**drifter**, a *Drosophila* POU-domain transcription factor, is required for correct differentiation and migration of tracheal cells and midline glia

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The *Drosophila drifter (dfr)* gene, previously referred to as *Cfla*, encodes a POU-domain DNA-binding protein implicated as a neuron-specific regulator in the developing central nervous system (CNS). We have isolated full-length *dfr* cDNA clones that encode a 46-kD protein containing the conserved POU-domain DNA-binding domain. The use of alternate polyadenylation sites produces two *dfr* mRNA transcripts that are first expressed in stage 10 embryos at 5- to 6-hr of development. A specific anti-dfr polyclonal antiserum generated against a *dfr*-glutathione S-transferase fusion protein recognizes a 46-kD protein on Western blots and has been used to analyze the cell-specific distribution of dfr protein during embryonic development. dfr protein is distributed in a complex expression pattern including the tracheal system, the middle pair of midline glia, and selected CNS neurons. We have carried out a genetic characterization of the *dfr* locus, previously localized to region 65D of the third chromosome, by generating a series of overlapping deficiencies between 65A and 65E1 that were used to isolate *dfr*<sup>ER2</sup>, an EMS-induced lethal allele. Analysis of *dfr*<sup>ER2</sup> mutant embryos shows a disruption of the developing tracheal tree as well as commissural defects in the developing CNS. Based on an examination of a cell-specific marker for tracheal cells and midline glia, these defects appear to be caused by a failure of these cells to follow their characteristic routes of migration. The *dfr*<sup>ER2</sup> tracheal phenotype is rescued by a *dfr* minigene present as a P-element transposon expressing wild-type *dfr* protein in tracheal cells. These results suggest that the dfr protein plays a fundamental role in the differentiation of tracheal cells and midline glia possibly by regulating the expression of essential cell-surface proteins required for cell–cell interactions involved in directed cell migrations.

**Key Words:** *Drosophila*; neuron; glia; POU domain; cell migration; trachea

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Numerous structural classes of DNA-binding proteins that function as transcriptional activators or repressors have been identified. One of the first such protein families to be recognized included regulators containing a conserved homeo domain DNA-binding domain [for review, see Scott et al. 1989]. Many members of this family were first characterized as essential embryonic pattern formation genes in *Drosophila melanogaster* [Scott et al. 1989] but have subsequently been shown to be both structurally and functionally conserved across species boundaries [McGinnis and Krumlauf 1992; Tabin 1992; Krumlauf 1993].

A subclass of homeo domain containing proteins designated as POU factors have been shown to play essential functions in cell-lineage determination in mammals and nematode [Herr et al. 1988; Ruvkun and Finney 1991]. The POU domain includes both a diverged homeo domain and an additional POU-specific domain that function together as a bipartite DNA-binding domain [Sturm and Herr 1988; Verrijzer et al. 1990; Assa-Munt et al. 1993; Li et al. 1993; Klemm et al. 1994]. Since recognition of the conserved POU-domain structure [Herr et al. 1988], the number of family members has expanded drastically with the identification of numerous murine POU factors [Scholer 1991; Hara et al. 1992; Stoykova et al. 1992], as well as additional genes from *Xenopus* [Agarwal and Sato 1991; Baltzinger et al. 1992; Frank and Harland 1992; Hinkley et al. 1992; Whitfield et al. 1993], zebrafish [Matsuzaki et al. 1992; Johansen et al. 1993; Takeda et al. 1994], nematode [Finney et al. 1988; Burglin et al. 1989] and human [Schreiber et al. 1993; Tobler et al. 1993].

Previously, we identified the *Cf1a* POU-domain gene from *Drosophila melanogaster* on the basis of its ability to bind to a neuron-specific regulatory element, element
Cf1a of the dopa decarboxylase (Ddc) gene [Johnson et al. 1989; Johnson and Hirsh 1990]. We have renamed Cf1a as the drifter (dfr) locus on the basis of results from analysis of an ethylmethane sulfonate (EMS)-induced lethal allele, dfr<sup>ER2</sup>, described in this paper. Partial Cf1a/dfr cDNAs have also been recovered, along with additional Drosophila POU-domain genes, in polymerase chain reaction (PCR)-based screens [Billin et al. 1991; Dick et al. 1991; Treacy et al. 1991]. After numerous screens, only four Drosophila POU-domain genes have been identified. In addition to Cf1a/dfr, these include two closely linked loci at 33F on the second chromosome, pdm1/dPOU-19 and pdm2/dPOU-28 [Billin et al. 1991; Dick et al. 1991; Prakash et al. 1992] and the I-POU/tl-POU gene located at 13C1-3 on the X chromosome [Treacy et al. 1991; 1992]. The relative simplicity of the Drosophila POU family may be an asset in the genetic analysis of POU–protein interactions during development, which may be highly conserved between species.

Many known POU factors are expressed in cell- and/or region-specific patterns within the developing central nervous system (CNS), suggesting an evolutionarily conserved role in neural development [He et al. 1989; Monuki et al. 1990; Collum et al. 1992; Andersen et al. 1993]. Using genetic analysis, antisense technology [Fuji and Hamada 1993], or overexpression experiments [Monuki et al. 1990; He et al. 1991], functional evidence has been gathered that suggests essential roles for various POU factors in CNS cell determination and/or function. The nematode gene unc-86 has been genetically dissected and shown to regulate the identity of neuroblast progeny [Finney and Ruvkun 1990]. Ubiquitous expression of the Drosophila POU–protein pdm-2/dPOU28 has been shown to alter the identity of neuronal precursor cells [Yang et al. 1993]. In addition, the generation of a dominant-negative phenotype by the ubiquitous expression of a truncated version of pdm-2/dPOU28 supports the hypothesis that this Drosophila POU gene is essential for specification of the NB4-2 neuroblast lineage [Bhat and Schedl 1994].

