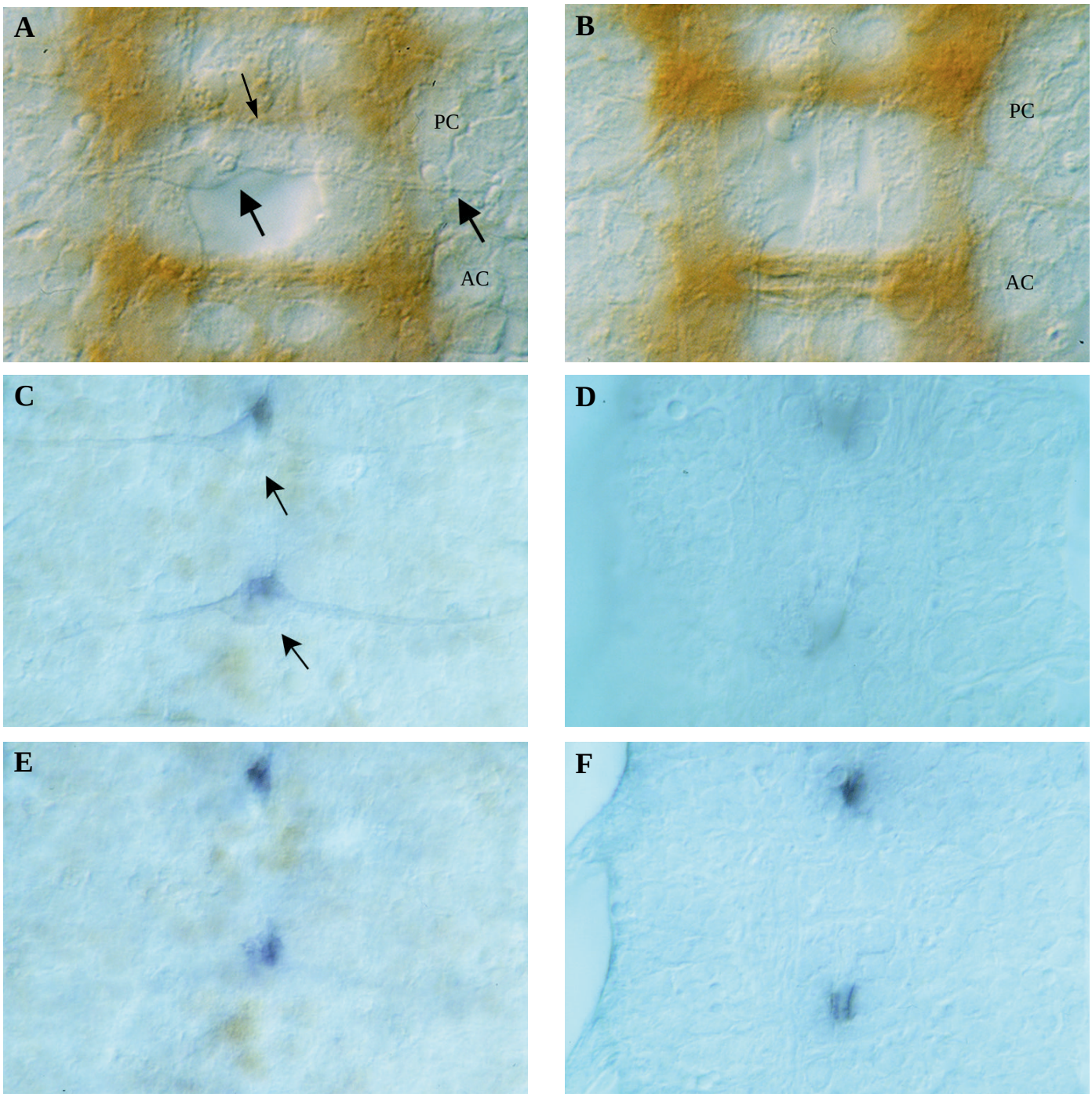


Fig. 5. Dorsal median cells are absent in embryos lacking *btn* function. Embryos in A,C and E are wild type or heterozygous for the *btn* gene and are homozygous *btn* mutants in B,D,F. (A,B) Embryos were stained with mAb BP102, dissected to reveal the CNS and viewed under Nomarski optics. DM cells and their processes (arrows) are located between the anterior (AC) and posterior (PC) commisures in wild type (A) but are absent in the *btn* mutant (B). (C-F) Whole embryos were double-stained with antibodies against glutactin (purple) and β-galactosidase (brown). The absence of β-galactosidase staining indicates a homozygous *btn* mutant embryo (see Materials and Methods). The view in C and D is in the focal plane of the DM cells and at a focal plane somewhat more ventral in E and F. Note the presence of DM cells (arrows) in wild type (C) but not in the *btn* homozygous mutant (D), while other anti-glutactin staining structures in the more ventral plane of focus are evident in both embryos (E,F). All embryos are at stage 14 and anterior is toward the top.

