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A new *Drosophila* homeobox gene, *bsh*, is expressed in a subset of brain cells during embryogenesis

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SUMMARY

Homeobox genes have been shown to control the determination of positional, tissue and cellular identity during the development of the fruitfly *Drosophila melanogaster*. Because genes involved in the determination of internal structures derived from neural, mesodermal and endodermal tissues may have been overlooked in conventional genetic screens, we undertook the identification of new homeobox genes expressed in these internal tissues. Here we describe the characterization of one of these new *Drosophila* homeobox genes, called *brain-specific-homeobox* (*bsh*). In embryos, *bsh* is expressed exclusively in the brain. bsh protein accumulates in approximately 30 cells in each brain hemisphere. One of these *bsh* expressing cells is closely associated with the terminus of the larval visual nerve (Bolwig's nerve). While deletions of chromosomal interval containing the *bsh* gene show no dramatic changes in embryonic brain morphology, the expression pattern of the *bsh* gene suggests that it may play a highly specialized role in the determination and function of cell type in the *Drosophila* brain.

Key words: homeobox, brain, central nervous system, Bolwig's nerve, *Drosophila*

INTRODUCTION

As in other metazoans, early events during the embryogenesis of the fruitfly *Drosophila melanogaster* direct cells into developmental pathways by position-specific determinative mechanisms. Through systematic genetic screens for mutants that alter the normal pattern of the cuticle of the *Drosophila* larva, many of the genes directing these events have been identified (Lewis, 1978; Kaufman et al., 1980; Nüsslein-Volhard and Wieschaus, 1980; Nüsslein-Volhard et al., 1984; Jürgens et al., 1984; Wieschaus et al., 1984). The discovery of these early patterning genes has opened the study of development to the increasingly sophisticated techniques of molecular biology, and over the last decade has led to an outline of the genetic mechanisms underlying early pattern formation in the *Drosophila* embryo (reviewed by Akam, 1987; Small and Levine, 1991; St. Johnston and Nüsslein-Volhard, 1992; Ingham and Martinez Arias, 1992).

A surprising discovery that emerged from these and subsequent studies was that a large number of early *Drosophila* patterning genes contain a highly conserved 180 bp sequence called the homeobox (reviewed by Scott et al., 1989; Dessain and McGinnis, 1992). Among them are the homeobox genes of the Antennapedia and Bithorax complexes (collectively called the HOM-C) which control segmental identity along the anterior-posterior axis; HOM-C genes have highly conserved vertebrate counterparts (the Hox genes), implicating conserved mechanisms of axial pattern formation in both insects and vertebrates (reviewed by McGinnis and Krumlauf, 1992). More diverged homeobox genes have been found in a wide variety of organisms as well as *Drosophila* and many have been implicated in the determination of positional, tissue and cellular identity.

The 60 amino acid homeodomain encoded by the homeobox motif has a helix-turn-helix structure similar to prokaryotic transcription regulators (Laughon and Scott, 1984; Otting et al., 1990; Kissinger et al., 1990). This domain mediates sequence specific DNA binding (Desplan et al., 1988; Hoey and Levine, 1988; Beachy et al., 1988; Affolter et al., 1990; Ekker et al., 1991; Dessain et al., 1992), and is very important for the biological specificity of the protein (Kuziora and McGinnis, 1989, 1991; Mann and Hogness, 1990; Gibson et al., 1990). It is thought that the products of homeobox genes control specific programs of development through the transcriptional regulation of target genes via the target specificity of the homeodomain. Thus, the differential expression of homeobox genes in different cells would result in a characteristic program of target gene expression which ultimately contributes to the determination of the position, morphology, and function of each cell in the animal.

The original genetic screens for *Drosophila* mutants with altered larval or adult cuticle patterns identified many homeobox genes involved in the development and organization of the epidermis. Some of these genes have since been found to have additional roles in the development of internal structures and germ layers. For example, besides specifying regional identity in the epidermis, HOM-C genes

are also involved in the specification of neuronal and mesodermal cell fates (Lewis, 1978; Hooper, 1986; Doe and Scott, 1988; Heuer and Kaufman, 1992; Immerglück et al., 1990, Reuter et al., 1990). The homeobox genes *fushi tarazu* (*ftz*) and *even-skipped* (*eve*), which have early functions in setting up the segmental pattern of the blastoderm, are also required for the specification of subsets of neurons in the central nervous system (CNS; Doe et al., 1988a,b). The *cut* gene and the homeobox genes at the *Bar* locus, originally identified for their effects on adult structures, have been shown to be involved in the determination of sensory organ identities in the embryo (Bodmer et al., 1987; Blochlinger et al., 1991; Higashijima et al., 1992a,b). Both *gooseberry* (Patel et al., 1989) and *orthodenticle* (Finkelstein et al., 1990) have been shown to have neural functions in addition to their epidermal functions, and most of the homeobox segmentation genes have a second phase of expression in different subsets of cells in the CNS, suggesting their involvement in neuronal cell lineage determination and differentiation events.

A number of homeobox genes in the nematode *Caenorhabditis elegans* are involved in specifying cell lineages and cell types in the nervous system. *mec-3* is required for the correct determination of touch receptor neurons (Way and Chalfie, 1988); *unc-86*, one of the POU class of homeobox genes, is required for the determination of a number of neuroblast lineages (Finney et al., 1988); and *unc-4* determines the pattern of synaptic input to specific motor-neurons (Miller et al., 1992).

Because many *Drosophila* genes dedicated to the determination of internal structures derived from neural, mesodermal and endodermal tissues may have little effect on the development of the larval cuticle, a significant number of important genes have probably been missed in conventional mutant screens. A 'reverse genetic' approach has been taken by many labs to identify such genes either by using the enhancer trap method (O'Kane and Gehring, 1987) screening for genes with tissue or cell-specific patterns of expression, or alternatively by cloning new genes homologous to members of gene families that have been strongly associated with developmental processes. Based on the hypothesis that more diverged members of the homeobox gene family remained to be discovered in *Drosophila* and that these genes may provide novel genetic functions in pattern formation and morphogenesis, screens have been undertaken to identify new members of the homeobox gene family using sequence homology (Levine et al., 1985; Macdonald and Struhl, 1986; Macdonald et al., 1986; Barad et al., 1988; Dalton et al., 1989; Kim and Nirenberg 1989; Billin et al., 1991; Treacy et al., 1991; Dessain and McGinnis, 1993). A number of homeobox genes have been isolated with novel expression patterns restricted to internal structures. Four are expressed in mesodermal cell lineages (*H2.0*, Barad et al., 1988; *S59*, Dorhmann et al., 1990; *msh*, Gehring, 1987; and *msh-2*, Bodmer et al., 1990); others have complex patterns, but are primarily expressed in the central nervous system (*zfh1* and *2*, Fortini et al., 1991, Lai et al., 1991; *prospero*, Doe et al., 1991, Vaessin et al., 1991; *Cf1-a*, Johnson and Hirsch, 1990; *I-POU*, Treacy et al., 1991; *pdm-2*, Billin et al., 1991; *57San*, *66Dem*, *90Bre*, *93Bal*, *94Che*, Dessain and McGinnis, 1993).

This paper describes a new *Drosophila* homeobox gene, called *brain-specific-homeobox* (*bsh*). *bsh* is the first known homeobox gene that is expressed exclusively in the developing brain. One of the *bsh*-expressing brain cells is closely associated with the terminus of the larval photoreceptor nerve (Bolwig's nerve). While deletions of the chromosomal interval containing the *bsh* gene show no dramatic changes in the morphology of the embryonic brain, the unique expression pattern of *bsh* protein in approximately 30 cells in each brain hemisphere suggests that *bsh* may play a role in the determination and function of cell types in the *Drosophila* brain.