Using a specific polyclonal antisera against the Drosophila dfr protein, we have examined its expression during embryonic development. Our results indicate that the dfr protein is expressed in the progeny of midline precursor cells of the developing CNS, in the progeny of other neuroblasts derived from the neurogenic ectoderm and throughout the tracheal system. We have genetically characterized the dfr gene by generating an EMS-induced lethal allele, dfr<sup>ER2</sup>. An examination of tracheal and CNS development in dfr<sup>ER2</sup> embryos suggests a requirement for dfr protein for the correct differentiation and/or migration of tracheal cells and midline glia in the CNS.

**Results**

**Characterization of full-length dfr cDNAs**

Using a 508-bp BamHI–PstI fragment from the original Cf1a/dfr lgt11 clone [Johnson and Hirsh 1990], we have isolated full-length cDNA clones by screening a 12- to 24-hr embryonic cDNA library. More than 20 independent cDNA clones were isolated and characterized by restriction mapping and sequence analysis. We have predicted translation to be initiated at a methionine codon located at 680 bp, although this does not correspond closely to the consensus translation initiation sequence suggested for D. melanogaster [Cavener 1987]. However, it is the first AUG in-frame with the conserved POU domain and three preceding AUGs at 207, 265, and 558 bp are followed immediately by in-frame stop codons. This corresponds exactly to the predicted reading frame reported by Billin et al. [1991] as deduced from a partial dfr cDNA sequence but differs markedly from the predicted reading frame reported by Treacy et al. [1991]. Because our sequence analysis does not alter the reported reading frame or protein-coding sequence reported by Billin et al. [1991], we have not included our sequence results here but have submitted the full-length cDNA sequence to GenBank.

We have designated two classes of inserts utilizing proximal and distal alternative polyadenylation sites as dfr<sub>a</sub> and dfr<sub>b</sub>, respectively. All clones appear to encode the same protein, and we have found no evidence indicating the use of alternative splicing pathways. As reported previously [Billin et al. 1991], we detect two mRNA transcripts of 3.4 and 3.5 kb on developmental Northern blots [data not shown] that now can be attributed to the utilization of alternative polyadenylation sites found in the dfr<sub>a</sub> and dfr<sub>b</sub> cDNAs. Both transcripts show identical expression patterns, first appearing at 5–6 hr of development, reaching a peak of expression between 10 and 13 hr and declining markedly by the end of embryogenesis [Billin et al. 1991; W. Johnson, unpubl.].

Results from primer extension analysis of dfr in vivo transcripts [data not shown] indicate that the dfr gene utilizes a single transcription start site that corresponds to the 5′ end of our longest dfr<sub>a</sub> cDNA inserts confirming that these are full-length cDNAs. At least three independently isolated clones contained inserts whose 5′ ends mapped to the same nucleotide. The full-length dfr<sub>a</sub> insert of 3548 bp contains a long open reading frame (ORF) that includes the highly conserved POU domain and encodes a 427-amino-acid protein with a predicted molecular mass of 46.0 kD. The predicted dfr<sub>a</sub> ORF is flanked by 679 bp of 5′ untranslated sequence and 1588 bp of 3′ untranslated sequence, which contains no consensus polyadenylation signals (AATAAA). dfr<sub>a</sub> cDNAs extend an additional 375 bp beyond the dfr<sub>a</sub> polyadenylation site. These full-length clones extend previously reported dfr sequences [Johnson and Hirsh 1990; Billin et al. 1991; Treacy et al. 1991] by 562 bp of 5′ untranslated sequence and 155 or 530 bp of 3′ untranslated sequence for the dfr<sub>a</sub> and dfr<sub>b</sub> transcripts, respectively.

**Analysis of dfr protein expression**

Staged Canton-S embryos were whole-mount labeled with polyclonal dfr–antiserum generated against a glutathione-S-transferase (GST)/dfr fusion protein as de-
scribed in Materials and methods. The dfr peptide fragment, containing amino acids 90–258, was selected to exclude the POU homeo domain, minimizing the cross-reactivity with other homeo domain proteins. The pre-absorbed antiserum recognized a single band at 46-kD on Western blots prepared from solubilized whole embryos and an embryonic nuclear extract containing Ddc element C-specific DNA-binding activity (Fig. 1). This agrees exactly with the predicted 46-kD derived from cDNA clones.

**dfr** protein expression is first apparent in early stage 10 embryos (Campos-Ortega and Hartenstein 1985) as epidermal labeling in two symmetrical rows of 10 cell patches corresponding to the developing tracheal placodes [Fig. 2A]. This corresponds to ~6 hr of development at 25°C. Expression continues in stage 11/12 embryos as the medial cells of each placode invaginate to form the deep tracheal pits [Fig. 2B]. As the germ band retracts during late stage 12, the tracheal pits are closed to the outside prior to fusing into transverse tubules [Manning and Krasnow 1993]. dfr expression serves as a marker for these extensive morphogenetic movements as the ring-like dfr expression around each tracheal pit is maintained in essentially all parts of the tracheal tree at the completion of germ-band retraction in stage 13 [Fig. 2C]. During germ-band retraction, significant epidermal expression of **dfr** protein begins, reaching a peak in the middle pair of midline glia (MGM).

