Tubulogenesis in Drosophila: a requirement for the trachealess gene product

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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-1α 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; Stamatoglou 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-β1 (TGF-β1), has been shown to inhibit tubulogenesis (Santos and Nigam 1993), and the addition of anti-TGF-β1 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...
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; Klambt 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 [Panz-er 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 *tracheless* (*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, *I(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 61CL2, three-letter divisions from the published position of *trh* in 61E–F [Jürgens et al. 1984]. In *I(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 *I(3)10512* line, remain clustered on the embryo surface in *I(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* mutant embryos, salivary glands also form closed sacs. This common phenotype suggests that *I(3)10512* is a *trh* allele. A closer examination of *I(3)10512* homozygous embryos revealed additional phenotypes overlapping those of *trh* 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* mutant embryo stained with α-CRB; [C] *trh* + embryo stained with α-β-gal; [D] *trh* homozygous mutant embryo stained with α-β-gal. The salivary gland duct stained 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* 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*) gives rise to high level β-gal stained 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*+ embryos the salivary gland duct is normal [thick arrows in C]. In *trh* 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 \( trh^1 \), l(3)10512, and deficiencies from the region. The results are summarized in Table 1 and Figure 2A. \( trh^1 \) and l(3)10512 fail to complement each other and \( Df(3L)jemc-E12 \). \( trh^1 \) 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 \( trh^1 \) mutant or the l(3)10512 mutant. In summary, 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 \( trh^3 \) (Table 1). The P-element insert was used to clone and characterize the \( trh \) gene (Fig. 2B).

### trachealess gene function

The P-element insert in the \( trh^3 \) 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

| × | D[3L]H5 | D[3L]H18 | D[3L]H2 | l(3)trh | l(3)trh | l(3)trh | l(3)trh | l(3)trh | l(3)trh |
|---|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| l(3)trh | 92 | 96 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| (137) | | | | | | | | | |
| l(3)trh | 108 | 96 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| (89) | | | | | | | | | |
| l(3)trh | 177 | 79 | 0 | 0 | 130 | 0 | 0 | 0 | 0 |
| (49) | | | | | | | | | |
| l(3)trh | 117 | 46 | 0 | 0 | 8 | 0 | 0 | 0 | 0 |
| (41) | | | | | | | | | |
| l(3)trh | 95 | 113 | 0 | 0 | 11 | 95 | 83 | 0 | 0 |
| (66) | | | | | | | | | |
| l(3)trh | 83 | 94 | 0 | 0 | 92 | 38 | 0 | 0 | 0 |
| (88) | | | | | | | | | |
| l(3)trh | 117 | 122 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| (23) | | | | | | | | | |
| l(3)trh-R12 | N.D. | N.D. | 0 | 46 | N.D. | N.D. | N.D. | N.D. | N.D. |
| (57) | | | | | | | | | |
| l(3)trh-R25 | N.D. | N.D. | 115 | 166 | N.D. | N.D. | N.D. | N.D. | N.D. |
| (127) | | | | | | | | | |
| trh-R3 | N.D. | N.D. | 16 | 88 | N.D. | N.D. | N.D. | N.D. | N.D. |
| (18) | | | | | | | | | |
| trh-R26 | N.D. | N.D. | 112 | 131 | N.D. | N.D. | N.D. | N.D. | N.D. |
| (96) | | | | | | | | | |
| trh-R63 | N.D. | N.D. | 73 | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |

*tr(h)* The original EMS-induced allele, formerly known as \( trh^{10} \) (Jürgens et al. 1984); \( trh^n \) the enhancer-trap insertion alleles, formerly known as l(3)10512. Results suggest an allelic series, decreasing by order of severity as follows: \( trh^1 = trh^6 > trh^7 > trh^3 > trh^5 > trh^4 \).

b) 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.
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 allele maps to a 7.2-kb EcoRI fragment that detects transcripts on Northern blots and in embryos. Three other genomic fragments (*) also detect trh transcripts in embryos and on Northern blots.

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 allele. In trh/+ embryos, β-gal expression is seen in all tracheal cells (Fig. 4A). In trh homozygous mutant embryos, β-gal expression is seen in a small number of cells that remain on the embryo surface at the approximate original position 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, trh, or trh, we see no evidence of tracheal pit formation, even at 1000× 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 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 EcoRI 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,F]. 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 ß-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 ß-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 ß-gal staining observed on the surface of trh^{3} homozygous mutant embryos [Fig. 4, cf. B and F]. We suggest that the ß-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
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^6, trh^7, trh^8, and trh^9), 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 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.
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 5. Tracheal pits do not form in trh mutant embryos. SEMs of wild-type [A] and trh [B] embryos are shown. Note the complete absence of tracheal pits in the trh 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 embryo [D]. Scale bars in A and B are 50 μm, and in C and D are 10 μm.

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.], hückebein [hkb] [Brömer 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

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 stained with α-β-galactosidase; [D,E,G,H] cuticle preparations of trh mutant first instar larvae; [F] late stage trh 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 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 digoxygenin-labeled \(trh\) genomic fragment detected with \(\alpha\)-digoxygenin antibody conjugated to alkaline phosphatase. (A) Wild-type embryo, ventral view; (B) \(Scr^{-}\) embryo, ventrolateral view; (C) \(fkh^{-}\) embryo, ventral view; (D) \(fkh^{-}\) 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 cosmide genomic libraries [Fig. 2B]. EcoRI 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 hybridized strongly to transcripts expressed in the trachea, salivary gland, filzkörper, and a subset of cells in the CNS, the same expression pattern observed with \(\beta\)-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-1α (HIF-1α) [Wang et al. 1995] and Single-minded (SIM), another \(Drosophila\) regulatory protein [Nambu et al. 1990, 1991]. Both HIF-1α and SIM encode related transcription factors. HIF-1α 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-1α 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.
shown is the genomic sequence and corresponding cDNA. The corresponding amino acid sequence is shown in the single-letter code.

