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Mechanisms of Development 91 (2000) 163–173

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Interaction between the bHLH-PAS protein Trachealess and the POU-domain protein Drifter, specifies tracheal cell fates

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Received 1 October 1999; received in revised form 4 November 1999; accepted 4 November 1999

Abstract

bHLH-PAS proteins represent a class of transcription factors involved in diverse biological activities. Previous experiments demonstrated that the PAS domain confers target specificity (Zelzer et al., 1997. Genes Dev. 11, 2079–2089). This suggested an association between the PAS domain and additional DNA-binding proteins, which is essential for the induction of specific target genes. A candidate for interaction with Trh is Drifter/Ventral veinless, a POU-domain protein. A dual requirement for Trh and Drifter was identified for the autoregulation of Trh and Drifter expression. Furthermore, ectopic expression of both Trh and Dfr (but not each one alone) triggered *trh* autoregulation in several embryonic tissues. A direct interaction between Drifter and Trh proteins, mediated by the PAS domain of Trh and the POU domain of Drifter, was demonstrated.

Keywords: bHLH; PAS; POU; Trachealess; Drifter; Tracheal development; *Drosophila*

1. Introduction

Basic helix-loop-helix (bHLH)-PAS (*P*er, *A*RNT, *S*im) proteins comprise a subset of the large class of bHLH transcription factors. The hallmark of these proteins is the presence of a PAS domain which includes a duplicated sequence of ~50 amino acids, shown to be important for protein–protein interactions (Nambu et al., 1991; Huang et al., 1993; Lindebro et al., 1995). Four major sub classes of bHLH-PAS proteins, which have distinct biological functions, have been identified in vertebrates to date. The Clock proteins, regulators of circadian rhythms (King et al., 1997); AhR protein induces the xenobiotic response to toxic chemicals (Burbach et al., 1992); Single minded (Sim) proteins shown to be important for specification of hypothalamic neurons mice (Michaud et al., 1998), and the Hypoxia inducible factor (HIF) proteins (HIF-1α, EPAS1 and HIF-3α) mediate the cellular and organismal hypoxic response (Maxwell et al., 1993; Wang and Semenza, 1993; Wang et al., 1995; Gu et al., 1998; Tian et al., 1998). bHLH-PAS proteins need to heterodimerize in order to induce transcription. In the latter three cases, the common heterodimeric partner is the bHLH-PAS protein ARNT (Wang et al., 1995; Ema et al., 1996, 1997; Hogenesch et al., 1997; Probst et al., 1997; Tian et al., 1997).

The diverse roles of bHLH-PAS proteins imply a broad range of non-overlapping target genes. Mammalian Sim induces the expression of the POU (*P*it, *O*ct, *U*nc) protein Brn2 in the hypothalamic nuclei (Michaud et al., 1998); HIF-1α induces an extremely broad range of target genes following hypoxia and insulin activation, including glycolytic enzymes, erythropoietin and VEGF (Maxwell et al., 1993; Wang and Semenza, 1993; Damert et al., 1997; Zelzer et al., 1998); EPAS1 appears to induce the expression of genes necessary for catecholamine biosynthesis in the organ of Zuckerkandl during hypoxia (Tian et al., 1998), and the target genes of HIF-3α have not been identified yet (Gu et al., 1998).

The corresponding difference in the DNA binding sites of the various heterodimers can not solely account for the different target genes: Since ARNT is the universal partner, the half site recognized by it on the DNA is always identical. The basic, DNA-binding domains of the three HIF proteins and the Sim proteins are extremely similar, and recognize the same consensus sequence (ACGTG). Therefore, another tier of regulation must exist, to define distinct and non-overlapping panels of target genes for each of these proteins.