MATERIALS AND METHODS

Library screens for genomic and cDNA clones

Phage clones that overlap the original *bsh* homeobox-containing clone λE86 (Dalton et al. 1989) were isolated from a library provided by A. Preiss (Preiss et al., 1985) consisting of partially digested *Sau*3A fragments of genomic *Drosophila melanogaster* DNA cloned into *Bam*HI digested λEMBL4 arms. Cosmid clones were isolated from a CoSpeR library containing *Sau*3A digested *Drosophila* genomic DNA which was constructed and kindly provided by J. Tamkun. Homeobox fragments were mapped in λE86 using reduced stringency Southern hybridization conditions as described in McGinnis et al. (1984a). cDNAs were isolated from libraries which were a generous gift from L. Kauvar and T. Kornberg (Poole et al., 1985). One *bsh* cDNA (cE86A) was isolated from a *Drosophila* λgt10 cDNA library derived from 3- to 12-hour-embryonic poly(A)$^+$ RNA (Kauvar's E6 library); a second cDNA (cE86C) was isolated from a library derived from late-third-instar poly(A)$^+$ RNA (Kauvar's I4 library). Exons were mapped onto genomic clones using high stringency hybridization conditions as described by McGinnis et al. (1984a).

In situ hybridization to polytene chromosomes

Preparation and hybridization to chromosomes was performed as described in Langer-Safer et al. (1982). DNA probes were labeled with biotin using the random-priming method of Feinberg and Vogelstein (1983). Enzymatic detection of biotinylated probes was done using a streptavidin/horseradish peroxidase conjugate (ENZO Biochemicals), followed by staining with diaminobenzidine.

RNA extraction and northern analysis

RNA extraction, electrophoresis, blotting, hybridization and washes were performed as previously described (Kuziora and McGinnis, 1988). A 1.8 kb *Eco*RI-*Sal*I genomic fragment from λE86 containing the *bsh* homeobox exon was used as a probe. Transcript size was estimated by running a lane with *Hin*dIII digested λ DNA as described in Maniatis et al. (1982).

In situ hybridization to tissue sections

Third instar larvae were frozen in Tissue-Tek (Miles Scientific) and cut into 8 μm sections which were placed on poly-lysine coated slides. After drying out on a hot plate at 45°C for 15 minutes, the slides were submerged in 4% paraformaldehyde/PBS for 20 minutes, rinsed in PBS and dehydrated through an ethanol series. The slides were then treated with pronase, acetylated and probed as described in Chadwick and McGinnis (1987). The probe used was a ^{35}S-labeled riboprobe synthesized from a pBluescript (Stratagene) clone containing the 1.8 kb *Eco*RI-*Sal*I genomic *bsh* homeobox exon fragment.

Sequencing

Overlapping restriction fragments of λE86 and the two *bsh* cDNAs, cE86A and cE86C, were subcloned in both orientations into M13 mp18 and mp19 (Messing, 1983), which were then used as single stranded templates for sequencing. Sequencing was performed by the dideoxynucleotide method (Sanger et al., 1977) using the protocol, reagents and a modified form of T7 polymerase from the 'Sequenase' kit of US Biochemicals. Both strands of cE86A and cE86C, and 2.6 kb of genomic DNA encompassing the *bsh* transcription unit were sequenced in their entirety.

Primer extension

Primer extension experiments were performed as described in Ausubel et al. (1987). An oligonucleotide primer was prepared that is complimentary to sequences 70-100 nucleotides from the 5′ end of cE86C (see Fig. 5). The primer was end-labeled with [γ-^{32}P]ATP (6000 Ci/mmol) using T4 polynucleotide kinase. Three ammonium acetate precipitations were performed to remove most of the unincorporated counts. Approximately 5×10^4 cts/minute of labeled primer was annealed to 10 μg of poly(A)$^+$ RNA derived from 12- to 24-hour embryos, and hybridized overnight in hybridization buffer (80% formamide, 40 mM Pipes pH 6.6, 400 mM NaCl, 1 mM EDTA pH 8) at 30°C after heating the mixture to 65°C. Extension of the primer was carried out at 42°C for 90 minutes using AMV reverse transcriptase, and the products were analyzed by autoradiography after running through a 5% polyacrylamide/8 M urea sequencing gel.

Preparation of bsh fusion protein and anti-bsh antiserum

The T7 expression system of Studier and Moffatt (1986) was used to produce bsh fusion protein in *E. coli.* A *Bam*HI fragment from the *bsh* cDNA cE86A containing the final two thirds of the *bsh* ORF was cloned into the *Bam*HI site of the expression vector pAR3039. The resulting plasmid, designated pAR E86B, has a T7 promoter in front of the codons for the first 13 amino acids of T7 gene *10*, followed by the codons for 136 amino acids of the *bsh* ORF. The expression plasmid pAR E86B was transformed into the *E. coli* strain BL21(DE3)pLysS which contains a T7 RNA polymerase gene under the control of the *lac* promoter (Studier and Moffatt, 1986). After induction with isopropyl-β-*D*-thiogalactopyranoside (IPTG; Sigma), amounts in the range of 10-20 mg/l of bsh fusion protein were obtained. Approximately 250 μg of fusion protein was cut out of an SDS-polyacrylamide gel, frozen, ground to a powder, resuspended in PBS and homogenized with an equal volume of Freund's complete adjuvant. This homogenate was injected into a New Zealand white rabbit at multiple subcutaneous sites. The rabbit was boosted after 6 weeks with 100 μg of bsh fusion protein which had been electroeluted from the SDS-polyacrylamide gel, precipitated with acetone, resuspended in PBS and mixed with Freund's incomplete adjuvant. After 8 weeks, the rabbit was bled, yielding serum that specifically interacted with bsh protein.

Immunohistochemical detection of proteins in embryos

Drosophila embryos were prepared and stained using HRP immunohistochemistry as described previously (Jack et al., 1988). Anti-bsh protein antiserum, which had been diluted 1:20 in PBS, was immunoabsorbed with fixed devitellinized 0- to 2-hour embryos and then used at a final dilution of 1:1000 to stain whole embryos. Goat anti-rabbit IgG antibodies conjugated to biotin (Jackson Immunoresearch) were used as the secondary antibodies followed by HRP detection with Vector Labs's ABC system with diaminobenzidine (DAB). To study the pattern of the developing nervous system in relation to the bsh pattern in both wild-type, deficiency, and mutant embryos, we double stained embryos with anti-bsh antiserum and a variety of antibodies that visualize components of the nervous system. The following antibodies were used. (1) Anti-horseradish peroxidase (anti-HRP) antibody recognizes a neural-specific carbohydrate moiety expressed on the surface of all neurons and axons (Jan and Jan, 1982; Snow et al., 1987); we used goat anti-HRP (Cappel) at a dilution of 1:1000, followed by donkey anti-goat IgG conjugated to biotin at 1:500 (Jackson Immunoresearch) and HRP detection. (2) mAb22C10 (Fujita et al., 1982; Zipursky et al., 1984), which recognizes a cytoplasmic antigen in all the PNS neurons as well as a subset of of neurons in the CNS, was used at a 1:50 dilution. (3) mAbBP102, which strongly stains the axons of the CNS with virtually no staining of neuron cell bodies and is an excellent marker for the pattern of commissures and connectives in the ventral cord and the brain (Elkins et al., 1990), was used at a 1:5 dilution. (4) mAb2D5, which recognizes the fasciclin III protein (Patel et al., 1987) and stains the membranes of a subset of neurons and axons in the CNS, as well as ectodermal and endodermal cells, was used at a 1:5 dilution. (5) mAbBP104, which recognizes a nervous system specific form of neuroglian (Hortsch et al., 1990) and stains the membranes of all neurons of the CNS and PNS, was used at a 1:5 dilution. The monoclonal antibodies used above were detected by a goat anti-mouse Ig antibody conjugated to biotin (Jackson Immunoresearch) followed by peroxidase labeling with the ABC kit and DAB staining. In all double staining experiments both anti-bsh antiserum and the co-labeling antibody were stained with DAB; bsh protein expressing cells are easily identified by their strong nuclear staining. Embryos were cleared in methyl salicylate and mounted in Permount (Fisher).

Digital optical microscopy

The high resolution composite image of the Bolwig's nerve (Fig. 8C) was taken using computer enhanced digital microscopy as described in Johansen et al. (1989). The composite image was made by tableting the in-focus areas of multiple averaged video-images taken at different focal plains. The image was photographed directly from a high-resolution video monitor using Kodak T-MAX 100 film.

P element construction and transformation

The P element vector phs-bshCaSpeR was constructed using the heat shock shuttle vector pHSBJ (Malicki et al., 1990; Jones and McGinnis, 1993). pHSBJ contains a polylinker located between *hsp70* promoter sequences and 3′ untranslated sequences of the *Adh* gene. The *bsh* cDNA cE86C was inserted into the *Eco*RI site in the polylinker of pHSBJ, and the resulting *bsh* heat shock cassette was excised with *Not*I and subcloned into the *Drosophila* transformation vector CaSpeR (Pirotta et al., 1988; see Fig. 9A). CaSpeR contains P element termini flanking both the polylinker and a functional copy of the *white* (*w*) gene.