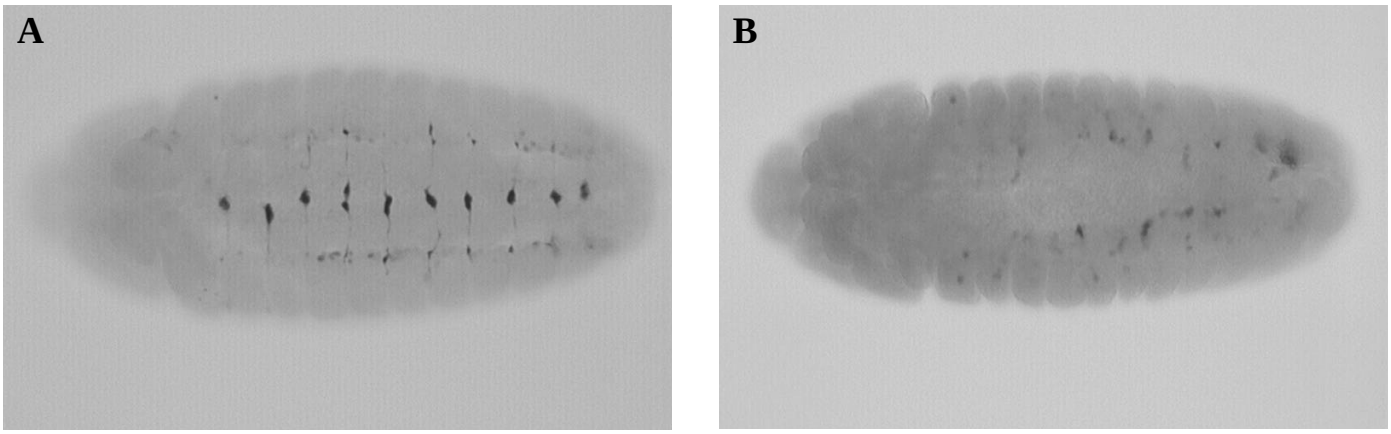


Fig. 6. Expression of the *btn*1.85-*lacZ* reporter is lost in *btn* mutant embryos. Ventral views of *btn*1.85-*lacZ* reporter expression as detected with an antibody against β-galactosidase (see Materials and Methods) are shown at stage 12-13 in wild-type (A) and *btn* mutant (B) embryos. Although expression is initiated normally in *btn* mutants (not shown), this expression is lost at later stages. In wild-type embryos (A), the long processes extending laterally from the cell bodies at the dorsal midline are clearly visible. in addition, weak expression not seen from the endogenous *btn* gene is consistently observed in cells flanking the CNS (compare Fig. 4A-E). In *btn* mutant embryos (B), the β-galactosidase expression characteristic of DM cells is absent, and only weak ectopic staining that may represent the remnants of DM cell precursors is observed.