In *Drosophila*, a single ARNT homologue (also termed Tango) was identified (Ohshiro and Saigo, 1997; Sonnenfeld et al., 1997; Zelzer et al., 1997). This protein is found in

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0925-4773/00/$ - see front matter
PII: S0925-4773(99)00295-6

the cytoplasm, and is transported to the nucleus only upon association with the relevant partner (Ward et al., 1998). Three partners for DARNT/Tango have been identified in *Drosophila*: Sima, Sim and Trh (Ohshiro and Saigo, 1997; Sonnenfeld et al., 1997). The role of Sima is still elusive; the protein accumulates under hypoxic conditions, suggesting it may be a component of the hypoxia-response factor (Nambu et al., 1996; Bacon et al., 1998). Sim is the regulator of midline cell fates. It induces a broad range of midline-specific target genes, and is autoregulated (Nambu et al., 1991; Wharton et al., 1994). Trh is a regulator of tracheal cell fates that also autoregulates its own expression, in parallel to the induction of tissue-specific genes (Isaac and Andrew, 1996; Wilk et al., 1996).

The basis for the tissue specificity of Sim and Trh was previously addressed, by monitoring the activity of chimeric proteins. It was found that whereas the similar basic regions of the two proteins recognize the same sequence on the DNA, the PAS domain is responsible for conferring target-gene specificity (Zelzer et al., 1997). We postulated that target specificity could result from PAS-mediated association with different factors.

In this work we searched for transcription factors that may cooperate with Trachealess during induction of tracheal-specific genes. We show that for some tracheal-specific genes, cooperation between Trh and the POU-domain protein Drifter is essential. A specific protein–protein interaction between the PAS domain of Trh and the POU domain of Dfr was demonstrated. This interaction may contribute to the specific induction of tracheal target genes by Trh and Dfr.

2. Results

2.1. Trh autoregulation requires Dfr, and vice versa

Transcription of the *trh* gene is autoregulated, thus maintaining its expression throughout tracheal development, after the initial cues that determine the position of the tracheal placodes have disappeared. However, several experimental results suggest that the Trh/ARNT heterodimer is not sufficient for autoregulation of the *trh* gene. First, examination of Trh-Sim chimeras demonstrated that target gene specificity is determined by the PAS domain, possibly through interactions with other proteins (Zelzer et al., 1997). Second, ubiquitous Trh can induce ectopic *trh* expression occasionally, at stage 11, but only at the position of tracheal pits in segments which do not normally form tracheal pits (Fig. 2B; Wilk et al., 1996), suggesting that additional protein(s) expressed in this pattern need to cooperate with Trh.

A candidate protein that may interact with Trh is the POU-domain protein Drifter/Ventral veinless (Dfr). This protein was previously shown to participate in tracheal morphogenesis (Anderson et al., 1995; de Celis et al., 1995). Initially, *dfr* is expressed in the ten tracheal placodes, as well as in the position of placodes in segments which normally do not produce tracheal pits (Anderson et al., 1995; de Celis et al., 1995). *dfr* mutations show a reduced expression of tracheal-specific genes such as *breathless* (*btl*), and accordingly exhibit migration defects that are reminiscent of the *btl* phenotype (Anderson et al., 1996). An important feature of both Trh and Dfr expression is their capacity to be autoregulated (Wilk et al., 1996; Certel et al., 1996). Once the exogenous cues that direct expression of these genes in the tracheal placodes diminish, expression is maintained by autoregulation.

Since, the *trh* and *dfr* genes themselves can be regarded as targets for Trh or Dfr, respectively, we tested whether autoregulation of each of the two genes requires both Trh and Dfr. Two phases of Trh expression have previously been defined; at stage 12 expression induced by exogenous cues is diminished and autoregulation ensues (Wilk et al., 1996). Staining for the Trh protein in *dfr* mutant embryos demonstrated that the initial phase of Trh expression in the placodes is normal. However, starting at stage 12 the levels of Trh are reduced, and are almost undetectable by stage 15 (Fig. 1A–C). Failure of the cells in the tracheal pits of *dfr* mutant embryos to express Trh is not due to the death of these cells. Previous examination of the tracheal pits of *dfr* mutant embryos has shown that the cells are viable and capable of secreting tracheal lumen material, regardless of their failure to migrate properly (de Celis et al., 1995). We can conclude that Dfr is required for the autoregulation, and hence the maintenance of *trh* expression.