To obtain transgenic lines, phs-bshCaSpeR was coinjected with the helper plasmid pπ25.7wc (Karess and Rubin,1984) into *Df(1)w, yw$^{67c23(2)}$* embryos as described by Rubin and Spradling (1982) and selected for rescue of the *w* phenotype. A single viable insert on the X chromosome designated *P[hsp70-bsh w$^+$]^A* was generated. Four additional strains, *P[hsp70-bsh w$^+$]^B* and *P[hsp70-bsh w$^+$]^C* on the second chromosome, and *P[hsp70-bsh w$^+$]^D* and *P[hsp70-bsh w$^+$]^E* on the third chromosome, were generated by transposition of *P[hsp70-bsh w$^+$]* using a genomic source of transposase (Robertson et al., 1988).

Fly strains

Fly culture and crosses were performed according to standard procedures. The wild-type stock was Oregon R. The deficiency strains *Df(2L)TW65*, *Df(2L)TW161*, and *Df(2L)E55* (Wright et al., 1976a)

were obtained from the Bowling Green Stock Center. *Df(2L)pr65* was a gift of B. Ganetzky (Brittnacher and Ganetzky, 1983) and *Df(2L)OD16* (Lindsley and Zimm, 1992) was donated by T. Wright. The mutants *l(2)37Fe* and *l(2)37Ff* (also called *l(2)E3* and *l(2)E43* respectively; Wright et al., 1976b) were obtained from the Bowling Green stock Center, and *l(2)37Fg* (also called *l(2)09*; Lindsley and Zimm, 1992) was obtained from T. Wright. The *screw* allele scw^{S12} and the *spitz* allele spi^{IIM105} were a gift of C. Nüsslein-Volhard, the *glass* allele gl^{2} was obtained from D. Kankel, and the strain used for P element transformations, $Df(1)w, yw^{67c23(2)}$, was obtained from V. Pirotta.

RESULTS

Isolation and molecular organization of the *bsh* gene

The *bsh* homeobox containing clone λE86 was previously isolated by Dalton et al. (1989; the *bsh* homeobox was originally called E86) in a screen of a *Drosophila* genomic library for sequences that cross-hybridize with the *even-skipped* (*eve*) homeobox under low stringency conditions. In situ hybridization to polytene chromosomes places λE86 at 38A1-3 on the left arm of the second chromosome (Dalton et al., 1989). We have isolated additional genomic clones λX3, λK1, and cosE86-12, that flank and overlap λE86, and which together include approximately 46 kb of contiguous genomic DNA (Fig. 1A).

To facilitate the genetic analysis of the *bsh* gene, which will be described later, a more detailed cytogenetic localization of the *bsh* genomic region was performed. Several deficiency chromosomes with breakpoints in the 38A region were tested for the presence or absence of the four genomic clones on the deleted region of the chromatid in polytene preparations. A summary of this analysis is shown in Fig. 2A. The entire 46 kb genomic region is contained within a small deficiency *Df(2L)OD16* (Lindsley and Zimm, 1992) which deletes the strong band at the beginning of 38A. The genomic region can be further delimited by its deletion in *Df(2L)TW65* (Wright et al., 1976a) whose proximal breakpoint is at 38A1, and its partial deletion in *Df(2L)pr65* (Brittnacher and Ganetzky, 1983) whose proximal breakpoint at 38A3 breaks within the DNA region where λE86 and λK1 overlap (see also Fig. 1A). The mapping of the *Df(2L)pr65* breakpoint to the overlapping DNA of λE86 and λK1 has allowed us to orient the molecular map along the chromosome with respect to the centromere (Fig. 1A).

The *bsh* homeobox was mapped by low stringency Southern hybridization with the *eve* homeobox probe (Dalton et al., 1989) to a 1 kb *Bam*HI-*Sal*I fragment near the center of λE86 (Fig. 1B). This fragment was sequenced and shown to contain the 180 nucleotide homeobox sequence in an apparent 296 nucleotide exon flanked by consensus splice donor and acceptor sequences (Mount, 1982). To determine the number and size of transcripts containing the *bsh* homeobox we probed an all stage northern blot (Fig. 3A). A single rare 2 kb transcript was detected throughout development beginning after six hours, with highest levels occurring between 6 and 24 hours of embryogenesis. The spatial distribution of *bsh* transcript expression was initially determined by in situ hybridization with ^{35}S-labeled anti-sense RNA probes to tissue sections of

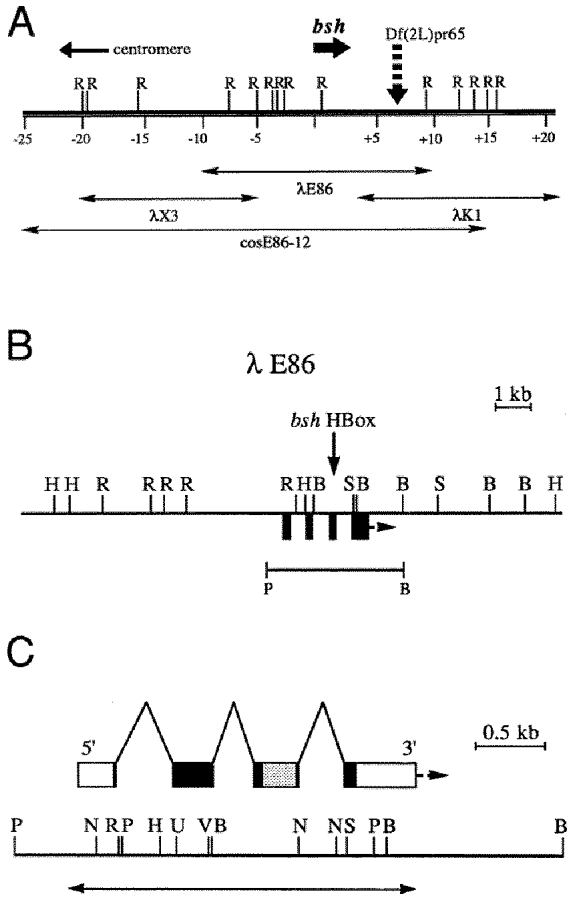


Fig. 1. Molecular organization of the *bsh* locus. (A) Molecular map of the *bsh* genomic region derived from four overlapping clones λE86, λX3, λK1 and cos E86-12 which are diagrammed below the restriction map. Restriction sites of *Eco*RI (R) are depicted as well as distances in kilobases from the predicted transcription start site of the *bsh* gene. The breakpoint of *Df(2L)pr65* maps to the overlap of λE86 and λK1 (dashed arrow). (B) Molecular map of the genomic clone λE86. The location of the *bsh* homeobox is indicated. Black boxes indicate the positions of *bsh* exons. The line below the map bounded by P and B (for *Pst*I and *Bam*HI) shows the region that is expanded in C. (C) Molecular map of the *bsh* transcription unit. The boxes indicate the position of exons, the unshaded parts indicate untranslated 5′ and 3′ regions, the black indicates the open reading frame, and the shaded box indicates the position of the homeobox. The arrow below the map shows the sequenced region (2672 base pairs) that is depicted in figure 5. Restriction enzymes: *Bam*HI (B); *Hin*dIII (H); *Nae*I (N); *Pst*I (P); *Eco*RI (R); *Sal*I (S); *Nru*I (U); and, *Eco*RV (V).

embryos, 3rd instar larvae, and adults. *bsh* transcripts can first be detected in very discrete patches in the procephalic lobe in stage 12 embryos (Campos-Ortega and Hartenstein, 1985) and transcripts are subsequently restricted to the brain lobes throughout embryonic development. A detailed description of the embryonic expression of bsh protein follows later in this paper. In 3rd instar larvae (Fig. 4A,B), transcripts are restricted to the developing optic lobes in cells that have just arisen from the inner and outer proliferation centers (Kankel et al., 1980). Later, adults show