**dfr** protein expression is almost completely masked in whole embryos during these stages until epidermal labeling in two symmetrical rows of 10 cell patches corresponding to the developing tracheal placodes [Fig. 2A]. This corresponds to ~6 hr of development at 25°C. Expression continues in stage 11/12 embryos as the medial cells of each placode invaginate to form the deep tracheal pits [Fig. 2B]. As the germ band retracts during late stage 12, the tracheal pits are closed to the outside prior to fusing into transverse tubules [Manning and Krasnow 1993]. dfr expression serves as a marker for these extensive morphogenetic movements as the ring-like dfr expression around each tracheal pit is maintained in essentially all parts of the tracheal tree at the completion of germ-band retraction in stage 13 [Fig. 2C]. During germ-band retraction, significant epidermal expression of **dfr** protein begins, reaching a peak in the middle pair of midline glia (MGM).

**tracheal placode expression of dfr protein** is followed shortly by additional expression in a row of cells extending down the midline of the extended germ band in the stage 10 embryo [Fig. 2A]. This expression corresponds to the mesectodermal or midline precursor cells whose progeny generate a population of midline neurons and glia, which play fundamental roles in the establishment of axonal commissures connecting the symmetrical halves of the CNS [Klambt et al. 1991]. Mesectodermal expression is not seen prior to germ-band extension and subsides by the end of stage 12 after germ-band retraction except for the maintenance of strong expression in the middle pair of midline glia (MGM).

No **dfr** expression is detectable in lateral segregating neuroblasts or their immediate progeny within the neurogenic ectoderm, which is undergoing a complex series of cell divisions and migrations during this period [Campos-Ortega and Hartenstein 1985]. However, **dfr** protein is eventually expressed in a relatively large number of lateral neuronal nuclei in the ventral nerve cord and brain lobes [Fig. 3AB]. Examination of the late embryonic nerve cord pattern reveals expression in a uniform repeated pattern of ~25 nuclei per neuromere grouped into five distinctive clusters: (1) a midline cluster of four nuclei containing the MGM and a pair of RP motoneurons, (2) two symmetrical mediolateral clusters that contain the paired serotonergic neurons; and (3) two symmetrical lateral clusters [Fig. 2B].

**Generation of dfr mutant allele, dfr**

The role of **dfr** protein during embryonic development was evaluated by the generation of an EMS-induced lethal allele, **dfr**<sup>E82</sup>. **dfr** was mapped previously by in situ hybridization to region 65D1-3 on the left arm of the third chromosome [Johnson and Hirsh 1990]. Because no useful deficiencies within region 65D were available previously, we initiated a genetic characterization of the **dfr** locus using X-ray mutagenic screens to generate a set of overlapping chromosomal deficiencies that remove portions of region 65D.

After examination of numerous P-transposon insertion strains by in situ hybridization to salivary chromosome squashes, no insertions within the **dfr** gene were

**Figure 1.** Western blot analysis of **dfr** protein. Solubilized 8- to 22-hr embryos [lane 1] and embryonic nuclear extract [lane 2] were separated by SDS-PAGE, electrottransferred to nitrocellulose, and probed with the dfr-antiserum as described in Materials and methods. Migration positions of prestained molecular weight standards are shown at left.
Figure 2. Embryonic expression of dfr protein. Whole Drosophila embryos were fixed and labeled with dfr-antiserum as described in Materials and methods. All embryos are magnified 188× and oriented with anterior to the left and posterior to the right unless indicated otherwise. (A) Stage 10 germ-band-extended embryo, dorsal view, showing initial dfr protein expression in the tracheal placodes (arrow). Expression is also seen in mesectodermal cells extending down the midline of the extended germ band [arrowhead]. (B) Stage 11/12 embryo, lateral view, showing dfr protein expression in cells surrounding the invaginating tracheal pits (arrow). (C) Lateral view of stage 13/14 embryo showing dfr protein expression in the epidermis. Branches of the tracheal tree, including the dorsal trunk and visceral branches [white arrowhead], are visible but mostly obscured by epidermal expression. High levels of expression are also seen within the oenocyte clusters of abdominal segments A1–A7 [open white arrow]. (D) Lateral view of stage 10/11 embryo head region showing strong dfr protein expression in cell placodes on dorsal and ventral sides of stomodeal opening [arrow].

Figure 3. Embryonic ventral nerve cord expression of dfr protein. Dissected ventral nerve cords were labeled with dfr-antiserum as described in Materials and methods and photographed using Nomarski optics. Nerve cords are oriented with anterior up and posterior down. (A) Late stage 15/16 ventral nerve cord [magnification 380×] showing dfr protein expression in a metamerically repeated pattern of neuronal nuclei. Expression is seen in three characteristic cell clusters, a midline cluster [arrowhead] between the anterior and posterior commissures including the middle pair of midline glia, a bilaterally symmetrical mediolateral cluster [solid thick arrow], which includes the serotonergic neurons, and a bilaterally symmetrical lateral cluster [open thick arrow]. (B) High magnification [950×] dorsal view of two neuromeres focused at the level of the anterior and posterior commissures. Note strong expression in the middle pair of midline glia [arrowhead] and the two RP neurons [arrows]. The mediolateral clusters containing the serotonergic neurons [solid thick arrows] and the lateral clusters [open thick arrows] are slightly out of focus in this focal plane. (C) High magnification [1900×] dorsal view of a dissected ventral nerve cord from the AA142 enhancer trap line [Klambt et al. 1991] that expresses β-galactosidase in the anterior and middle pairs of midline glia. Embryos were double labeled with dfr-antiserum [brown] and β-galactosidase antiserum [blue] as described in Materials and methods. Coexpression of dfr protein and β-galactosidase in the same pair of cells at the midline [dark brown cells] confirms the cell-specific expression of dfr protein in the middle pair of midline glia. [ac] Anterior commissure; [pc] posterior commissure; [lc] longitudinal connectives.
nated as Df(3L)W5.4 revealed that this chromosome carries a large deficiency extending from the proximal region of 65A to 65E1 (Fig. 4E). In addition, homozygous mutant Df(3L)W5.4 embryos express no detectable dfr protein (data not shown).