Absence of *btn* function results in loss of the dorsal median cells

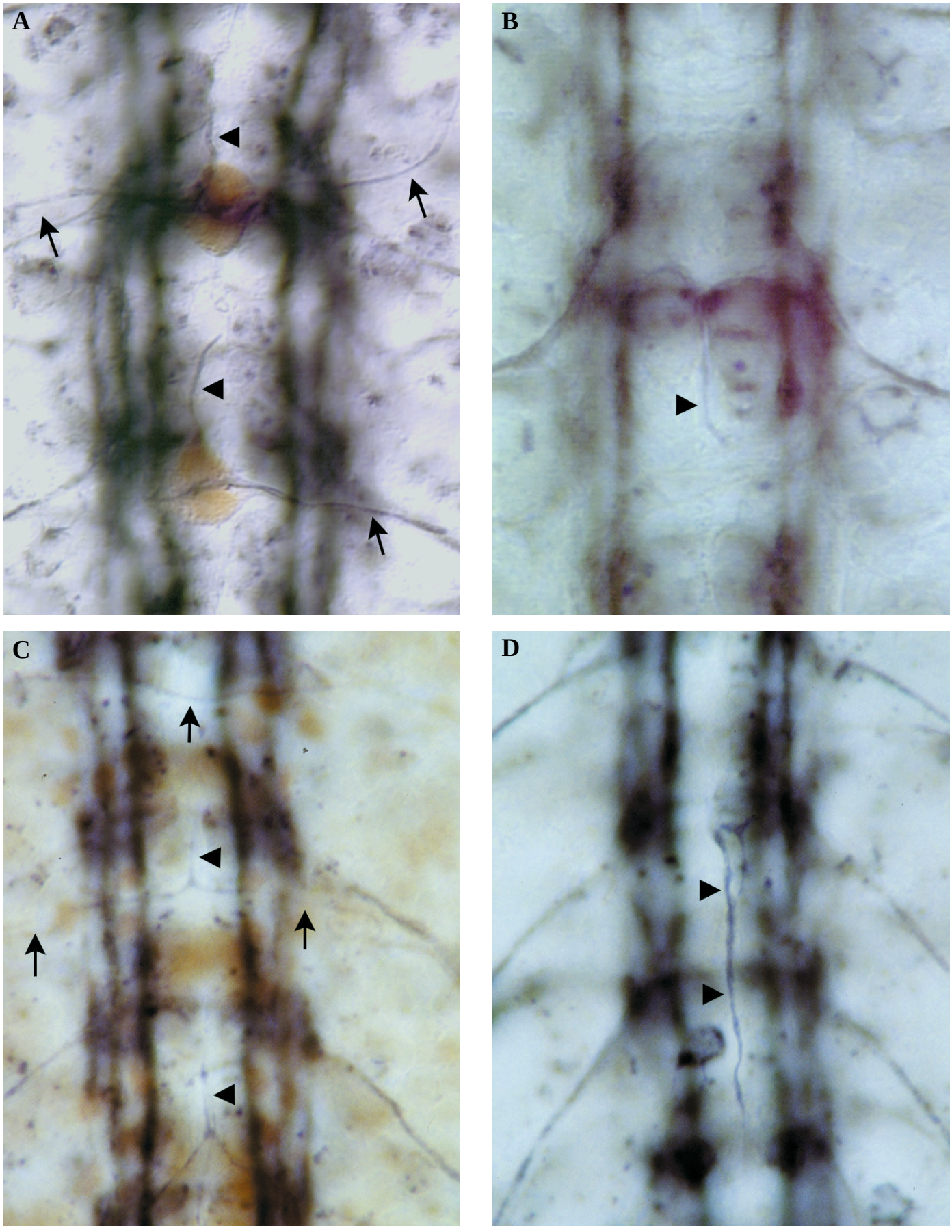
Localization of the *btn* gene to polytene chromosome region 94B by in situ hybridization (data not shown) revealed an absence of nearby mutations or deficiencies, but did permit identification of a promising strain carrying the single P-element insertion P[rib7(94B)] (Karpen et al., 1988). The site of the [rib7](94B) insertion was mapped by Southern hybridization and sequence analysis to a location 1717 bp upstream from the *btn* translation initiation codon (Figs 1, 2A; see Materials and Methods). This insertion, which carried a ry^+ eye color marker but otherwise was associated with no phenotype, was exposed to transposase from the P[Δ2-3] to generate *btn* mutations for phenotypic analysis. Of approximately 65 ry^- excision events, two were associated with homozygous lethality; one of these, designated *btn*, carried a deletion from 0 to +3.2 kb (see Fig. 1). This region includes the entire *btn* transcription unit, and this deletion therefore constitutes a null mutation.

