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Control of *Drosophila* tracheal branching by the novel homeodomain gene *unplugged*, a regulatory target for genes of the bithorax complex

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SUMMARY

We have identified a novel *Drosophila* homeodomain gene, *unplugged (unp)*, whose function is required for formation of the tracheal branches that penetrate the CNS. In *unp* mutant embryos the segmentally repeated ganglionic branches stall and fail to penetrate the CNS and the segment-specific cerebral branch and associated cerebral anastomosis fail to form. Expression of *unp* in the founder cells for the cerebral branch within the first tracheal metamere is repressed in posterior segments by *Ubx* and other bithorax complex genes. This pattern of expression and homeotic gene regulation is reproduced by an unusual 2.7 kb *cis*-regulatory sequence located downstream of the *unp* transcription unit. Since the *unp* protein is localized to the nucleus of tracheal precursor cells as they migrate and extend, *unp* protein appears to play a regulatory role in neural branching of the tracheae, and the segment-specific aspects of these neural branching patterns appear to be generated by homeotic regulation of *unp* expression.

Key words: *Drosophila* development, homeotic, homeodomain, gene regulation, tracheal development

INTRODUCTION

A key turning point in the early understanding of *Drosophila* homeotic gene function was the shift from analysis of partial loss-of-function mutations in adults to analysis of null mutations associated with embryonic or larval lethality. In the first major description of such lethal homeotic phenotypes, Lewis (1978) relied heavily upon branching patterns within the tracheal system as segment-specific landmarks that allowed him to formulate an insightful model relating the functions of homeotic genes to their evolution and chromosomal organization. Much has been learned in the interim about homeotic selector (HOM) gene function. HOM genes thus have been shown to contain the homeodomain, a conserved 60 residue motif that mediates sequence-specific DNA binding, and HOM genes in *Drosophila* and in other animals are thought to specify the characteristics of spatial units along the anteroposterior axis via the transcriptional regulation of target genes (for review see McGinnis and Krumlauf, 1992). Despite these advances and despite the identification of several potential HOM gene targets in *Drosophila*, however, no downstream targets have been described that are specifically involved in the segment-specific branching of the tracheal system.

Although many genes are known to be expressed within the developing tracheal system (reviewed by Manning and Krasnow, 1993), most of these genes are also expressed in a variety of other tissues, thus complicating the analysis of their function and regulation. Two genes whose functions appear to be specifically concerned with tracheal development are *breathless* (*btl*) and *pointed* (*pnt*). The *pnt* gene encodes an *ets* domain-containing transcription factor and is expressed in the tracheal placodes and in the developing tracheal branches (Klämbt, 1993). The *btl* gene encodes the *Drosophila* homologue of the FGF receptor and also is expressed in tracheal precursors during invagination and branching. Although mutations in both of these genes block migration and branching of the tracheal precursors, these effects appear to be general and are difficult to relate to the segment-specific aspects of tracheal morphogenesis that are presumed to be under homeotic gene control.

We present here the identification and molecular and genetic characterization of *unplugged* (*unp*), a gene required for formation of specific tracheal branches including some segment-specific branches. The *unp* locus was identified on the basis of the segment-specific expression of β-galactosidase from a *lacZ* enhancer detector P element. The *unp* gene encodes a novel homeodomain protein required for the development of the segmentally reiterated ganglionic branches, which fail to penetrate the CNS in the absence of *unp* function. In addition, *unp* function is specifically required for development of the cerebral branch, a tracheal branch uniquely derived from the first thoracic segment. Our study suggests that *Ultrabithorax* (*Ubx*) and other homeotic genes restrict the formation of the cerebral branch to the first thoracic segment by repressing the expression of *unp* in more posterior segments; in addition we have identified an unusual 2.7 kb enhancer region downstream of the *unp* gene that reproduces *unp* gene expression, including homeotic gene regulation.

MATERIALS AND METHODS

Fly stocks

The fly lines 1912, f85 and E22 were kindly provided by C. Doe, A. Spradling (Karpen and Spradling, 1992) and C. Goodman respectively. The *Ubx*^{922} and *Df(Ubx*^{109}*)* stocks were originally from E. Lewis (Lewis, 1978), the triple mutant stock (*Ubx*^{MX12} *abd-A*^{M1} *Abd-B*^{M8}) was obtained from M. Bienz (Casanova et al., 1987; Bienz and Tremml, 1988), and the *pnt*^{9J} allele was obtained from the Indiana Stock Center.

Isolation of *unp* genomic sequences and cDNAs

Genomic DNA flanking the 5′ end of the P element in *f85* line was recovered by plasmid rescue (Mlodzik and Hiromi, 1992). The resulting DNA fragment was used to isolate two overlapping genomic clones carrying 28 kb of contiguous DNA. Various restriction fragments of the genomic DNA were digoxigenin-labeled and hybridized to whole embryos. The 5 kb *Eco*RI fragment (–2.5 to –7.5 in Fig. 1) gave an RNA expression pattern similar to that of β-galactosidase activity. This fragment was subsequently used to screen a cDNA library from 4- to 8-hour embryos (Brown and Kafatos, 1988). Eight positive clones were recovered from screening about 10^6 colonies. The largest of these (p*unp*-1), 1.7 kb in size, was chosen for sequence determination on both strands by the Sanger dideoxy method (Sanger et al., 1977) using Sequenase v2.0 (US Biochemicals).

Mapping and mutagenesis of the *unp* gene

Hybridization to polytene chromosome squashes was as described by Langer-Safer et al. (1982) using the alkaline phosphatase-based DNA detection system (GIBCO/BRL). A probe was generated from 1.7 kb *unp* cDNA using the random hexamer priming kit (Boehringer Mannheim) in the presence of biotinylated dUTP (ENZO Biochemicals). The *unp* sequences hybridized near polytene division 45C.

The precise locations of the three P element lines were determined by isolation of sequences flanking the f85, E22 and 1912 inserts using inverse PCR (Ochman et al., 1990). Briefly, two primers, GTATACTTCGGTAAGCTTCGGCTAT and CGAAATGCGTCGTTTAGAGCAGCAG, 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. 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 1912 was mobilized by exposure to transposase from the P[Δ*2-3 ry*$^+$] *Sb* chromosome. Excision events were identified by loss of the *w*$^+$ marker present in the 1912 line. Twelve homozygous lethal lines were recovered from approximately 230 excision event; eleven excisions contain DNA lesions associated with large deletion beginning in the element and extending to the left or right as represented by the alleles *unp*^{r1} and *unp*^{r221} respectively in Fig. 1. One allele, *unp*^{r37}, deleted about a 1.5 kb of sequence that extends from the 5′ end of the P element to a region just beyond the *Spe*I restriction site in the third exon. The extent of the deletions were inferred by Southern hybridization to DNA digested with restriction endonucleases from heterozygous *unp* individuals using various *unp* cDNA and genomic probes flanking the P element.

Analysis of *unp* RNA expression

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

Production of *unp*-specific antiserum

The *unp*-specific antibody was generated by immunization of mice with the recombinant protein containing a glutathione-S-transferase fusion to residues 26-476 of the *unp* protein. The induction and purification of the glutathione-S-transferase fusion protein were carried out according to the instructions provided by the pGex vector supplier (Amrad, Melbourne, Australia).

P element-mediated transformation

Genomic fragments (see Fig. 1) were cloned into the pCasperAUG-β-gal vector (Thummel et al. 1988) and injected into w^{1118} embryos as described (Rubin and Spradling, 1982).

In situ hybridization and immunohistochemistry

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).

Embryos and larvae were fixed and immunostained as described (Patel, 1994), using a 1:300 dilution of secondary antibody conjugated to horseradish peroxidase (Jackson Laboratory). The signal for the antibody reaction was intensified with 0.03% $NiCl_2$ except for double staining, where $NiCl_2$ was omitted in the second reaction. Primary antibodies were used at the following dilution: mouse anti-β-galactosidase (Promega), 1:1000; anti-engrailed mAb 4D9, 1:3; mouse anti-unp (preabsorbed with embryos), 1:400. The stained embryos were cleared in 50% glycerol followed by 70% glycerol. In some cases, the embryos were flattened prior to photomicroscopy by dissection on a clean slide using a tungsten needle. Photography was with a Zeiss Axiophot microscope.

RESULTS

Isolation and organization of the *unplugged* gene

We identified the *unplugged* (*unp*) gene by systematic screening of P element enhancer detector lines for β-galactosidase expression patterns suggestive of regulation by the homeotic gene *Ultrabithorax* (*Ubx*). Line f85, which carries an insertion in the *unp* locus, was found to have β-galactosidase expression in a pattern restricted to the lateral ectoderm of the first thoracic segment, suggesting that *Ubx* may negatively regulate expression in more posterior segments. The f85 line also displayed a segmentally repeated pattern of expression in the neuroblasts and the neurons (see below).