In the case of Dfr, a distinct 514 bp fragment has been defined as the *dfr*-autoregulatory element, which begins to drive Dfr expression at stage 11/12 (Certel et al., 1996). This fragment also confers expression in the oenocytes. In *trh* mutant embryos, *lac*Z expression driven by this fragment in the oenocytes was retained, but completely abolished in the trachea (Fig. 1E,F). Again, the absence of expression in the tracheal placodes which fail to invaginate in the *trh* mutant background, is not due to death of these cells. Staining of *trh* mutant embryos with anti-Dfr antibodies (Fig. 1G) or with a probe detecting *dfr* RNA (Llimargas and Casanova, 1997), revealed the early, Trh-independent phase of expression up to stage 11. The uninvaginated placode cells in *trh* mutant embryos are thus intact, but fail to express the *dfr* autoregulation reporter. These experiments demonstrate that Trh and Dfr are required simultaneously for the autoregulation of Trh and Dfr themselves.

The dual requirement for Trh and Dfr in *trh* autoregulation was examined by following the capacity of ectopic expression of both genes to induce the *trh 1-eve-1* enhancer trap. Ubiquitous Dfr expression is not sufficient to induce ectopic *1-eve-1* expression (Fig. 2A). Ubiquitous Trh induces ectopic *trh* expression occasionally, at stage 11, but only at the position of tracheal pits in segments which do not normally form tracheal pits (Fig. 2B; Wilk et al.,