To study the role of *btn* in DM cell development, we immunostained embryos from *btn* heterozygous parents and dissected to expose the CNS. The monoclonal antibody BP102 (Patel, 1994) was used to highlight the commissures and longitudinal connectives of the CNS and antibodies directed against β-galactosidase were used to identify homozygous *btn* embryos (by absence of β-galactosidase expression from a P-element reporter on the balancer chromosome; see Materials and Methods). In wild-type and heterozygous embryos, DM cells are located between commissures along the segment boundary, and are clearly visible under Nomarski optics (Fig. 5A). In all 20 of the *btn* homozygotes identified by absence of β-galactosidase staining, however, DM cells were completely absent (Fig. 5B). To rule out the possibility of artifacts generated during dissection of *btn* mutants, embryos were viewed without dissection after immunostaining with anti-glutactin antibody to visualize the DM cells. Homozygotes were once again identified by use of a β-galactosidase-marked balancer and the DM cells were absent in all homozygous mutants (Fig. 5C,D). Fig. 5E,F illustrate the staining by glutactin of a structure located just ventral to the DM cells. This staining was unaffected by the *btn* mutation and thus serves as a positive control for glutactin staining. The name *buttonless* for this mutation derives from the apparent loss of DM cells, which resemble a row of buttons when present.

btn function is required for DM cell differentiation

To understand how *btn* is involved in DM cell determination or differentiation, we examined the effects of the *btn* mutation upon expression of DM cell markers. Although several *lacZ* reporter strains express β-galactosidase in the DM cells, the expression patterns are complex or not well characterized with respect to the early stages of DM cell formation (Hartenstein and Jan, 1992; Nelson and Laughon, 1993; Wharton and Crews, 1993). We therefore constructed and characterized a reporter using promoter elements from the *btn* gene itself. This construct, P[*btn*1.85-*lacZ*], contained 1.85 kb of *btn* genomic sequence, including upstream sequences and the first 32 codons of the *btn* ORF as an in-frame fusion to *lacZ*. Three independent transgenic lines harboring this construct generated β-galactosidase expression in an embryonic pattern nearly indistinguishable from that of the *btn* RNA (Figs 4F-H, 6A). These strains were also useful for characterization of larval *btn* expression, which is very similar to that of the embryo, including cells with triangular morphology in the posterior segment (Fig. 4H). In embryos and larvae, the β-galactosidase expression is cytoplasmic, suggesting that the presumed nuclear localization signal for the *btn* protein is situated carboxy-terminal to the first 32 residues.

Expression of this reporter in *btn* mutant embryos (identified by absence of the marked balancer chromosome) is like that in wild type at stage 11, when normal *btn* expression first appears in the DM cell precursors (see Fig. 4A). Shortly thereafter, however, the level of β-galactosidase expression declines in the mutants relative to wild type. In addition, DM precursor


A
B
C
D

cells in *btn* mutants fail to maintain the characteristic position and shape of the DM cells (Fig. 6A,B), although the ultimate fate of these precursor cells in *btn* mutant embryos is uncertain since β-galactosidase expression from the reporter construct is lost by stage 14. These results indicate that *btn* is required for DM cell differentiation. In contrast, initial commitment to the DM cell fate occurs in the absence of *btn* function, as indicated by normal initiation of *btn* reporter expression in *btn* mutant embryos.

The role of dorsal median cells in transverse nerve pathfinding

Having developed the ability to ablate the DM cells in *Drosophila* genetically, we looked to cellular studies of several other larger insects for clues regarding potential function of the DM cells. The muscle pioneer cells of the grasshopper, like *Drosophila* DM cells, are also mesodermal in origin, have large cell bodies and long processes, and occur in pairs immediately dorsal to each segment of the developing CNS (Ho et al., 1983). In contrast to *Drosophila* DM cells, the muscle pioneers of the grasshopper appear to serve as a site of recruitment for other mesodermal cells that eventually form the transverse muscle, a muscle not seen in *Drosophila*. In *Manduca*, a pair of non-neuronal cells that look like muscle pioneer cells also occur dorsal to the CNS at the posterior edge of a cluster of non-neuronal cells called 'the strap' (Carr and Taghert, 1988). What is common to these cells in the grasshopper and *Manduca* is that they appear early during development and are thought to prefigure the growth of the transverse nerve, which courses laterally toward the periphery in each segment.

