Tubulogenesis in *Drosophila*: a requirement for the *trachealess* gene product

Daniel D. Isaac and Deborah J. Andrew¹

Department of Cell Biology and Anatomy, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205-2196 USA

The *trachealess* (*trh*) gene of *Drosophila* is required for embryonic tube formation. In *trh* mutants, tube-forming cells of the salivary gland, trachea, and filzkörper fail to invaginate to form tubes and remain on the embryo surface. We identified a P-element insertion that disrupts *trh* function and used the insert to clone and characterize *trh*. *trh* is expressed in the salivary duct, trachea, and filzkörper primordia, and expression persists in these cells throughout embryogenesis. *trh* expression in the salivary duct is controlled by the homeotic gene, *Sex combs reduced* (*Scr*), and by another salivary gland gene, *fork head* (*fkh*). *trh* is homologous to two transcription factors: the human hypoxia-inducible factor-1 α and the *Drosophila* Single-minded protein.

[Key Words: Tubulogenesis; *Drosophila*; *trachealess* gene; transcription factors]

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To form organs and tissues in a developing embryo, distinct cell populations must coordinate their growth, differentiation, and movement in three dimensions. For example, sheets of epithelial cells can become organized as tubes, forming large simple structures such as the neural tube, or smaller, more intricate structures such as the branching ducts of various glands. At least three general mechanisms are known for tube formation (Ettensohn 1985; Bard 1992). One mechanism involves cell shape changes driven by the contraction of microfilaments at the apical surfaces of epithelial cells, deforming them so that their bases are wide and apices are narrow. This mechanism is thought to drive neural tube formation along the dorsal midline of most vertebrate embryos. A second mechanism, known as canalization, involves the formation of a lumen in an elongated array of polarized cells. Canalization is used during neural tube formation in bony fishes, blood capillary formation, and proximal tube formation arising from induced metanephric mesenchyme. The third mechanism for tube formation involves cell migration, where cells move away from one another while maintaining the integrity of the epithelium to form a blind-ended tubular extension. This mechanism is driven by cell growth and/or cell division and is thought to involve interactions between the epithelia and underlying mesenchymal cells (for review, see Ettensohn 1985; Gumbiner 1992).

In vitro studies of tissue culture cells have implicated a number of growth factors and/or their corresponding

receptors in tubulogenesis (for review, see Zarnegar and Michalopoulos 1995). For example, scatter factor/hepatocyte growth factor (SF/HGF) can induce Madin-Darby canine kidney (MDCK) epithelial cells (Montesano et al. 1991a,b) and a number of other cell types (Rosen et al. 1991; Bussolino et al. 1992; Grant et al. 1993; Cantley et al. 1994; Galimi et al. 1994; Karp et al. 1994; Stamato-glou and Hughes 1994; Schmidt et al. 1995; Soriano et al. 1995; Uehara et al. 1995) to form tubes when grown in reconstituted matrices such as basement membrane gels or collagen. Epidermal growth factor (EGF) and transforming growth factor- α (TGF- α) can induce tube formation in murine inner medullary collecting duct kidney (mIMCD-3) cells (Barros et al. 1995) and in human omental microvascular endothelial (HOME) cells (Ono et al. 1992). In primary rat organ culture, the addition of anti-TGF- α inhibits tubulogenesis within the metanephric blastema (Rogers et al. 1992), further supporting a positive regulatory role for this molecule in tube formation. A different growth factor, transforming growth factor- β 1 (TGF- β 1), has been shown to inhibit tubulogenesis (Santos and Nigam 1993), and the addition of anti-TGF- β 1 antibodies to primary rat kidney cultures accelerates tubulogenesis (Rogers et al. 1993). Correspondingly, the addition of TGF- α or EGF restores tubulogenic activities of growth factor-depleted matrigel cultures of baby mouse kidney cells, whereas the addition of TGF- β inhibits tube formation in the same system (Taub et al. 1990). Other molecules that either induce or inhibit tubulogenesis include basic fibroblast growth factor (bFGF), (Montesano et al. 1986), both α and γ interferons (Maheshwari

¹Corresponding author.

et al. 1991), protein kinase C (Kinsella et al. 1992), and epimorphin, a novel 150-kD protein expressed on the surface of mesenchymal cells of mouse embryonic tissues (Hirai et al. 1992). A *Drosophila* FGF-receptor gene, known as *breathless* (*btl*), is required for tubulogenesis in the embryonic trachea (Glazer and Shilo 1991; Klamt et al. 1992; Reichman-Fried et al. 1994). In *btl* mutants, the initial tracheal invaginations appear normal, but branch migration does not occur. The loss-of-function phenotype is completely rescued by the global production of wild-type *btl* and is rescued partially by activated forms of other receptor tyrosine kinases including DFGF-R2, EGF-R, *sevenless*, and *torso*. The roles of growth factors and their receptors in tube formation link this morphogenetic process to cell division and/or cell growth.

The studies described above have established the importance of growth factors and their corresponding receptors in stimulating tube formation (Birchmeier and Birchmeier 1993). However, the molecules that determine whether or not cells have the capacity to form tubes have not been identified. Growth factors such as SF/HGF, EGF, and TGF- α can only induce tube formation in immortalized cultured cells or primary cells that would have normally formed tubes in vivo. To identify the molecules that program epithelial cells to form tubes, we must turn to manipulable systems where tube formation can be studied in vivo using genetics and molecular biology.

Epithelial tube formation is an essential feature of development in *Drosophila*, particularly in forming organs of the respiratory system (trachea and filzkörper), urinary system (Malpighian tubules), and digestive system (esophagus, gastric caeca, and the salivary gland duct). Among these organs, the salivary gland duct is particularly interesting because salivary gland formation is linked to the expression of a single homeotic gene, *Sex combs reduced* (*Scr*). Homeotic genes encode transcription factors that bind to specific DNA sequences (Levine

and Hoey 1988; Scott et al. 1989). In embryos mutant for *Scr*, no salivary glands form, and in embryos that express SCR ectopically, salivary glands form in new places (Panzer et al. 1992; Andrew et al. 1994). Therefore, *Scr* appears to direct salivary gland formation by regulating the transcription of downstream genes whose products uniquely define the salivary gland. Few downstream targets of *Scr*, or any other homeotic genes, have been identified (Andrew and Scott 1992). Our efforts to isolate downstream targets of *Scr* led us to *tracheiless* (*trh*). We show here that *trh* is positively regulated by *Scr* in the embryonic salivary gland, and that *trh* encodes a transcription factor essential for forming a distinct subset of tubes during *Drosophila* embryonic development.

Results

A P-element insert in cytological region 61C1,2 disrupts trh function

We identified a P-element insert, *l(3)10512*, in an enhancer-trap screen for genes expressed in the embryonic salivary gland (D.J. Andrew, unpubl.). We mapped this single P-element to cytological region 61C1,2, three-letter divisions from the published position of *trh* in 61E–F (Jürgens et al. 1984). In *l(3)10512* homozygous embryos, we observe that the salivary glands form closed sacs that are not connected to the foregut (Fig. 1A,B). The salivary duct cells, which express β -galactosidase to high levels in the *l(3)10512* line, remain clustered on the embryo surface in *l(3)10512* homozygotes instead of forming the tubular ducts that normally connect the salivary glands to the foregut (Fig. 1C,D). In data not shown, Younossi-Hartenstein and Hartenstein (1993) reported that in *trh*¹ homozygotes, salivary glands also form closed sacs. This common phenotype suggests that *l(3)10512* is a *trh* allele. A closer examination of *l(3)10512* homozygous embryos revealed additional phenotypes overlapping those of *trh*¹ mutant embryos (Jürgens et al. 1984; see below).

