

Function of the *Drosophila* POU domain transcription factor Drifter as an upstream regulator of Breathless receptor tyrosine kinase expression in developing trachea

Michael G. Anderson¹, Sarah J. Certel², Kaan Certel², Tzumin Lee³, Denise J. Montell³ and Wayne A. Johnson^{1,2,*}

¹Department of Physiology and Biophysics and ²Genetics PhD Program, University of Iowa, College of Medicine, Iowa City, Iowa 52242, USA

³Department of Biological Chemistry, Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, MD 21205, USA

*Author for correspondence

SUMMARY

Organogenesis of the *Drosophila* tracheal system involves extensive directed cell migrations leading to a stereotypic series of interconnected tubules. Although numerous gene products have been shown to be essential for tracheal morphogenesis, direct functional relationships between participants have not been previously established. Both the *breathless* gene, encoding a *Drosophila* fibroblast growth factor receptor tyrosine kinase homologue, and the POU-domain transcription factor gene, *drifter*, are expressed in all tracheal cells and are essential for directed cell migrations. We demonstrate here that ubiquitously expressed Breathless protein under control of a heterologous heat-shock promoter is able to rescue the severely disrupted tracheal phenotype associated with *drifter* loss-of-function mutations. In the absence of Drifter function, *breathless* expression is initiated normally but transcript levels fall drastically to undetectable levels as tracheal differentiation

proceeds. In addition, *breathless* regulatory DNA contains seven high affinity Drifter binding sites similar to previously identified Drifter recognition elements. These results suggest that the Drifter protein, which maintains its own expression through a tracheal-specific autoregulatory enhancer, is not necessary for initiation of *breathless* expression but functions as a direct transcriptional regulator necessary for maintenance of *breathless* transcripts at high levels during tracheal cell migration. This example of a mechanism for maintenance of a committed cell fate offers a model for understanding how essential gene activities can be maintained throughout organogenesis.

Key words: organogenesis, signal transduction, cell migration, transcription, *breathless*, *drifter*, *Drosophila*, trachea

INTRODUCTION

Genetic pathways controlling cell-specific differentiation and organogenesis normally involve early acting transcription factors that can profoundly affect the fate of individual cells (Davis et al., 1987; Nambu et al., 1991; Affolter et al., 1994; Halder et al., 1995). The detailed mechanisms by which transcription factors exert such major influences remain poorly characterized but are central to a thorough understanding of organ development. The process of tracheal morphogenesis in *Drosophila* is an excellent system for elucidation of these complex mechanisms. *Drosophila* tracheal cells complete their final mitotic cell divisions during stage 10 of embryogenesis and subsequently undergo extensive directed cell migrations to form an array of interconnected epithelial tubules (Manning and Krasnow, 1993). Establishment of tracheal tubules is thought to occur in three morphologically distinct phases corresponding to the formation of primary, secondary and terminal branches (Manning and Krasnow, 1993; Samakovlis

et al., 1996). The earliest phase consists of stereotypic growth and fusion of primary tracheal branches, which normally begins during embryonic stage 11 and continues through stage 15. During the remainder of embryogenesis and continuing into larval stages, finer secondary and terminal branches extend from existing primary branches.

The *Drosophila* POU domain transcription factor Drifter (DFR) has been shown to play an essential role in the earliest phase of tracheal cell migration (Anderson et al., 1995). In the absence of DFR function, tracheal cells appear to be designated normally but do not migrate, resulting in a failure to form defined primary tracheal branches (Anderson et al., 1995). Consistent with an essential role in tracheal cell migration, *dfr* expression is maintained at high levels throughout tracheal morphogenesis utilizing an autoregulatory mechanism (Certel et al., 1996). The *dfr* gene is only one of a number of *Drosophila* genes required for directed tracheal cell migrations (Bray and Kafatos, 1991; Glazer and Shilo, 1991; Klämbt, 1993; Affolter et al., 1994; Guillemain et al., 1996; Isaac and

Andrew, 1996; Wilk et al., 1996), although the exact functional relationships between these multiple gene activities remain largely uncharacterized.

The *Drosophila* Breathless (BTL) receptor tyrosine kinase is also required for directed tracheal cell migrations and establishment of primary tracheal branches (Glazer and Shilo, 1991; Klämbt et al., 1992; Reichman-Fried et al., 1994). The *btl* gene encodes a fibroblast growth factor receptor (FGF-R) homologue (Glazer and Shilo, 1991) believed to initiate an intracellular signal transduction cascade similar to the well-characterized receptor tyrosine kinase pathways required for vertebrate cell proliferation (Schlessinger and Ullrich, 1992), *Drosophila* photoreceptor induction (Rubin, 1991) and *Caenorhabditis elegans* vulval development (Kayne, 1994). In all of these systems, cell surface receptors initiate a signaling cascade mediated by the activities of RAS, RAF and MAP Kinase ultimately affecting the activity of nuclear transcription factors (Pulverer et al., 1991; O'Neill et al., 1994; Beitel et al., 1995).

The *btl* gene exhibits a restricted embryonic expression pattern limited to mesectodermal progeny and tracheal cells (Glazer and Shilo, 1991), both of which coexpress *dfr*. DFR protein is also seen in a number of other tissues including the central nervous system, hindgut and epidermis (Anderson et al., 1995). The mutant phenotypes of *btl* and *dfr* are essentially identical with severe loss-of-function alleles displaying an absence of tracheal cell migration (Glazer and Shilo, 1991; Scholz et al., 1993; Anderson et al., 1995). Based upon these similar functional characteristics, we have tested the possibility that *dfr* and *btl* may interact within a common genetic pathway in which the DFR transcription factor could act to directly regulate expression of the *btl* gene or, alternatively, DFR protein expression or function could be activated as a downstream effector of the BTL receptor tyrosine kinase signal transduction cascade.

Results presented here demonstrate that the first of these possibilities is correct. We observe a striking rescue of tracheal cell migration in *dfr* mutant embryos when BTL is ubiquitously expressed under the control of a heterologous heat-inducible promoter. Although initial *btl* transcript levels appear transiently unaffected by severe *dfr* mutations, maintenance of high level *btl* expression requires wild-type *dfr* function. Furthermore, our characterization of *btl* regulatory sequences has identified seven high affinity DFR recognition elements implying direct transcriptional regulation of *btl* expression by DFR protein. These results establish an essential functional relationship between the *dfr* and *btl* genes during tracheal cell differentiation and suggest a mechanism involving separable regulatory events necessary for the specification and maintenance of the tracheal cell phenotype.

MATERIALS AND METHODS

Drosophila stocks

Flies were raised on standard cornmeal-yeast-agar medium. All stocks and balancer chromosomes not specifically mentioned in the text are described in Lindsley and Zimm (1992). All genetic crosses were performed at 25°C unless otherwise specified. Stages of embryonic development are according to Campos-Ortega and Hartenstein (1985).