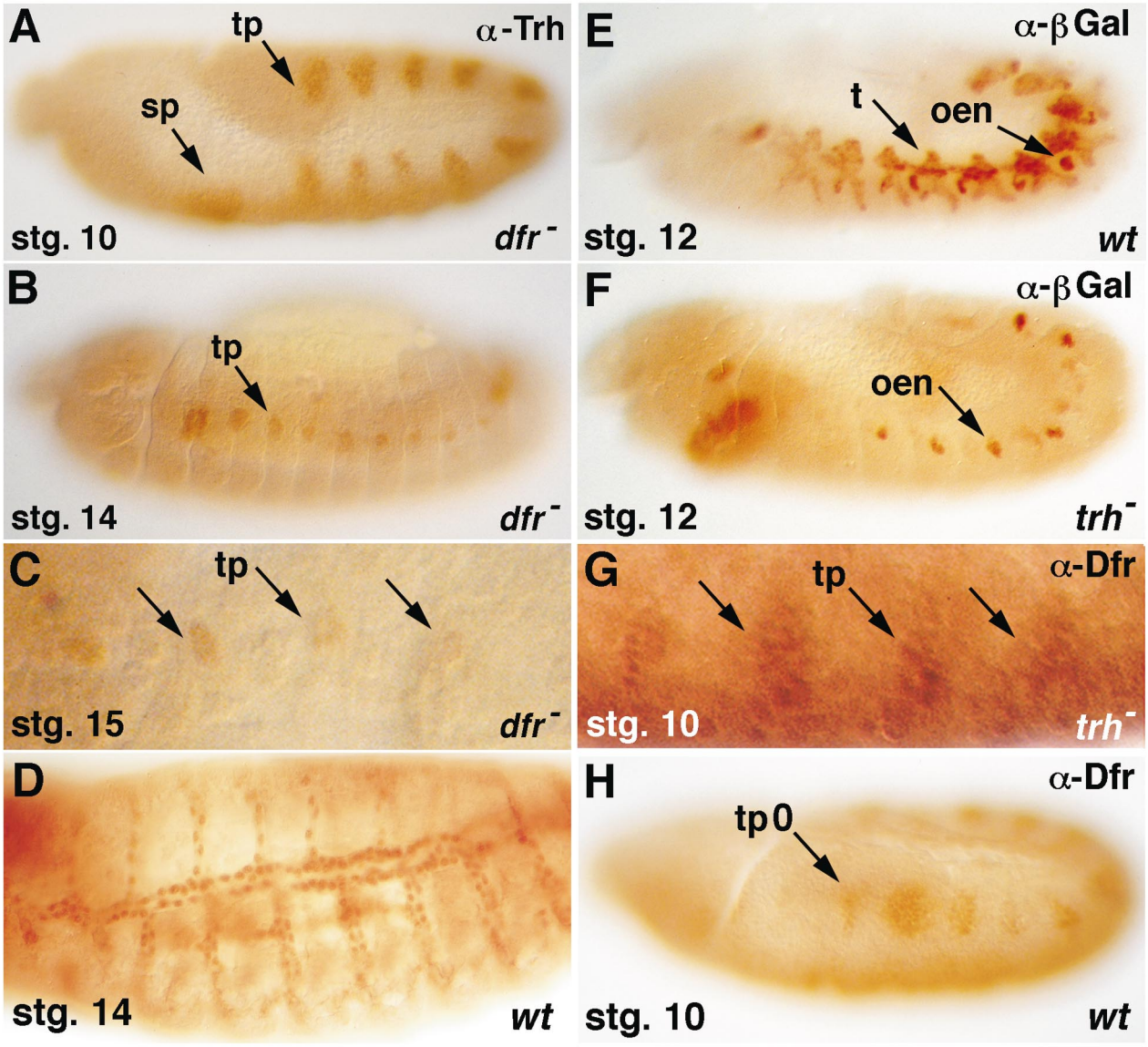

Fig. 1. Trh and Dfr participate in the autoregulation of each other. (A) In stage 10 *dfr* mutant embryos, the initial expression of Trh in the tracheal placodes (tp) and salivary placodes (sp) is normal. (B,C) At stages 14 and 15, only residual Trh expression is detected in the tracheal pits, indicating that Dfr is required for autoregulation of Trh. (D) In contrast, wt stage 14 embryos display pronounced Trh expression in all tracheal branches. (E) In wt stage 12 embryos, a construct containing the 514 bp *dfr* autoregulatory sequence drives *lac*Z expression in the trachea (t) and oenocytes (oen). (F) In *trh* mutant embryos at the same stage, only expression of *lac*Z in the oenocytes can be detected, implying that Trh is required for autoregulation of Dfr. (G) At stage 10, normal expression of Dfr in the tracheal placodes of *trh* mutant embryos can be detected. (H) In wt stage 10 embryos, Dfr is expressed in the ten tracheal placodes, as well as in additional placodes which will not give rise to trachea (tp 0).

1996). This induction of *1-eve-1* expression in the extra pits may be due to cooperation of ectopic Trh with Dfr, which is normally expressed in these placodes (Anderson et al., 1995; de Celis et al., 1995). In contrast, misexpression of both Trh and Dfr gave rise to multiple patches of *1-eve-1* expressing cells, randomly distributed in the ectoderm and head (Fig. 2C,D). Thus, in many cells, simultaneous Trh and Dfr expression is sufficient to induce *trh* autoregulation.

2.2. Trachealess associates with Drifter

We examined the possibility of physical interaction between the Trh and Dfr proteins. Full length Dfr was generated in bacteria as a GST-fusion, immobilized on glutathione-agarose beads, and assayed for its ability to retain in vitro translated, ^{35}S-labeled, bHLH-PAS proteins. The panel of proteins tested includes *Drosophila* Trh, Sim,