To initiate the molecular characterization of *unp*, DNA flanking the 5′ end of the P element was recovered by plasmid rescue from the f85 line, and subsequently was used to isolate genomic DNA from a phage library (see Materials and Methods). The presence of the *unp* transcription unit in the isolated genomic DNA was confirmed by RNA in situ hybridization using various digoxigenin-labeled restriction fragments (Fig. 1). The 5 kb *Eco*RI fragment (Fig. 1; –2.5 to –7.5) that contains part of the *unp* transcription unit was used to isolate six cDNAs 1.7 kb in size, in reasonable agreement with a 1.8 transcript size as determined by northern blot hybridization (see below). Direct comparison between the *unp* cDNA and genomic sequences revealed that the transcription unit of *unp* is organized in 3 exons and separated by two introns with sizes 3.5 kb and 62 bp respectively (Fig. 1). Sequence analysis of the first intron of *unp* also revealed that it contains a transposable element; this 1360 element is moderately repetitive and has copy numbers ranging from 25 to 30 within the *Drosophila* genome (Kholodilov et al., 1988). Whether presence of the 1360 element in the *unp* locus has any functional significance is unknown.

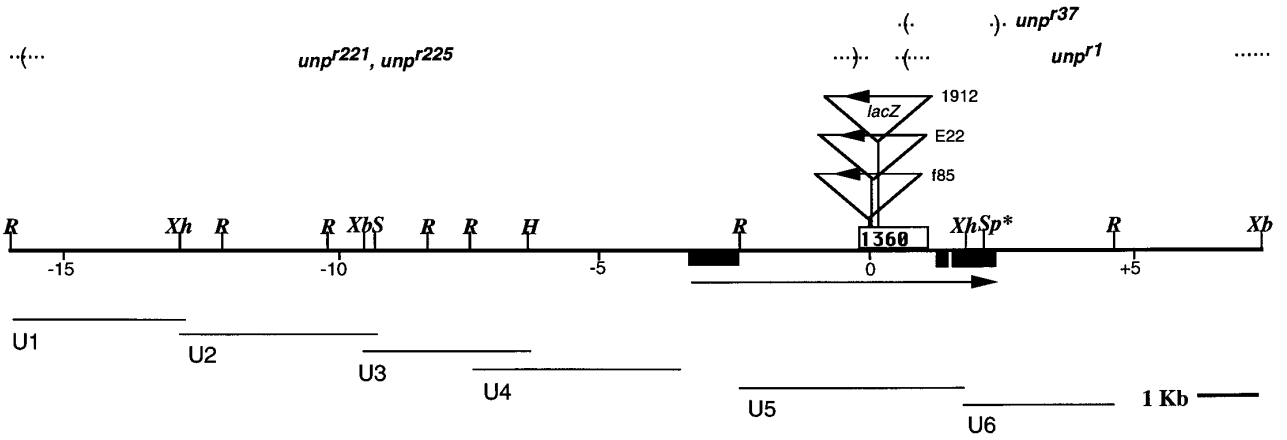

Fig. 1. Genomic map of the *unplugged* locus. Genomic DNA at the *unplugged* (*unp*) locus is represented by the thick line with the 0 coordinate (in kilobases) corresponding to the site of insertion for the P element f85. The insertion sites of the two other P elements, E22 and 1912, are also indicated. Only one relevant *Spe*I restriction site is shown (asterisk). The exons of *unp* are represented by the filled boxes, with the direction of transcription indicated by an arrow. The location of the 1360 element is indicated by an open box. The extent of DNA lesions for the four deletion mutations, which were obtained from imprecise excision of the 1912 P element, are represented by parentheses with the uncertainty of the boundaries indicated by the dotted lines. The six thin lines below the *unp* genomic map represent the DNA fragments used in reporter constructs to define enhancer region of the *unp* gene (see text). Abbreviations: H, *Hin*dIII; R, *Eco*RI; S, *Sac*I; Sp, *Spe*I; Xb, *Xba*I; Xh, *Xho*I.

The *unp* cDNA also permitted us to localize the *unp* gene to polytene chromosome 45C by in situ hybridization (data not shown). We have since obtained two additional enhancer detector lines with similar polytene localizations, 1912 and E22, and have mapped the P element insertion sites of all three lines by sequence analysis of rescued genomic DNA (see Materials and Methods). Interestingly, all three P elements are inserted within the 1360 element at intervals of 5 bp and 52 bp, respectively, between F85, E22 and 1912 (Fig. 1).

unp encodes a novel homeobox protein

The longest methionine-initiated open reading frame (ORF) within the *unp* cDNA extends from positions 126 to 1586 and has the capacity to encode a protein of 486 amino acid residues (Fig. 2). The sequence surrounding the first methionine in the ORF is a reasonable match with the *Drosophila* translational initiation consensus sequence C/A-A-A-A/C (Cavener and Ray, 1991), and this methionine codon is preceded by termination codons in all three frames. A stretch of 24 adenines at the 3′ end of the cDNA is preceded by a perfect match to the consensus polyadenylation signal AAUAAA (Proudfoot, 1991).

A protein data base search revealed that the *unp* ORF contains a homeodomain (residues 319-378; see Fig. 2) belonging to a family that includes several vertebrate homeobox genes (Fig. 3). The *unp* homeodomain shares amino acid identities ranging between 90 and 93% with homeodomains of the *CHox7* gene in chicken (Fainsod and Greunbaum, 1989), the *HOX7Q* and *GBX2* genes in human (Matsui et al., 1993), partial sequence of the *MMoxA* gene in mouse (Murtha et al., 1991), the *XlHox7a* and *XlHox7b* gene in *Xenopus* (King and Moore, 1994), the *G9* gene in goldfish (Levine and Schechter, 1993), and the *Hrox7* gene in abalone (Degnan and Morse, 1993). Little is known about the expression and function of these vertebrate homologs; however, the *MMoxA* and *G9* genes were initially isolated from brain libraries and thus may be involved in brain development or function.

One of two introns in the *unp* transcription unit interrupts homeodomain coding sequences at a location first noted in *labial*-class homeobox genes (Fig. 2; Mlodzik et al., 1988; Diederich et al., 1989). This location, between Gln 44 and Val 45, is conserved for introns of many other homeodomain genes of different species (Fig. 3), but is distinct from the location of the intron that interrupts homeodomain coding sequences in *engrailed* and related genes.

Outside of the homeodomain, *unp* shares no significant homology with other known proteins in the data base. However, Pro/Gln-rich regions are found amino terminal to the homeodomain, with one particular region from residues 111 to 142 comprising 52% proline and glutamine residues (Fig. 2). Pro/Gln-rich regions are found in many proteins that are capable of transcriptional activation (for review see Mitchell and Tjian, 1989; Gerber et al., 1994).

Embryonic expression of *unp*

Northern hybridization using *unp* cDNA as a probe detected a 1.8 kb transcript size that correlates reasonably well with the size of the cDNA (Fig. 4). The *unp* transcript is first detectable in 4- to 8-hour embryos and expression is maintained throughout embryogenesis. Following a transient decrease in transcript levels during larval periods, levels increase during the pupal and adult stages. In addition to the expected transcript, a 2.8 kb band is also observed at the third instar larval stage. We cannot distinguish between the possibilities that this novel band represents a product of alternative splicing or a cross-hybridizing transcript.