Smaller overlapping deficiencies within the region were recovered in an X-ray-induced F2 lethal screen for chromosomes failing to complement Df(3L)W5.4. [see Materials and methods]. We have used two of the resulting deficiency chromosomes, Df(3L)XB70 and Df(3L)XAS96, in the identification of an EMS-induced lethal dfr allele. Analysis of salivary chromosome squashes from Df(3L)XB70/+ larvae shows the loss of a single band at 65D1-3 (Fig. 4C). In addition, homozygous Df(3L)XB70 embryos do not express the dfr protein (Fig. 4A,B) indicating that Df(3L)XB70 is a small deficiency that removes dfr. Df(3L)XAS96, extending from 64DE to 65C1, does not remove dfr but overlaps with the distal half of Df(3L)W5.4 (Fig. 4E), making it useful in complementation crosses to eliminate non-dfr lethal complementation groups.

We have tested 3300 EMS-mutagenized third chromosomes in an F2 lethal screen for chromosomes failing to complement Df(3L)W5.4. The resulting 41 lethal chromosomes were tested in complementation crosses and grouped into 15 lethal complementation groups (Fig. 4E).

**Figure 4.** Genetic characterization of region 65D. (A) Lateral view of a wild-type stage 11 whole-mount embryo labeled with dfr-antiserum. Characteristic high levels of dfr protein in cells surrounding the invaginating tracheal pits are indicated by the arrow. (B) Lateral view stage 11 homozygous Df(3L)XB70 embryo labeled with dfr-antiserum. No dfr protein is detectable. (C) Salivary chromosome squash from Df(3L)XB70/+ third instar larva aligned with a schematic representation of region 65 of the third chromosome adapted from the original reference map of C.B. Bridges as found in Sorsa (1988). The loss of a single band at 65D is indicated by the arrowhead. (D) Lateral view stage 11 homozygous dfr<sup>682</sup> embryo labeled with dfr-antiserum. Barely detectable levels of dfr protein are seen at the position of the tracheal pits. (E) Schematic representation of deficiencies and lethal complementation groups identified within region 65 of the third chromosome. Region 65 of the left arm of the third chromosome is represented by the stippled line. Hatched lines underneath represent three deficiency chromosomes generated in X-ray mutagenic screens with the regions uncovered by the deficiencies indicated by brackets. EMS lethal mutations uncovered by Df(3L)W5.4 grouped into lethal complementation groups are shown at the top with brackets indicating those that complement (Proximal W5.4 Group) or failed to complement (Distal W5.4 Group) Df(3L)XAS96. Lines also failing to complement Df(3L)XB70 are grouped separately and designated as the XB70 Group. The location of the dfr locus is indicated by the arrow at 65D1-3.
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Five lethal lines complemented all other isolated alleles and are shown as unique complementation groups. The recovery of numerous single noncomplementing alleles indicates that our screen may not have saturated the large chromosomal region uncovered by Df(3L)W5.4.

By testing representative alleles from each of the identified lethal complementation groups for their ability to complement Df(3L)XAS96 and Df(3L)XBB70 (Fig. 4E), we were able to eliminate all but groups VII, VIII, and IX as candidate dfr alleles. Members of group VII failed to complement a lethal allele of the pale locus identifying these alleles as mutations in the tyrosine hydroxylase gene [Neckameyer and Quinn 1989]. The remaining members of groups VIII and IX were each tested for their ability to express the dfr protein by whole-mount labeling with dfr antiserum. All members of group VIII expressed dfr protein at normal levels, however, the single allele E82 of group IX produced homozygous embryos that express dfr protein at barely detectable levels (Fig. 4D). On the basis of these results, we tentatively identified this mutation as a lethal allele of dfr designated as dfrE82. The mutant phenotype of homozygous dfrE82 embryos is essentially identical to that seen in dfrDf(~82)/Df(3L)XBB embryos suggesting that this is a null mutation; however, the persistence of very low levels of dfr protein makes this a tentative assessment until additional alleles have been further characterized. Because we have not yet definitively ruled out the presence of any additional lethal mutations unassociated with the dfr locus but carried on the dfrE82 chromosome, all of the phenotypic analysis described below was performed on dfrE82/Df(3L)XBB70 embryos.

dfrE82 embryos display severe tracheal defects

We have utilized a P-element transposon expressing β-galactosidase as a cell-specific marker to detect disruptions of the developing tracheal tree in dfrE82 embryos (Fig. 5). This marker transposon, RX-dfr-lacZ, contains a 2.4-kb fragment of dfr genomic regulatory DNA fused to a truncated Hsp70 promoter and lacZ-coding sequences (see Materials and methods; Fig. 5A). Whereas a complete description of our analysis of dfr regulatory sequences will be published elsewhere, transformant flies carrying this fusion gene show strong β-galactosidase expression in the developing tracheal tree and lateral oenocytce clusters but not in the stage 10/11 mesoderm or the epidermis where the dfr protein is also normally expressed at high levels during stages 13 and 14 (Fig. 5B). The absence of epidermal expression, which often masks the internalized tracheal cells when labeling with the dfr antiserum (see Fig. 2C), makes this transposon a useful marker for the observation of tracheal development. In addition, RX-dfr-lacZ is expressed in the middle (MGM) and anterior (MGA) pairs of midline glia [Klambt et al. 1991] of the ventral nerve cord (Fig. 5C,D), allowing us to use the same marker for analysis of midline glia migration and its effect on commissure formation. RX-dfr-lacZ contains no dfr-coding sequence and therefore should be incapable of a partial rescue of the mutant phenotype. dfrE82/Df(3L)XBB70 embryos were identified as those failing to express dfr protein when double labeled with dfr antiserum and a rabbit polyclonal antiserum against β-galactosidase [Cappel].