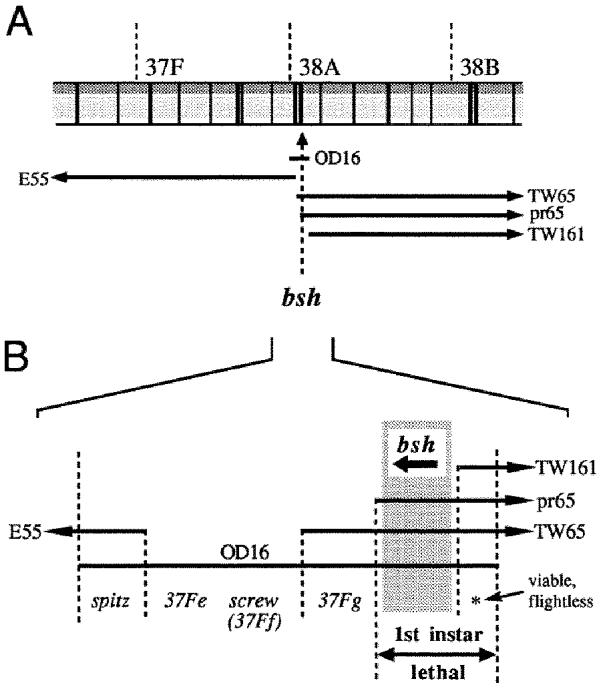


Fig. 2. Cytogenetic and complementation maps of the 38A chromosomal region. (A)Cytogenetic representation of the salivary gland chromosome region around 38A showing the location of *bsh* clones (dashed arrow) with respect to deficiency breakpoints. The chromosomal regions deleted by each deficiency are depicted by horizontal bars with arrows beneath the diagram of the polytene chromosome. *bsh* hybridizes to the strong bands at the beginning of 38A (38A1-3) and is deleted by *Df(2L)OD16*, *Df(2L)TW65* and *Df(2L) pr65*. (B) Complementation map of the *bsh* region. Deficiency breakpoints (dashed vertical lines) are ordered with respect to the ability of each deficiency (horizontal bars) to complement known lethal mutant alleles (in italics, described more fully in Materials and methods). In situ hybridization places the *bsh* transcription unit between the breakpoints of *Df(2L)pr65* and *Df(2L)TW161* (shaded region) eliminating the possibility that *bsh* corresponds to one of these lethals. The smallest deletion interval that eliminates *bsh* (the overlap of *Df(2L)OD16* and *Df(2L)pr65*) produces a first instar larval lethal phenotype.

very low levels of labeling over the cell body layer of the first optic ganglia (the lamina; data not shown). In both the larva and adult there is no significant labeling above background levels outside of the brain hemispheres. While we were able to detect bsh transcripts in the larval and adult brain, we have not obtained convincing staining of bsh protein using anti-bsh antiserum in the larva or adult, which is apparently due to the very low levels of bsh protein expression during these stages.

Based on the northern and in situ analysis we expected that copies of *bsh* messenger RNAs would not be abundantly represented in cDNA libraries. We used a 0.6 kb *Bam*HI-*Nae*I fragment containing the entire homeobox exon to screen three embryonic cDNA libraries constructed by L. Kauvar (Poole et al., 1985). From an estimated 1,000,000 cDNA clones we were able to isolate a single cDNA with an insert of 1.1 kb (designated cE86A). This cDNA was then used to screen through additional libraries; one more cDNA was isolated from a late 3rd instar library with an insert of 1.2 kb (designated cE86C). cE86C differs from cE86A by being 0.2 kb longer on its 5′ end and 0.1 kb shorter on its 3′ end. Hybridization of the two *bsh* cDNAs to genomic clones placed exonic sequences in the center of λE86 (Fig. 1B,C).

In order to determine accurately the structure of the locus, the entire 2.6 kb genomic region encompassing the four *bsh* exons (Fig. 1C) and the two *bsh* cDNAs (cE86A and cE86C) were sequenced using single-stranded dideoxynucleotide sequencing (Fig. 5). Aligning the cE86C sequence with the genomic sequence indicates the presence of three introns, all of which are flanked with excellent matches of consensus donor and splice exceptor sequences (Mount, 1982). The 5′ end of cE86A starts 18 nucleotides 5′ of the acceptor site of the second exon and is present in the genomic sequence. cE86A is likely to represent an incompletely processed and truncated RNA based on the northern data, in which only one species of messenger RNA is detected and the observation that the sequence immediately 5′ of the end of cE86A present in the genomic sequence is not capable of continuing the open reading frame, nor is there a consensus translation start or splice acceptor sequence in the vicinity.

To provide evidence of whether the 5′ end of cE86C is at or near the transcription start site, a primer extension experiment was performed on embryonic mRNA. Using a 30-mer homologous to the opposite strand of sequences from 70 to 100 nucleotides from the 5′ end of the cE86C (underlined in Fig. 5) we detected a single primer extension product of 119 nucleotides (Fig. 3B). If the 5′ end of this extension product represents the legitimate transcription start site it would extend the transcript another 19 nucleotides 5′ to the end of cE86C. Neither of the *bsh* cDNAs have 3′ poly(A) tracts or polyadenylation signals. This signal presumably lies further 3′ in the genomic DNA, accounting for the remaining difference between the combined size of the cDNAs and the size of the message predicted by northern blots.

The longest open reading fame (ORF) of cE86C is in frame with the homeobox codons. Starting from the first methionine codon (ATG; which is preceded by an in frame stop codon) to the first stop codon (TGA), the ORF spans 678 nucleotides and would encode a protein of 226 amino acids. Fig. 6A shows a schematic representation of the predicted protein. The homeodomain is in the carboxy-terminal half of the protein. Sequences outside of the homeodomain have no significant homology to known proteins, but contain a number of proline-rich stretches, a feature common to other transcription factors (Mitchell and Tjian, 1989).

A comparison of the bsh homeodomain with other published homeodomains reveals no strikingly close relatives in *Drosophila melanogaster* or in more divergent species. Fig. 6B shows a comparison of the bsh homeodomain to five of the most similar homeodomain sequences in *Drosophila*. bsh is most closely related to *Antennapedia*-like homeodomains, having the highest degree of similarity in the third helix region. The *Bar* class of homeodomains (Higashijima et al., 1992a) have the highest degree of sim-

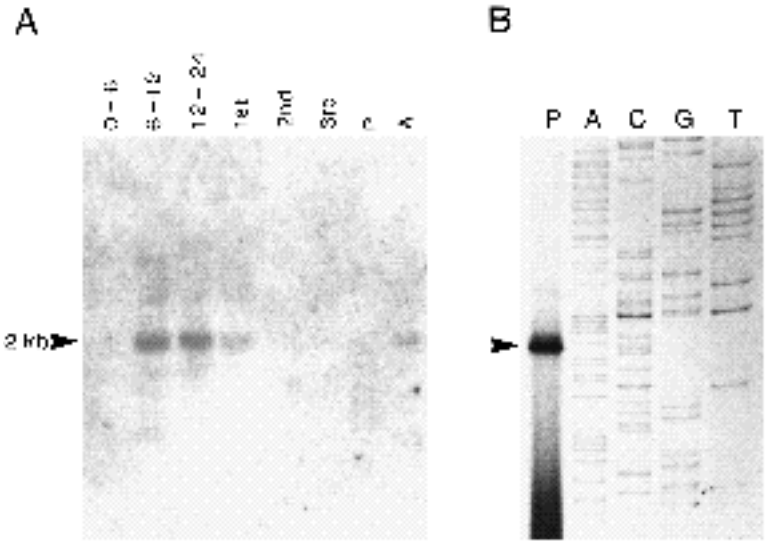
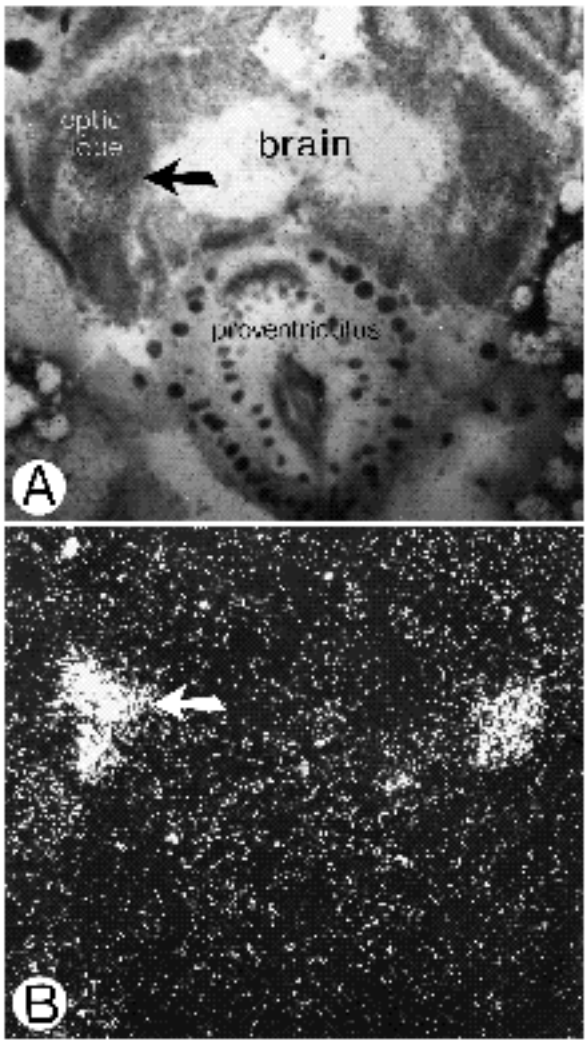