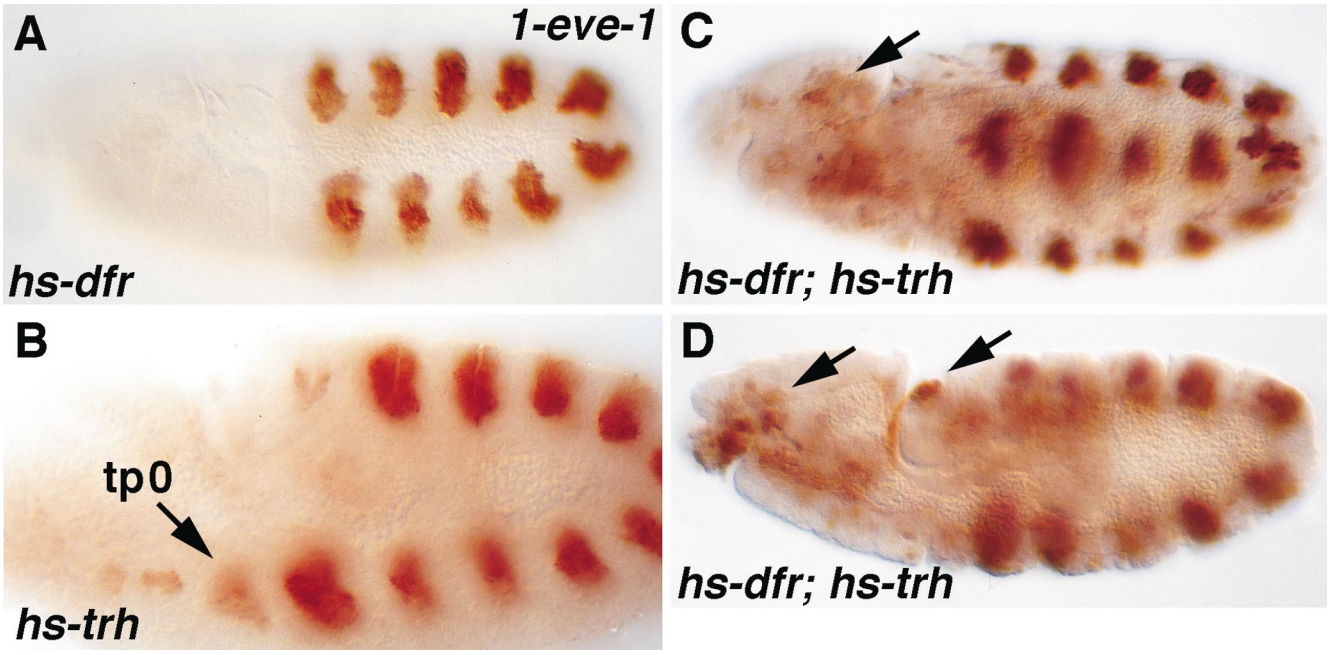

Fig. 2. Trh and Dfr are sufficient to induce *trh* autoregulation. (A) At stage 11, the *trh 1-eve-1* enhancer trap is expressed in ten tracheal pits on each side of the embryo. This pattern is not altered following induction of the *hs-dfr* construct at 2.7 h after egg lay. (B) Induction of *hs-Gal4/UAS-trh* leads to ectopic expression of *1-eve-1* only in the placodes which normally also express Dfr. (C,D) Simultaneous induction of the *hs-dfr* and *hs-Gal4/UAS-trh* transgenes results in ectopic patches of cells expressing *1-eve-1* (arrows).

Sima and ARNT proteins, and human HIF-1α protein (Fig. 3). Only Trh was co-precipitated with GST-Dfr, and this association was maintained at a concentration of 300 mM NaCl (marked by an asterisk). We can therefore conclude that Trh interacts avidly with Dfr in a specific manner.

2.3. Interaction of PAS and POU domains

Our previous work has demonstrated that the PAS domain confers target-specificity of Trh. Replacement of the PAS domain with that of Sim was sufficient to convert the target specificity of the protein to that of Sim (Zelzer et al., 1997). It was therefore important to test if the PAS domain of Trh is sufficient to mediate the interaction with Dfr. GST-Dfr was indeed capable of precipitating a deleted protein containing only the PAS domain of Trh (Fig. 4). In order to identify the region within Dfr which is responsible for binding the PAS domain of Trh, a GST fusion construct containing only the POU domain of Dfr was used (Turner, 1996). This construct retained the ability to precipitate full length Trh, as well as only the PAS domain of Trh, indicating that Trh recognizes the POU domain of Dfr (Fig. 4). We can conclude that the PAS domain of Trh is necessary for conferring specific interaction to the POU domain of Dfr.