In wild-type embryos, during late stage 12 and early stage 13, cells of the tracheal placodes invaginate to form the tracheal pits, which are then closed to the exterior of the organism before the extension of dorsal and lateral tracheal branches and fusion of internalized tracheal cells to form the dorsal trunk [Manning and Krasnow 1993] (Fig. 6A,C). These morphogenetic events result from the directed migration of postmitotic tracheal cells from within the tracheal pits to new positions as part of the extended tracheal tree. dfrDf(~82)/Df(3L)XBB70 embryos show normal tracheal pit formation during late stage 11 and early stage 12. However, by the end of germ-band retraction in stage 13, while the lateral branches appear relatively normal, the dorsal and visceral branches of the tracheal tree are absent and cell migrations required for dorsal trunk formation do not occur (Fig. 6B,D). As mutant embryos progress to stage 15, by which time the majority of dfrE82 mutant embryos have died, cells of the lateral branches continue to migrate and occasionally accomplish homotopic fusions; however, the final lateral branch structures are usually drastically abnormal. The dorsal and visceral branches and dorsal trunk are never successfully formed even in later embryos. The oenocytes, which strongly express RX-dfr-lacZ, are present in mutant embryos but often fail to form tight clusters and are randomly displaced, sometimes breaking into smaller clusters of two to three cells each (Fig. 6B).

The prohibitive size of the complete dfr transcription unit, as judged by results from dfr-lacZ fusion genes [R.J. Shrigley, unpubl.], has thus far made it impossible to accomplish a complete transposon rescue of the dfr mutant phenotype. However, the identification of tissue-specific dfr enhancer sequences has allowed us to successfully rescue the dfrE82 tracheal phenotype using a dfr-minigene transposon. The RX-dfr minigene [Fig. 7A] expresses the wild-type dfr cDNA under the control of the same 2.4-kb fragment of tracheal- and MGM-specific dfr genomic regulatory sequence used in the marker transposon RX-dfr-lacZ (Fig. 5A). The complete absence of epidermal expression from the RX-dfr minigene allowed us to easily distinguish between wild-type heterozygous dfrE82/TM6B, Tubby (Tb) ebony (e) causal embryos and phenotypically rescued homozygous dfrE82 embryos by labeling staged embryos with dfr antiserum. Heterozygous embryos labeled with dfr antiserum show pronounced epidermal labeling in stage 13–14, which almost completely masks tracheal expression when viewed in whole mounts (see Fig. 2C). In sharp contrast, when expressed in a homozygous dfrE82 genetic background, the RX-dfr minigene provides the only source of dfr protein expressed in the same tracheal- and MGM-specific pattern seen for β-galactosidase expression from RX-dfr-lacZ (see Fig. 7B,C). Examination of homozygous RX-dfr, dfrE82 embryos labeled with dfr antiserum shows a complete rescue of dorsal trunk formation as well as visceral and dorsal branch migration (Fig. 7C),
indicating that the tracheal defects associated with dfr^{E82} are caused by a loss of function mutation in the dfr locus.

**Defects in midline glia migration in dfr^{E82} embryos**

The wild-type embryonic ventral nerve cord is composed of ~200 neuronal cell bodies on each side of the bilaterally symmetrical thoracic and abdominal neuromeres. These neurons extend axons that eventually form a uniform ladderlike structure of commissures and longitudinal connectives at the midline of the ventral nerve cord (Fig. 8A; Klambt et al. 1991). The anterior and posterior commissures of each neuromere are normally cleanly separated by a group of mostly identified neurons and glia, including one pair of midline glia, the MGM, as well as the RP1 and RP3 neuronal cell bodies. Examination of dissected ventral nerve cords from dfr^{E82}/Df(3L)XBB70 embryos reveals the presence of a single fused commissure in each neuromere [Fig. 8B,C]. Axon tracts were labeled using antiserum against horseradish peroxidase (anti-HRP), which recognizes an antigen present on the surface of all neuronal membranes (Jan and Jan 1982). In addition to the fused commissure phenotype, widespread defects in longitudinal connectives are also seen, indicating a more extensive malformation of axon tracts. The severity of longitudinal defects varies significantly between neuromeres, ranging from the complete absence of connectives [Fig. 8B] to an asymmetric distribution of longitudinal fascicles with few tracts on one side and an
excess on the other (Fig. 8C). Whereas the appearance of fused commissures could be directly attributable to MGM defects caused by \textit{dfr\textasciitilde82}, the failure of longitudinal connective formation suggests more extensive defects resulting from a loss of \textit{dfr} function. The RX--\textit{dfr} rescue transposon, which is expressed in tracheal cells after stage 10 and the MGM from stage 13 on, was unable to rescue the \textit{dfr\textasciitilde82} CNS phenotype. Note that this rescue transposon does not restore stage 10–12 mesectodermal or late neuronal \textit{dfr} expression. This indicates that the lack of early mesectodermal and/or neuronal \textit{dfr} expression may make a significant contribution to the mutant phenotype.