Fig. 3. Analysis of *bsh* transcripts. (A) Northern analysis of *bsh* mRNA expression throughout the *Drosophila* life cycle. Poly(A)$^+$ RNA from, 0- to 6-hour, 6- to 12-hour and 12- to 24-hour embryos, from first (1st), second (2nd) and third (3rd) instar larvae, from pupae (P) and from adults (A) was tested; 10 μg of RNA was loaded in each lane. The position of the 2 kb *bsh* transcript is marked with an arrow. (B) Primer extension analysis. The autoradiogram shows the primer-dependent extension product (lane P) from a primer complementary to sequences 70-100 nucleotides from the 5′ end of the *bsh* cDNA cE86C (underlined in Fig. 5), which has been hybridized to poly(A)$^+$ RNA from 12- to 24-hour embryos. Lanes A, C, G and T are dideoxy-chain termination sequencing tracks from a genomic subclone primed with the same primer, and therefore show sequences from the 5′ region of the *bsh* transcription unit. The extension product (arrow) is 119 nucleotides in length and extends to a C residue which is depicted as +1 in Fig. 5.

ilarity to bsh (38 out of 60) but differ significantly by having an amino acid difference in the highly conserved third helix (Phe replaces Tyr).



Localization of bsh protein during embryogenesis

To study the spatial expression of *bsh* in more detail we generated anti-bsh protein antibodies. Polyclonal rabbit antiserum was raised against a bsh fusion protein (see Materials and methods). This antiserum specifically recognizes bsh protein in embryos; animals in which the *bsh* gene is deleted (*Df(2L)OD16*, *Df(2L)TW65*, *Df(2L)pr65* homozygotes and trans-heterozygotes (see Fig. 2) fail to stain (data not shown).

Fig. 7 shows the immunostaining patterns of anti-bsh antiserum in wild-type embryos from about 7 hours to 15 hours of development (stages 11-16 of Campos-Ortega and Hartenstein, 1985). bsh staining is confined to the nuclei of cells. It first appears in four widely separated cells, two in each procephalic lobe just before the beginning of germ band retraction at around 7 hours of development (stage 11; Fig. 7A,B). These cells appear to be one or two cell layers beneath the epidermis. Judging by their position and size they are likely to be cells whose lineage is derived from the neurogenic ectoderm (Hartenstein and Campos-Ortega, 1984). At this time in development neuroblasts, glioblasts and the progenitors of other neuronal support cells are segregating from the epidermis of the procephalic neurogenic region; bsh expressing cells lie just below the neurogenic layer. In subsequent stages the number of bsh expressing cells increases in two bilateral clusters adjacent to the original two cells (Fig. 7C-F).

After germ band retraction, head movements reorganize the shape of the procephalic lobe (at the end of stage 13). The two halves of the procephalic lobe round up and rotate inwards to produce the two lobes of the brain (the suprae-

Fig. 4. Expression of *bsh* transcripts in the optic lobes of the 3rd instar larva. Frozen sections of climbing 3rd instar OregonR flies were probed with ^{35}S-labeled antisense RNA transcribed from a 1.8 kb genomic fragment containing the *bsh* homeobox. (A) Bright-field and (B) dark-field photomicrographs of a horizontal medial section of a 3rd instar showing a section through the brain. *bsh* transcripts (arrows) are detected only in the developing optic lobes (anterior is up).

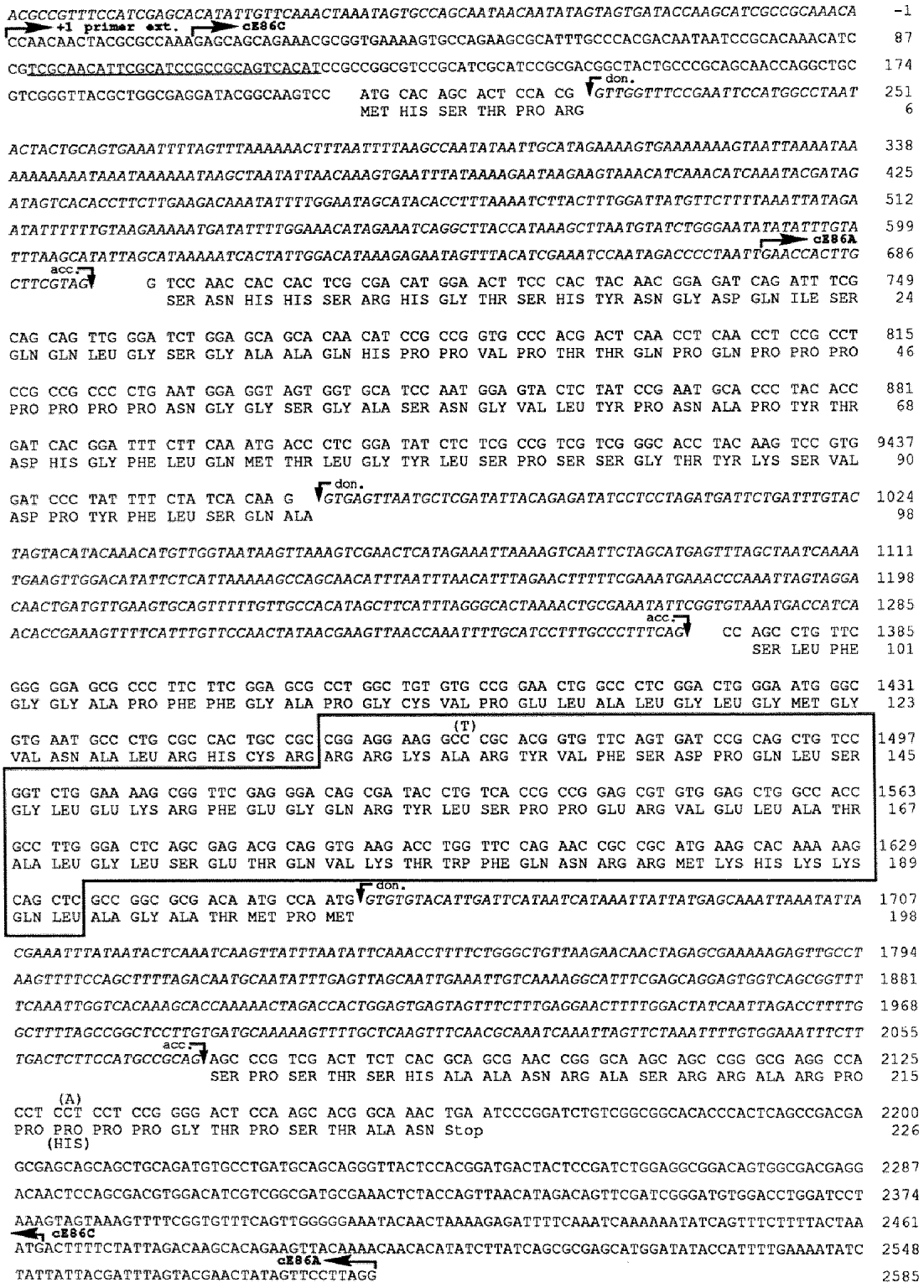


Fig. 5. Nucleotide and predicted amino acid sequence of the *bsh* gene. The entire sequence of the 2672 nucleotide genomic region encompassing the four *bsh* exons is shown. The introns and 5′ non-transcribed sequences are depicted in italics Donor (don.) and acceptor (acc.) splice sites are marked as determined by sequencing cDNAs and by conformation to consensus sequences (Mount, 1982). The homeodomain region is outlined. The start and end points of the two *bsh* cDNAs (cE86A and cE86C) are depicted with arrows. The predicted end point of the primer extension product is shown (+1 primer ext.) and the complementary sequence to the primer is underlined. Positions in which cE86C differs in sequence to both the genomic and cE86A sequences are shown in parentheses above the nucleotides; in one case the polymorphism causes a change in amino acid sequence (Pro to His at position 117). Genbank accession number is L06475.