Figure 1. In *trh* mutants, the salivary duct cells fail to invaginate to form the tubes that would normally connect the salivary gland secretory cells to the foregut. (A) Wild-type embryo stained with α -CRB; (B) *trh*⁵ homozygous mutant embryo stained with α -CRB; (C) *trh*^{3/+} embryo stained with α - β -gal; (D) *trh*³ homozygous mutant embryo stained with α - β -gal. The salivary gland duct stains with α -CRB antibody in wild-type embryos (thick arrow in A). No α -CRB staining in the region of the salivary duct is detected in the *trh*⁵ mutant embryo (thick arrow in B) or in the other six *trh* alleles examined. The salivary gland secretory cells in the *trh* mutants are closed where they would normally fuse with the duct cells (arrows in B). The P-element insert (*trh*³) gives rise to high level β -gal staining in the entire salivary gland (C,D), in both secretory (arrowheads point to secretory cells, which are below the plane of focus) and duct cells (thick arrows), the filzkörper (open arrows in D), and a subset of cells in the CNS (thin arrows in C,D). In the *trh*^{3/+} embryos the salivary gland duct is normal (thick arrows in C). In *trh*³ homozygous mutant embryos, the salivary duct cells fail to invaginate and remain on the embryo surface (thick arrow in D).

To test whether the *l(3)10512* stock carries a loss-of-function mutation in *trh*, we performed a series of complementation tests between *trh¹*, *l(3)10512*, and deficiencies from the region. The results are summarized in Table 1 and Figure 2A. *trh¹* and *l(3)10512* fail to complement each other and *Df(3L)emc-E12*. *trh¹* and *l(3)10512* complement *Df(3L)AR11*, *Df(3L)27-3*, *Df(3L)AR14-8*, and *Df(3L)AR12-1*, placing *trh* in the region from 61A to 61C3,4 [Fig. 2A]. This region includes the insertion site of the single P-element in the *l(3)10512* stock, supporting the hypothesis that the P-element insertion into region 61C1,2 created a new loss-of-function *trh* allele.

To determine whether it is the P-element insert in *l(3)10512* that disrupts *trh* function, and not a second independent mutation on the same chromosome, we excised the P element in the *l(3)10512* stock and screened for a loss of the *ry⁺* eye color marker contained within the P element. We recovered a large number of viable *ry⁻* excisions as well as several lethal *ry⁻* excisions. Complementation tests and phenotypic analysis of ten of these lines indicated that five of the *ry⁻* excision lines had lost the P-element with concomitant restoration of *trh* function (Table 1). The remaining five *ry⁻* lines were *trh⁻* and in some cases had weaker phenotypes than the original *trh¹* mutant or the *l(3)10512* mutant. In sum-

mary, our genetic and cytological analysis shows that *l(3)10512* maps to the *trh* locus, that it fails to complement *trh*, and that excision of the P-element restores *trh* function. We conclude that the P-element insertion in *l(3)10512* creates a new loss-of-function allele of *trh*, which we will refer to as *trh³* (Table 1). The P-element insert was used to clone and characterize the *trh* gene (Fig. 2B).

trh is required for tube formation

The P-element insert in the *trh³* allele gives high-level β -gal expression in the entire salivary gland, in both duct and secretory cells (Fig. 1C,D). However, only the duct cells are abnormal in *trh* mutants; these cells fail to invaginate to form tubes and remain on the surface (Fig. 1D). The secretory cells of the salivary gland invaginate normally and express all of the tested salivary gland markers but are no longer attached to the foregut through the salivary duct. The salivary gland secretory cells in *trh* mutants form a self-contained sac (Fig. 1B). An analysis of *trh* RNA expression by whole mount in situ hybridization using a genomic probe from the cloned gene reveals that although *trh* transcripts are present in the entire salivary gland primordia by stage 9

Table 1. Complementation tests among *trh* mutant alleles, excision revertants, and deficiencies

(×)	Percent survival (no.) ^b							
	Df(3L)H5	Df(3L)H18	Df(3L)H2	<i>l(3)trh¹</i>	<i>l(3)trh³</i>	<i>l(3)trh⁴</i>	<i>l(3)trh⁶</i>	<i>l(3)trh⁷</i>
<i>l(3)trh¹</i> ^a	92 (137)	96 (75)	0 (97)	—	—	—	—	—
<i>l(3)trh³</i> ^a	108 (89)	96 (179)	0 (278)	0 (264)	—	—	—	—
<i>l(3)trh⁴</i>	177 (49)	79 (53)	0 (38)	0 (148)	130 (92)	—	—	—
<i>l(3)trh⁵</i>	117 (41)	46 (100)	0 (29)	0 (164)	8 (320)	0 (97)	—	—
<i>l(3)trh⁶</i>	95 (66)	113 (159)	0 (99)	0 (260)	11 (282)	95 (104)	83 (72)	—
<i>l(3)trh⁷</i>	83 (88)	94 (125)	0 (94)	0 (217)	0 (215)	92 (104)	38 (56)	0 (57)
<i>l(3)trh⁸</i>	117 (23)	122 (208)	0 (34)	0 (111)	0 (398)	0 (98)	0 (101)	0 (53)
<i>l(3)trh-R12</i>	N.D.	N.D.	0 (57)	46 (163)	N.D.	N.D.	N.D.	N.D.
<i>l(3)trh-R25</i>	N.D.	N.D.	115 (127)	166 (85)	N.D.	N.D.	N.D.	N.D.
<i>trh-R3</i>	N.D.	N.D.	16 (18)	88 (34)	N.D.	N.D.	N.D.	N.D.
<i>trh-R26</i>	N.D.	N.D.	112 (96)	131 (158)	N.D.	N.D.	N.D.	N.D.
<i>trh-R63</i>	N.D.	N.D.	N.D.	73 (76)	N.D.	N.D.	N.D.	N.D.

^a(*trh¹*) The original EMS-induced allele, formerly known as *trh^{SD}* (Jürgens et al. 1984); (*trh³*) the enhancer-trap insertion alleles, formerly known as *l(3)10512*. Results suggest an allelic series, decreasing by order of severity as follows: *trh¹* = *trh⁸* > *trh⁷* > *trh³* > *trh⁶* > *trh⁵* > *trh⁴*.

^b(0%) A complete failure to complement; (100%) complete complementation. (Numbers >100% simply indicate that the relative survival of the tested genotype is better than their balancer-carrying siblings.) (no.) The number of adult animals scored in all classes. Data were pooled from pair matings in both directions with respect to the gender of the parents. (N.D.) Not done.

A Genetics of *trh* region

Figure 2. (A) *trh* maps to region 61C1,2 on chromosome 3. In situ hybridization to polytene chromosomes was done using a biotin-labeled probe containing sequences in the insertion element (*Casper*- β -gal, which contains the *white* gene and β -gal gene coding regions). The chromosome in situ map *trh* to cytological region 61C1,2, consistent with the failure of *Df(3L)emc-12* to complement *trh* mutations. *Df(3L)AR11*, *Df(3L)27-3*, *Df(3L)AR14-8*, and *Df(3L)AR12-1* complement *trh* mutations. (B) *trh* transcripts span ~12 kb. The top line is a restriction map of the cloned region. The insertion in the *trh*³ allele maps to a 7.2-kb *Eco*RI fragment that detects transcripts on Northern blots and in embryos. Three other genomic fragments (*) also detect *trh* transcripts in embryos and on Northern blots.