To further investigate the possibility of some defect in the differentiation, survival, or migration of midline glia, we have analyzed the position of midline glia in the \textit{dfr\textasciitilde82}/\textit{Df}/(3L)\textit{XBB}70 mutant background by examining dissected nerve cords from embryos carrying the RX--\textit{dfr-lacZ} marker transposon, which expresses \(\beta\)-galactosidase in the MGM and MGA. Mutant embryos were identified by their defective tracheal phenotype, which previously had been shown to be associated with the absence of \textit{dfr} protein in double-labeling experiments. RX--\textit{dfr-lacZ} expression is seen in a highly uniform metameric pattern within the ventral nerve cord of wild-type embryos (Fig. 9A). Analysis of ventral nerve cords derived from RX--\textit{dfr-lacZ}, \textit{dfr\textasciitilde82}/\textit{Df}/(3L)\textit{XBB}70 embryos (Fig. 9B) indicates that the MGM and MGA are still present but fail to migrate to their normal positions within each neuromere, resulting in an essentially random distribution of RX--\textit{dfr-lacZ}-expressing cells along the midline. This mismigration is not limited by neuromere boundaries as indicated by the formation of six- to seven-cell clusters in some neuromeres while others may contain no midline glia. Mutant nerve cords contain approximately the same number of RX--\textit{dfr-lacZ} expressing cells as in wild type, suggesting that \textit{dfr\textasciitilde82} has no major effect upon the survival of midline glia but must fundamentally alter an inherent mechanism regulating directed glial migration.

In addition to CNS defects at the midline, we have observed occasional alterations in growth cone guidance as axon tracts exit the ventral nerve cord through the segmental and intersegmental nerves of mutant \textit{dfr\textasciitilde82}/\textit{Df}/(3L)\textit{XBB}70 embryos (data not shown). Axons often appear misguided with extensive bending and looping, which often results in the axon tract reversing direction and actually migrating back into the nerve cord. This could be a cell autonomous defect of the migrating axons or a secondary defect resulting from the loss of substrates such as the tracheal branches, which are thought to normally participate in the guidance of growth cone movements (Giniger et al. 1993; Younossi-Hartenstein and Hartenstein 1993). Although this phenotype is not rescued by the RX--\textit{dfr} minigene, suggesting that it may be independent of the tracheal phenotype, we have not yet definitively distinguished between these two possibilities.

Discussion

Colocalization of Drosophila POU factors

Mammalian members of the POU-domain family have been shown to associate as heterodimers via their POU
Drosophila dfr gene required for cell migration

The developing tracheal system expresses dfr protein at high levels beginning with the early designation of tracheal placodes and continuing throughout embryogenesis. The mechanism by which postmitotic cells of the tracheal placodes follow a specified migration path to form an interconnected grid of tubular epithelia is thought to involve the interaction of cell-surface proteins with specific marker proteins expressed on adjacent cells [Klambt et al. 1991; Manning and Krasnow 1993]. The same fundamental mechanisms of cell recognition may be functioning in the directed migration of other diverse cell types such as mammalian neural crest cells [Bronner-Fraser 1993], metastatic tumor cells [Weinstat-Saslow and Steeg 1994] and follicle cells of the Drosophila ovary [Rorth and Montell 1992].

Here, we have focused on a phenotypic characterization of the dfr^E82 allele because of its behavior genetically as a null allele. However, we have subsequently generated three additional dfr loss-of-function alleles that show the same tracheal and glial cell migration mutant phenotype with varying degrees of severity [M. Anderson, unpubl.]. The close correlation between the cell- and tissue-specific expression of dfr protein and mutant phenotypes resulting from its absence implies an essential function in these cells. On the basis of this migration-defective phenotype, we propose that whether by direct interaction or secondarily by alteration of a transcriptional program of differentiation, it is likely

dfr Function in cell migration

function may be regulated by the formation of inhibitory heterodimers with another Drosophila POU domain gene product, I-POU [Treacy et al. 1991, 1992]. In light of these results, a comparison of dfr expression patterns with those of other Drosophila POU genes analyzed previously using whole-mount in situ hybridization [Billin et al. 1991; Dick et al. 1991; Treacy et al. 1991] reveals significant differences that have broad implications concerning the potential formation of POU–protein heterodimers. The two Drosophila POU genes, dPOU19/pdml and dPOU28/pdm2 [Billin et al. 1991; Dick et al. 1991], are initially expressed in stage 5 cellular blastoderm embryos in circumferential stripes reminiscent of certain segmentation genes. In germ-band extended embryos, dPOU19/pdml and dPOU28/pdm2 transcripts are found in a large subset of neuroblasts and ganglion mother cells segregating from the neurogenic ectoderm but not in neuronal progeny or the mesectodermal cells. These two transcripts are also seen in certain sensory organs of the CNS. In contrast, dfr protein expression is not detectable in segregating neuroblasts of the neurogenic ectoderm or cells of the CNS. I-POU transcripts are restricted to the developing CNS and may be present in a subset of dfr-expressing cells [Treacy et al. 1991]. Thus, although it is possible that dfr and pdml/pdm2 protein expression could overlap if pdml protein levels persisted long enough, only the dfr protein and I-POU show definite spatial and temporal patterns of expression suggestive of potential heterodimer formation.
Commissural defects caused by dfr~82. Dissected ventral nerve cords from wild-type and dfr~82/Df(3L)XBB70 embryos carrying the RX-dfr-lacZ marker transposon. Embryos were double labeled with anti-HRP (light purple) and anti-β-galactosidase (brown) antibodies as described in Materials and methods. Mutant embryos were identified based on their defective tracheal phenotype which had been shown previously to correlate with the absence of dfr protein. Each panel shows two neuromeres with the positions of anterior and posterior commissures indicated by thin arrows and the middle pair of midline glia (MGM) by the arrowhead. Lateral labeling with anti-β-galactosidase is in cells of the tracheal ganglionic branches that have migrated into the nerve cord by this stage. The thinning or complete absence of longitudinal connectives are marked with thick arrows. All cords are viewed from the dorsal surface at 950× magnification and positioned with anterior up and posterior down. [A] Wild-type stage 15 RX-dfr-lacZ ventral nerve cord. Note the uniform spacing of anterior and posterior commissures within each neuromere and the central position of MGM pairs. [B] dfr~82/Df(3L)XBB70 ventral nerve cord with a single fused commissure within each neuromere (thin arrows). Note the absence of MGM in the upper fused commissure and the severe thinning of both longitudinal connectives (thick arrows). [C] dfr~82/Df(3L)XBB70 ventral nerve cord from a different embryo than B. Commissures of the upper neuromere appear fused even though the midline glia are present. Commissures of the lower neuromere are separated but misshapen with the labeled MGM shifted out of their normal midline position and the anterior commissure appearing thinner than the posterior commissure. In addition, asymmetrical thinning of longitudinal connectives is indicated by the thick arrow. Note the pronounced thickening of the opposite longitudinal connective between each neuromere.