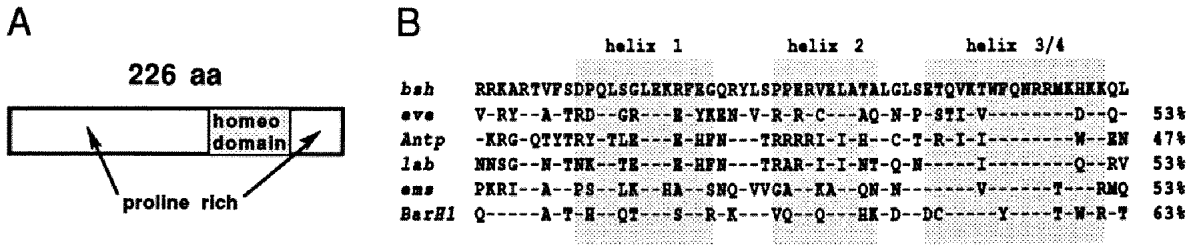


Fig. 6. bsh protein and homeodomain. (A) A schematic diagram of the predicted bsh protein. (B) Comparison of the bsh homeodomain sequence to sequences of related homeodomains. Only amino acids that differ from thebsh sequences are shown, and the percentage identity to bsh is listed at the end of each sequence. The positions of putative helix regions are shaded. References for sequences: *eve*, Macdonald et al. 1986, Frasch et al., 1987; *Antp*, McGinnis et al., 1984b, Scott and Weiner, 1984; *lab*, Mlodzik et al., 1988; *ems*, Dalton et al., 1989; and, *BarH1*, Higashijima et al., 1992a.

sophageal ganglia). The number of bsh expressing cells continues to increase during this period (Fig. 7G-I), and by the beginning of stage 15, the brain is almost completely formed and the bsh cells are arranged in their final pattern (Fig. 7J-L).

In stage 16 embryos (Fig. 7M-O) morphogenesis is essentially complete. Approximately 30 bsh-expressing cells can be counted in each brain hemisphere. These cells are clustered in four separate regions of the brain. The cells that were originally in the anteriormost cluster in stages 11-13 become situated in the dorsal brain (Fig. 7M,N) while the posterior-most cluster becomes situated in the ventrolateral posterior of the brain (Fig. 7M,O). A third small cluster of cells can be seen in the center of each brain hemisphere and a single bsh expressing cell can be found isolated at the ventral most portion of the brain (arrow in Fig. 7J,M).

We cannot say with certainty what range of cell types express bsh protein. Some of the cells expressing bsh protein also express the antigen for the monoclonal antibody mAb22C10 (Fujita et al., 1982; Zipursky et al., 1984) which stains a subset of neurons in the central nervous system. In addition, in serial plastic sections of anti-bsh-stained embryos we have observed processes leaving the cell bodies of a number of bsh expressing cells (data not shown). While it is likely that some of the bsh expressing cells are neurons, it cannot be ruled out that all are glia or other non-neuronal support cells whose numbers and types have not been well explored in the brain of the *Drosophila* embryo (see Jacobs et al., 1989).

A bsh-expressing cell is closely associated with the terminus of Bolwig's nerve

In an attempt to understand more about the organization of bsh-expressing cells we performed double staining experiments on embryos with anti-bsh antiserum and a variety of neuronal markers. The neuron-specific mAb22C10 stains the cytoplasm of all neurons in the peripheral nervous system (PNS) as well as a small number in the central nervous system (CNS). Double staining with mAb22C10 and anti-bsh antibodies allows one to visualize the relationship of bsh-expressing cells with axons that innervate the brain from the PNS.

One of the most prominent PNS structures that innervate the brain is the nerve bundle of the larval photoreceptor organs (Bolwig's organs). A pair of Bolwig's organs can be found in the cephalopharyngeal region of the embryo; bundles of 12 axons and surrounding glial elements leave each organ and make synaptic contacts with the a subset of cells in the prospective optic lobes of each brain hemisphere (Steller et al., 1987). This axon bundle, known as Bolwig's nerve, appears to contact a bsh-expressing cell (Fig. 8). Fig. 8A shows a stage 16 embryo stained with bsh antiserum; a solitary bsh cell on the ventral most portion of the brain hemisphere lies near the anlagen of the optic lobes. When double stained with mAb22C10 and anti-bsh antiserum both Bolwig's nerve and bsh-expressing cells can be visualized (Fig. 8B,C). Bolwig's nerve traverses the ventral surface of the brain hemisphere and appears to terminate at the ventral bsh-expressing cell (Fig. 8C).

Deletions of the *bsh* gene cause no obvious morphological defect

As described above, the *bsh* gene is deleted by a number deficiencies that break in the 38A, chromosomal region (Fig. 2A). The small deficiency *Df(2L)OD16*, which deletes the large band at the beginning of 38A, is known to contain at least four previously isolated lethal complementation groups. Complementation analysis combined with the

Fig. 7. Expression of bsh protein during embryogenesis. bsh protein was detected by immunoperoxidase staining; the cephalic regions of stage 11 through stage 16 embryos are depicted, with anterior to the left. (A,B) Lateral and dorsal views, respectively, of a stage 11 embryo just before the beginning of germ band retraction. Note staining in two widely separated nuclei on both sides of the procephalic lobe. (C,D) Lateral and dorsal views of a stage 12 embryo during germ retraction. The number of bsh staining cells increases in two bilateral clusters. (E,F) Lateral and dorsal views of a stage 13 embryo, after germ band retraction is complete. (G,H,I) Lateral view and two dorsal views, of a stage 14 embryo during head involution. The two dorsal views are in different focal planes with H being more superficial than I. Notice that the two halves of the procephalic lobe begin to round up to form the two brain lobes. The number of bsh expressing cells continues to increase. (J,K,L) Lateral view and two dorsal views in different focal plains (K is more superficial than L) of a stage 15 embryo. The brain lobes are almost completely formed and bsh cells are in their final pattern. (M,N,O) Lateral view and two dorsal views (N is more superficial than O) of a stage 16 embryo. The arrow in J and M indicates a bsh expressing cell which contacts the Bolwig's nerve (see Fig. 8).

in situ hybridization data (described earlier) enabled us to order the position of these lethals with respect to deficiencies that break in *Df(2L)OD16* and the location of the *bsh* gene. Fig. 2B shows the results of the complementation analysis. The *bsh* gene maps to the region between the distal breakpoints of *Df(2L)pr65* and *Df(2L)TW161*. This precludes the possibility that the previously isolated lethal mutations map in *bsh*. In further support of this conclusion, we have detected normal bsh protein expression in embryos of each one of these lethal lines.