Immunohistochemistry and in situ hybridization

Staged *Drosophila* embryos were labeled using a modification of protocols previously described (Mitchison and Sedat, 1983; Johnson, 1992). β -galactosidase (β -gal) was detected using a rabbit polyclonal antiserum (Cappel) at a 1:500 dilution in PBT (1× PBS, 0.5% bovine serum albumin, 0.2% Triton X-100). Tracheal lumen were visualized with a rabbit polyclonal antiserum (TL-1) raised against the Punch protein used at a 1:1000 final dilution (Chen et al., 1994; Samakovlis et al., 1996). The TL-1 antiserum was generously provided by Nir Hacohen and Mark Krasnow. DFR protein distribution was visualized by labeling with a polyclonal DFR-antiserum generated against a glutathione-s-transferase-DFR fusion protein (Anderson et al., 1995). After repeated washes with PBT, primary antibody was detected using the Vectastain ABC kit (Vector Laboratories) and horseradish peroxidase immunohistochemistry following protocols recommended by the manufacturer. Embryos were mounted in 70% glycerol/1× PBS and photographed on a Nikon Optiphot microscope using Nomarski optics. Homozygous embryos were identified by double-labeling embryos with β -gal antiserum to detect the absence of the *ftz*-derived zebra stripe expression pattern produced from the *TM3, ftzlacZ* third chromosome balancer. *btl* transcript levels were assayed by whole-mount in situ hybridization with digoxigenin (DIG)-incorporated probes visualized with alkaline phosphatase-conjugated anti-DIG antibody as previously described (O'Neill and Bier, 1994). Antisense *btl*-specific DIG-UTP RNA probes were generated by in vitro transcription of a 2.2 kb *btl* cDNA fragment kindly provided by Benny Shilo (Glazer and Shilo, 1991; Klämbt et al., 1992). The *btl* cDNA fragment encodes the carboxy-terminal region of the BTL protein from the fourth immunoglobulin-like domain to the translation stop. Homozygous embryos were identified by detecting the absence of β -gal protein derived from the *TM3, ftzlacZ* balancer prior to in situ hybridization.

Heat-shock procedure for *hs-btl* and *hs-dfr* transformants

The generation of transgenic flies expressing either BTL (Murphy et al., 1995) or DFR (Certel and Johnson, 1996) under the control of the heat-inducible *hsp70* promoter has been previously described. Embryos produced from *hs-btl; dfr^{E82} / TM3, Sb ftzlacZ* *e* adults were collected on yeast grape agar plates for 1 hour at 25°C, aged for 4 hours at 25°C, heat-shocked for 45 minutes at 37°C, and returned to 25°C for 7-8 hours prior to fixation and labeling with the tracheal-specific anti-Punch antibody. For experiments utilizing the *hs-dfr* transposon, embryos laid by *hs-dfr; Df(3L)XBB70 / TM3, Sb ftzlacZ* *e* adults were collected for 4 hours at 25°C, aged for 8-9 hours at 25°C, heat-shocked for 1 hour at 37°C, followed by 30 minutes at 25°C prior to fixation and labeling with *btl*-specific DIG-UTP RNA probes. Control embryos were raised over the same time frame at 25°C but not heat-treated. Homozygous mutant embryos were identified by detecting the absence of β -gal protein derived from the *TM3, ftzlacZ* balancer.

DNaseI protection assay

Glutathione-s-transferase-DFR (GST-DFR) fusion proteins were produced in protease-deficient Epicurian Coli BL21 cells (Stratagene) using protocols recommended by Pharmacia for use of the pGEX vector. A 1087 bp fragment of *dfr* cDNA sequence extending from the *Bam*HI site at +949 bp to a synthetic *Eco*RI site at +2035 bp was used to construct the GST-DFR fusion gene in pGEX-1. This fragment encodes the carboxy-terminal 338 amino acids of the DFR protein including the entire *dfr* POU domain. Bacterial strains carrying the GST-DFR plasmid were induced with 0.4 mM isopropyl- β -D-thiogalactopyranoside (IPTG), lysed by sonication and GST-DFR protein purified using glutathione-agarose beads (Sigma). Purified GST-DFR fusion protein yielded a single band on SDS-Polyacrylamide gels with the expected molecular mass of 65×10³ M_r.

Concentrations of purified proteins were quantitated using the Bio-Rad protein assay system and used in DNaseI protection assays as

previously described (Johnson et al., 1989; Johnson and Hirsh, 1990). Single-end-labeled footprinting probes were generated from subclones of *btl* 5' regulatory DNA using polynucleotide kinase and [γ - 32 P]ATP following previously described protocols (Johnson and Hirsh, 1990; Certel et al., 1996). Individual fragments used as probes consisted of a 511 bp *Sau3AI* fragment (-2263 bp to -1752 bp), a 497 bp *Sau3AI* fragment (-1752 bp to -1255 bp), a 372 bp *Sau3AI-HindIII* fragment (-1255 bp to -883 bp), a 560 bp *HindIII-Sau3AI* fragment (-883 bp to -323 bp) and a 367 bp *Sau3AI-ApoI* fragment (-323 bp to +45 bp). DNaseI digestion fragments were separated on 7% sequencing gels, dried and exposed to Kodak X-OmatAR X-ray film.

RESULTS

Tissue-specific expression of *dfr* and *btl*

The *dfr* and *btl* genes are expressed in both tracheal and mesectodermal cells with essentially identical temporal and spatial patterns (Fig. 1). Both genes are among the earliest tracheal-specific genes to be expressed in the bilaterally symmetrical series of tracheal placodes seen in stage 10 embryos. Placode formation represents the first indication of tracheal cell specification. In addition to tracheal expression, *dfr* and *btl* are coexpressed in developing mesectoderm, seen as a stripe of cells located medially along the ventral surface of stage 9/10 embryos and in an anterior cluster of cells associated with the

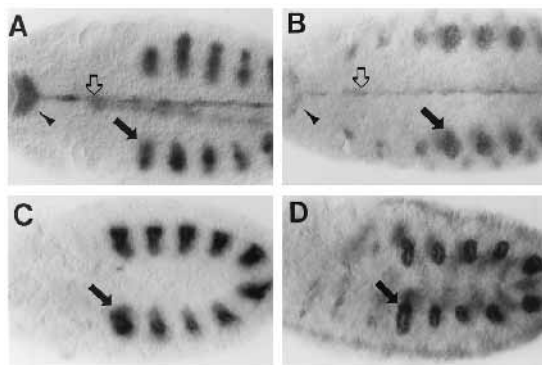


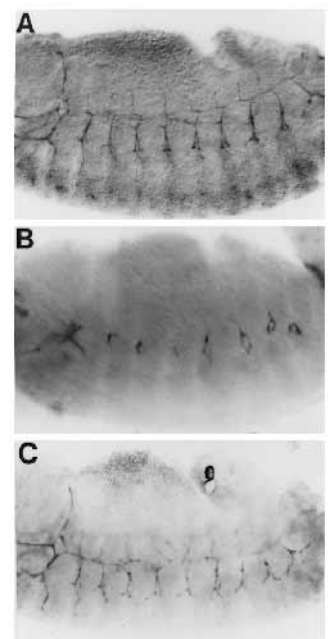
Fig. 1. Coexpression of *dfr* and *btl*. Wild-type expression patterns were visualized by either whole-mount in situ hybridization with a DIG-labeled *btl* RNA probe (A,C) or whole-mount immunohistochemistry using DFR-specific antiserum (B,D). All embryos are oriented with anterior to the left. (A) Ventral view of a stage 10 wild-type embryo showing *btl* transcripts first detected in a medial line of cells corresponding to the mesectoderm (hollow arrow) and a cluster of cells, which in later stages will eventually become associated with the stomodeal opening (arrowhead). Also visible are both rows of bilaterally symmetrical tracheal placodes expressing *btl* at high levels (solid arrow). (B) Ventral view of a stage 10 embryo labeled with DFR antiserum showing the overlapping expression of DFR protein in mesectoderm (hollow arrow), the stomodeal cluster (arrowhead) and the tracheal placodes (solid arrow). Note that DFR protein is also detected in a small number of cells that do not express *btl* transcripts. (C) Lateral view of a late stage 11 embryo showing *btl* transcripts in the invaginating tracheal pits (solid arrow). Throughout the remainder of embryogenesis, all tracheal cells will continue to express *btl* as they migrate during tracheal morphogenesis. (D) Lateral view of a late stage 11 embryo labeled with DFR antiserum showing coexpression of DFR protein in all cells of the tracheal pits (solid arrow).

stomodeal opening (Fig. 1A,B). Subsequent to stage 10 in wild-type embryos, tracheal expression of both genes is maintained at high levels throughout embryogenesis as cells invaginate and migrate into the embryo to form the complex tracheal tree (Fig. 1C,D). We have previously shown that the maintenance of tracheal *dfr* expression is dependent upon a tissue-specific autoregulatory enhancer containing multiple variant DFR recognition elements (Certel et al., 1996). However, the transcriptional regulation of tracheal *btl* expression has not been previously characterized. Although tracheal expression is maintained, mesectodermal expression of both genes is restricted to a subset of midline cells by early stage 13 with *dfr* expression limited to the middle pair of midline glia and *btl* restricted to the posterior pair of midline glia (Klämbt et al., 1992; Anderson et al., 1995).