Commissural defects similar to those seen in dfr~82 embryos are often associated with mutants that affect either the survival or migration of the MGM such as spitz (spi), Stat (S), and rhomboid (rho) [Klambt et al. 1991]. In each of these previously characterized mutants, the commissures are formed but exist as a single fused bundle of axons [Klambt et al. 1991]. This is assumed to result from the failure of the MGM to undergo a directed posterior migration from an initial anterior position, over the top of the newly formed anterior commissure to take their final position between the two commissures [Klambt et al. 1991].

The dfr protein is expressed in a cluster of cells associated with the anterior and posterior commissures of the ventral nerve cord beginning in stage 13 embryos. This cluster includes the MGM, derived from the mesectoderm, and a pair of RP motoneurons, derived from bilaterally symmetrical segregating neuroblasts of the neurogenic ectoderm [Fig. 3]. A significant earlier component of dfr expression is seen in all mesectodermal cells, including the midline glia, beginning in germ-band-extended stage 10 embryos and lasting until late that the dfr protein may ultimately regulate the expression of one or more cell-surface proteins involved in cell adhesion or signal transduction. Loss of that regulation may result in the defective tracheal and glial cell migrations seen in dfr~82. Whether the interaction between dfr protein and genes encoding cell-surface proteins is direct or more global in nature cannot be determined definitively at our current level of analysis. The identification of cell- and tissue-specific targets of the dfr protein should allow a more detailed description of dfr function in cell migration.

Aberrant migration of midline glia in dfr~82/Df(3L)XBB70 ventral nerve cord. Dissected ventral nerve cords from wild-type and dfr~82/Df(3L)XBB70 embryos carrying the RX-dfr-lacZ marker transposon. Embryos were labeled with anti-β-galactosidase antibodies as described in Materials and methods. Mutant embryos were identified based on their defective tracheal phenotype that had been shown previously to correlate with the absence of dfr protein. Cords are viewed from the dorsal surface at 400× magnification and positioned with anterior up and posterior down. [A] Wild-type stage 14/15 RX-dfr-lacZ ventral nerve cord. Midline glia are distributed with highly uniform metameric spacing within each neuromere. [B] dfr~82/Df(3L)XBB70 ventral nerve cord. Labeled midline glia are randomly clustered along the midline often forming clusters of six to seven cells within a single neuromere (thin arrow).
The Drosophila dfp gene required for cell migration

Drosophila stocks

Flies were raised on standard cornmeal/yeast/agar medium. All stocks and balancer chromosomes not mentioned specifically in the text are described in Lindsley and Zimm [1992]. All genetic crosses were performed at 25°C unless specified otherwise.

DNA manipulation and cDNA characterization

Restriction mapping, subcloning, and other DNA manipulations were performed as per Maniatis et al. [1989], except as described. Restriction enzymes were purchased from New England Biolabs or Boehringer Mannheim Biochemicals and used as suggested by the supplier. Full-length dfp cDNAs were isolated from a 12- to 24-hr embryonic cDNA library prepared in the pNB40 vector by Nick Brown [Brown and Kafatos 1988]. Before transformation, the library was size selected for inserts larger than 2 kb by separation on low melt agarose. The library was plated according to transformation in Library Efficiency DHS+ competent cells [BRL, Gaithersburg, MD]. Colony lifts were prepared and hybridized as described previously [Brown and Kafatos 1988] using a 508-bp BamHI-PstI fragment derived from the original dfp/Cfla kgtll phage insert [Johnson and Hirsh 1990] as 32p-labeled probe. Screening of ~6 x 10^5 colonies yielded 36 positive plasmids containing inserts ranging from 2.5 to 3.7 kb; 20 of these were selected for further characterization. Positive pNB40 inserts were transferred to the Bluescript KS vector for restriction mapping and sequencing. DNA sequences were determined by single-stranded dideoxy nucleotide sequencing techniques, using modified T7 DNA polymerase (Ta-
Antiserum production

A GST/dfr fusion protein was produced by cloning the dfr 508-bp BamHI–PstI fragment into the pGEX-1 polylinker as a BamHI–EcoRI fragment (Smith and Johnson 1988). Fusion at the in-frame BamHI site should produce a protein with 169 amino acids of dfr peptide (amino acids 90–258) coupled to the carboxy-terminal end of the GST protein. The resulting peptide contains 30 amino acids from the amino-terminal end of the POU-specific domain but does not contain the POU homoeodomain. Production of the 45-kD fusion protein was induced in Escherichia coli DH5 cells with IPTG, protein isolated, and antiserum generated using protocols described previously in detail (Johnson 1992).