To learn more about spatial expression of *unp*, a digoxigenin-labeled cDNA probe was used to perform whole-mount in situ hybridization. The *unp* expression pattern was also analyzed with transgenic embryos carrying *unp* enhancer/reporter constructs (see below) and by immunolocal-

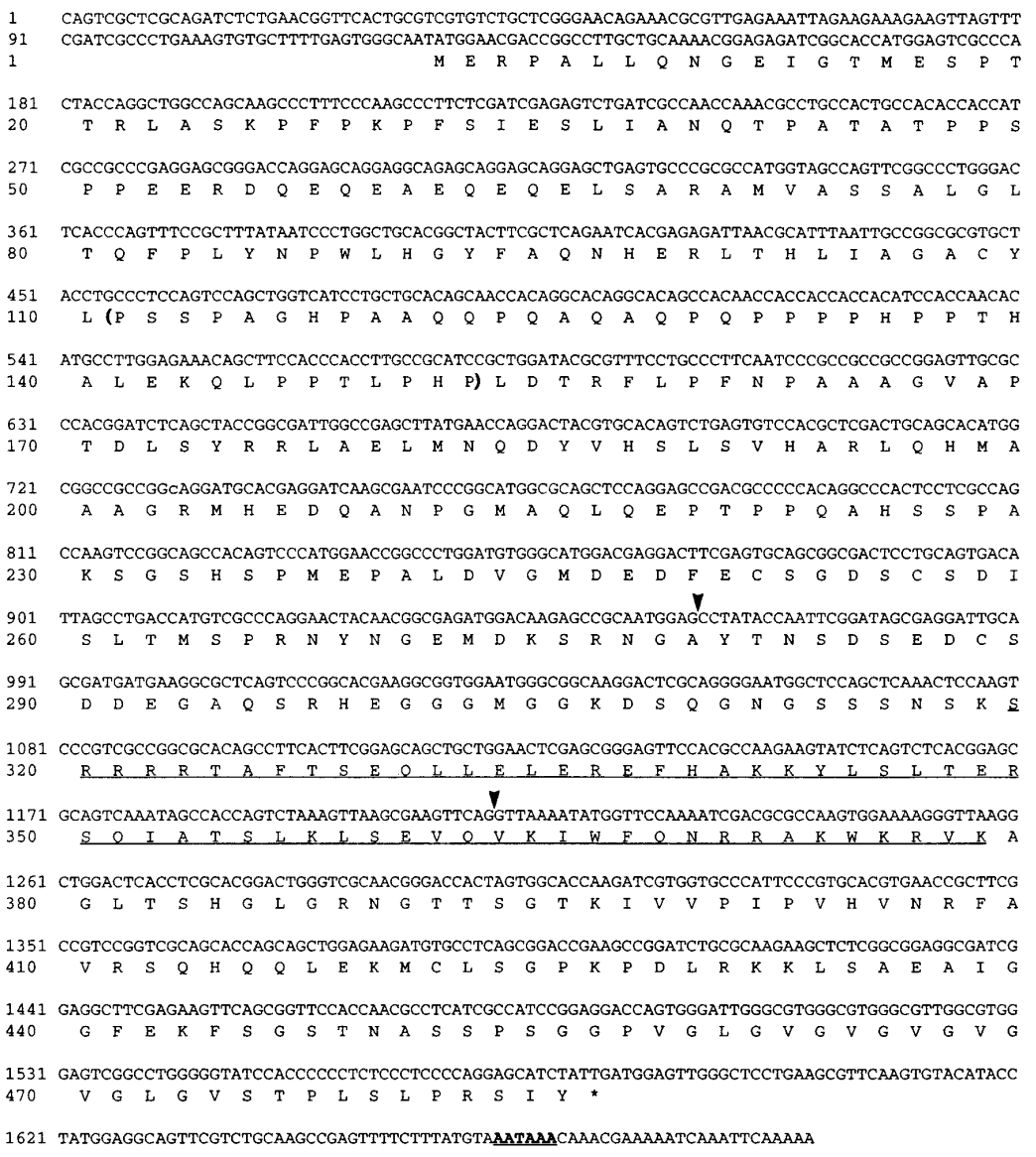

Fig. 2. Sequence of the *unp* gene and its deduced protein product. The *unp* transcription unit includes two introns at the locations indicated by arrowheads within the open reading frame. The glutamine- and proline-rich region at the amino terminus is indicated by the parentheses. Homeodomain residues near the carboxy terminal half of the protein are underlined. The consensus polyadenylation signal is represented by the bold letters.

The accession number for *unp* sequence is U35427.

ization using unp-specific antiserum. All three expression patterns gave indistinguishable results, although expression in the CNS is relatively weaker in the transgenic embryos.

unp expression first appears at stage 8 (3-3.5 hours of development; Campos-Ortega and Hartenstein, 1985) in the midline of the central nervous system (CNS), at a location corresponding to a subset of neuroblasts (Fig. 5A). These neuroblasts divide during germband extension to generate sibling neuroblasts and neurons that largely correspond to *engrailed*-expressing cells within the CNS (Fig. 5B-F; Doe 1992). As the germband retracts, midline CNS expression begins to fade (Fig. 5I and J), and by stage 14 the CNS expression is restricted to a few cells in each segment (Fig. 5M-O).

Outside the CNS, *unp* expression is first observed in two clusters of ectodermal cells located laterally within the labial and first thoracic (T1) segments of stage 9 embryos (Fig. 5B). During germband extension *unp* expression continues in T1 and rapidly diminishes in the labial segment (Fig. 5C,D). By stage 11, the lateral cells are recognizable as 15-20 *unp*-expressing cells around the anterior part of the first tracheal pit (Fig. 5E,F). As the germband retracts, these cells begin to migrate anterodorsally with expression restricted to 5-6 cells (Fig. 5G,H). By stage 13, the expression is detected in a few cells close to the dorsal midline of the embryos (Fig. 5K,L); these cells appear to form long cytoplasmic connections that prefigure the cerebral branches of the tracheal system (Fig. 5L; see below).

As the germband retracts a new expression domain within the invaginated tracheal pits appears on each side of the CNS in segments T1-A7 (open triangles in Fig. 5G,H). Expression in this domain is restricted to a few cells per hemisegment, which may represent the precursors of the ganglionic branches of the tracheal system (see below). During germband retraction, these precursor cells extend ventrally and dorsally (Fig. 5I-K). By stage 14, the ganglionic branch in each hemisegment consists of 7-9 *unp*-expressing cells whose cell bodies appear to form a continuous chain that penetrates the CNS of stage 14 embryos (Fig. 5M,N). No RNA or protein expression of *unp*

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          |----Helix 1---|    |-Helix 2-|     |-----Helix 3----|
        10          20          30          40          50          60
         |           |           |           |     ↓     |           |
SRRRRTAFTS EQLLELEREF HAKKYLSLTE RSQIATSLKL SEVQVKIWFQ NRRAKWKRVK   unp

---------- -------K-- -C-------- -----HA--- ---------- ----------   CHox7
---------- -------K-- -C-------- -----HA--- ---------- ----------   HUMHOX7Q
N--------- -------K-- -C-------- -----HA--- ---------- ----------   GBX2
                    -- -C-------- -----HA--- ---------               X1Hox7a
                    -- -C-------D G----HA--- ---------               X1Hox7b
                       -C-------- -----HA--- -------                 G9
                        C-------- -----HA--- ------                  MMoxA
                        S-------- -----H---- ----                    Hrox7
RK-G-QTY-R Y-T----K-- -FNR--TRRR -IE--HA-C- T-R-I----- ---M---KEN   Antp


                                             ↓
P--L---Y-N T------K-- -FN---CRPR -IE--A--D- T-R---V--- ---M-H--QT   pb
                                             ↓
P--A-----Y ---VS--NK- KTTR---VC- -LNL-L--S- T-T------- ---T---KQN   NK-1
                                             ↓
V-KK-KPYSK F-T----K-- LFNA-V-KQK -WEL-RNDQ- T-R------- ---M-N-KNS   Abd-B
                                             ↓
NNSG-TN-TN KQ-T---K-- -FNR--TRAR -IE--NTDQ- N-T------- ---M-Q-KRV   lab
                                             ↓
M-KP--IYS- L--QQ-N-RL QRTQ--A-P- -AEL-A--G- TQT------- ---S-Y-KMM   Dll
                                             ↓
RKWN--VFSL M-RRG--KS- QSQ--VAKP- -RKL-DA-S- TDA------- ---M--RQEI   AHox1
                                             ↓
NGTN--N--T H--T---K-- -TAYLVNR-R -TE--SN-D- Q-A------- ---M-E-KRE   ceh-13
                                             ↓
I-KP--IYS- L--QA-N-R- QQTQ--A-P- -AEL-A--G- TQT------- -K-S-F-KLM   Dlx
                    ↓
EK-P---FS- ---AR-K--- NENR--TERR -QQLSSE-G- N-A-I----- -K-A-I-KEN   en
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Fig. 3. The *unp* homeodomain is a member of a divergent homeodomain family. Amino acid sequence of the *unp* homeodomain is aligned with those of the chicken *CHox7* gene (Fainsod and Greunbaum, 1989), the human *HOX7Q* and *GBX2* genes (Matsui et al., 1993), the mouse *MMoxA* (Murtha et al., 1991) and *Dlx* (Price et al., 1991) genes, the *Xenopus XlHox7a* and *XlHox7b* genes (King and Moore, 1994), the goldfish *G9* gene (Levine and Schechter, 1993), the abalone *Hrox7* gene (Degnan and Morse, 1993), the acscidian *Ahox1* gene (Saiga et al., 1991) and the *Drosophila Antp*, *pb*, *NK-1*, *lab*, *Dll*, and *en* genes (Cribbs et al., 1992). A dash indicates residues identical to those in *unp*. Three regions corresponding to the α-helical regions from the crystallographically derived structure of the e*ngrailed* homeodomain are indicated above (Kissinger et al., 1990). The arrows denote the position of introns residing between residues 44 and 45 of the third helix or within the codon for amino acid 17 (*en*) of the first helix. The top alignment includes closely related homeodomains from a variety of species; the bottom alignment includes homeodomains whose coding sequences are interrupted by introns. Note that coding sequences for the *en* homeodomain are interrupted at a different location.