Rescue of the *dfr* tracheal mutant phenotype by ubiquitous BTL expression

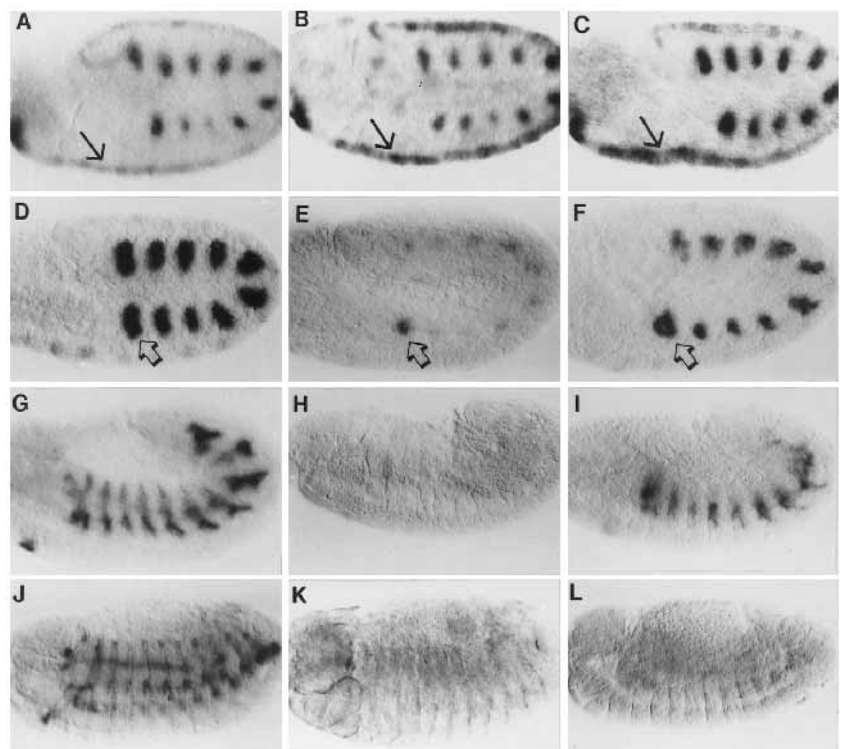
Ubiquitous expression of BTL protein under control of the heat-inducible *hsp70* promoter is able to rescue the tracheal mutant phenotype associated with *btl* mutations when transgenic embryos are exposed to a single heat-shock treatment at 4 hours of development (Murphy et al., 1995). We tested whether ubiquitous BTL expression under these conditions could suppress the *dfr* mutant phenotype in the trachea or CNS. Non-heat-treated homozygous *dfr*^{E82} embryos are characterized by a severely disrupted tracheal phenotype with limited tracheal cell migration and the absence of primary branch formation (Anderson et al., 1995). Heat-treated *hs-btl*; *dfr*^{E82} embryos display a striking restoration of the normal tracheal phenotype evident by the greater extent of tracheal cell migration, well defined tracheal branches and proper branch fusions (Fig. 2C). In contrast, ventral nerve cord

Fig. 2. Rescue of tracheal cell migration in homozygous *dfr*^{E82} mutants by ubiquitous BTL expression. Late stage 13 embryos containing *hs-btl* were labeled with anti-Punch antibody to visualize the tracheal lumen. Embryos are all shown in a lateral view with anterior to the left and dorsal up. (A) In wild-type embryos, the highly uniform metameric repeated branches of the tracheal tree are visible with a completed dorsal trunk and well-defined lateral and visceral branches. (B) Non-heat-shocked homozygous *hs-btl*; *dfr*^{E82} embryos show no tracheal cell migration resulting in severely deformed trachea lacking recognizable branching. (C) Homozygous *hs-btl*; *dfr*^{E82} embryos



subjected to a 45 minute heat shock at 4 hours of development show a significant restoration of wild-type morphology as seen by extensive cell migrations and several well-defined branches. Note formation of the dorsal trunk and restoration of lateral branches.

Fig. 3. *btl* transcript levels in *dfr* loss-of-function backgrounds. Lateral view of staged embryos labeled with DIG-labeled *btl* RNA probes. All embryos are shown with anterior to the left and dorsal up. Expression of *btl* transcripts in wild-type (A,D,G,J), homozygous *Df(3L)XBB70* (B,E,H,K), and *dfr^{E82} / Df(3L)XBB70* (C,F,I,L) embryos. (A-C) Stage 10 embryos showing *btl* transcripts in the tracheal placodes and mesectoderm (thin arrows). (D) Early stage 12 wild-type embryo expressing *btl* at high levels throughout the tracheal pits. (E,F) Early stage 12 *dfr* mutant embryos displaying altered levels of *btl* expression. The second through tenth tracheal pits show markedly reduced *btl* levels especially evident at this stage in *Df(3L)XBB70* homozygotes. Tracheal metamere 1 retains *btl* transcripts longer than the other tracheal metameres (open arrows). (G) Wild-type late-stage 12 embryo. (H) Late-stage 12 homozygous *Df(3L)XBB70* embryo with *btl* transcripts reduced to undetectable levels. (I) Late-stage 12 *dfr^{E82} / Df(3L)XBB70* embryo also showing a marked reduction in *btl* transcript levels. (J) Late-stage 13/14 wild-type embryo showing the expression of *btl* transcripts throughout the well-developed tracheal branches. (K) Homozygous *Df(3L)XBB70* and (L) *dfr^{E82} / Df(3L)XBB70* embryos at stage 13 showing no detectable *btl* expression.



defects associated with mismigration of mesectodermal progeny in *dfr^{E82}* embryos appear to be largely unaffected by *hs-btl* (data not shown). Identical results were also obtained using an additional *ems*-induced allele, *dfr^{B157}*, which also displays a severely disrupted tracheal phenotype (M. G. Anderson, unpublished results). These results suggest that tracheal defects associated with *dfr* loss-of-function mutations may result from a failure to express the *btl* gene.

Analysis of *btl* transcript levels in *dfr* mutant backgrounds

We have used whole-mount in situ hybridization to examine *btl* expression in a series of genetic backgrounds providing varied levels of DFR activity produced by combinations of the previously characterized *dfr^{E82}* and *Df(3L)XBB70* chromosomes. The *ems*-induced *dfr^{E82}* mutation is caused by a 553 bp deletion in the *dfr* 5' untranslated region, which severely reduces the transcriptional efficiency of the mutated gene allowing production of only trace amounts of functional protein (Anderson et al., 1995; Certel et al., 1996). *Df(3L)XBB70* is a small X-ray-generated deficiency at region 65D uncovering three embryonic lethal complementation groups including *dfr* (Anderson et al., 1995). Examination of the *dfr^{E82}/Df(3L)XBB70* heteroallelic combination along with the respective homozygotes allowed the construction of a dose-response relationship between DFR activity levels and the expression of *btl* mRNA transcripts.