Western blotting

Protein samples were separated on 15% SDS–polyacrylamide gels and electrotransferred to nitrocellulose in 25 mM Tris, 192 mM glycine, and 20% methanol using a Bio-Rad Trans-blot cell. Embryonic nuclear extract was prepared as described previously (Johnson et al. 1989). Dechorionated whole 8- to 22-hr embryos were homogenized in SDS loading buffer. The nitrocellulose membrane was blocked in 0.2% Tween 20 in PBS for at least 3 hr followed by incubation with the preabsorbed antiserum at a final dilution of 1:3000 for 2 hr. Primary antibodies were detected using the Vectastain ABC kit (Vector Laboratories) and HRP immunohistochemistry following protocols suggested by the manufacturer. Double-labeled ventral nerve cords were obtained by incubating fixed whole embryos with mouse monoclonal anti-b-galactosidase and rabbit anti-HRP primary antibodies. After washing, embryos were incubated with alkaline phosphatase-conjugated anti-rabbit and biotinylated anti-mouse secondaries followed by streptavidin–HRP complex. Color was developed first using 4-nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indoly-phosphate (BCIP) in alkaline phosphatase buffer containing 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 5 mM MgCl2, and 0.1% Tween 20. Embryos were then transferred to 1× PBS for color development in 0.5 mg/ml of diaminobenzidine/0.3% H2O2. Embryos were either whole mounted in 70% glycerol/1× PBS or embryonic CNS was dissected in 70% glycerol/PBS using electrolytically sharpened tungsten needles. Mounted embryos were examined and photographed on a Nikon Optiphot microscope using Nomarski optics. Homozygous embryos were identified by double-labeling with b-galactosidase antiserum to determine the presence or absence of the TM3, ftz-lacZ third chromosome balancer.

Mutagenic screens

F1 transgenic screen

Male flies from the w; P[walter,w+]65D/TM3, Sb e strain (Gloor et al. 1991) were X-ray mutagenized (4000 rads) and mated in mass matings to virgin w; mwh red e females. Scoring of 53,304 X-ray mutagenized P[walter,w+]65D chromosomes yielded 30 white-eyed progeny of which 8 were lethal mutations. F1 progeny were scored for eye color and white-eyed flies mated to the w;TM3, Sb e/TM6, Tb e double-balancer strain to establish balanced mutant strains.

X-ray-induced F2 lethal screen

To obtain smaller deficiencies overlapping Df(3L)W5.4, homozygous mwh red e males were X-ray mutagenized (4000 rads) and mass-mated to +/TM6, Tb e virgin females. Tb e males resulting from this cross were mated individually to virgin w; Df(3L)W5.4/TM6, Tb e females and vials scored for the absence of non-Tb pupae indicating a failure to complement the Df(3L)W5.4 chromosome. Vials producing no non-Tb pupae were used to establish working stocks by recovering Tb e males and females. Established mutant lines were re-crossed to w; Df(3L)W5.4/TM6, Tb e females to verify their failure to complement. Approximately 8130 mutagenized chromosomes were scored for failure to complement Df(3L)W5.4, producing 19 noncomplementing lethal lines. All lethal chromosomes were tested for visible chromosomal abnormalities by the analysis of orcin-stained salivary chromosome squashes using techniques described previously (Ashburner 1989). Lethal chromosomes were also tested for removal of the dfr gene using dfr–antiserum. Staged embryos [5–8 hr] were collected from population cages of each lethal strain maintained over the TM6, Tb e balancer chromosome and labeled as whole mounts with dfr–antiserum. During this stage of development, every embryo should express the dfr protein in a characteristic pattern. Labeled embryos were examined to determine whether a significant percentage of stage 10 or later embryos did not produce dfr protein.

EMS-induced F2 lethal screen

Male mwh red e flies were fed EMS in 1% sucrose as described previously (Grigliatti 1986) and mated in mass culture to h/TM6, Tb e virgin females. Male Tb e F1 progeny were mated...
in individual matings (3300 crosses) with Df(3L)W5.4/TM6, Tbk
virgin females and incubated at 29°C to optimize for the re-
covery of temperature-sensitive alleles. Presence of the domi-
nant Tb marker allowed scoring of vials for the distinctive Tb
phenotype. Vials containing wild-type F2 pupae were dis-
carded. Eclosing F2 progeny were collected from vials contain-
ing only Tb F2 pupae and ebony progeny were intermated to
establish stable mutant lines. After retesting for lethality over
the Df(3L)W5.4 chromosome, the remaining 41 lines were
tested in complementation crosses.

P-element transformation

Methods for P-element integration were essentially as described
previously [Spradling 1986]. The RX-dfr-lacZ plasmid was in-
jected into y ac w; Sb ry 5°6 P[y+Δ2-3]/TM6, Ultrabitho-
rax [Ubx] embryos. Transformant flies were identified by
screening for the appearance of w+ eye color in the progeny of
injectees crossed to w~118 adults. Five independent transfor-
mant strains were established for each fusion construct. Strains
were confirmed to contain single copy inserts of the appropriate
P-element vector by Southern blot analysis of genomic DNA.

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