The transverse nerve of *Manduca* carries the axons of two motor neurons and 16 neuroendocrine neurons (Carr and Taghert, 1988); a significant proportion of these axons enter the transverse nerve after first coursing posteriorly along the midline within the median nerve, then bifurcating laterally when they encounter the transverse nerve. In *Drosophila* the median nerve also appears to grow posteriorly along the midline and then bifurcates to contribute to the transverse nerve, which also has been shown to carry axons of identified neurosecretory cells (Zitnan et al., 1993). The median nerve and the transverse nerve can both be visualized by antibody staining for Fasciclin II (Grenningloh et al., 1991; Van Vactor et al., 1993), and we have used this antibody in combination with anti-β-galactosidase antibodies in embryos carrying the *btn-lacZ* reporter to simultaneously mark these nerves and the DM cells (Fig. 7A). This double stain confirms that the median nerve bifurcates at the point where it encounters the DM cell bodies and that the path followed by the transverse nerve precisely follows the path of the DM cell processes. Anti-Fasciclin II staining of an embryo not carrying the *btn-lacZ* reporter (Fig. 7C) more clearly illustrates the bifurcation of the median nerve at precisely the point where it meets with and contributes to the transverse nerve; the morphology of these nerves in relation to the DM cells is schematically illustrated in Fig. 8A. The close correspondence between the paths of the DM cell processes and the transverse nerve, particularly in the posterior segments where the DM cell processes form a triangular shape, strongly supports the notion that DM cells may aid in transverse nerve pathfinding.

If DM cell processes serve as a guiding substratum for transverse nerve outgrowth, loss of DM cell processes should disrupt formation of the transverse nerve. Indeed, we find that transverse nerves are not present in *btn* mutant embryos at stage 14, with only the median nerve visible at its outgrowth stage (Fig. 7B). By stage 16, abnormally long and thick axon bundles expressing Fasciclin II can be seen extending along the midline at the dorsal surface of the ventral cord (Fig. 7D). These bundles, although of varying lengths and thicknesses, were observed reproducibly in all 15 mutant embryos characterized at this later stage of development. We presume that these bundles result from outgrowth and fasciculation of axons

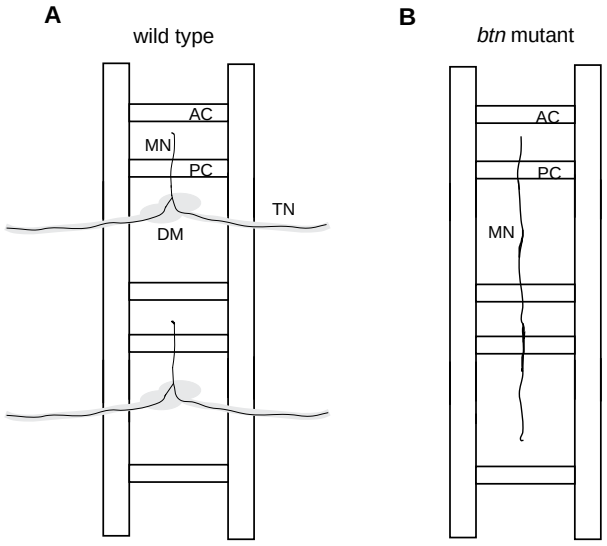


Fig. 8. Schematic representation of median nerve bifurcation and transverse nerve outgrowth in wild-type and *btn* mutant embryos. The DM cells are shaded in wild type and absent in the *btn* mutant; in the *btn* mutant the transverse nerve is also missing and growth of the median nerve is deranged (see text and Fig. 7). Abbreviations: AC, anterior commissure; PC, posterior commissure; MN, median nerve; TN, transverse nerve; DM, dorsal median cells.

Fig. 7. Absence of the DM cells disrupts median nerve bifurcation and transverse nerve pathfinding. Embryos were double-stained with anti-Fasciclin II (purple product) and anti-β-galactosidase (brown product) antibodies, dissected to expose the CNS and viewed under Nomarski optics. (A) A stage 16 wild-type embryo carrying the *btn*1.85-*lacZ* reporter. Note the bifurcation of the median nerve (arrowheads; purple stain) at the DM cell bodies (brown), and the close association between DM cell processes and the transverse nerve (arrow). (B-D) Embryos are from parents carrying a *btn* mutation over a chromosome marked with a β-galactosidase reporter (see Materials and Methods); absence of β-galactosidase antibody staining (brown product) therefore indicates a *btn* homozygous mutant embryo (B,D). (B) A *btn* mutant embryo at stage 14. Note the absence of the transverse nerve, while the midline branch of the median nerve remains visible. (C) A wild-type embryo at stage 16. Note that staining by anti-Fasciclin II highlights the median nerve (arrowheads) and its bifurcation as it joins the transverse nerve (arrows). (D) A *btn* mutant embryo at stage 16. Note the absence of the transverse nerve, with *fasciclin II*-expressing nerve bundles wandering in the midline of the CNS (arrowhead; compare to B); these nerve bundles probably represent the continued outgrowth and fasciculation of the median nerve originating in several adjacent segments (see text and Fig. 8). Anterior is toward the top in all panels.