Wild-type embryos express *btl* transcripts at high levels in all tracheal cells as they migrate from stage 10 placodes through successive phases of branch formation (Fig. 3A,D,G,J). In *dfr* mutant backgrounds, initial stage 10 *btl* expression is unaffected in both trachea and mesectoderm (Fig. 3A-C). However, in contrast to wild-type embryos, *btl* tran-

scripts are significantly reduced beginning in stage 12 and rapidly fade to undetectable levels. *Df(3L)XBB70* homozygotes, representing a *dfr* null phenotype, show a rapid reduction in *btl* expression easily visible by early stage 12 with *btl* transcripts undetectable after late stage 12 (Fig. 3E,H,K). The same progressive loss of *btl* expression is observed in the *dfr^{E82}/Df(3L)XBB70* heteroallelic combination but occurs at slightly later developmental stages with detectable staining persisting until stage 13 (Fig. 3F,I,L). The rate of decline in *btl* transcripts is directly proportional to levels of functional DFR protein suggesting that the maintenance of *btl* transcript levels is directly dependent upon DFR.

Our examination of *btl* transcripts in *dfr* mutant embryos has detected an interesting phenomenon in which the first tracheal metamere (Tr1) retains high levels of *btl* expression significantly longer than other tracheal metameres (Fig. 3D-F; hollow arrow). This labeling pattern is consistently observed in both homozygous *Df(3L)XBB70* embryos and heteroallelic *dfr^{E82}/Df(3L)XBB70* embryos throughout stage 12, which coincides with the time overall *btl* levels are in rapid decline (Fig. 3E,F,I). Tr1 contains approximately 150 cells per hemisegment compared to an average of about 90 cells in Tr2-Tr10 (Manning and Krasnow, 1993). However, the markedly higher levels of *btl* expression in Tr1 are not observed in wild-type embryos (Fig. 3A,D,G,J). In addition, *dfr* mutant embryos labeled with other tracheal-specific markers do not reveal an observable loss of cells from Tr2-Tr10 (Anderson et al., 1995) suggesting that variations in cell number are not likely to be fundamentally responsible. Since this distinctive difference in residual *btl* expression occurs in the absence of DFR function, it may reflect tracheal metamere-specific differences in DFR-independent upstream regulators necessary for the initiation of *btl* expression. Our results at this point do not allow us to dis-

tinguish between this possibility and a number of other potential causes.

Direct binding of DFR protein to *btl* regulatory sequences

We have previously described three related but distinct DFR recognition elements found within either the *dfr* autoregulatory enhancer (Certel et al., 1996) or the neuron-specific enhancer of the *dopa decarboxylase* (*ddc*) gene (Johnson and Hirsh, 1990). The DFR binding elements DFRE1 and DFRE2 have been shown to mediate separable tissue-specific functions within the *dfr* autoregulatory enhancer (Certel et al., 1996). DFRE1 (TAATGATATGC) can be classified as a functional TAATGARAT element similar to those found in regulatory sequences of the Herpes Simplex Virus immediate early genes (Herr and Cleary, 1995). DFRE2 (ATGCAAAT) is a consensus octamer element identical to elements known to bind the POU domain (Singh et al., 1986). Element C (CATAAAT) was shown to be essential for neuron-specific expression of the *ddc* gene (Johnson et al., 1989).

We have examined more than 2.2 kb of *btl* 5' flanking DNA to identify DFR recognition elements capable of mediating direct regulation of *btl* expression by the DFR protein. A series of single-end-labeled restriction fragments derived from *btl* 5' flanking sequences between the predicted transcription start site and a *Bam*HI restriction site at -2263 bp was analyzed in DNaseI protection assays using a glutathione-s-transferase (GST)-DFR fusion protein. The GST-DFR protein containing the carboxy-terminal 338 amino acids of the DFR protein, including the entire POU domain, was bacterially expressed and purified using glutathione-agarose. GST-DFR was shown to bind with high affinity to all previously identified DFR recognition elements (data not shown).

Eight distinct DFR binding elements (DBEs) were detected within *btl* 5' regulatory sequences and shown to bind GST-DFR with high affinity (Figs 4, 5). One of these sites, DBE4, binds with relatively low affinity and shows no apparent relationship to known DFR recognition elements other than an AT-rich character. However, as shown in Fig. 5B, the seven high affinity DBEs are similar to previously identified DFR recognition sites and can be placed into two groups containing those closely related to the DFRE2 consensus octamer element (DBE6 and DBE8) or those related to the variant *ddc* Element C (DBE1, DBE2, DBE3, DBE5 and DBE7). The derived consensus sequence for the Element C group [$^{C/A}$ AT (N)₀₋₂ A/T AAT; see Fig. 5B] is identical to the consensus binding site for the Brn-2 protein, a vertebrate POU domain transcription factor highly homologous to DFR (Li et al., 1993). DBE3 shows an unusually large region of DNase protection, which may be due to the presence of two closely associated DFR recognition elements. Although the similarity of identified DBEs to known DFR recognition sites provides evidence that these sites are relevant to *btl* expression, they cannot be definitively evaluated for functional significance based solely upon their ability to bind the DFR protein.

Restoration of tracheal *btl* expression by ubiquitous DFR protein

We have examined the ability of ubiquitous DFR expression produced from a heterologous heat-shock promoter to restore *btl* expression in *dfr* null mutant embryos. By providing

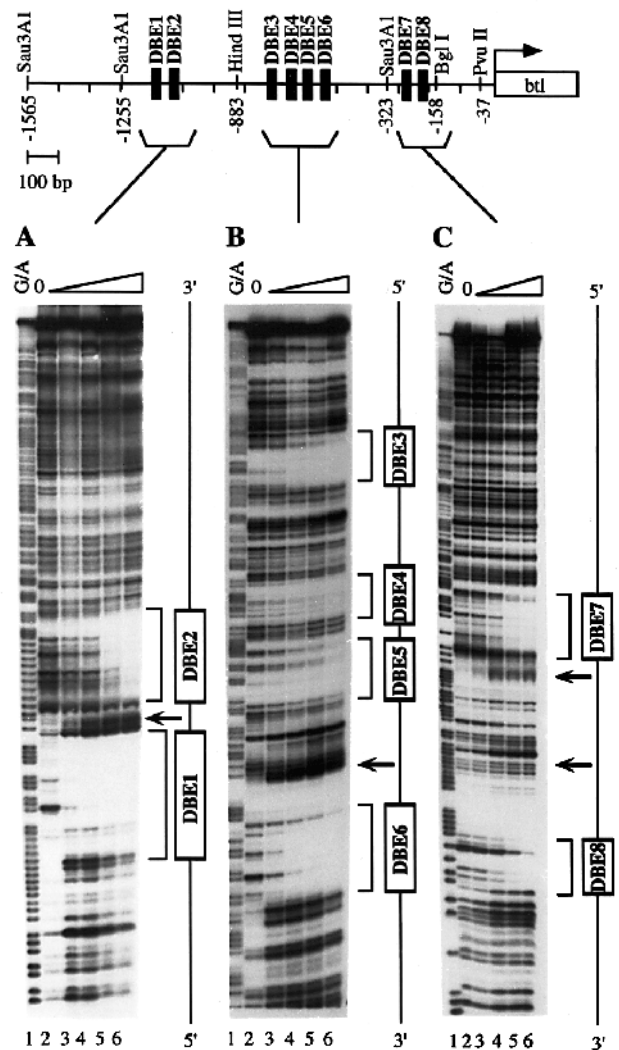
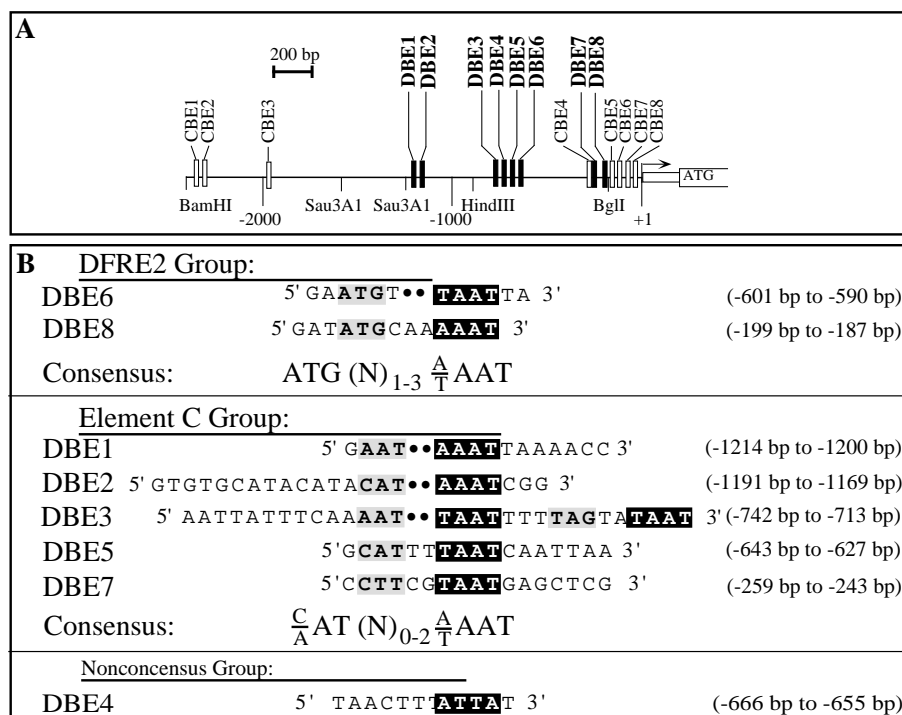