outside the CNS can be detected in later stage embryos (Fig. 5O).

unp expression marks two neural branching patterns of the tracheal system

To determine the tissue types of cells expressing *unp* outside the CNS, we have performed double labeling experiments using unp-specific antiserum and other antibodies that recognize different tissue types in the embryos. A β3-tubulin antibody (Kimble et al., 1990) as well as the 22C10 antibody (Zipursky et al., 1984), which highlight muscle and peripheral nervous system (PNS) cells respectively, fail to co-localize with unp-specific antiserum, indicating that cells expressing *unp* are not muscle or PNS (data not shown).

The elongated morphology of *unp*-expressing cells resembles the morphology of cells in the developing tracheal system. Indeed, double-labelling with unp-specific antiserum and 2A12, a monoclonal antibody that specifically highlights the lumen of the tracheal system, demonstrates that most *unp*-expressing cells outside the CNS also express the 2A12 antigen (Fig. 6). On the ventrolateral side of each hemisegment, the *unp* protein accumulates in the nuclei of 7-9 cells overlapping with the 2A12 antigen in the ganglionic and lateral branches of the tracheal system (Fig. 6A and B). The organization of ganglionic branches differs between thoracic and abdominal segments, and this difference is reflected by the *unp* expression pattern (Fig. 6A).

On the dorsal side of stage 13 embryos, *unp* protein accumulates in 5-6 nuclei overlapping with 2A12 antigen in the cerebral branch of the first tracheal metamere (Fig. 6C; Manning and Krasnow, 1993). By stage 14, the cerebral branch courses posteriorly and medially such that it lies close to the dorsal midline of T2 (Fig. 6D). Thus, *unp* expression outside of

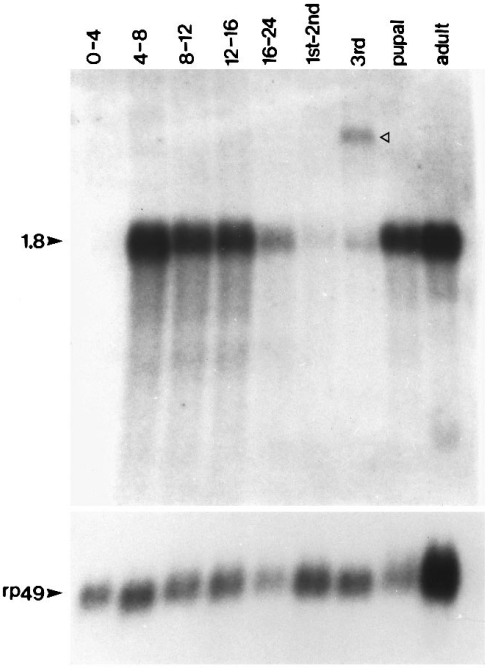

Fig. 4. Temporal profile of the *unp* transcript. Each lane contains 5 μg of poly(A)$^+$ RNA derived from several embryonic stages (stage given in hours after egg-laying), in two larval stages, and in the pupal and adult stages, as indicated. A single 1.8 kb band was detected throughout development except in the 0- to 4-hour stage, with peak expression observed in 4- to 8-hour embryos. Lower-level expression is detected during late embryonic to larval stages. A 2.5 kb band is also detected during third instar larval period (open triangle). The lower panel shows the same blot subsequently hybridized with the *Drosophila rp49* gene.

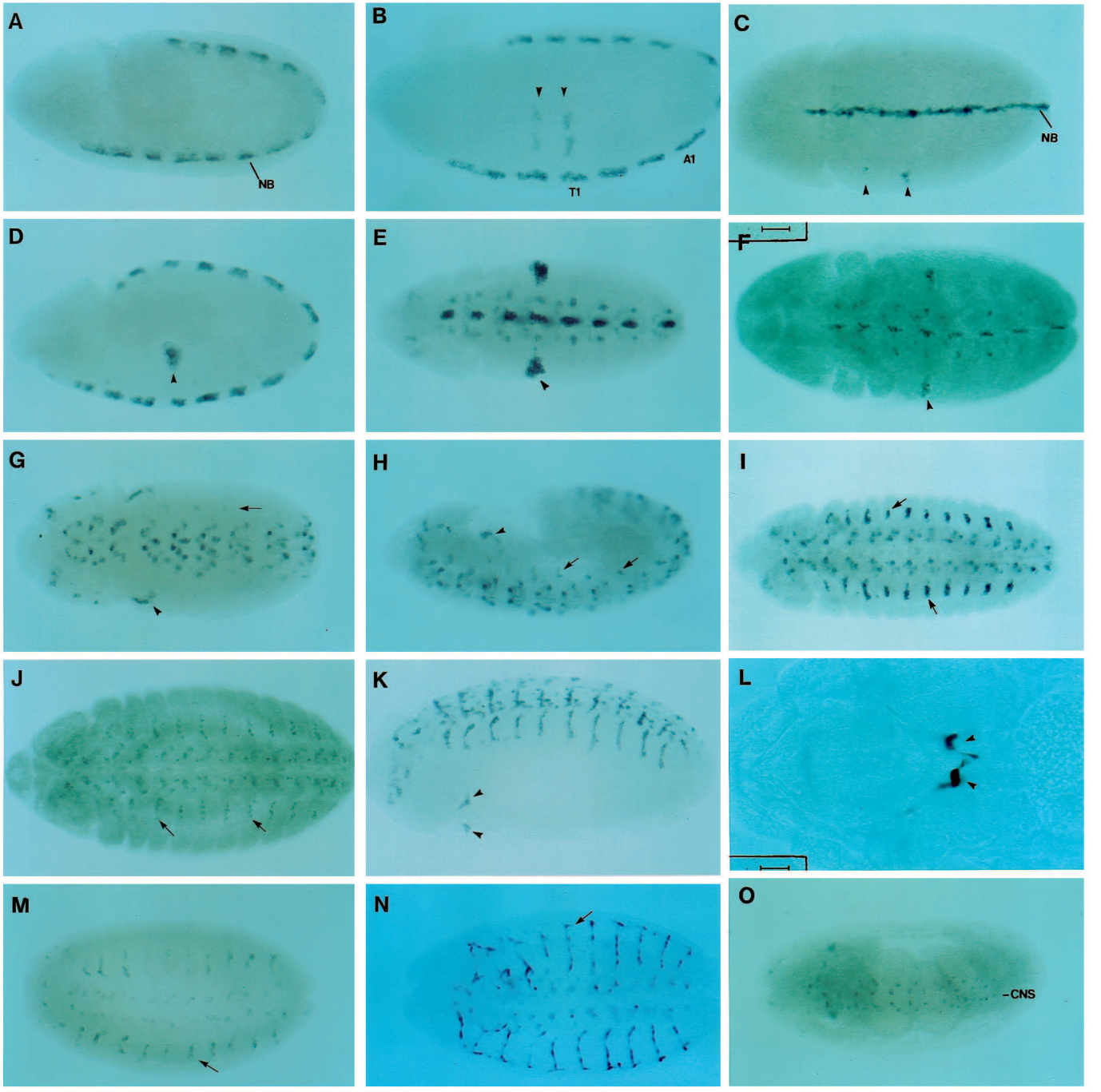

Fig. 5. Embyonic patterns of *unp* gene expression. The localization of *unp* transcripts at various stages was visualized by in situ hybridization to whole embryos using digoxigenin-labeled *unp* cDNA as a probe, except for F, J, L and N, which show embryos immunostained with an antibody against *unp* protein (F,J) or against β-galactosidase (L,N; embryos are carrying the *unpU6-lacZ* reporter construct described in the text). (A) Lateral view of a stage 8 embryo showing the expression of *unp* in midline neuroblasts. (B,C) Lateral and ventral views of a stage 9 embryos. Arrowheads mark the two groups of *unp*-expressing ectodermal cells in the labial and first thoracic segments. (D) Lateral view of a stage 10 embryo. Note the increase in the expression in T1 and a corresponding loss of expression in the labial segment. (E,F) Ventral views of stage 11 embryos showing that the distributions of *unp* RNA (E) and protein (F) are identical. Note that lateral expression at this stage is restricted to cells surrounding the anterior half of the first tracheal pit (arrowhead). (G,H) Ventral and ventrolateral (H) views of stage 12 embryos during germband retraction. The lateral cells in T1 begin to migrate anteriorly and dorsally (arrowheads). A new ventral expression domain that flanks the CNS is indicated by arrows. (I-J) Ventral view of stage 13 embryos showing that the RNA expression pattern (I) is identical to the protein distribution pattern (J). Note the extension of the ventral cells toward the CNS (arrows). (K) Dorsolateral view of a stage 13 embryo illustrating the migration of lateral cells from both sides to the dorsal midline (arrowheads; embryos shown with dorsal side down). (L) Higher magnification view of the dorsal cells originating from both lateral T1 regions (arrowheads). (M,N) Ventral views of stage 14 embryos. The 7-9 ventral cells extending into the CNS are clearly visible. (O) Ventral view of a stage 16 embryo showing persistent expression of *unp* in the CNS. All embryos are oriented with anterior to the left and dorsal up (except K).