2.4. Tracheal and midline regulation of rhomboid expression

Heterodimers of Single minded or Trachealess with ARNT recognize the same DNA binding consensus, and yet activate distinct midline or tracheal target genes, respectively. In order to decipher the molecular basis for this regulation, we chose to dissect the regulatory region of

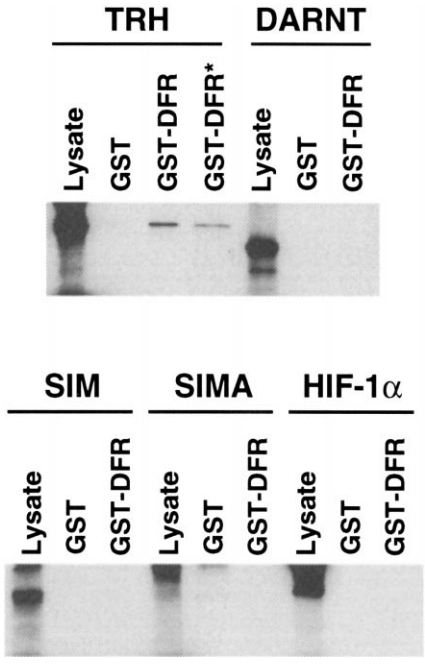

Fig. 3. Specific interaction between Trh and Dfr proteins. Interaction between Trh and Dfr proteins was examined by GST 'pull down' experiments. A GST-Dfr protein containing the entire Dfr protein, was capable of precipitating *in vitro* translated Trh, but not DARNT, Sim, Sima or HIF1α. The interaction with Trh was maintained at 0.3 M NaCl (asterisk). The size of the major band in each of the lysates corresponds to the expected size of the respective protein produced.

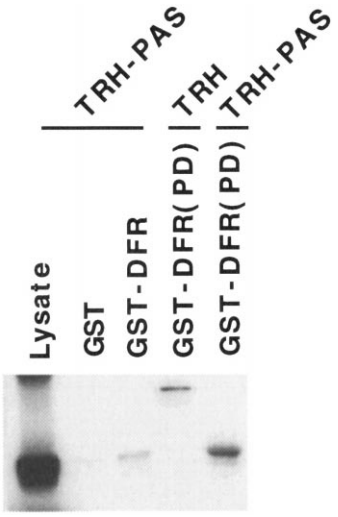

Fig. 4. Interaction of Trh-PAS domain with Dfr-POU domain. Further analysis identified the regions within Trh and Dfr responsible for their interaction. GST-Dfr precipitated a protein containing only the Trh-PAS domain. Within Dfr, the necessary region was assigned to the POU domain, by demonstrating that a GST-Dfr protein containing only the POU domain was capable of precipitating full length Trh, or the Trh-PAS domain.

the *rhomboid* (*rho*) gene. Rho functions as a regulator of processing of the EGF receptor ligand Spitz (Schweitzer et al., 1995; Golembo et al., 1996), and is expressed at embryonic stage 9/10 in the midline glial cells, as well as in cells positioned at the center of the tracheal placodes (Bier et al., 1990; Wappner et al., 1997). The parallel expression of *rho* in the tissues where Sim and Trh are functional, suggested that it may be a transcriptional target of these two bHLH-PAS proteins. Dissection of its regulatory region may thus provide insights to tissue-specific regulation of gene expression by Sim and Trh.