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8CG ATT GAG GGC TAG ATT GCC GAT CCC GAT CCG TAT TGC GTG ACC CCT ACA TCG CTG GCT GGA
```

A large number of regulatory proteins and are thought to be involved in the regulation of gene expression. This region of homology, referred to as the HST domain, is found in the Drosha and Pasha proteins.

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ATC TAT G GGC CTG TCG CAG GTG ATG ACG GGT AGC ATC CAC TAT ATC GAT AAC T V
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Our results suggest that this domain plays a crucial role in the regulation of gene expression in flies. Further studies are needed to understand the exact mechanism by which this domain functions.

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CTG GGC CTG AGC CTA ACA AGC GGC GGC GGC GGC GGT GGC GGT GGA AGC TCG AGC AGC GGC GGA GGA GCA
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Discussion

**trh and the control of tubulogenesis**

The trh gene is essential for the formation of three different tubes in the Drosophila embryo: the trachea, filzkörper, and salivary duct. In the absence of trh function, the cells that normally form these tubes fail to invaginate and remain clustered at their site of origin. The absence of any morphological evidence of invagination in trh mutants, as well as the lack of detected transcripts in trh mutants, suggests that trh is required for the initial events of tube formation. The persistent expression of trh during the embryonic development of normal trachea and salivary duct suggests that trh is required continuously for the cell migrations that characterize later stages of tube formation.

Because trh is expressed very early in embryogenesis, we suggest that it may directly regulate the ability of cells to form tubes. trh transcripts can be detected in tracheal precursor cells as early as embryonic stage 8, which is at least an hour before these cells show any sign of differentiation.

Figure 8. trh encodes two alternative proteins of 925 and 896 amino acids. An additional exon is found in some cDNAs at nucleotide position 1627 to 1710. Shown is the genomic sequence and corresponding cDNA. The corresponding amino acid sequence is shown in the single-letter code. The numbers indicate position within the largest composite cDNA.
Figure 9. TRH is homologous to HIF-1α and SIM. (A) CLUSTAL V alignment of all three proteins (Higgins 1993). The asterisk (*) indicates identities; the period (.) indicates conserved residues found in all three proteins. Additional identities and conserved residues are evident when only two of the three proteins are compared. The bHLH regions of TRH, HIF-1α, and SIM, indicated with italics, are amino-terminal. The PAS domains, PAS-A and PAS-B, which are conserved among TRH, HIF-1α, and SIM, as well as the Period protein and the aryl hydrocarbon receptor, are underlined. (B) An alignment of the domains common to TRH, HIF-1α, and SIM. In addition to the bHLH and PAS domains is a short (15 amino acid) region of homology found between the PAS-A and PAS-B regions and another homologous region of ~50 amino acid carboxy-terminal to the PAS-B region, the HST domain. (B, bottom) The percent identity/percent similarity between TRH and HIF-1α, TRH and SIM, and SIM and HIF-1α in the bHLH, PAS-A, PAS-B, and HST domains.

morphological indications, such as placode formation, that they will give rise to trachea. trh expression in the salivary duct and filzkörper also significantly precedes tube formation in these organs. In the absence of trh function, none of these cells form tubes suggesting an absolute requirement for trh in this process. It will be exciting to learn whether the ectopic expression of trh results in the formation of additional tubes in the embryo. The lag time between the appearance of trh transcripts and the resulting cell movements suggests that the timing of ectopic trh expression may be critical to the ability of trh to induce extra tubes.

Each of the trh-requiring tubes derives from ectoderm and serves as a passageway for air (trachea and filzkörper) or liquid secretions (salivary gland duct). Structurally, the major branches of the trachea and salivary duct are similar, both form striated tubes that are quite alike in appearance at the light microscope level. Correspondingly, a number of genes or markers are expressed in both tissues, including trh, bil, mAb68G5D3, and crb. The filzkörper have not been so well studied, but it will be interesting to determine whether they share molecular and cellular features common to the salivary duct and trachea.

Other embryonic tubes, such as the Malpighian tubules, gastric caeca, and esophagus are unaffected by trh mutations and trh is not expressed in these cells. Other molecules, perhaps related to trh, may control the formation of these internal tubes. It will be interesting to test whether trh has any tube-inducing activities in
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, unpub]. 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 trh+ 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-3a (HNF-3a) [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 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, MA68G5D3 and rp395, whose expression in the trachea is dependent on trh. Mutations in the genes corresponding to MA68G5D3 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 MA68G5D3 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; Klambt et al. 1992]. In btl mutants the trachea invaginates, 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 Ni gar 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-1α, 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-1α 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-1α 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-1α or another closely related gene. HIF-1α 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-1α may be involved in regulating the formation of the oxygen delivery system in mammals. HIF-1α 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-1α. 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)trh5) 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 Zimm [1994]. The EMS allele, trh114 (formerly known as trhSD) 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 fkh2, were obtained from the Indiana Stock Center. hkb mutants [Brommer et al. 1994] were obtained from Q. Chu-LaGraff and C. Doe [University of Illinois, Urbana]. Loss-of-function mutations in the dCREB-A and jaland genes were isolated in this laboratory and will be published elsewhere [D.J. Andrew, unpubl.]. H5–SCR and Scf 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 invading 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 Axiopt. 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 μg) 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 cosmids 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%,1, made at 14°C.

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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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