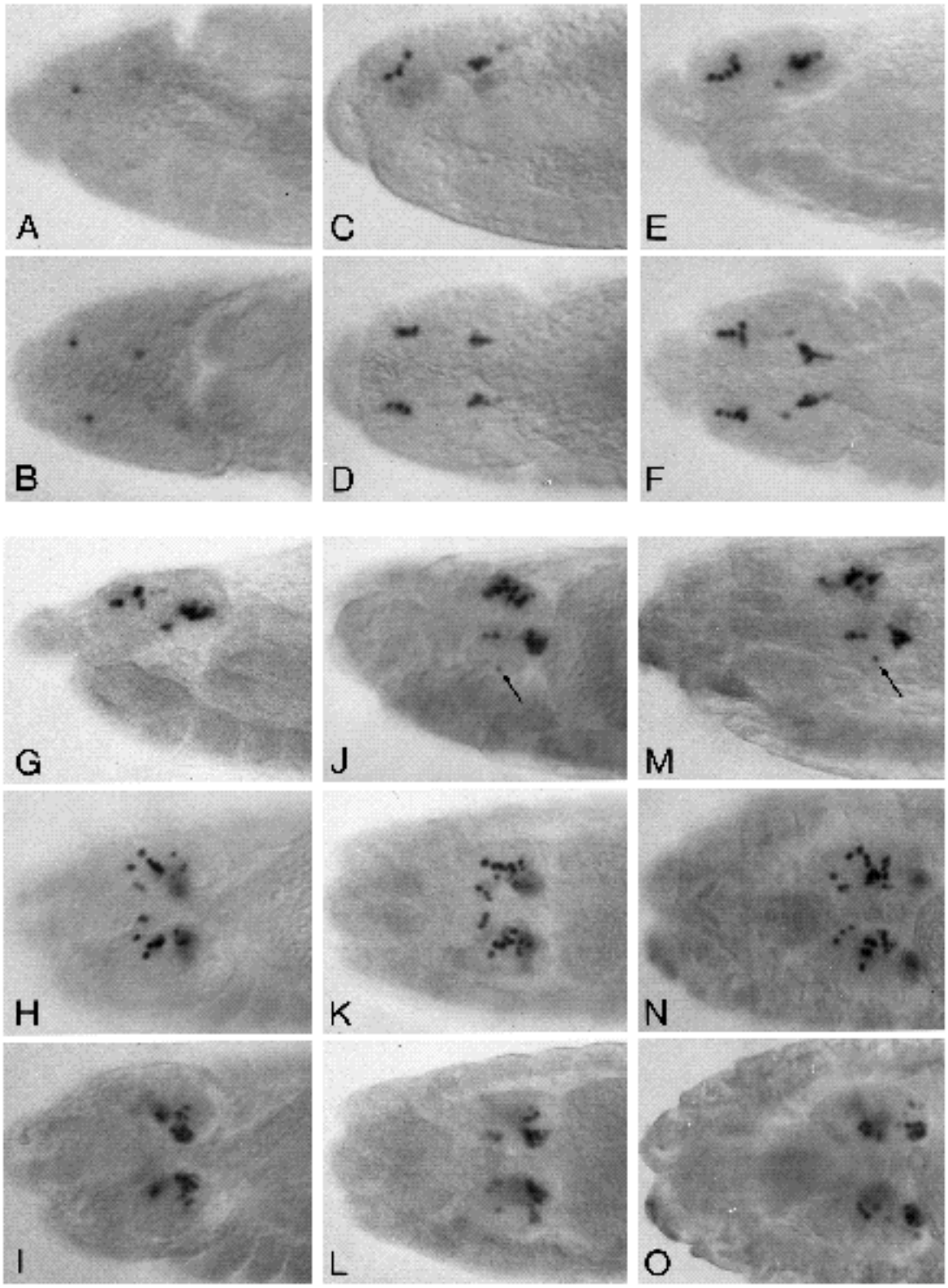


Fig. 7

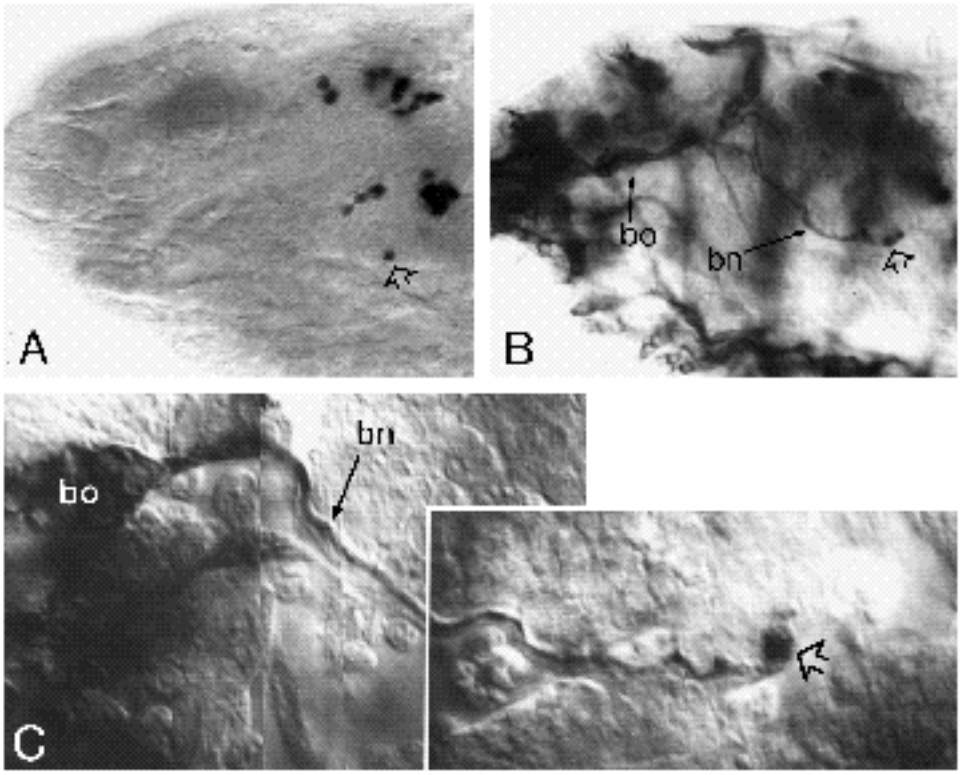


Fig. 8. A bsh expressing cell is closely associated with the terminus of Bolwig's nerve. (A) Lateral view of the cephalic region of a stage 16 embryo stained with anti-bsh antiserum. The open arrow points to a solitary bsh-expressing cell in the ventral-most region of the brain hemisphere. (B) Lateral view of a stage 16 embryo double-stained with anti-bsh antiserum and mAb22C10. mAb22C10 stains all peripheral neurons and a subset of central neurons; the larval photoreceptors or Bolwig's organ (bo) can be visualized. The axon bundle leaving Bolwig's organ called Bolwig's nerve (bn) can be seen traversing the ventral surface of the brain hemisphere and appears to terminate at the bsh expressing cell (open arrow). (C) A high resolution composite image of an embryo similar to the one in B, taken with computer enhanced digital microscopy at higher magnification. Bolwig's nerve (bn) appears to contact the bsh expressing cell (open arrow). Anterior is to the left; dorsal is up.

Animals trans-heterozygous for *Df(2L)OD16* and *Df(2L)pr65* are deleted for a small interval that contains the *bsh* gene (see Fig. 2), and can be identified by their lack of staining with anti-bsh antiserum. These deletion mutants die soon after emerging as 1st instar larvae. Whether loss of bsh function contributes to the lethal phenotype is as yet unknown, as other genes besides *bsh* may be eliminated in the transheterozygotes.

We have used a variety of nervous system markers to determine if the structure of the brain is grossly altered in embryos carrying deletions of *bsh. Df(2L)OD16/ Df(2L)pr65* trans-heterozygotes (hereafter called '*bsh*-interval deletion embryos') were double stained with antibodies that visualize many aspects of the brain (see Materials and methods for probes used) and anti-bsh antiserum which allowed us to identify embryos lacking bsh protein. We also performed serial plastic sections through the brains of both wild type and *bsh*-interval deletion embryos. In *bsh*-interval deletion embryos the overall structure of the brain appears to be normal at this crude level of analysis; stage 16 embryos have no obvious alterations in the size and morphology of the brain hemispheres, and the size and shapes of the commissures also appear normal.

On a superficial level it appears that *bsh* is not required for the proper pathfinding of Bolwig's nerve. Under the light microscope Bolwig's nerve appears normal in *bsh*-interval deletion embryos. As shown by Steller et al. (1987) Bolwig's nerve sends out axons to the developing ventral brain at stage 12, which is prior to the appearance of bsh expression in the same region. In light of these observations we wondered if innervation of Bolwig's nerve is required for the activation of *bsh* expression in the ventral brain. To test this hypothesis we looked at bsh protein expression in *glass*^{2} mutants in which the development of Bolwig's organ does not occur (Moses et al., 1989). In *glass*^{2} mutants, despite the absence of Bolwig's nerve, *bsh* expression is normal. Thus contact from Bolwig's nerve is not required for the activation of *bsh* protein expression in the ventral cell that is associated with the terminus of the photoreceptor nerve.