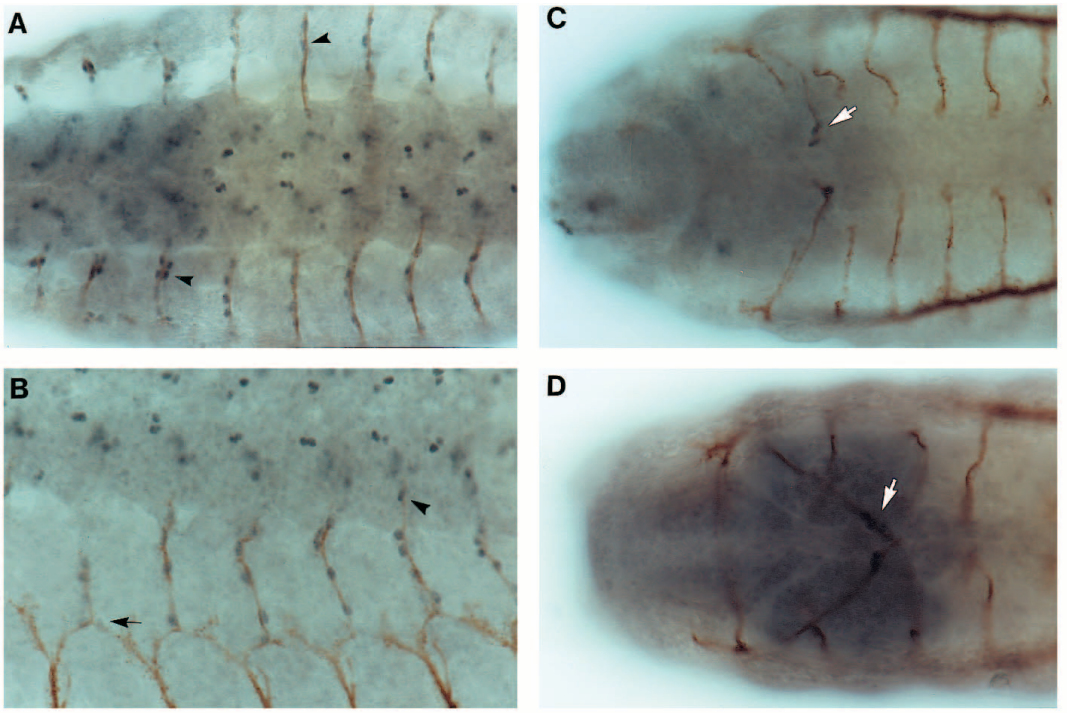

Fig. 6. Expression of *unp* in cells of the developing cerebral and ganglionic tracheal branches. Embryos were double-stained with antibodies against *unp* (dark purple product) and the 2A12 monoclonal antibody that stains the lumen of the tracheae (brown product). (A) Ventral view of a stage 14 embryo. The ventral cells that accumulate *unp* protein (nuclear stain) overlap with the lumen of the ganglionic branches (brown), as indicated by the arrowheads. (B) Higher magnification view of a dissected embryo illustrates the *unp* protein accumulation in cells of the ganglionic branches that extend into the CNS (arrowhead) and in cells of the lateral branches (black arrow). (C) Dorsal view of a stage 13 embryo. The overlapping distribution of the *unp* protein (purple) and 2A12 antigen (brown) in the developing cerebral branch is indicated by a white arrow. (D) Dorsal view of a stage 14 embryo. The *unp* protein accumulates in 4-5 nuclei within the caudal end of the cerebral branch (white arrow).

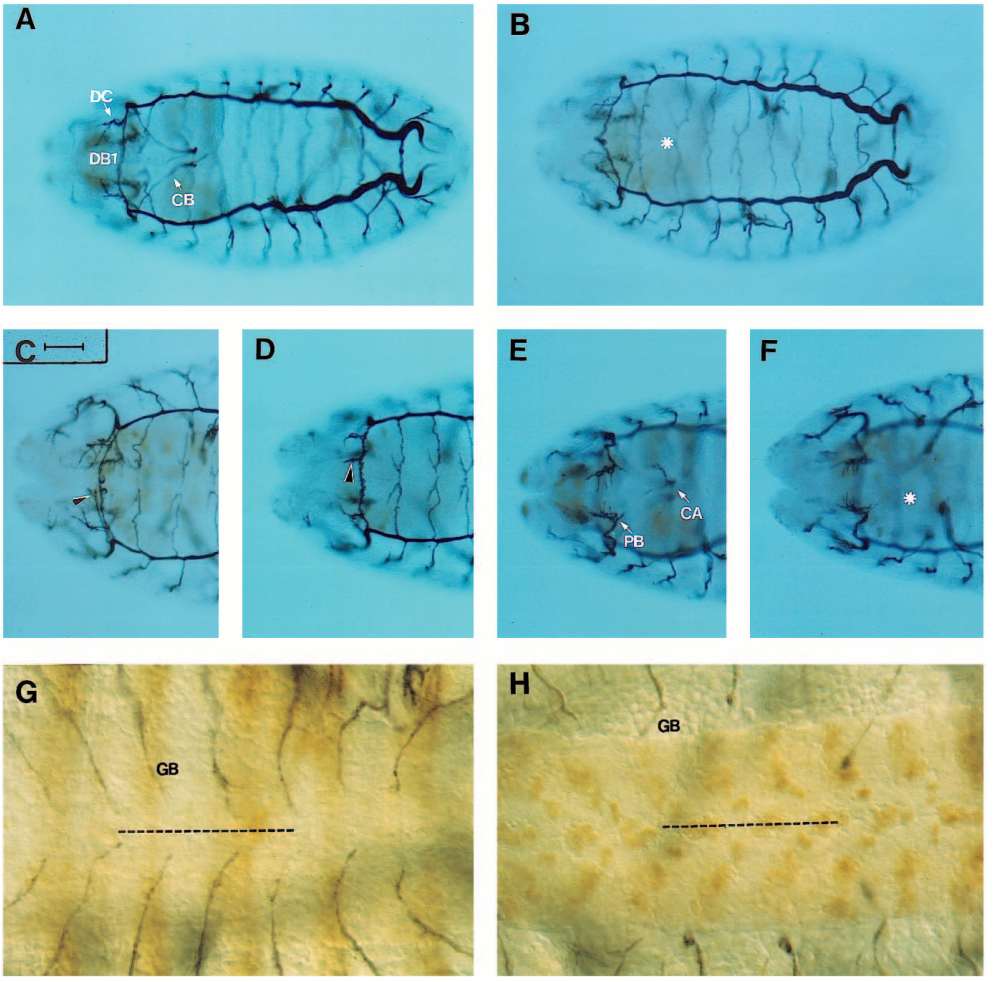

Fig. 7. *unp* controls the development of specific tracheal branching patterns. The tracheal branching patterns in all the panels were visualized by 2A12 antibody staining of stage 15 embryos. A-F are dorsal views and G-H are ventral views. The wild-type embryo in A is photographed in a focal plane that illustrates the normal morphology of the dorsal cephalic branch, dorsal branch 1, and the cerebral branch. Note the absence in *unp* mutant embryos of the cerebral branch (B, asterisk) with frequent occurrence of ectopic dorsal branch-like (C; arrowhead) and dorsal cephalic-like (D; arrowhead) tracheal outgrowths. In a more ventral plane of focus, the normal mophology of the cerebral anastomosis and pharyngeal branch are apparent (E); the cerebral anastomosis is missing in *unp* mutant embryos (F, asterisk). The ganglionic branches normally extend into the CNS in a stereotyped pattern (G), and this pattern is altered in *unp* mutant embryos (H), with branches stalling as they reach the CNS. Dotted lines indicate the midline of the CNS. Abreviations: CB, cerebral branch; DB1, dorsal branch 1; DC, dorsal cephalic branch; GB, ganglionic branch; PB, pharyngeal branch.