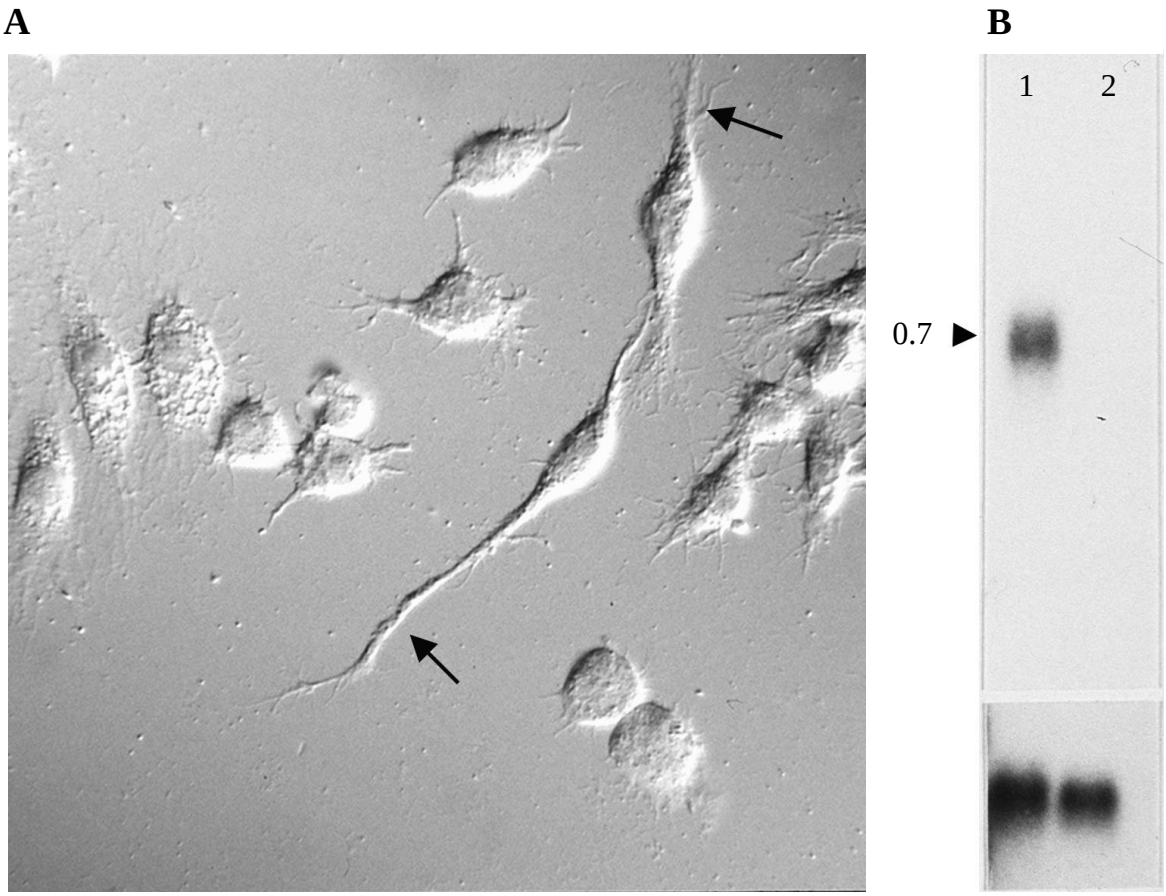


Fig. 9. A cultured *Drosophila* cell line with characteristics like those of embryonic DM cells. (A) Cells from the *Shibire* cultured cell line viewed under Nomarski optics. Note processes that resemble those of the DM cells (arrow). (B) Northern blot hybridization to RNA from two *Drosophila* cultured cell lines. The 0.7 kb *btn* transcript is observed in RNA from the *shibire* cell line (1) but not in RNA from the Schneider cell line (2).

that contribute to the median nerve in multiple segments (Fig. 8B). These results thus suggest that DM cells are necessary for bifurcation of the median nerve and lateral outgrowth of the transverse nerve.