Fig. 4. DFR protein binding to *btl* regulatory sequences. (Top) Schematic representation of *btl* 5' flanking sequences showing the positions of DFR binding elements (DBE) indicated as rectangles numbered from 5' to 3' with DBE1 located most distal and DBE8 closest to the transcription start site (arrow). (Below) Autoradiographs produced from DNaseI protection assays showing eight distinct regions of *btl* 5' flanking DNA protected by increasing concentrations of purified GST-DFR fusion protein. Protected regions are indicated by brackets and hypersensitive sites by arrows. (A) *btl* probe -1255 to -883 bp, (B) *btl* probe -882 to -323 bp, (C) *btl* probe -322 to +45 bp. Lane 1 of each gel, Maxam-Gilbert purine cleavage reaction; lane 2, no protein control; lane 3, 36 ng protein; lane 4, 108 ng protein; lane 5, 216 ng protein; and lane 6, 360 ng protein.

ectopic DFR at a relatively late stage of development when *dfr* mutant embryos normally express no detectable *btl* transcripts, these experiments should demonstrate a direct response of the *btl* transcription unit to the DFR transactivator. We have utilized a previously characterized *hs-dfr* transposon shown to produce ubiquitous high levels of DFR protein in response to heat shock (Certel and Johnson, 1996). In the absence of heat-shock, stage 13 homozygous *hs-dfr; Df(3L)XBB70* embryos show no detectable *btl* expression (Fig. 6A). However, after receiving a single heat-shock 30 minutes prior to fixation and

Fig. 5. Sequence alignment of Drifter binding elements identified in *btl* 5' regulatory DNA. (A) Schematic representation of *btl* 5' regulatory sequence with the transcription startsite indicated by the arrow and the translation startsite by ATG. Identified DBEs are indicated by black boxes labeled as DBE1 through DBE8 and the previously characterized CBEs by white boxes labeled as CBE1 through CBE8. (B) Sequence alignment of DBE elements classified as most similar to either the consensus octamer DFRE2 or the variant Element C found within the *ddc* neuron-specific enhancer. Nucleotides predicted to be bound by the DFR POU_S or POU_H are highlighted with gray or black boxes, respectively. The single low affinity site DBE4 is also included as the sole member of a nonconsensus group showing limited similarity to known DFR recognition elements. The location of each DBE is indicated at right as a range of nucleotides relative to the *btl* transcription startsite.



labeling, similarly staged *hs-dfr; Df(3L)XBB70* embryos display a significant induction of tracheal-specific *btl* transcripts (Fig. 6B). Although, as expected, the single pulse of heat-induced DFR expression at this late stage was unable to rescue the *Df(3L)XBB70* tracheal mutant phenotype (Reichman-Fried et al., 1994; Reichman-Fried and Shilo, 1995), the close temporal correlation between DFR expression and the reappearance of *btl* transcripts is consistent with a direct regulatory relationship. DFR-induced *btl* expression is limited to tracheal cells despite the ubiquitous presence of DFR protein. These results suggest that DFR is capable of restoring tracheal *btl* expression but may require the function of an additional tracheal-specific coactivator. This observation is consistent with our previous demonstration that when expression of

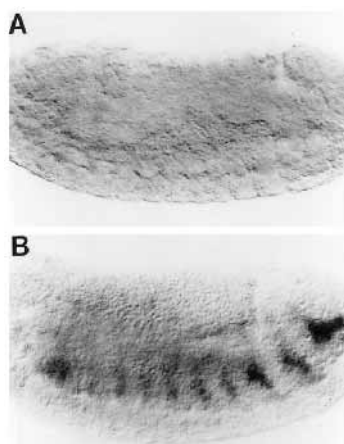
dfr-lacZ transgenes containing tracheal autoregulatory enhancer sequences is examined in embryos ubiquitously expressing DFR protein, a tissue-specific expression pattern is maintained (Certelet et al., 1996).

DISCUSSION

The *dfr* and *btl* genes are both expressed throughout tracheal differentiation and have been shown to be essential for tracheal cell migration (Glazer and Shilo, 1991; Anderson et al., 1995). As demonstrated here, the severe tracheal defects seen in homozygous *dfr*^{E82} embryos can be rescued by ubiquitous BTL expression suggesting that the *dfr* loss-of-function phenotype results from a failure to express BTL. After early stage 12 of embryogenesis, which coincides with the time tracheal cells normally begin migrating, *dfr* mutant embryos produce drastically reduced levels of *btl* transcript. Prior to this point, however, *btl* expression levels are near normal, even in the absence of DFR activity. This implies that tracheal-specific expression of both *btl* and *dfr* is dependent upon separable regulatory events necessary for initiation (Fig. 7A) and maintenance (Fig. 7B) of expression.

Several genes affecting embryonic pattern formation are expressed in tracheal cell precursors and are excellent candidates for initiators of early tracheal-specific gene expression. Previous results have shown that permutations of the anterior-posterior axis mediated by *wingless* or the dorsal-ventral axis by *decapentaplegic* can alter the tracheal expression boundaries of DFR and other early acting tracheal-specific proteins such as Trachealeless (TRH) (de Celis et al., 1995; Isaac and Andrew, 1996; Wilk et al., 1996). Therefore, it is likely that the initiation phase of expression utilizes transient pattern formation signals from both major *btl* developmental axes. In addition, the extended retention of *btl* transcripts in Tr1 of *dfr*

Fig. 6. Restoration of tracheal *btl* transcripts by ubiquitous DFR expression. Staged embryos were labeled by in situ hybridization using a DIG-labeled *btl* RNA probe. Embryos are shown as a lateral view with anterior to the left and dorsal up. (A) Non-heat-shocked stage 13 homozygous *hs-dfr; Df(3L)XBB70* embryo showing undetectable levels of *btl* transcript. (B) Stage 13 homozygous *hs-dfr; Df(3L)XBB70* embryo after exposure to a single one hour heat-shock 30 minutes prior to fixation and labeling. Induced *btl* transcripts are visible as metamericly repeated dark patches representing deformed tracheal tissue.



