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

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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; Guillemin 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; Klabunde 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 Lindsey 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 antisemur (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 antisemur (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 antisemur was generously provided by Nir Hacohen and Mark Krasnow. DFR protein distribution was visualized by labeling with a polyclonal DFR-antisemur generated against a glu-tathione-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-ida grouse immunochemistry 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 antisemur to detect the absence of the *fx*-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 *dfr*-specific DIG-UTP RNA probes were generated by in vitro transcription of a 2.2 kb *dfr* cDNA fragment kindly provided by Benny Shilo (Glazer and Shilo, 1991; Klabunde et al., 1992). The *dfr* 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 (Cerlet and Johnson, 1996) under the control of the heat-inducible hsp70 promoter has been previously described. Embryos produced from *hs-btl, dfr* / *TM3, Sb ftzlacZ* adults were collected on yeasted 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)Xrb870 / TM3, Sb ftzlacZ* adult 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.

**DNasel 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 BamHI site at +949 bp to a synthetic EcoRI 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 65×10^3 M.<sup>*</sup>

Concentrations of purified proteins were quantitated using the Bio-Rad protein assay system and used in DNasel protection assays as
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 expressed 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 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 tissuespecific 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 (Klambt 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 dfrE82 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; dfrE82 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

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**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 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).
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<sup>E82</sup> / Df(3L)XBB70 (C,F,IL) 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<sup>E82</sup> / 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<sup>E82</sup> / Df(3L)XBB70 embryos at stage 13 showing no detectable btl expression.

defects associated with mismigration of mesectodermal progeny in dfr<sup>E82</sup> embryos appear to be largely unaffected by hs-btl (data not shown). Identical results were also obtained using an additional ems-induced allele, dfr<sup>B157</sup>, 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<sup>E82</sup> and Df(3L)XBB70 chromosomes. The ems-induced dfr<sup>E82</sup> 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 5D uncovering three embryonic lethal complementation groups including dfr (Anderson et al., 1995). Examination of the dfr<sup>E82</sup>/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 transcripts 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<sup>E82</sup>/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<sup>E82</sup>/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 di-
tistinguish 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 (Johnston and Hirsh, 1990). The DFR binding elements DFR1 and DFR2 have been shown to mediate separable tissue-specific functions within the *dfr* autoregulatory enhancer (Certel et al., 1996). DFR1 (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). DFR2 (ATGCAAAT) is a consensus octamer element identical to elements known to bind the POU domain (Singh et al., 1986). Element C (CATAAAAT) 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 *BamHI* 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 DFR2 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 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 (Cerlet 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 Drf 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 dalm neuron-specific enhancer. Nucleotides predicted to be bound by the DFR POUS or POUH 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.

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 metamerically repeated dark patches representing deformed tracheal tissue.

dfr-lacZ transgenes containing tracheal autoregulatory enhancer sequences is examined in embryos ubiquitously expressing DFR protein, a tissue-specific expression pattern is maintained (Certel 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 homzygous dfrE82 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 Tracheless (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 developmental axes. In addition, the extended retention of btl transcripts in Tr1 of dfr
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* 5′ 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 (POUs) and the POU-homeodomain (POUH) connected by a variable linker region (Herr and Cleary, 1995). Both the POUs and POUH 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 POUH recognizes the ATG /AAT sequence normally found at the 3′ end of most POU recognition elements (Klemm et al., 1994; Herr and Cleary, 1995). The POUs binds to the ATGC sequence located at the 5′ end of the octamer element. However, the relative orientation and spacing of the POUs and POUH 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, DFR2, 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 POUs subelement (ATGC) is inverted relative to the POUH binding sequences. This inverted sequence could potentially alter the relative orientations of the DNA-bound POUs and POUH 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 DFRB1 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 DFRB1 and DFRB2 (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 TTL 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 TTL is a very interesting one in which evolutionary pressures appear to have provided a mechanism for TTL autoregulation by proxy utilizing the autoregulated DFR protein.

**Ability of tracheal cells to migrate versus tracheal differentiation**

Activation of the TTL signal transduction pathway and the resulting patterned tracheal cell movements are thought to be primarily dependent upon the restricted distribution of an endogenous TTL ligand. This is based upon the observation that ubiquitous expression of wild-type TTL 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 TTL results in disruption of migration patterns (T. Lee and D. Montell, unpublished data). Therefore, the DFR-dependent maintenance of cell-specific TTL 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 TTL 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 TTL-dependent tracheal cell migration and TTL-independent differentiation.

Expression of the TTL receptor may be associated with regulated cell migration in a number of different tissues as demonstrated by the characterization of TTL 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 TTL 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 TTL 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 TTL function (Samakovlis et al., 1996). This group includes the ecdysone domain transcription factor Pointed (PNT) (Klambt, 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 TTL signal transduction cascade in tracheal cells. The modified PNT protein could, therefore, play a role as a potential mediator of certain TTL-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 TTL 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/TTL 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 breastless 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.).

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(Accepted 5 September 1996)