A cultured cell line representative of the DM cell type

The *btn* gene was isolated from a cell line of unusual morphology that was established from *shibire* mutant embryos (Kosada and Ikeda, 1983; Simcox et al., 1985). These cells display a monopolar or bipolar cell morphology with extended processes that resemble those of DM cells (Fig. 9A). Since the initial source of *btn* sequences was a cDNA library made from cells artificially induced to express *Ubx* protein, we confirmed by northern blotting that *btn* mRNA is normally expressed in the *shibire* cell line and does not require transcriptional activation by *Ubx* protein (Fig. 9B). In contrast, the *btn* transcript is not expressed in the Schneider line 2 cells, which display a characteristic round morphology (Schneider, 1972). Given the exquisite specificity of *btn* expression in the embryo and the obvious morphological similarities between DM cells and the *shibire* cells, we suggest that the *shibire* cell line may be representative of DM cells in embryos.

DISCUSSION

Initiation and maintenance of *btn* gene expression in DM cell precursors

The absence of DM cells in *btn* mutant embryos indicates an essential role for *btn* function at some stage during the development of these cells. This role appears not to be the initial commitment to DM cell fate since expression of the *btn-lacZ* reporter gene is initiated normally in *btn* mutants. Expression of the *btn-lacZ* reporter gene represents the earliest sign of commitment to DM cell fate, and we thus conclude that activities other than those encoded by *btn* are required for initial commitment to the DM cell fate. The *twist* (*twi*) gene appears to be a good candidate for encoding such an activity since it encodes a member of the helix-loop-helix family of transcription factors (Thisse et al., 1988) and is expressed in cells of the mesodermal bridge (Bate, 1993). Consistent with this idea, we find a total of 6 consensus binding sites (CTNNAG; Ip et al., 1992) for the *twi* protein within a 300 bp region of genomic sequence immediately upstream of the *btn* cDNA, and a total of 10 sites within the larger 1.7 kb segment that suffices to confer DM cell-specific expression. Transcription factors encoded by other genes such as *dorsal* and *tinman* (Azpiazu

and Frasch, 1993; Bodmer et al., 1990; Ip and Levine, 1992; Steward, 1987), which are involved in early specification of mesodermal fates, may also be involved in *btn* activation, but their expression in cells of the mesodermal bridge has not been documented clearly.

The expression within the mesoderm of regulators, such as *twist*, *dorsal* and *tinman*, is far too general to account for the highly specific and restricted pattern of *btn* gene expression. A candidate gene for a role in restriction of *btn* expression is suggested by the work of Hartenstein et al. (1992), who observed that DM cells differentiate in approximately twice the wild-type number in *Notch* mutant embryos. The phenocritical period for hypertrophy of the DM cells in the temperature-sensitive *Notch* mutant occurs between 4 and 6 hours after fertilization (Hartenstein et al., 1992), which coincides with and possibly precedes the time when *btn* is first expressed. These observations suggest that lateral inhibition mechanisms associated with the function of *Notch* and other neurogenic genes may restrict the number of cells initially committed to the DM cell fate, possibly by restricting expression of *btn*.

Although initiation of *btn-lacZ* reporter expression does not require wild-type *btn* function, later maintenance of *btn* expression does. Because we were unable to trace the fate of the DM cell precursors in *btn* mutant embryos, we can not rule out the possibility that loss of *btn* reporter expression in *btn* mutants may be an indirect result of a series of other events, such as failure to differentiate and ultimately death and phagocytosis of the DM cell precursors. The alternative possibility, that maintenance of *btn* gene expression requires a shift to autologous regulation by the *btn* product, is consistent with the extremely A/T rich character of genomic sequences immediately upstream of the *btn* cDNA and with the presence of many motifs reminescent of homeodomain binding sites (for review see Levine and Hoey, 1988; McGinnis and Krumlauf, 1992).