B Molecular map of the *trachealess* gene

(Fig. 3C,H), *trh* expression in the secretory cells disappears during stage 12 (Fig. 3E). Only the salivary duct cells continue to express *trh*, consistent with their requirement for *trh* function (Fig. 3F,L). The β -gal expression from the enhancer-trap allele, therefore, does not faithfully reproduce the expression pattern of the endogenous *trh* gene. There are two likely reasons for the difference between *trh* mRNA expression and the P-element β -gal expression. The P-element insertion may disrupt *cis*-acting elements that are required to turn off *trh* in the secretory cells at later stages. Alternatively, the β -gal protein may be stable and persist in cells that no longer express the mRNA.

In tracheal cells, *trh* mRNA expression begins at embryonic stage 8, preceding tracheal pit formation by more than an hour and continues throughout embryogenesis (Fig. 3A–L). We also observe β -gal expression in the trachea with the P-element insertion *trh*³ allele. In *trh*³/+ embryos, β -gal expression is seen in all tracheal cells (Fig. 4A). In *trh*³ homozygous mutant embryos, β -gal expression is seen in a small number of cells that remain on the embryo surface at the approximate original posi-

tion of the tracheal placodes (Fig. 4B). This result suggests that in *trh* mutants, the tracheal cells, like those of the salivary duct, simply fail to invaginate to form tubes.

In another *Drosophila* mutant, *btl*, the tracheal cells invaginate to form tracheal pits but do not undergo the subsequent extensive migrations characteristic of tracheal cells (Glazer and Shilo 1991; Klambt et al. 1992). To determine whether *trh* mutants initiate any steps in tracheal tube formation, we examined *trh* mutant embryos by scanning electron microscopy (SEM). In embryos homozygous for *trh*¹, *trh*³, or *trh*⁸, we see no evidence of tracheal pit formation, even at 1000 $\times$ magnification, suggesting that *trh* is required at the earliest stages of tracheogenesis (Fig. 5). This result contrasts with that of Younossi-Hartenstein and Hartenstein (1993), who suggest that in *trh*¹ mutant embryos, tracheal pits form but fail to elongate and migrate. Because we do not have conditional *trh* alleles, we cannot determine whether the continued expression of *trh* is necessary for subsequent migration events. However, *trh* is expressed in the trachea throughout embryogenesis (Fig. 3), and *trh*- β -gal expression in the larval trachea can be

Figure 3. *trh* is expressed in the tube-forming cells of the salivary duct (sd), trachea (tr), filzkörper (fk), and CNS. Shown are lateral views of embryos of increasing age: [A–F] and ventral views of embryos of increasing age (G–L). Embryos were hybridized with a digoxigenin-labeled genomic *Eco*RI 3.8-kb fragment that includes most of the *trh* coding region. Transcripts are first detected at embryonic stage 8 in the tracheal primordia (A,G). Transcripts begin to appear in the salivary gland (sg) primordia by late stage 8, early stage 9 (B), coincident with SCR expression (not shown). By stage 10, the intensity of *trh* expression in the trachea correlates with the relative number of cells that contribute to trachea in each metamere (C,H). Also by stage 10, we can detect high level *trh* expression in the entire salivary gland. As the salivary gland cells begin to invaginate, *trh* expression begins to disappear with persistent salivary expression remaining only in duct cells (D,E,I). The duct cells are the most ventral cells of parasegment 2 that express *trh*, the salivary cells that have the higher levels of *trh* at stage 12 (J). Filzkörper expression becomes obvious by stage 11 (I). Expression in the trachea, salivary duct, and filzkörper persists for as long as it is possible to detect transcripts by whole mount in situ hybridizations (E,F,K,L). By late stage 13, we also begin to detect *trh* mRNA in a subset of cells in the CNS. The CNS staining in the supraesophageal ganglion (spg) is apparent in F and K and correlates with the α - β -galactosidase staining in the CNS detected from the P-element insertion in the *trh*³ allele. The regular pattern of CNS staining in later embryos is shown in L. (dt) Dorsal trunk of the trachea; (fk) filzkörper; (gb) ganglionic branches of the trachea; (sd) salivary duct; (sg) salivary gland; (spg) supraesophageal ganglia; (tr) trachea; (vb) visceral branches of the trachea. Embryos were staged according to Campos-Ortega and Hartenstein (1985): A is stage 8, B and G are stage 9, C and H are stage 10, D and I are stage 11, E and J are stage 12, F and K are stage 14, and L is stage 15.

detected as late as the third instar (data not shown). The early and persistent expression of *trh* in the trachea and early defects seen by SEM suggest that *trh* functions very early in the trachea and may be required continuously in this tissue.

We find that *trh* is required for the expression of at least two tracheal markers: mAb68G5D3, an antibody that recognizes an unknown antigen on invaginating epithelia (Giniger et al. 1993), and enhancer-trap line *rp395* (Fig. 4C,D), a line that normally expresses β -gal to high levels in all tracheal cells beginning in mid-stage 11, when tracheal pits are first visible, and continuing throughout embryogenesis. These markers were not expressed at any stage in tracheogenesis in *trh* mutant embryos suggesting that the corresponding genes are dependent on *trh* for their expression in the trachea.

We also looked at expression of CRUMBS (CRB), an integral membrane protein that specifies apical identity to the plasma membrane (Tepass et al. 1990a,b; Wodarz et al. 1995). Although CRB expression also largely disappears in the trachea, we do see some expression of CRB in the lateral regions of several abdominal segments (Fig. 4F). This CRB staining coincides with the β -gal staining observed on the surface of *trh*³ homozygous mutant embryos (Fig. 4, cf. B and F). We suggest that the β -gal-positive cells on the surface of *trh*³ embryos are tracheal cells, that these cells retain their ability to induce CRB expression, but have lost the ability to invaginate to form tubes. We also looked at expression of DRIFTER (DFR), a transcription factor expressed and required in both tracheal cells and in midline glial cells for their proper migration (Anderson et al. 1995). At later stages of

Figure 4. In *trh* mutant embryos the tracheal cells do not invaginate to form tubes. (A) *trh*^{3/+} embryo stained with β -galactosidase; (B) *trh*³ homozygous mutant embryo stained with β -galactosidase; (C) *+/+*; *rp395* embryo stained with β -galactosidase; (D) *trh*¹; *rp395* homozygous mutant embryo stained with β -galactosidase; (E) *+/+* embryo stained with α -CRB; (F) *trh*⁸ homozygous mutant embryo stained with α -CRB. The embryos are either heterozygous (A) or homozygous (B) for the inactivating P-element insertion in *trh*³. The embryos in C and D carry a homozygous viable P-element insertion known as *rp395*. In otherwise wild-type embryos (C), β -gal nuclear staining in *rp395* can be detected in the salivary gland secretory cells, every tracheal cell (arrows), midline cells of the CNS, and a subset of cells of the gut endoderm (not visible in these embryos). In *trh*¹ homozygous embryos (D) no tracheal staining is observed at any stage in embryogenesis (arrows) although other aspects of the *rp395* staining pattern are identical to wild-type embryos. Limited α -CRB tracheal staining is seen in *trh*⁻ embryos (F) in regions corresponding to the tracheal precursor cells that fail to invaginate in *trh*³ mutant embryos (open arrows in B). Other aspects of CRB staining in other invaginating ectoderm is unchanged (except in the salivary gland duct).