the CNS is restricted to cells of the cerebral and ganglionic branches of the tracheal system during embryonic development.

unp is required for the development of the tracheal branches

To study the function of the *unp* gene, the 1912 line carrying a P element insertion in the first intron of *unp* was exposed to transposase from the P[Δ2-3] element to generate mutations for phenotypic analysis. Of approximately 230 excision events, 12 were associated with homozygous lethality; the extents of chromosomal deletions in four of these excisants are as shown in Fig. 1. Since lethal alleles unp^{r1}, unp^{r221} and unp^{r225} have DNA lesions extending beyond the *unp* transcription unit, we concentrated our phenotypic analysis on the unp^{r37} allele. The DNA lesion associated with this allele begins in the 5′ end of the P element and extends to the region close to a *Spe*I restriction site in the third exon (see Materials and Methods). Thus, the unp^{r37} deletion removes all of exon 2 and part of exon 3, including the entire homeodomain sequence. Interestingly, the mutation still retains *lacZ* expression in embryos, consistent with our findings that the major regulatory sequences for *unp* expression are located downstream of the *unp* transcription unit (see below).

Specific expression of *unp* in neural branches of the tracheal system suggests that it may play a role in tracheal development. Indeed, tracheal staining of unp^{r37} homozygous mutant embryos (identified by the absence of a marked balancer chromosome; see Materials and Methods) with antibody 2A12 revealed the absence of the entire cerebral branch (Fig. 7A,B), with occasional ectopic branches in the first tracheal metamere (Fig. 7C,D; arrowhead). In addition, the cerebral anastomosis, which normally is associated with the cerebral branch is also absent (compare Fig. 7E,F). A specific defect is also observed in the ganglionic branches, which in most cases extend only partially and fail to penetrate the CNS (compare Fig. 7G and H). Similar effects on the cerebral branch and anastomosis and on ganglionic branches were observed with the unp^{r225} and unp^{r1} alleles (data not shown). The specific defects observed in the *unp* mutants are consistent with the *unp* protein distribution and suggest a specific role for *unp* in the formation of tracheal branches that penetrate the CNS. Despite these tracheal defects, about 3-5% of homozygous unp^{r37} flies, under uncrowded culture conditions, eclose to adulthood; these escapers exhibit an upheld wing phenotype (data not shown).

Regulation of *unp* expression by genes of the bithorax complex

Lewis (1978) showed that appropriate development of segment-specific tracheal structures requires the function of the homeotic genes of the bithorax complex (BX-C). As described above, *unp* expression highlights portions of the tracheal system that penetrate the CNS, including the cerebral branch specific to T1 (Fig. 8A). To test the possibility that genes in the BX-C play a role in regulating *unp* expression, we examined the distribution of *unp* transcript in $Ubx^{9.22}$, in *Df109* (lacking *Ubx* and *abd-A* functions; Karch et al., 1985), and in Ubx^{Mx12} $abd\text{-}A^{M1}$ $Abd\text{-}B^{M8}$ triple mutants (lacking function of all three BX-C genes; Casanova et al., 1987). In *Ubx* mutant embryos additional *unp* expression is observed in cells surrounding the tracheal pits of T2 and T3, indicative of a role for *Ubx* in repression of *unp* in the posterior segments and consistent with homeotic transformation in *Ubx* mutants of posterior T2 and T3 towards a T1 identity (Fig. 8B). In *Df109* embryos extra patches of *unp*-expressing cells around the tracheal pits extend posteriorly to A7, indicating a role of *abd-A* in the repression of *unp* expression in the abdominal segments (data not shown). The homeotic gene *abd-B* probably contributes to the repression of *unp* expression in A7, since slightly elevated expression in A7 is observed in the triple mutants as compared to the *Df109* embryos (Fig. 8C).

3′ region of *unp* confers *Ubx*-mediated repression of a reporter gene

To identify *cis*-regulatory regions that are responsible for the normal *unp* expression in embryos, six restriction fragments encompassing 20 kb of *unp* genomic sequence were tested for their ability to direct expression of a *lacZ* reporter gene containing a minimal hsp70 promoter (Thummel et al., 1988). The region far upstream of the *unp* transcription unit, represented by fragment U1 in Fig. 1, gave a restricted *lacZ* expression pattern in the dorsal vessel (data not shown), although this pattern does not correspond to any known *unp* product distribution. The U2 and U3 fragments that are located immediately downstream of the U1 fragment did not generate any detectable *lacZ* expression, and the promoter proximal fragment U4 (see Fig. 1 for location) gave weak but nonspecific *lacZ* expression in embryos (data not shown), suggesting that a general enhancer-like element may be present in the fragment. Finally, the U5 fragment in the first intron does not show any detectable *lacZ* expression.

The only region capable of driving a *lacZ* reporter gene expression similar to the *unp* protein distribution is located at the 3′ end of the *unp* transcription unit. In 10 out 10 independent transgenic lines, the 2.7 kb fragment (U6 in Fig. 1) gave characteristic *unp*-like expression in the CNS and the cerebral and ganglionic branches of the tracheal system during embryogenesis (see Fig. 5L,N). This fragment also gave *unp*-like expression in the cells around the first tracheal pit at the germband extended stage (Fig. 9A). Thus, the results indicate that a 2.7 kb fragment of *unp* 3′ flanking sequence contains most of the *cis*-regulatory elements for the normal *unp* expression in embryos.

To ascertain that the 2.7 kb fragment contains regulatory elements that can mediate homeotic control of gene expression, we examined expression of the U6-*lacZ* construct in $Ubx^{9.22}$ mutant embryos and found that the *lacZ* reporter gene is derepressed in the T2 and T3 segments in a manner similar to the endogenous gene (Fig. 9B). Expression in T2 and T3 continues as the cells extend to the dorsal side of the embryo (Fig. 9D). We note that the cells at T2 and especially at T3 do not extend as far dorsally as the cells at T1. The ultimate fate of cells in T2 and T3 in *Ubx* mutant embryos is uncertain since the expression diminishes in later stages; these cells nevertheless appear committed to cerebral branch fates, since ectopic cerebral-like branches in T2 and T3 can be detected with tracheal-specific 2A12 antibody in *Ubx* mutant embryos (Fig. 9F).

The *pointed* gene acts upstream of *unp* in regulating branching morphogenesis

The specific branching defects observed in *unp* mutant embryos prompted us to examine the relationship between *unp* and *pointed* (*pnt*), a gene encoding a member of the *ets* family

of transcription factors; *pnt* is expressed in tracheal placodes and in the developing tracheal branches (Klambt, 1993). In the absence of *pnt* gene function, tracheal cells fail to migrate and branches do not extend to target tissues (Klambt et al., 1992). In particular, stalling of the ganglionic branches at the ventral oblique musculature in hypomorphic *pnt* embryos is reminiscent of the most extreme phenotype observed in *unp* embryos.

To determine the regulatory relationship between *pnt* and *unp* genes we immunostained *pnt* homozygous embryos with *unp*-specific antibody to follow the fate of the ganglionic branches. At stage 12.5, ganglionic branch precursor cells migrating toward the CNS are clearly visible in wild-type embryos (Fig. 10A). In *pnt* mutant embryos no migratory cells that are expressing *unp* protein can be detected (Fig. 10B); however, a few precursor cells accumulating low levels of *unp* protein occasionally can be identified (Fig. 10B; asterisk). By stage 14, the ganglionic branches of normal embryos are well developed, as is evident from a group of 8 to 9 *unp*-expressing cells along the ventrolateral region of each hemisegment (Fig. 10C; also see Fig. 5). In *pnt* mutant embryos, only 3 to 4 *unp*-expressing cells can be detected (Fig. 10D); these *unp*-expressing cells are clustered in a group suggesting that the precursor cells remain immobile and fail to extend from the tracheal pits. These results suggest that the ganglionic branch phenotype in *pointed* embryos may be in part due to a loss or reduction of *unp* gene expression and failure of *unp*-expressing cells to extend into the CNS.