Wild-type *btn* function is also required for DM cell differentiation, and we therefore presume a second regulatory function of the *btn* protein product to be the activation, whether direct or indirect, of genes required for the differentiation and function of DM cells. Such genes might encode specialized cytoskeletal proteins and cell surface proteins involved in the outgrowth and maintenance of the unusually large DM cell processes. In addition, other target genes for *btn* might encode extracellular matrix proteins and cell surface proteins that play a later role in axon guidance (see below). Expression of *btn* thus appears to serve the dual functions of autogenous maintenance and implementation of the DM cell differentiation pathway. The execution of both functions by a single regulatory protein represents an economical mechanism for simultaneously maintaining and implementing a differentiated state.

A simple cellular cue for transverse nerve pathfinding

The evidence indicative of a role for DM cells in axon guidance is threefold. First, the extending processes of DM cells are readily visible at stage 12 and are complete by stage 14, the earliest stage at which the transverse nerve can be detected (see Fig. 6A, data not shown). DM cell differentiation thus precedes the bifurcation of the median nerve and lateral outgrowth of the transverse nerve. Second, simultaneous labeling of DM cells with β-galactosidase and of the transverse nerve with anti-Fasciclin II antibodies demonstrates that axons of the transverse nerve grow in close association with processes of the DM cells (Fig. 8A). Finally, genetic ablation of the DM cells results in a failure of the median nerve to bifurcate and in loss of the transverse nerve. The DM cell thus represents a single identified cell capable of serving as a template for guidance of axonal growth.

The mechanisms by which bifurcation of the median nerve and lateral outgrowth of the transverse nerve are guided may involve recognition of cell-surface cues from DM cell processes. The expression by DM cells of many extracellular matrix proteins including laminin (Montell and Goodman, 1989), glutactin, entactin and collagen IV (Olson et al., 1990) is consistent with this possibility. *Drosophila* laminin in particular can promote cell adhesion and differentiation of primary *Drosophila* embryo cells (Volk et al., 1990). Similarly, vertebrate laminin has been shown to interact with cell surface receptors such as integrin receptors and with other extracellular matrix proteins to promote adhesion, neurite outgrowth, differentiation and other functions (for review see Adams and Watt, 1993).

In addition to extracellular matrix proteins, DM cells also express neuroglian, a member of the immunoglobulin superfamily with homology to the vertebrate neural adhesion molecule L1 (Moos et al., 1988; Rathjen and Schachner, 1984). L1 can interact with N-CAM (neural cell adhesion molecule), another member of the immunoglobulin superfamily (Barthels et al., 1987; Cunningham et al., 1987; Hoffman et al., 1982) with similar structural organization to *Drosophila* Fasciclin II and 25-30% amino acid identity in the extracellular domains (Grenningloh et al., 1990). The homologies between these interacting vertebrate molecules and their *Drosophila* counterparts suggest the possibility that Fasciclin II expressed on axons of the median and transverse nerves may interact with neuroglian expressed on the surfaces of DM cell processes to promote adhesion and guide axon outgrowth. Loss of this interaction in the *btn* mutant might also account for the abnormal path and thickness of the median nerve, since in the absence of neuroglian the homophilic adhesion properties of Fasciclin II (Grenningloh et al., 1990) might lead to abnormal fasciculation of axons expressing it.

Isolation of the *btn* gene has provided a unique view of the commitment and differentiation of an unusual and highly specialized group of cells originating from within the mesoderm. Mutations in the *btn* gene have further provided us with the means to cleanly ablate DM cells, and this in turn has revealed the role of DM cells as simple cellular cues in axonal pathfinding. Other demonstrations of axonal guidance by single identified cells generally have relied upon laser ablation in organisms with large, easily identified cells and relatively simple nervous systems. In the grasshopper, for example, laser ablation of a specific muscle pioneer cell in limbs (Ball et al., 1985) or of the segmental boundary cell and the intersegmental nerve root glia (Bastiani and Goodman, 1986) cause the axons of specific identified neurons to be misrouted. In the leech ablation of the axonal runway cell results in loss of the sex nerve (Jellies and Kristan, 1988). Alongside these others, the role of DM cells in axonal pathfinding provides one of the simplest examples of axonal guidance by a single identified cell yet described. The identification of a cultured cell line representative of DM cells should provide novel opportunities for study of the cellular and molecular properties that underlie the

unique morphology and function of DM cells. In addition, tests of specific gene function in DM cells should be facilitated by genetic tools such as the *btn* mutation and the DM cell-specific promoter element.

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