development, we fail to detect obvious expression of DFR in the trachea; however, at early stages, prior to tracheal cell invagination, expression of DFR in *trh* mutants is indistinguishable from wild type (data not shown). The DFR positive cells of the trachea fail to invaginate in *trh* mutants. Thus, the defect in *trh* mutant embryos is not a defect in the identity of tracheal cells (because they express other tracheal genes), but rather a defect in the ability of the tracheal precursors to organize tubes. The downstream markers that are dependent on *trh* for their expression are thus likely to correspond to genes required for tube formation or to genes expressed as a consequence of tubulogenesis.

trh is also required in the tubes that connect the trachea to the posterior spiracle (Fig. 3; Jürgens et al. 1984). *trh* RNA expression in these cells is first seen during embryonic stage 11 (Fig. 3I). These *trh*-expressing tubes form the filzkörper, which are large chambers lined by cuticular threads that function as air filters for the trachea (Fig. 6C,F). In almost all larvae homozygous for the more severe *trh* mutations (*trh*¹, *trh*⁸, *trh*⁷, *trh*³, and *trh*⁶), the filzkörper completely fail to elongate and remain clustered near the posterior spiracular opening (Fig. 6C,E,F). In larvae mutant for the weaker *trh* alleles (*trh*⁴ and *trh*⁵) there is a range of phenotypes; the filzkörper sometimes completely fail to elongate, but more frequently, the filzkörper either elongate unilaterally (Fig. 6G) or bilaterally and frequently break (Fig. 6H). Although the filzkörper fail to elongate in *trh* mutants, the cells still secrete the cuticle that normally lines this structure (Fig. 6C,E,F). This observation suggests that, as with the trachea, the identity of these cells is not disrupted in *trh* mutants; these cells have simply lost the ability to form (and perhaps maintain) tubes.

trh is expressed and required in many but not all cells that form tubes. Other embryonic tubes, such as the esophagus, gastric caeca, and Malpighian tubules, do not express detectable levels of *trh* (Fig. 3) and are unaffected by *trh* mutations (data not shown). We also detect *trh* mRNA in cells that do not form tubes; a subset of cells in the central nervous system (CNS) express *trh* (Figure 3F,K,L).

trh expression in the salivary gland duct is controlled by *Scr*, *fkh*, and *dpp*

We first identified the P-element insertion allele of *trh* because of its early strong β -gal expression in the entire embryonic salivary gland (Fig. 1C). To test whether *trh* could be controlled by *Scr* in the salivary gland, we looked at *trh* expression in embryos that lack *Scr* function and in embryos where SCR protein is expressed in new places. In *Scr* null homozygous embryos, we see a complete loss of *trh* expression in parasegment 2, the region where salivary glands normally form (Fig. 7B). In embryos where SCR is expressed ubiquitously, using a heat shock promoter to drive expression of an *Scr* cDNA (HS-SCR), we observe ectopic expression of *trh* more anteriorly in parasegments 0 and 1 (data not shown). We also looked at the expression of *trh* in embryos mutant for *teashirt* (*tsh*). In *tsh* mutants, we see a posterior expansion of *trh* expression into parasegment 3 (Fig. 3F), coincident with the changes in *Scr* expression observed in *tsh* mutants (Fasano et al. 1991; Andrew et al. 1994). Thus *trh* expression in the salivary gland is activated by the homeotic gene *Scr*. These results also suggest that SCR activation of *trh* is prevented in posterior segments

Figure 5. Tracheal pits do not form in *trh* mutant embryos. SEMs of wild-type (A) and *trh*³ (B) embryos are shown. Note the complete absence of tracheal pits in the *trh*³ mutant embryo. C and D are higher magnifications focusing on the segment containing the eighth tracheal metamere in wild type (C) and in the *trh*³ embryo (D). Scale bars in A and B are 50 μ m, and in C and D are 10 μ m.

by the trunk gene *tsh*, as was observed with another salivary gland gene, *dCREB-A* (Andrew et al. 1994).

trh is initially expressed in the entire salivary primordia but is shut off in the secretory cells during stage 12 (Fig. 3E). To test whether any of the known early salivary gland genes whose expression is limited to the secretory cells could be shutting *trh* off in these cells, we looked at *trh* RNA expression in embryos mutant for *fork head* (*fkh*) (Jürgens and Weigel 1989), *dCREB-A* (D.J. Andrew and S. Smolik, unpubl.), *hückerbein* (*hkb*) (Brönner et al. 1994) and *jalapeño* (*jal*) (D.J. Andrew, unpubl.). *trh* expression in embryos mutant for *dCREB-A*, *hkb*, and *jal* was indistinguishable from wild type. In *fkh* homozygous embryos, however, *trh* RNA persists in the entire salivary gland through late stages of embryogenesis (Fig. 7C) until the *fkh*-requiring secretory cells disappear during stages 14/15. In *fkh* mutant embryos, the salivary secretory cells fail to invaginate and eventually disappear (Jürgens and Weigel 1989; J. Aishima and D.J. Andrew, unpubl.). Thus, *trh* salivary gland expression at later stages is limited to the salivary duct through repression by *fkh* in the secretory cells. In *fkh* mutants, the salivary duct cells also fail to invaginate to form tubes (Fig. 7D), but do occasionally invaginate to form an internalized teardrop-shaped structure. *fkh* is unlikely to be required in the duct cells for tube formation because

fkh is not expressed in these cells (Panzer et al. 1992). We think that if the secretory cells do not invaginate, then mechanical constraints may prevent subsequent duct cell invagination.

trh expression in all segments is limited dorsally by the gene *decapentaplegic* (*dpp*). In *dpp* mutant embryos, expression of *trh* in the salivary gland is observed in all ectodermal cells that express *Scr* giving rise to a band of *trh* expression encircling parasegment 2 (Fig. 7H). In the

Figure 6. Filzkörper do not elongate in *trh* mutants. (A,B) cuticle preparations of wild-type first instar larvae; (C) late stage embryo, heterozygous for *trh*³ stained with α - β -galactosidase; (D,E,G,H) cuticle preparations of *trh* mutant first instar larvae; (F) late stage *trh*³ homozygous mutant embryo stained with α - β -gal. In wild-type embryos, *trh*-expressing cells are normally organized into tubes that connect the trachea to the posterior spiracle, the only functional spiracle in first instar larvae (C). In *trh*³ homozygous embryos, we detect *trh*- β -galactosidase staining in the same precursors, but the cells fail to organize into a tube (F). In *trh* mutant larvae, we observe either a complete failure of the filzkörper to elongate (C,E,F), partial elongation (G), or elongation and breakage (H).