DISCUSSION

The role of *unp* in development of the tracheal branches

The *Drosophila* tracheal system is organized in a network of tubular branches that meet the respiratory needs of appropriate target tissues. The branches of the tracheal system originate from ten pairs of tracheal placodes located on the lateral side of segments T2 through A8 of stage 10 embryos (Hartenstein and Jan, 1992; Manning and Krasnow, 1993). During development, founder cells of the tracheal placodes extend and migrate via stereotyped paths to establish specialized tracheal branching patterns. Cells of the cerebral and ganglionic branches that specifically accumulate *unp* protein are the major tracheal cells known to target the brain and ventral nerve cord of the CNS (Manning and Krasnow, 1993). The absence of the cerebral branch and associated cerebral anastomosis and the abnormal development of the ganglionic branches in *unp* mutant embryos indicate that *unp* plays an important role in the formation of all the major neural branches of the tracheal system.

The expression of *unp* in founder cells of the cerebral branch within the first tracheal placode suggests an early role during branch development. *unp* function, however, is most likely not involved in the initial commitment to founder cell fates, since expression of the *lacZ* reporter gene by the enhancer trap is maintained in *unp* mutant embryos (data not shown). Instead *unp* appears to be involved in branching morphogenesis by regulating cell migration or extension, and in the absence of such function the founder cells either die or adopt other branch patterns. This is consistent with the observation that in *unp* mutant embryos the absence of the cerebral branch is occasionally accompanied by the presence of an ectopic branch in the first tracheal metamere. The appearance of this ectopic branch resembles that of the dorsal branch or of the dorsal cephalic branch, which like the cerebral branch originate from the first tracheal placode (Manning and Krasnow, 1993).

In addition to the cerebral branch, *unp* is also expressed in cells of the ganglionic branches, but here expression occurs much later (stage 9 vs stage 12) suggesting that *unp* may have secondary functions during ganglionic branch development. Consistent with this view is the observation that the ganglionic branches develop but fail to extend consistently to the CNS in *unp* mutant embryos (see Fig. 7F). This phenotype is reminiscent of the hypormorphic alleles of *pnt* and *breathless* mutants (Klambt et al., 1992). *breathless* encodes a *Drosophila* homologue of the fibroblast growth factor (FGF) receptor, and its expression in the developing tracheal system is required for the migration of tracheal cells. Thus, the observed *unp* phenotype appears to be consistent with the role of *unp* in the specification of tracheal cell migration or extension.

Regulation of tracheal branch development

A limited number of target genes has been characterized whose functions correlate with HOM gene regulation in *Drosophila.* These genes, initially identified either by their mutant phenotypes or homology to other gene families, encode distinct proteins whose functions are associated with development of many different structures. For example, repression of the homeobox gene *Distal-less* and activation of the helix-loop-helix containing transcription factor *nautilus* in abdominal segments by genes of the bithorax-complex (BX-C) are required for proper development of limb and somatic muscle respectively (Paterson et al., 1991; Vachon et al., 1992; Michelson, 1994); activation of another homeobox gene *empty spiracles* in the 8th abdominal segment by *Abdominal-B* is required for development of the fillzkorper (Jones and McGinnis, 1993); and activation of the *Drosophila TGF-β* homologue *decapentaplegic* by *Ultrabithorax (Ubx)* is required for gut morphogenesis (Immergluck et al., 1990; Panganiban et al., 1990; Reuter et al., 1990; Capovilla et al., 1993; Hursh et al., 1993; Sun et al., 1995). Other targets of *Ubx* have also been isolated by either immunoprecipitation of protein-DNA complexes from native chromatin (Gould et al., 1990) or UV-crosslinked chromatin (Graba et al., 1992) using a *Ubx*-specific monoclonal antibody. Although these methods suggest direct interaction between *Ubx* protein and the cognate target sequences, it is difficult to determine the significance of the *Ubx* regulation due to the absence of obvious mutant phenotypes associated with these target genes (Brookman et al., 1992; Gould and White, 1992; Nose et al., 1992; Strutt and White, 1994).

Our results indicate that restricted expression of *unp* in the cerebral branch founder cells requires normal function of genes in the BX-C. In the absence of these homeotic genes, the expression of *unp* expands more posteriorly to the abdominal segments. This is consistent with the notion that *Ubx* controls tracheal development by regulating the expression of target genes (Lewis, 1978). Since *unp* encodes a transcription factor and is required for cerebral branch development, we suggest that normal restriction of cerebral branch development to T1 is mediated by *Ubx* repression of *unp*. This repression is mediated by the 2.7 kb fragment located downstream of the *unp* transcription unit. Further dissection of this regulatory

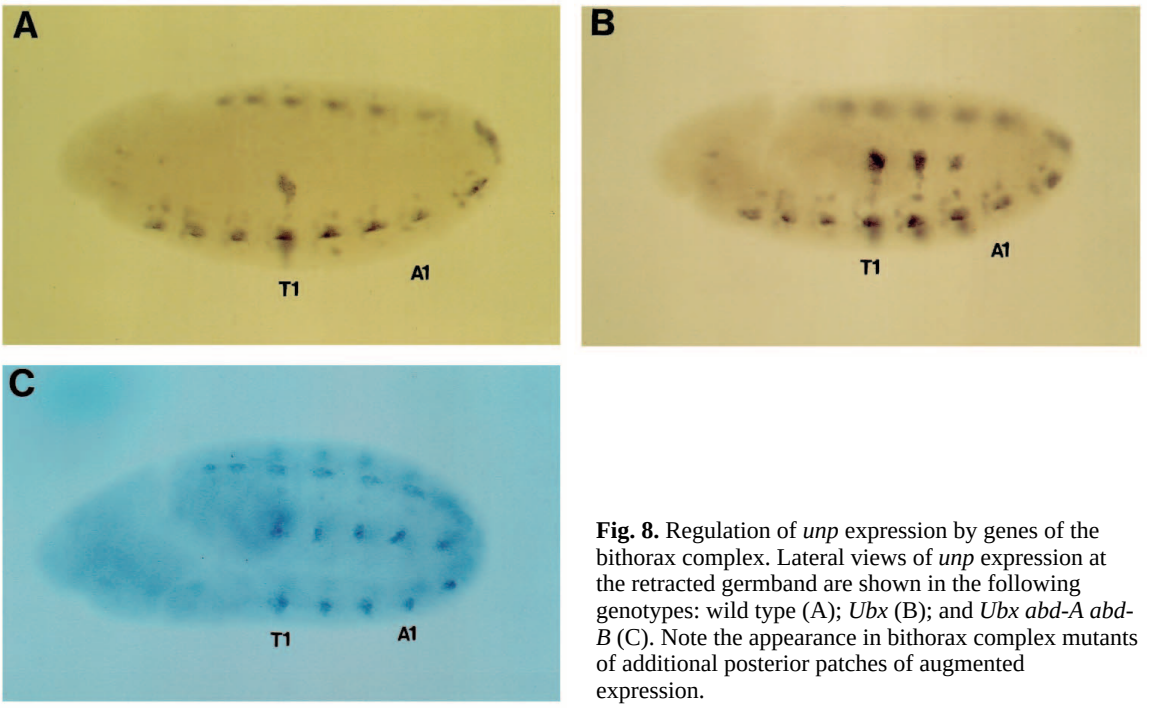

Fig. 8. Regulation of *unp* expression by genes of the bithorax complex. Lateral views of *unp* expression at the retracted germband are shown in the following genotypes: wild type (A); *Ubx* (B); and *Ubx abd-A abd-B* (C). Note the appearance in bithorax complex mutants of additional posterior patches of augmented expression.