Transient ectopic expression of bsh protein has no detectable phenotype

To assess the phenotypic consequences of transient ectopic *bsh* expression throughout the nervous system we constructed transgenic fly lines which carry a *bsh* cDNA (cE86C) fused to the *hsp70* promoter (Fig. 9). Six independent lines were generated from a single original insertion, in which abundant levels of bsh protein can be detected throughout the embryo after a one hour heat shock (Fig. 9B). A number of different heat shock regimes were applied to *hsp70-bsh* embryos, including multiple 1 hour heat shocks during the period of nervous system development. Embryos that are treated with multiple heat shocks are for the most part viable and produce healthy adults, showing no significant difference in viability when compared to a control strain (the parental strain *Df(1)w, yw*$^{67c23(2)}$) treated with the same regime. Embryos that have been treated with up to three 1 hour heat shocks and stained after 15 hours of development (when the nervous system is almost fully

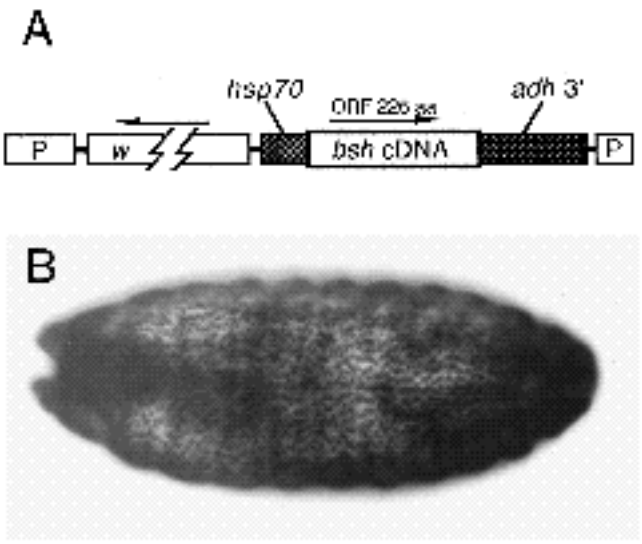


Fig. 9. Ectopic expression of bsh protein from an *hsp70-bsh* fusion gene. (A) Diagram of the *hsp70-bsh* fusion gene P element construct used to make *hsp70-bsh* lines. The *bsh* cDNA cE86C is fused to heat inducible *hsp70* promoter sequences and 3′ untranslated sequences of the *Adh* gene containing polyadenylation signals. P denotes the P element terminal sequences and *w*, the *white* gene used to select for transformants; these sequences are derived from the CaSpeR vector (Pirotta et al., 1988). (B) A ventral view of an *hsp70-bsh* embryo stained with anti-bsh antiserum shortly after a 1 hour heat shock. Note abundant levels of bsh protein detected throughout the embryo.

formed) show normal staining patterns of anti-bsh antiserum and the neural markers mAb22C10, mAbBP102, and anti-HRP (see Materials and methods). 1 hour heat shocks repeated at 12 hour intervals to *hsp70-bsh* animals during larval and pupal development results in viable adults which appear healthy and are capable of flight. However, they have not been rigorously tested for other behavioral abnormalities. While we have not yet detected any phenotypes produced by ectopic expression of bsh protein in *hsp70-bsh* lines, it should be pointed out that we have no evidence that a functional bsh protein is produced by our construct.

DISCUSSION

In this paper we have presented a molecular analysis of a new *Drosophila* homeobox gene, determined its pattern of expression in the embryo, and initiated a genetic analysis of its function. This homeobox gene, called *bsh*, is the first described to be expressed exclusively in the embryonic brain (the supraesophageal ganglion). bsh protein is found in a very restricted pattern, expressed in approximately 30 cells in each brain hemisphere. This expression pattern suggests that *bsh* may play a highly specialized role in the differentiation and function of neural cell types in this complex part of the nervous system.

The *bsh* homeobox was isolated by low stringency hybridization to the *eve* homeobox (Dalton et al., 1989) despite the fact that the genes share only 53% amino-acid identity over the homeodomain region. The ability to pick up *bsh* using the *eve* probe may be due to the highly conserved third helix codons which results in a 24 nucleotide stretch of perfect sequence identity (Macdonald et al., 1986; Frasch et al., 1987). While no highly conserved relatives have yet been discovered in *Drosophila* or other organisms, bsh shares the most similarity to Antennapedia-class homeodomains in the third helix region, the so called 'recognition helix' where the protein interacts with the major groove of DNA (Treisman et al., 1989; Kissinger et al., 1990). The Bar class of homeodomains (Higashijima et al., 1992a) are most similar to bsh, sharing 63% amino-acid identity, although they differ in the most highly conserved part of the third helix by a single amino acid substitution. With a predicted size of 226 amino-acids, bsh is a small protein in comparison to other *Drosophila* homeoproteins (Tomlinson et al., 1988).

It is at present difficult to propose a model for the regulatory mechanisms that would activate *bsh* in such a discrete set of cells in the brain. Little is known about the genetic mechanisms that control the development of the procephalic region. One attractive hypothesis that we explored was that *bsh* might be activated in discrete cells by inductive events caused by the innervation of the brain from the peripheral nervous system. This model has been suggested to function in the development of the optic ganglia in the imaginal visual system in which photoreceptor cell innervation into the optic lobes has been proposed to induce the differentiation of ganglion mother cells into neurons (Meinhertzhagen, 1975; Meyerowitz and Kankel, 1978; Kankel et al., 1980; Fishbach and Technau, 1984; Selleck and Steller, 1991). The observation that a *bsh* expressing cell is closely associated with the terminus of the larval photoreceptor nerve (Bolwig's nerve) led us to test whether the activation of *bsh* in that cell represented an analogous, or indeed homologous, innervation-induced differentiation event in the embryonic brain. We have found that *bsh* activation is normal in the absence of innervation from Bolwig's nerve, showing that *bsh* expression is not under the control of photoreceptor axon induced activation.

In order to gain some insight into the function of the bsh protein in the brain, we have examined the brains of embryos in which the *bsh* gene is genetically deleted, and in which *bsh* has been transiently ectopically activated. The possibility that *bsh* expression may be required for the proper targeting of the axons of the Bolwig's nerve was explored. In *bsh*-interval deletion embryos, Bolwig's nerve appears normal and thus at the gross level does not seem to require *bsh* expression. We have so far detected no morphological defects associated with the deletion of the *bsh* gene. Nor does the transient ectopic expression of the bsh protein throughout the embryo have an obvious morphological effect.

Given what we know about the function of other homeobox genes in the nervous system, it is plausible that *bsh*-interval deletion embryos have defects too subtle to detect without appropriate markers. In *Drosophila* the function of homeobox genes in the CNS has been best explored in the cases of *ftz* and *eve* (Doe et al., 1988a,b). In embryos mutant for *ftz* nervous system expression, for seven different neuronal types examined that normally express *ftz*, only one type of neuron had altered neuronal morphology. The altered

RP2 neurons changed their axonal pathways as if they had assumed the identity of sibling RP1 neurons. In the absence of *eve* function in the CNS similar transformations were observed, resulting in aberrant axonal morphologies in the aCC neurons and the RP2 neurons. Both the *cut* and the *Bar* genes in *Drosophila* and the *mec-3* and *unc-86* genes in *C. elegans* have been shown to be involved in similar differentiation decisions in which one neural cell type is changed into another (Bodmer et al., 1987; Blochlinger et al., 1991; Higashijima et al., 1992a,b; Way and Chalfie, 1988; Finney et al., 1988). If *bsh* functions in a similar way, the transformation of some *bsh* cells into other cell types in *bsh* mutants may have little effect on the overall morphology and structure of the brain that would allow us to detect such change without appropriate markers.

Another possibility is that *bsh* is not required for the actual morphology of brain cells, the axonal pathways, etc., but instead regulates expression of neurotransmitters, receptors, channels, synaptic specializations, or other effector molecules that are critical to the physiologic function of particular neural cells. Such functions would be missed in our analysis. Finally, it is possible that *bsh* represents a redundant or an obsolete gene. The continuing searches for enhancer trap lines, mAbs and other probes with specific patterns of expression in the nervous system, and the identification of neural effector genes whose patterns overlap *bsh* expression, will generate the markers enabling the elucidation of *bsh* genetic functions.

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