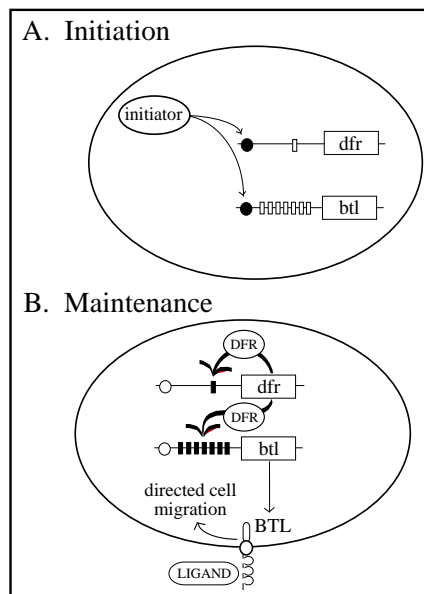


Fig. 7. Maintenance of BTL expression in migrating tracheal cells by autoregulated DFR. (A) Both *btl* and *dfr* gene expression is initiated by an as yet unidentified transient pattern formation signal. (B) DFR protein maintains its own expression through a tracheal-specific autoregulatory enhancer. Sustained levels of DFR protein maintain high levels of the BTL receptor tyrosine kinase by direct transcriptional activation mediated by multiple high affinity DFR recognition elements within *btl* 5' regulatory sequences. This coordinated relationship between the *dfr* and *btl* genes provides sustained high levels of BTL receptor tyrosine kinase for detection of extracellular cues necessary for directed tracheal cell migrations.

mutant embryos suggests that the exact combination of initiator pattern signals may vary from segment to segment. Although upstream regulators have not been definitively identified, the initial tracheal expression of both *btl* and *dfr* may result from the same patterning signals.

Once the transient initiation signal has subsided, our results suggest that the activated BTL receptor tyrosine kinase is unable to either directly or indirectly activate its own expression. However, maintained expression of the BTL receptor is necessary not only for primary branch formation but also for activation of genes required for formation of the secondary and terminal tracheal branches (Samakovlis et al., 1996). Consequently, the *btl* gene must rely upon autoregulated DFR protein to sustain expression during later developmental stages. The presence of seven high affinity DFR recognition elements within *btl* regulatory sequences is consistent with the DFR protein acting as a direct transcriptional regulator necessary for maintenance of tracheal *btl* expression. There does not appear to be a DFR requirement for either initiation or maintenance of mesectodermal *btl* expression. Although our results demonstrate that at least one essential function of DFR in migrating tracheal cells is to maintain high levels of the BTL receptor tyrosine kinase at the cell surface, our experiments do not rule out the possibility that DFR protein may regulate additional tracheal-specific target genes or that the activated BTL signal transduction cascade might eventually feed back to regulate DFR activity.

DFR recognition elements

The POU domain DNA-binding motif has been shown to display a profound flexibility for binding to variant recognition elements (Li et al., 1993; Cleary and Herr, 1995; Herr and Cleary, 1995). This is thought to be due primarily to the bipartite structure of the POU domain itself, which consists of the POU-specific domain (POU_S) and the POU-homeodomain (POU_H) connected by a variable linker region (Herr and Cleary, 1995). Both the POU_S and POU_H are necessary for high affinity recognition of specific sequence elements. This distinctive flexibility in site recognition results in a number of related POU-domain binding elements which, although variant, show certain common characteristics (Herr and Cleary, 1995). Studies of Oct-1 binding to the ATGCAAAT octamer element suggest that the POU_H recognizes the ^A/_T AAT sequence normally found at the 3' end of most POU recognition elements (Klemm et al., 1994; Herr and Cleary, 1995). The POU_S binds to the ATGC sequence located at the 5' end of the octamer element. However, the relative orientation and spacing of the POU_S and POU_H recognition elements can vary markedly between binding sites showing comparable levels of binding affinity for the same POU domain protein (Li et al., 1993; Cleary and Herr, 1995; Herr and Cleary, 1995). This somewhat promiscuous ability of the POU domain to recognize variant sequence elements raises concern over the relevance of DFR recognition elements identified solely by their ability to bind the DFR protein in DNA-binding assays. Lacking *in vivo* studies demonstrating the functional significance of DFR recognition elements detected in *btl* 5' regulatory sequences, we have presented evidence showing that these sites are very similar to previously characterized binding sites identified for the DFR protein.

Two of the DFR recognition elements identified in *btl* regulatory sequences (DBE6 and DBE8; see Fig. 5) are similar to the consensus octamer element, DFRE2, found within the *dfr* autoregulatory enhancer (Certel et al., 1996). The remainder can be closely aligned with the previously characterized DFR binding site designated as Element C within the neuron-specific enhancer of the *ddc* gene (Johnson and Hirsh, 1990). Both Element C and the new binding sites described here conform to a consensus sequence [^C/_A AT (N)₀₋₂ ^A/_T AAT] in which the presumed POU_S subelement (ATGC) is inverted relative to the POU_H binding sequences. This inverted sequence could potentially alter the relative orientations of the DNA-bound POU_S and POU_H protein domains. Such site-dependent alterations in DNA-bound protein conformation have been shown to contribute to the tissue-specific regulation of target genes by the mammalian POU domain factor Pit-1 by altering protein-protein interactions (Holloway et al., 1992). In addition, the same variant sequence conforms to the consensus recognition sequence for the mammalian POU-III class transcription factor Brn-2 (Li et al., 1993).

It is significant that none of the identified *btl* DBEs can be classified as a TAATGARAT element similar to the DFRE1 sequence element found within the *dfr* autoregulatory enhancer and previously shown to specifically mediate DFR transactivation in the middle pair of midline glia of the ventral nerve cord (Certel et al., 1996). The absence of TAATGARAT-like DFR response elements within a tracheal-specific target gene is consistent with the previously characterized functional characteristics of DFRE1 and DFRE2 (Certel et al., 1996).

Although the functional significance of the *btl* DBE sites remains to be tested, the structural conservation of these sites compared to previously characterized DFR recognition elements is consistent with DFR function as a direct regulator of *btl* expression in the developing trachea.

Autoregulation as a mechanism for commitment to tracheal differentiation

Autoregulation of tissue-specific transcription factors has been observed for a number of genes involved in the commitment of cells to organogenesis and may be a fundamental component of numerous regulatory pathways. This includes certain genes thought to function as master regulators of tissue-specific differentiation such as *single-minded* (Nambu et al., 1991), *myoD* (Thayer et al., 1989) and *pit-1* (Rhodes et al., 1993; Chen et al., 1994). It is reasonable to assume that the maintained expression of certain essential regulators may be necessary for the irreversible commitment of cells to a particular tissue phenotype.

Recent observations suggest that the autoregulation of early acting genes may also be an important characteristic of transcription factors expressed throughout the process of tracheal differentiation. The helix-loop-helix transcription factor TRH, which is also expressed continually throughout tracheal morphogenesis, has been shown to regulate its own expression in trachea after stage 12 (Wilk et al., 1996). Tracheal cells do not migrate in homozygous *trh* mutants, however, certain tracheal markers continue to be expressed indicating that the cells have assumed tracheal fates (Isaac and Andrew, 1996; Wilk et al., 1996). Expression of DFR is unaffected in *trh* mutant embryos, suggesting that the *dfr* gene is not regulated by TRH (Isaac and Andrew, 1996). It is also unlikely that DFR directly regulates *trh* since the *trh* mutant phenotype is more severe than the *dfr* mutant phenotype and becomes apparent at an earlier developmental stage (Isaac and Andrew, 1996; Wilk et al., 1996). While the precise relationship of these two genes remains to be determined, it appears that the presence of both is required continually throughout tracheal morphogenesis.