Figure 7. *trh* expression in the salivary duct is controlled by *Scr*, *tsh*, *fkh*, and *dpp*. Embryos were hybridized with a digoxigenin-labeled *trh* genomic fragment detected with α -digoxigenin antibody conjugated to alkaline phosphatase. (A) Wild-type embryo, ventral view; (B) *Scr*⁻ embryo, ventrolateral view; (C) *fkh*⁻ embryo, ventral view; (E) wild-type embryo, lateral view; (F) *tsh*⁻ embryo, lateral view; (G) *dpp*⁻ embryo, lateral view; (H) *dpp*⁻ embryo, lateral view. Note the loss of only salivary gland staining in the *Scr*⁻ embryo (B) compared with wild type (A,E). In *tsh*⁻ embryos, *trh* expression in the region of the salivary gland expands into parasegment 3 (F) coincident with the expansion of SCR protein. In *fkh*⁻ embryos, *trh* expression persists in the secretory cells of the salivary gland (C). Although this embryo is not germ-band shortened, it is older than stage 12 because staining in the CNS, not visible in wild-type embryos until stage 13, can be detected. The salivary duct does not invaginate in *fkh* mutants, nor do the tracheal cells complete their migrations (D). In *dpp*⁻ embryos, tracheal expression expands around the dorsal surface of the embryo (G,H) and salivary gland expression spans the entire ectoderm of parasegment 2 (H).

tracheal forming segments, *trh* is expressed in a thin arc around each segment in *dpp* mutant embryos (Fig. 7G,H). Only the most ventral ectodermal cells in these segments do not express *trh* in *dpp* mutants.

trh is expressed in the embryonic salivary duct, trachea, filzkörper, and CNS (Fig. 3). In each primordia a distinct set of regulators controls *trh* expression. In the salivary gland, *trh* is initially activated by SCR (Fig. 7B,F) and is further limited to the salivary duct through repression by FKH (Fig. 7C,D), another transcription factor expressed in only the secretory cells of the salivary gland. *trh* expression in the trachea and CNS is likely to be under the control of the segment polarity genes because of the reiterated pattern of expression from segment to segment. The limited expression of *trh* to the 10 segments that form trachea and the salivary gland-forming segment suggests that *trh* is also regulated by homeotic genes other than *Scr*. Expression of *trh* in the filzkörper is also likely to be under the control of one or more homeotic gene. In every segment, *trh* expression is limited to more ventral regions by the dorsal determinant, *dpp* (Fig. 7G,H).

trh encodes a protein with homology to bHLH-PAS transcription factors

To isolate the *trh* gene, we cloned DNA flanking the *trh*³ P-element insert from both phage and cosmid genomic libraries (Fig. 2B). *Eco*RI restriction fragments from the cloned region were then used to screen for *trh* transcripts both by Northern blots and by whole-mount in situ hybridization to embryos. Two genomic fragments hybrid-

ized strongly to transcripts expressed in the trachea, salivary gland, filzkörper, and a subset of cells in the CNS, the same expression pattern observed with β -gal staining of the *trh*³ insertion line. DNA fragments that hybridize to mRNA were used to screen several embryonic cDNA libraries. We isolated one 0.6-kb cDNA from the Tamkun 0- to 22-hr embryonic cDNA library and 17 cDNAs ranging in size from 1.8 to 3.2 kb from the K. Zinn 9- to 12-hr embryonic cDNA library. We sequenced both genomic DNA and several of the longest cDNAs (Fig. 2B).

The largest *trh* open reading frame in the sequenced region spans 2775 nucleotides and encodes a putative 925-amino-acid protein (Fig. 8). A smaller transcript omits exon 6 deleting 29 amino acids of the coding region. A search of available protein data bases with TRH reveals extensive homologies between *trh* and two other proteins: the human Hypoxia-inducible factor-1 α (HIF-1 α) (Wang et al. 1995) and Single-minded (SIM), another *Drosophila* regulatory protein (Nambu et al. 1990, 1991). Both HIF-1 α and SIM encode related transcription factors. HIF-1 α is a protein required for transcriptional activation of the erythropoietin gene in response to low oxygen tension (Wang et al. 1995). SIM is required for the development of neurons, glia, and other nonneuronal cells that derive from the midline of the embryonic CNS (Nambu et al. 1990, 1991). TRH, SIM, and HIF-1 α are homologous in two regions found in other regulatory proteins: (1) the basic helix-loop-helix (bHLH) region, including the basic region, which in other bHLH proteins directly contacts DNA, and the HLH region, which directs dimer formation (Murre et al. 1989; Davis et al.