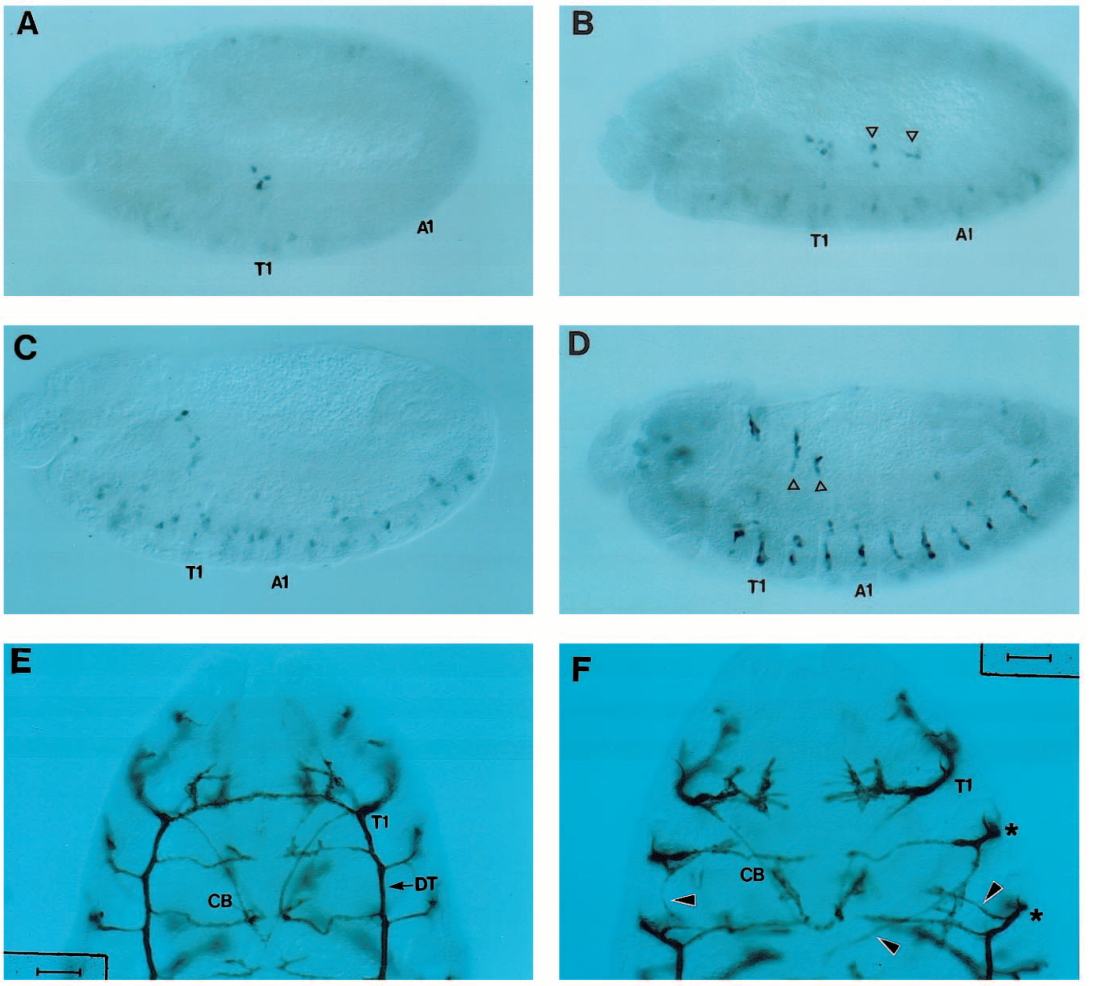

Fig. 9. A downstream regulatory region of the *unp* locus reproduces normal expression, including homeotic regulation. A-D show the expression of a *lacZ* reporter (*unpU6-lacZ*) carrying the U6 segment downstream of the *unp* locus in wild-type (A,C) and *Ubx* mutant embryos (B,D). β-galactosidase antibody staining patterns are shown in embryos during extended (A,B) and retracted (C,D) germband strages. Open triangles in the *Ubx* mutant embryos indicate additional patches of expression resulting from the appearance of ectopic cerebral branch (CB) precursor cells. E and F display the 2A12 antibody staining pattern in wild-type (E) and *Ubx* mutant (F) embryos at stage 14. Note the presence of ectopic cerebral branch-like structures (arrowheads) originating from T2 and T3 (asterisks) in the *Ubx* mutant embryos (F). Note also the the absence of dorsal trunk segments (DT) projecting anteriorly at T2 and T3, consistent with a general transformation of tracheal branches at T2 and T3 toward a T1 identity.

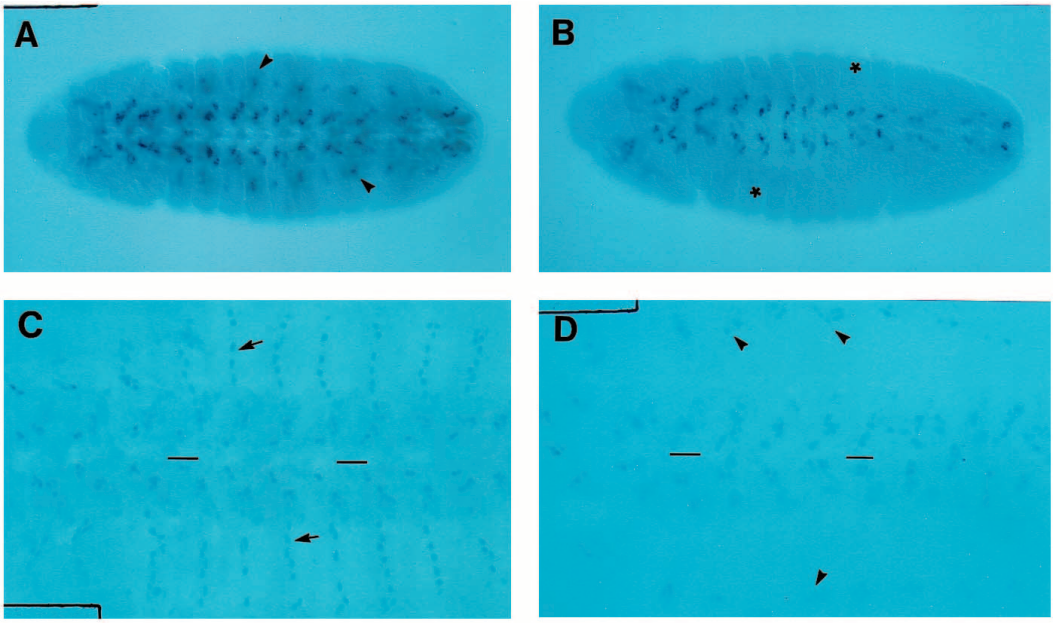

Fig. 10. Alteration of *unp* protein distribution in *pnt* mutant embryos. Wild-type (A,C) or *pnt* mutant (B,D) embryos were immunostained with *unp*-specific antiserum. During germband retraction, the *unp* protein normally accumulates in cells of the ganglionic branches (A; arrowheads); in *pnt* mutant embryos, however, the *unp* protein levels are barely detectable (B; asterisks). After germband retraction, each ganglionic branch consists of 7-9 cells that traverse the ventral oblique muscles and penetrate into the CNS (C; arrows). In *pnt* mutant embryos, only 4-5 precursor cells can be observed to accumulate low levels of *unp* protein (D; arrowheads), and these cells fail to extend into the CNS. Note the abnormal pattern of *unp* expression in the CNS of *pnt* mutants (B,D), consistent with other CNS defects such as collapsed commissures seen in *pnt* mutant embryos. The lines in C and D indicate the midline of CNS.

region will be necessary to establish whether repression by *Ubx* protein is direct or mediated by another transcription factor.

As mentioned above *unp* does not appear to be required for initial commitment to a tracheal branch cell fate. Thus, initiation of *unp* expression must be mediated by other transcription factors. There are three observations to suggest that *pnt* is involved in regulating *unp* gene expression in the ganglionic branches. First, the branching defects observed in *pnt* embryos are more severe than those of *unp* embryos. Second, *pnt* is expressed in the tracheal founder cells including the cells that later give rise to the ganglionic branches, while *unp* expression in the ganglionic branches does not occur until founder cells have been established. *pnt* expression thus clearly precedes *unp* expression. Third, the *unp* protein levels and the number of *unp*-expressing cells in the ganglionic branches are greatly reduced in *pnt* mutant embryos. These observations, however, do not indicate that the *pnt* protein is the only regulator of *unp* gene expression, nor even that this regulation is direct. Indeed, several other transcription factors known to be expressed during early tracheal development are candidates for regulators of *unp* gene expression, although their precise role in regulating branching morphogenesis is not clear, since their complex embryonic expression patterns obscure the determination of primary versus secondary effects (Manning and Krasnow, 1993).

In conclusion our studies demonstrate that *unp* is essential for normal development of the tracheal branches that penetrate the central nervous system, possibly by regulating genes involved in tracheal cell migration and extension. Regulation of the *unp* gene requires normal function of the *pnt* gene in a general manner, whereas segmentally specific aspects of unp expression are established through repression by the homeotic genes of the bithorax complex. Recent elucidation of FGF receptor function in airway branching of mouse lung buds resembles that of *Drosophila* FGF receptor in tracheal formation, suggesting the existence of parallel molecular pathways that operate in morphogenesis of branched tubular structures in insects and mammals (Peters et al., 1994; Klambt et al., 1992). It would be interesting to know whether this parallel extends to the control of specific branch formation by homeodomain proteins, as we have found for neural branching of tracheae in *Drosophila*.

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