Transcriptional regulators such as DFR and TRH can easily autoactivate; however, other genes encoding cell surface receptors, such as BTL or cytoplasmic signaling molecules, lack the ability to directly maintain their own expression. Although these proteins could potentially feed back to indirectly influence their own transcriptional activity, they presumably must often rely upon other means to sustain expression levels throughout development. Consequently, the functional relationship between DFR and BTL is a very interesting one in which evolutionary pressures appear to have provided a mechanism for BTL autoregulation by proxy utilizing the autoregulated DFR protein.

Ability of tracheal cells to migrate versus tracheal differentiation

Activation of the BTL signal transduction pathway and the resulting patterned tracheal cell movements are thought to be primarily dependent upon the restricted distribution of an endogenous BTL ligand. This is based upon the observation that ubiquitous expression of wild-type BTL does not deleteriously affect tracheal cell migration (Reichman-Fried et al., 1994; Murphy et al., 1995) but ectopic expression of a constitutively active form of BTL results in disruption of migration

patterns (T. Lee and D. Montell, unpublished data). Therefore, the DFR-dependent maintenance of cell-specific BTL expression provides cells with the ability to respond to extracellular migratory cues but does not account for all aspects of tracheal differentiation. This is readily apparent in tracheal cells lacking BTL function, which still express a number of tracheal-specific marker genes (Samakovlis et al., 1996) even though they are unable to migrate. These results imply the existence of separable regulatory pathways mediating BTL-dependent tracheal cell migration and BTL-independent differentiation.

Expression of the BTL receptor may be associated with regulated cell migration in a number of different tissues as demonstrated by the characterization of BTL function in ovarian follicle cells. Expression of the *btl* gene in ovarian follicle cells is dependent upon function of the *Drosophila* C/EBP homologue encoded by the *slow border cells* (*slbo*) gene (Murphy et al., 1995). The *Drosophila* C/EBP protein is necessary for proper migration of a subset of ovarian follicle cells referred to as border cells, which normally migrate from the anterior tip of the developing egg chamber to a position over 15 cell diameters away at the oocyte-nurse cell boundary (Montell et al., 1992; Montell, 1994). Recent results have shown that lethal alleles of *btl* are dominant enhancers of some *slbo* alleles and that ubiquitous BTL expression can rescue border cell migration defects associated with *slbo* mutants (Murphy et al., 1995). In addition, a series of eight C/EBP binding elements (CBEs) were previously identified within the same 2.2 kb region of *btl* regulatory sequence shown here to bind DFR protein (Murphy et al., 1995). The seven DBEs identified here are distinct from the eight previously identified CBEs (Murphy et al., 1995) and, based upon DNA-binding activity alone, we have no evidence that the DFR and C/EBP binding sites functionally interact. These results suggest, however, that the DFR and C/EBP proteins may play parallel roles in the trachea and ovary respectively and that the BTL protein is a central component of a universal mechanism utilized for the developmental control of cell migration.

However, the distinction between regulators of cell migration and mediators of tracheal cell differentiation may not be clear since certain tracheal-specific genes necessary for formation of secondary and terminal branches do require BTL function (Samakovlis et al., 1996). This group includes the ets-domain transcription factor Pointed (PNT) (Klämbt, 1993; Scholz et al., 1993) previously shown to be phosphorylated by MAP-kinase in response to activation of the Sevenless receptor tyrosine kinase in the *Drosophila* eye (Brunner et al., 1994). The PNT protein presumably functions in a similar manner as a downstream effector of the BTL signal transduction cascade in tracheal cells. The modified PNT protein could, therefore, play a role as a potential mediator of certain BTL-dependent components of tracheal differentiation.

BTL-independent tracheal differentiation could result from activation of additional target genes by tracheal-specific DFR protein, although this possibility seems unlikely considering the nearly complete restoration of tracheal morphology in *dfr* mutants by ubiquitous BTL expression alone (Fig. 2). Alternatively, these components of tracheal cell identity could be derived in response to expression of TRH, which is thought to function in parallel to the DFR/BTL pathway (Isaac and Andrew, 1996; Wilk et al., 1996). Regardless of the specific

regulator(s) involved, specification of tracheal cell identities is undoubtedly a multicomponent process involving a relatively large number of gene products (Samakovlis et al., 1996). Results presented here, demonstrating a requirement for DFR protein in the maintenance of *btl* expression, represent an initial step in understanding the complex functional relationship between these essential participants in tracheal organogenesis.

We thank Nir Hacohen and Mark Krasnow for tracheal-specific antibodies and Benny Shilo for *breathless* cDNA. For helpful comments on the manuscript, we also thank Greg Petersen and Robert Watson. This work was supported by Public Health Service grant NS28743 (W. J.) from the National Institute of Neurological Disorders and Stroke and by NIH Grant DK25295 (W. J.).