*cDNA begins		intron/exon 9 start	
CCG GGA CCG ACC GGA TAC CCG ACT CCG TCG GCG ACG GTA CTC CCG ACT COT COT CAC AAT ACT CCG TAG GCG TAC TAC GTA CTT COT ATT	90	GAC CTA ACC CTA TGA CAG TAG TAT ATA TTA AAA TCT GAT ACT CAC AAG GGA ATG AEA ATT CTT CTT ATT TTGATCTTTAG TGA ATC GAG AAG	2829
CCG TAT TCC GTT GTC AGC GTC ATC AGC TGC AGT TTT ACG TAC GGA GCG CCG TCG AAG TGT GTC COT ATA TCG GTA GGC CAT CCG TTT	180		
CGA TAG CCG GTT AAT GCG AAG CAG GAT GCG TGC TGC GGA ATT GCG AGG TCT CAT TCC GGA GAG CCG AGG AGC AAC GGA CCG TAC	270		
TGC ATG CCG GCG TAG TTT GCG CAT CCG GAT CCG TAT TGC GTG ACC COT ACA TGC CTT GCG GGA TTC GAG GAT AAC TAA AGA CCG GTA CCA	360	GCT CAG GTG TTG ACC GGA TAC TAC CCA ATG AAC AAG AGC GGA GGC TAC ACT TGG CTC CAG ACC TGT GCC ACT GTG CTC CCG ACA	2115
CAC CCG COT GTG TGA TTA COT TTA TGT GAG CCG AAG AAC AAA AGT ATG CCG CTT TCA AAT GGA ATG CCA CCG CCG CTA GAA TGC CCG	450	G Q V L T G Y Y R L M N K S G G Y T W L Q T C A T V V C S T	
CAA GCG AAC TGC AAT AAC AAG TGA AGT GCG AAG TAG TTA AAT ACT TGC TGA GAT CAT CAG GCT TCC AAT AAG AAA AAG TCA	540		
GCC CCA GGA TTT CTA TGT TCG CTT ACC AGG CAG CCG TCG CCA TGG ACT ACG CCG GAT AAC CCA CCG CCG ACA TCC CCG AAG	630	end exon 9 /intron	
CGA CAT GCG CAG CAT GGC ATC TCT GGC ATC CCG GTG CCG TTT ACC CAG CAG TGG ATG GTG CCG ACG CAG CAG CTT TGC CCG ATC CCA	720	AAG AAC GCG GAT GAG CAG AAC ATC ATC GTG GTG AAC TAT GTG ATC GCG GTGGTGT GTA GGA TAA GGA ATT ACG AAG GTA AAA TCC ACT AAA	2186
M P A V P F T T H S W M V P T Q D L C A M P	810	K N A D E Q N I T C V N Y V I S	
exon 1 end /intron		intron/exon 10 start	
CCC TAC AAC AAA ATG ACG GGA CAG CAG CAG CCG GGA ATG CAG CCG CAG CCA CAG CCG CCG CCG CCG GTTAGTAGCC.....	887	AAC CTC TTT CACCTACG C AAC CCG GAG AAC GAG AAC ATG ATA CTG GAT TGC TGC CAA CTG CAA CCC AGT CCG AGC ATT AAG CAG CAG	2226
P Y N K M T G H Q P P G A G N H A Q Q Q P L E P G		N R E N E N M I L D C Q L E P S P D S I K H E	
intron/exon 2 start		intron/exon 11 start	
.....TCCTGACTTCGAG C ATC CTG GAG CCG CCG AAG GAG AAG TCG AGG CAG CCG AGA TCG CCG CCG GGA AAG AAG TAC GAG TTC	968	GAG GGA CCG GGT AAC CAA AAA AAC AGC CCG CCG TCA GGA GGA GAT GCC AGT GGC GGA AAC TCT CAG CTG AGC GCG CAG ATG AAG	2329
I L E L R K E K S R D A A R S R R G X G N Y E F		E G L G W M D K S S C S P G G D A S G C E A G E N S H L S A G D M K	
TAC GAG CTA GCG AAG ATG CTT CCG CCG GGA GCG ATC ACG CAG CCG CAG AAG GCG TCG ATC ATA GGA CTTG ACC ATC AGC TAC CTA CTG	1058	CTT ACA TCG CCG AAA AAC CAT TGA GAG CCG CAT TCC CAC CCG AGG AGT GCG AGC GCG TCC CAT GGA ACT TGA ATG AAG AAC	2439
Y E L A K M L P I P A A I T S Q L D K A S I I R L T I S Y L		L N S P R K Y T C G S H S H R G R G A S A A A S H G S S M N S	
exon 2 end /intron		intron/exon 12 start	
AAG CCG AGG GAT TTC TCG CAG CCG GAT CCG AAG TCG AAT CCG GAA CCG TCT ACG AGC AGT AAG CTA AAA A GTAGTAA.....CCC	1123	CTC AAC ATC ATC GAT AGC CCG ACT CCG GTG GTG AAG CAG TCT GGA GTG CCG CCG ACC ACA ACG GCG ACT CTA GTC CCG CCA	2589
K L R D S F S G H G D P P W T R E A S S S K L K		L T W I E K D S P T P L G V E I D S G C L G V L P V A T T V A T P V P A	
intron/exon 3 start		intron/exon 13 start	
ATTCCTTCCTGAG G TGC GCG GCG ATT COT CCG ACG CCG GCT GTT GAT TTT GAG CAA CAT CAG GCG ACC CAG CTA AAA A GTAGTAA.....CCC	1191	GCC ACT CTA CCG CAG TCC ACC AAG CAG GGA AAA AAC AAG CCG TCG CAG CCG GAG CAG CAG CAG TAT CAG CAG GAT ATA TCC	2599
S A A I R R S P A V D L F E Q H Q G T H I L Q		A T P P V Q S T T K R K R K T R A S Q H A E D Q G G E Q V I S	
intron/exon 4 start		intron/exon 14 start	
.....GGGATTCATTCCTGAG TCG CCG GAT GGT TTT GCG CTA CCG GTG CCG GGA CCG CCG TTT CTT TAT ATT TCC CAG AGC GTG TCC	1260	GAG CCG CTT CTT GCA CCG ATG CCA ACA ATG CAA CCG CAT CAG CCG CCG ACC CCG ACG CCG TTA CCG TCC GTT GAT CAG CCG CCG	2689
S L D G F A L A V A A D D G R F L Y I S T Y S		E Q P L P K I P T M E Q R D Q Q P R S L R P S I V D E Q P P S	
ATC TAT TTT GCG CCG TCG CCG GAT GAG ATG AGC AGC ACG ATC TTC CAG TAT ATC CAG CCG GAT CAG TCG GAG ATC GCG CAG C	1350	TCC GCG GCG CAG CCG TCG CCG AAG CAT CAG CCG CCG ATC CAG CAT CTT CCG CCG CCG CCG GCG GCG GCG GCG GCG GCG CCG	2779
I Y L G L S Q V E M T G S S I F D Y I N Q A R D H X E A D C		S A A D S A V K D L E Q A M S K H L P S P A A V V S V A P P	
CTG GCG CCG ACG CTA ACG GCG GCG GCG GGT GCG GGT ACG ACG TCG ACG ACG GCG GGA GGA GCG GGA GCG GGT GGT A	1440	AAT ACT TGT TTT AGC CAG CCG TCT CTT CCG CAG CAA CAA CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG	2869
L G L S L T A S G G G G G G G S S G G G G G G G G G G A G G G M		N T D F S A D S L L K K Q Q Q Q Q Q Q Q L D P N E K S S T I Q W	
exon 4 /intron		intron/exon 15 start	
GCC TCA CCG ACT TCC GGA CCG TCC CAG GAT GGA ACG GCG ACG ACG ACG ACG ACG ACG ACG ACG ACG ACG ACG ACG ACG ACG	1504	CTC GAG TCC TAG GAG CAA TAC CTT CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG	3139
A S P T S G A S D G S G T H G T N N P D		E S S Y E N Q Y L Q L H S A A S G G H P G G K T S A D A F	
intron/exon 5 start		intron/exon 16 start	
DNA.....CTTTTACTCTGAG TT CCG GCG TCG ATC CAA GGT TCG AGC AGC GCG TAC GAT CCA GCG TTT TGT GTC COT ATG	1575	ACC AAT CTT GCG TGA ACA TCC GCG GCG TAT CAG AGC TCC AAT CAG TAT CAG AAC GCG AAT CCG CCG CCG CCG CCG CCG CCG	3229
V A A S M T Q A S T S G Y K G Y D R S F C V R H		T N L V S Y E N Q Y L Q L H S A A S G G H P G G K T S A D A F	
AAG TCC ACG CCG ACG CCG GCG TGT CAG CTT AAA TCC TCA CCG GAT CCG GTAGTTTC.....291 nt: intron DNA.....CATCA	1626	TGC AAT CAA CCG GGT GAG GCG GCG TGA CCG GCG TCC AAC GGG GGA TAC CAG TAC TCC GCG CCG CAG CTA GTC TGT TCC AGG CAG	3319
K S T L T K R G C H F K S S G Y R		N Q P G C A A A P V L A S N G G Y D Y A P D A T L R G G Q Y A T	
intron/alternative exon 6 start		intron/exon 17 start	
CTGCTATCTACAG GCG AGC GAT CCA ACG AGC AAT TCC AAC AAG GGT AAC AAT COT AGT AAC AAT COT AAC AAT GGT AAC AAT CCG GCG TCA	1705	TCA TCC CAG CCG CTT GCG CCG	3409
A S D A T S N C N H G N H A S N H A K N V K N P G S		S G D V G C A A A P L P L K Q Q A S Y T Q A S Y T Q A S Y T Q A S Y T Q A S Y T Q A S Y T Q A S Y T Q A S Y T Q A S Y T Q A S Y T	
end exon 6 /intron		intron/exon 18 start	
AAC TAT TCG GTA COT ATC AAT TGA AAT CAG TAC TGT TGT ATG CTA AAA TCG943 nt: intron DNA.....TGG CTT CAG TTT GAA CCG	1714	GAG GGT GCG GTT AAC TAC AGT AAT CTC GAG CCG CAG TAC TTT CCG AGC CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG	3499
N Y S		G V Y T Y S N L D Q P Q Y F A P H S S F H L Y N K G S P A	
intron/exon 7 start		intron/exon 19 start	
TTGAGC GTG GTT CTT CTT TGC TGC AAG CTT CCG CAG TAC ACA TTT TCC CAG AAT CCG AAA TCG CAG CAG CCG CAG CAG CCG CAG CAG	1801	ACC GCG TCC TAC CAG CCG CCG TCC TGT GAG CTT CAG CCA GGG GGA GGT CCG CCG ATC CTT GCA CCA GCA CTA CCA CCA	3589
V V L L L L K L R P Q Y T F S H R K S Q P P L L G N V		S G W Y S T P S	
GCC CCG GCG ACG CCG	1891	CCA CCA CTA TCA TCA ACA CCG ATC ACC GGT CAG CAG CCG CAG CAG CCG CAG CAG CCG CAG CAG CCG CAG CAG CCG CAG CAG	3679
A L A I A L P P S V N E I R L E C D M F V T R V N F D L A		TAG GAT GGT TTT TAT CCG CCA AAA GGG TAA TGG ATA CAG CCA CCA TGT CTT ATT AAG CCG CCG CCG CCG CCG CCG CCG	3769
exon 7 /intron		intron/exon 20 start	
GTA GCG CAG	1918	TTG CAG TCC CTA AAA CAG	3859
V A H C E P R		ATC AAC TCA AAA CAG	3949
GAT CTT CCG CAG TAT TCC CCG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG		ATT AAG CAA CAG	4039
D L D Y S P E D L V N K S L Y S L C H A E D A N R L R K S		ATC AAC TCA AAA CAG	4129
exon 8 /intron		intron/exon 21 start	
CCC TAC AAC AAA ATG ACG GGA CAG CAG CAG CCG GGA ATG CAG CCG CAG CCA CAG CCG CCG CCG CCG GTTAGTAGCC.....	2018	AAA TGG TAC TTT CAG CTT TTT TTA TCA ACA ATT AAA AAA AAA AAA ATT AAA ATT CCT AAA TAC CAA AGT AGG CCA AAG GAG AAG TTT CCA	4684
P Y N K M T G H Q P P G A G N H A Q Q Q P L E P G			

these cells. Using a number of expression systems currently available for *Drosophila*, it should be possible to express *trh* in internal tissues and see if extra tubes form.

We also detect *trh* expression in a subset of cells in the CNS. In *trh* mutants the CNS has subtle defects in axonal morphology (D.D. Isaac and D.J. Andrew, unpubl.). We are now testing whether these defects are caused by a failure of *trh*-expressing cells in the CNS to migrate. This result would provide a molecular link between cell migration and tube formation. One of the three general mechanisms for tubulogenesis involves cell migration with the maintenance of intercellular contacts. *trh* function in nerve cells, which do not have the adhesive properties of epithelial cells, may be simply to induce migration.

Regulation of *trh* expression

trh expression in the salivary duct begins late in embryonic stage 8 and depends on the homeotic gene *Scr* (Figs. 3 and 7). In *Scr* mutants, *trh* is never expressed in parasegment 2 where salivary glands form. In embryos where *Scr* is expressed in new places, either in *tsh*⁻ embryos or in induced HS-SCR embryos, corresponding changes in *trh* expression are observed. Initially, *trh* is expressed in the entire salivary primordia, in both duct and secretory cells, consistent with a simple model of *trh* activation by *Scr*, and repression of that activation by *dpp* (Fig. 7) (Panzer et al. 1992; Andrew et al. 1994). At later stages, *trh* expression is restricted to the duct cells. Here, we have shown that another gene, *fkh*, represses *trh* expression in the secretory cells of the salivary gland. *fkh* is expressed in only the secretory cells of the salivary gland. *fkh* encodes a transcription factor related to the mammalian gene *hepatocyte nuclear factor-3α* (*HNF-3α*) (Weigel et al. 1989; Weigel and Jäckle 1990), so the repression of *trh* expression by *fkh* may be direct.

We propose that *Scr* activates expression of *trh*, *fkh*, and other salivary gland genes in PS2, except in dorsal regions where *dpp* is expressed (Fig. 10) (this work; Panzer et al. 1992; Andrew et al. 1994). *dorsal* (*dl*) and the *spitz*-group genes prevent expression of *fkh* (and perhaps other salivary gland genes expressed in only the secretory cells) in the most ventral regions of the salivary primordia (the cells that form the duct) (Panzer et al. 1992). *trh* is not directly affected by *dl* or the *spitz*-group genes and is therefore initially expressed in both salivary gland secretory cells and duct cells. Once *fkh* is expressed by the secretory cells, it acts to prevent *trh* expression in those cells. Thus, *trh* expression is limited to the duct, whereas *fkh* is limited to the secretory cells. Based on the loss-of-function phenotype, *fkh* is required in the secretory cells for their invagination, whereas *trh* is required in the duct cells for their invagination. To determine if *Scr* directly activates *trh* expression, and if *fkh* directly represses *trh* expression, we must see if the SCR or FKH proteins bind directly to *trh* DNA.

The earliest expression of *trh* in the trachea appears in 10 stripes, suggesting possible regulation by the segmentation hierarchy. The earliest expression of *trh* is not

Figure 10. Model for salivary gland gene expression. *Scr* is the only known positive inducer of salivary glands. Both *dpp* and *tsh* prevent activation of salivary gland genes by *Scr* (this work; Panzer et al. 1992; Andrew et al. 1994). *trh* is induced in the entire salivary gland primordia early. *fkh* is induced only in the secretory cells, because of repression by *dorsal* (*dl*) and the *spitz*-group genes (Panzer et al. 1992). Once *fkh* is induced in the secretory cells, it shuts off *trh* in these cells. Persistent expression remains of *trh* in the duct cells and of *fkh* in the secretory cells. In the cells where *trh* and *fkh* are expressed, each gene is required for the invagination of those cells.

uniform in each tracheal-forming segment; expression is high in metamere 1 and low in metamere 3 consistent with the number of cells that form trachea in each segment. Tracheal metamere 1 contributes ~150 cells per hemisegment, and metamere 3 contributes ~50, whereas all others contribute ~90 cells (Manning and Krasnow 1993). The absence of *trh* expression in some anterior segments as well as differences in numbers of cells that express *trh* in adjacent segments suggests that *trh* expression in tracheal tissues is also regulated by homeotic genes (Fig. 7). During stages 9 and 10, the stripes of tracheal expression become more oval, a shape change fueled perhaps by an increased affinity among *trh*-expressing cells for one another. The striped domains of *trh* expression in the tracheal precursors are even more apparent in *dpp* mutants where *trh* expression expands into the entire dorsal axis.

Downstream of *trh*

The homology of TRH to other bHLH transcription factors suggests that TRH may function as a regulator of transcription. To our knowledge, *trh* is the first transcription factor known to control tubulogenesis in any system. We identified two markers, MAb68G5D3 and *rp395*, whose expression in the trachea is dependent on *trh*. Mutations in the genes corresponding to MAb68G5D3 and *rp395* have not yet been isolated. Because *rp395* is an enhancer-trap insert, it should be possible to make mutations in the corresponding gene and

assay for tracheal phenotypes. Both MAb68G5D3 and *rp395* represent potential downstream target genes that could be directly regulated by *trh*.

We are very interested in an additional gene, *btl*, which may be directly controlled by *trh*. *btl* encodes a bFGF receptor that is expressed early and continuously in tracheal cells (Glazer and Shilo 1991; Klamt et al. 1992). In *btl* mutants the trachea invaginate, but the cells remain near the site of invagination, suggesting a role for *btl* in facilitating migration. bFGFs, as well as other growth factors such as TGF- α and HGF/SF, have been implicated in tube formation in mammalian systems and are thought to be produced by the local inducing mesenchyme (Montesano et al. 1986; Ono et al. 1992; Rogers et al. 1992; Santos and Nigam 1993; Barros et al. 1995; Zarnegar and Michalopoulos 1995). Receptors for these growth factors must be expressed in the responding epithelial cells. Transcription factors, such as *trh*, may determine whether epithelial cells can form tubes by controlling the expression of receptors for the appropriate growth factors.