REFERENCES

- Affolter, M., Nellen, D., Nussbaumer, U. and Basler, K. (1994). Multiple requirements for the receptor serine/threonine kinase *thick veins* reveal novel functions of TGF beta homologs during *Drosophila* embryogenesis. *Development* **120**, 3105-3117.
- Anderson, M. G., Perkins, G. L., Chittick, P., Shrigley, R. J. and Johnson, W. A. (1995). *drifter*, a *Drosophila* POU-domain transcription factor, is required for correct differentiation and migration of tracheal cells and midline glia. *Genes Dev.* **9**, 123-137.
- Beitel, G. J., Tuck, S., Greenwald, I. and Horvitz, H. R. (1995). The *Caenorhabditis elegans* gene *lin-1* encodes an ETS-domain protein and defines a branch of the vulval induction pathway. *Genes Dev.* **9**, 3149-3162.
- Bray, S. J. and Kafatos, F. C. (1991). Developmental function of Elf-1: an essential transcription factor during embryogenesis in *Drosophila*. *Genes Dev.* **5**, 1672-1683.
- Brunner, D., Ducker, K., Oellers, N., Hafen, E., Scholz, H. and Klämbt, C. (1994). The ETS domain protein pointed-P2 is a target of MAP kinase in the sevenless signal transduction pathway. *Nature* **370**, 386-389.
- Campos-Ortega, J. A. and Hartenstein, V. (1985). *The Embryonic Development of Drosophila melanogaster*. Berlin: Springer-Verlag.
- Certel, K., Anderson, M. G., Shrigley, R. J. and Johnson, W. A. (1996). Distinct variant DNA-binding sites determine cell-specific autoregulated expression of the *Drosophila* POU domain transcription factor Drifter in midline glia or trachea. *Mol. Cell. Biol.* **16**, 1813-1823.
- Certel, S. J. and Johnson, W. A. (1996). Disruption of mesectodermal lineages by temporal misexpression of the *Drosophila* POU-domain transcription factor, drifter. *Dev. Genet.* **18**, 279-288.
- Chen, X., Reynolds, E. R., Ranganayakulu, G. and O'Donnell, J. M. (1994). A maternal product of the Punch locus of *Drosophila melanogaster* is required for precellular blastoderm nuclear divisions. *J. Cell Sci.* **107**, 3501-3513.
- Cleary, M. A. and Herr, W. (1995). Mechanisms for flexibility in DNA sequence recognition and VP16-induced complex formation by the Oct-1 POU domain. *Mol. Cell. Biol.* **15**, 2090-2100.
- Davis, R. L., Weintraub, H. and Lassar, A. B. (1987). Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell* **51**, 987-1000.
- de Celis, J. F., Llimargas, M. and Casanova, J. (1995). ventral veinless, the gene encoding the Cfla transcription factor, links positional information and cell differentiation during embryonic and imaginal development in *Drosophila melanogaster*. *Development* **121**, 3405-3416.
- Glazer, L. and Shilo, B. Z. (1991). The *Drosophila* FGF-R homolog is expressed in the embryonic tracheal system and appears to be required for directed tracheal cell extension. *Genes Dev.* **5**, 697-705.
- Guillemin, K., Groppe, J., Ducker, K., Treisman, R., Hafen, E., Affolter, M. and Krasnow, M. A. (1996). The *pruned* gene encodes the *Drosophila* serum response factor and regulates cytoplasmic outgrowth during terminal branching of the tracheal system. *Development* **122**, 1353-1362.
- Halder, G., Callaerts, P. and Gehring, W. J. (1995). Induction of ectopic eyes by targeted expression of the *eyeless* gene in *Drosophila*. *Science* **267**, 1788-1792.
- Herr, W. and Cleary, M. A. (1995). The POU domain: Versatility in transcriptional regulation by a flexible two-in-one DNA-binding domain. *Genes Dev.* **9**, 1679-1693.
- Holloway, J. M., Szeto, D. P., Scully, K. M., Glass, C. K. and Rosenfeld, M. G. (1992). Pit-1 binding to specific DNA sites as a monomer or dimer determines gene-specific use of a tyrosine-dependent synergy domain. *Genes Dev.* **9**, 1992-2006.
- Isaac, D. D. and Andrew, D. J. (1996). Tubulogenesis in *Drosophila*: A requirement for the *trachealless* gene product. *Genes Dev.* **10**, 103-117.
- Johnson, W. A. (1992). Characterization of Neuron-Specific Transcription Factors in *Drosophila melanogaster*. *Methods Neurosci.* **9**, 362-380.
- Johnson, W. A. and Hirsh, J. (1990). Binding of a *Drosophila* POU-domain protein to a sequence element regulating gene expression in specific dopaminergic neurons. *Nature* **343**, 467-470.
- Johnson, W. A., McCormick, C. A., Bray, S. J. and Hirsh, J. (1989). A neuron-specific enhancer of the *Drosophila* dopa decarboxylase gene. *Genes Dev.* **3**, 676-686.
- Kayne, P. S. (1994). Knocking down a pathway to build it up. *Trends Genet.* **10**, 145-147.
- Klämbt, C. (1993). The *Drosophila* gene *pointed* encodes two ETS-like proteins which are involved in the development of the midline glial cells. *Development* **117**, 163-176.
- Klämbt, C., Glazer, L. and Shilo, B. Z. (1992). *breathless*, a *Drosophila* FGF receptor homolog, is essential for migration of tracheal and specific midline glial cells. *Genes Dev.* **6**, 1668-1678.
- Klemm, J. D., Rould, M. A., Aurora, R., Herr, W. and Pabo, C. O. (1994). Crystal structure of the Oct-1 POU domain bound to an octamer site: DNA recognition with tethered DNA-binding modules. *Cell* **77**, 21-32.
- Li, P., He, X., Gerrero, M. R., Mok, M., Aggarwal, A. and Rosenfeld, M. G. (1993). Spacing and orientation of bipartite DNA-binding motifs as potential functional determinants for POU domain factors. *Genes Dev.* **7**, 2483-2496.
- Lindsley, D. L. and Zimm, G. G. (1992). *The Genome of Drosophila melanogaster*. San Diego, California: Academic Press.
- Manning, G. and Krasnow, M. A. (1993). Development of the *Drosophila* tracheal system. In *The development of Drosophila melanogaster* (ed. M. Bate and A. Martinez-Arias), Vol. 1, pp. 609-685. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.
- Mitchison, T. J. and Sedat, J. (1983). Localization of antigenic determinants in whole *Drosophila* embryos. *Dev. Biol.* **99**, 261-264.
- Montell, D. J. (1994). Moving right along: regulation of cell migration during *Drosophila* development. *Trends Genet.* **10**, 59-62.
- Montell, D. J., Rørth, P. and Spradling, A. C. (1992). *slow border cells*, a locus required for a developmentally regulated cell migration during oogenesis, encodes *Drosophila* C/EBP. *Cell* **71**, 51-62.
- Murphy, A. M., Lee, T., Andrews, C. M., Shilo, B. Z. and Montell, D. J. (1995). The *Breathless* FGF receptor homolog, a downstream target of *Drosophila* C/EBP in the developmental control of cell migration. *Development* **121**, 2255-2263.
- Nambu, J. R., Lewis, J. O., Wharton, K. J. and Crews, S. T. (1991). The *Drosophila single-minded* gene encodes a helix-loop-helix protein that acts as a master regulator of CNS midline development. *Cell* **67**, 1157-1167.
- O'Neill, E. M., Rebay, L., Tjian, R. and Rubin, G. M. (1994). The activities of two Ets-related transcription factors required for *Drosophila* eye development are modulated by the Ras/MAPK pathway. *Cell* **78**, 137-147.
- O'Neill, J. W. and Bier, E. (1994). Double-label in situ hybridization using biotin and digoxigenin-tagged RNA probes. *Biotechniques* **17**, 874-875.
- Pulverer, B. J., Kyriakis, J. M., Avruch, J., Nikolakaki, E. and Woodgett, J. R. (1991). Phosphorylation of *c-jun* mediated by MAP kinases. *Nature* **353**, 670-674.
- Reichman-Fried, M., Dickson, B., Hafen, E. and Shilo, B. Z. (1994). Elucidation of the role of *breathless*, a *Drosophila* FGF receptor homolog, in tracheal cell migration. *Genes Dev.* **8**, 428-439.
- Reichman-Fried, M. and Shilo, B. Z. (1995). *Breathless*, a *Drosophila* FGF receptor homolog, is required for the onset of tracheal cell migration and tracheole formation. *Mech. Dev.* **52**, 265-273.
- Rhodes, S. J., Chen, R., DiMattia, G. E., Scully, K. M., Kalla, K. A., Lin, S. C., Yu, V. C. and Rosenfeld, M. G. (1993). A tissue-specific enhancer confers Pit-1-dependent morphogen inducibility and autoregulation on the *pit-1* gene. *Genes Dev.* **7**, 913-932.
- Rubin, G. M. (1991). Signal transduction and the fate of the R7 photoreceptor in *Drosophila*. *Trends Genet.* **7**, 372-377.
- Samakovlis, C., Hacohen, N., Manning, G., Sutherland, D. C., Guillemin, K. and Krasnow, M. A. (1996). Development of the *Drosophila* tracheal system occurs by a series of morphologically distinct but genetically coupled branching events. *Development* **122**, 1395-1407.
- Schlessinger, J. and Ullrich, A. (1992). Growth factor signaling by receptor tyrosine kinases. *Neuron* **9**, 383-391.

- Scholz, H., Deatrick, J., Klaes, A. and Klämbt, C.** (1993). Genetic dissection of *pointed*, a *Drosophila* gene encoding two ETS-related proteins. *Genetics* **135**, 455-468.
- Singh, H., Sen, R., Baltimore, D. and Sharp, P. A.** (1986). A nuclear factor that binds to a conserved sequence motif in transcriptional control elements of immunoglobulin genes. *Nature* **319**, 154-158.
- Thayer, M. J., Tapscott, S. J., Davis, R. L., Wright, W. E., Lassar, A. B. and**

- Weintraub, H.** (1989). Positive autoregulation of the myogenic determination gene MyoD1. *Cell* **58**, 241-248.
- Wilk, R., Weizman, I. and Shilo, B. Z.** (1996). *tracheiless* encodes a bHLH-PAS protein that is an inducer of tracheal cell fates in *Drosophila*. *Genes Dev.* **10**, 93-102.

(Accepted 5 September 1996)