TRH, HIF-1 α , and SIM encode a family of closely related DNA-binding transcription factors. The three proteins are highly conserved in their bHLH and PAS domains, and the homology extends into the region carboxy-terminal to the PAS domain (the HST domain) (Fig. 9B). Whereas it is clear that TRH is more related to the HIF-1 α and SIM proteins than these proteins are to each other, it is less clear which of the two is most similar to TRH. However, HIF-1 α and TRH are identical in the DNA-binding region of the bHLH domain. It would be exciting to find that the vertebrate ortholog of TRH is HIF-1 α or another closely related gene. HIF-1 α activity is induced in response to hypoxia and activates transcription of erythropoietin. One homeostatic response to hypoxia is neovascularization, the formation of blood vessels (White et al. 1992). The trachea is the oxygen delivery system in *Drosophila*; it is enticing to speculate that HIF-1 α may be involved in regulating the formation of the oxygen delivery system in mammals. HIF-1 α expression patterns may reveal a role for this gene in the formation of tubes during early mammalian embryogenesis.

Conclusion

In this work we have isolated six new alleles of the *trh* gene and have shown that this gene is required in *Drosophila* embryos for the formation of three embryonic tubes. We have demonstrated that *trh* may encode a transcription factor very closely related to two known proteins, *Drosophila* SIM and Human HIF-1 α . We find that *trh* is expressed prior to any morphological changes preceding tube formation and is continuously expressed in these tube-forming cells. We have shown that the expression of *trh* in the salivary gland duct is controlled by at least three different regulators, *Scr*, *fkh*, and *dpp*, and that, in turn, *trh* regulates several downstream genes. *trh* represents the first potential downstream target gene for *Scr* with a distinct role in the morphological changes

involved in organ formation. The link between *Scr* and *trh*, and *trh* with its downstream target genes, brings us closer to an understanding of how homeotic genes control the formation of distinct organs in the different body segments.

Materials and methods

Fly stocks

Line *l(3)10512* (= *l(3)trh³*) is from the enhancer-trap P-element stock collection from the Spradling laboratory and is marked with the *ry⁺* gene (Karpen and Spradling 1992). *l(3)trh⁴*, *l(3)trh⁵*, *l(3)trh⁶*, *l(3)trh⁷*, and *l(3)trh⁸* are *ry⁻* excision derivatives of *l(3)10512* that fail to complement a *trh* deficiency and *l(3)trh¹*. *l(3)trh-R12* and *l(3)trh-R25* are lethal *ry⁻* excision derivatives that complement *l(3)trh¹*. *trh-R3*, *trh-R26*, and *trh-R63* are fully viable *trh⁺ ry⁻* excision derivatives. Excision mutagenesis was carried out as described in Hamilton and Zinn (1994). The EMS allele, *trh¹* (formerly known as *trh^{5D}*) was provided by S. Beckendorf (University of California, Berkeley).

rp395 is a viable enhancer-trap insertion line identified in the Goodman–Rubin laboratory enhancer-trap screen that does not correspond to any currently known gene. *rp395* was used in this study because it exhibited strong nuclear β -gal staining throughout the entire embryonic tracheal system.

Loss-of-function *fkh* alleles, *fkh¹* and *fkh⁶*, were obtained from the Indiana Stock Center. *hkb* mutants (Brönnner et al. 1994) were obtained from Q. Chu-LaGriff and C. Doe (University of Illinois, Urbana). Loss-of-function mutations in the *dCREB-A* and *jal* genes were isolated in this laboratory and will be published elsewhere (D.J. Andrew, unpubl.). *HS-SCR* and *Scr^d* are described in Zeng et al. (1993) and Lindsley and Zimm (1992).

Deficiency stocks from cytological region 61 were kindly provided by H. Ellis (University of Georgia, Athens) and J. Posakony (University of California, San Diego).

Antibodies and embryo staining

The Engrailed (En) mouse monoclonal antibody (mAb4D9) was supplied by T. Kornberg (University of California, San Francisco) (Patel et al. 1989). The α -CRB monoclonal antibody was provided by E. Knust (University of Cologne, Germany) (Tepass et al. 1990a) and stains the apical surface of the salivary gland and trachea (as well as other invaginating ectoderm) from the initiation of the invagination of these tissues (embryonic stage 11) throughout embryogenesis. α -HRP, α -BP102, and α -DFR antisera (Anderson et al. 1995) were generously provided by M. Anderson and W. Johnson (University of Iowa, Iowa City). The mouse monoclonal antibody to β -gal was purchased from Promega Biotec (Madison, WI).

Embryo staining was performed as described (Reuter et al. 1990). Homozygous mutant embryos were identified by the absence of staining with the β -gal antibody, which selectively stains embryos carrying the balancer chromosome *lacZ* insert or by morphological criteria. Antibody-stained embryos were visualized and photographed by Nomarski optics using a Zeiss Axiophot. Ektachrome 64 Tungsten slide film and Ektar 25 print film (Kodak) was used for photography.

Whole-mount in situ hybridizations

Whole-mount in situ hybridization to embryos was done as outlined in Tautz and Pfeifle (1989), with the following changes:

Formaldehyde was used in place of paraformaldehyde, and levamisole was omitted from the staining reaction. Fragment DNA (0.5–1 μ g) was labeled by random priming overnight at 14°C.

Cuticle preparations

Cuticle preparations were done as described in Andrew et al. (1994). Preparations were examined using either phase or dark-field optics and photographed with Kodak TMAX 100 print film.

SEMs

Embryos for SEM were prepared as described in Sweeton et al. (1991).

Cloning and molecular characterization of trh

DNA flanking the original P-element insert was isolated by plasmid rescue (Hamilton and Zinn 1994). The ~1-kb genomic fragment was then used to screen isogenic cosmid and phage libraries (Tamkun et al. 1991). Exon-containing genomic fragments were identified by both Northern analysis and whole mount in situ hybridization to embryos. The hybridizing fragments were used to screen several embryonic cDNA libraries. cDNAs were isolated successfully from the Tamkun 0- to 22-hr embryonic library (Tamkun et al. 1991) and from the K. Zinn 9- to 12-hr embryonic library (Zinn et al. 1988). At each step of the cloning we performed in situ hybridization to chromosomes to verify that clones mapped to the 61C region of chromosome 3 and in situ hybridizations to embryos to be sure that the cDNAs corresponded to the *trh* gene.

Sequencing was done using the method of Sanger et al. (1977) with sequencing kits from U.S. Biochemical (Cleveland, OH). Sequencing primers were designed based on sequence information generated from the ends of the clones as well as sequence derived from convenient deletions made with internal restriction sites. Both strands of the DNA were sequenced. Regions of GC compression were also sequenced using dITP, a nucleotide analog for dGTP. Urea-polyacrylamide gels (7 M/6%), made with the Long Ranger Gel solution (FMC Bioproducts, Rockland, ME), allowed sequence resolution of 300–450 nucleotides per reaction.

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Note added in proof

The sequence data described in this paper have been submitted to the EMBL/GenBank data libraries under accession nos. BankIt 25704-U42699.

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D D Isaac and D J Andrew

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