In *trh* mutant embryos, expression of *rho* in the tracheal placodes is abolished (Fig. 7B). Similarly, in *sim* mutant embryos, expression of *rho* in the midline is eliminated (Nambu et al., 1990; Zhou et al., 1997). To determine if *rho* expression is regulated by direct binding of Sim and Trh, we dissected a 762 bp fragment of the *rho* 5′ regulatory region, which was previously shown to be sufficient for midline and tracheal expression (Ip et al., 1992; and Fig. 7A). The sequence of this fragment (provided by T. Ip and M. Levine; shown on Fig. 5) contains four sites with the Sim/Trh (ST) binding consensus. Similar sites have previously been shown by in vivo and in vitro analysis to represent the binding sites for Sim/ARNT or Trh/ARNT heterodimers (Wharton and Crews, 1993; Zelzer et al., 1997; Ohshiro and Saigo, 1997; Sonnenfeld et al., 1997).

The 762 bp *rho* regulatory region was further dissected, and the capacity of smaller fragments to induce in embryos midline or tracheal expression was followed (Figs. 6 and 7). The following conclusions were obtained: Sim/Trh binding sites STc and STd are neither sufficient (construct 3) nor necessary (construct 6) for tracheal or midline expression. In contrast, Sim/Trh binding sites STa and STb are essential for midline and tracheal expression. Construct 3, in which STa and STb were eliminated, failed to induce any expression, whereas addition of STa and STb to the fragment tested in construct 3, rescued both midline and tracheal expression (constructs 4 and 5). Construct 6, which shows expression in both tissues and contains STa and STb, but not STc and STd, confirms that the two latter sites are not required. However, binding of bHLH-PAS proteins is not sufficient, and the two functional STa and STb sites are unable to promote expression on their own (construct 7).

Distinct cis elements appear to be required to promote midline vs. tracheal expression. Two different additional sequences, each of which is sufficient to promote midline expression in conjunction with STa and STb were identified (constructs 2, 4 and 5). For tracheal expression, one set of sequences, which is present on the 3′ *Ssp*I-*Eco*RI fragment, is required in conjunction with STa and STb (constructs 2, 4 and 5). This region can be narrowed down to the 110 bp fragment between the *Ssp*I site and the position of STc (construct 6). The inability of the STc and STd sites to promote tracheal expression together with the same fragment (construct 3), may be explained by different spacing or orientations of the sites.

The 110 bp fragment was narrowed further. Removal of a 44 bp fragment abolished tracheal expression, thus pointing to essential binding site(s) in the fragment removed (Fig. 8). A potential binding site for Dfr (termed Db) is found in this 44 bp fragment (Fig. 5). It contains the consensus POU-homeo domain site (TAAT), and a less conserved POU-specific domain site (ARAT) (Ryan and Rosenfeld, 1997). A similar sequence in the *dfr*-autoregulatory region was shown to be bound by Dfr (Certel et al., 1996). In *dfr* mutant embryos, expression of the *rho* reporter is abolished in the trachea but not in the midline (Llimargas and Casanova, 1997; and Fig. 8D). However, a construct containing the

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   1 GAATTCCAGCTCGAGTGTTTTGGGTGGCTGGGATTGCTTTGGTACGGTGG
  51 CTGACCTTGCCAGTGCCAGTGGGTCCATGTCCGTGGAGTGCTGGCAATGC
 101 GTGTCTGGCACAATGAAAACGCAGCAATGGAGGATGGAGAAACTCGACCT
                STa
 151 GAGGGAACGTGGGAAAAAAGGATTTACGATGAAGATG AGCTGAGAAACTC
                  STb
 201 CGAGCTGATGACGTGCCTGGTCATGGGCATGTAGTGCAGCAACTGTGTTT
                                          Da
 251 TCGTATCTGCTTACCTGTTGCACGCACATGTTAAAAAGTCATCATAAATC

 301 TCAGTGGACGAGGACGAGGACTCTGAATCTGTTTTGCCTCTCTTTTGCGC

 351 CCTGTGAAAATATTTTCACTTTTCATGCGGGAGTTTTCATGCCGCCGAAG
                                      Db
 401 GTCGATGAGGATGACTGGCCAGGATGGGTATATCTATCTATTAGATCTGT
                        STc           STd
 451 GTAAATTCCCAGCTTATCATGCAAAACACGTAACCACGTCGTCTCGATCC

 501 TTTCGGCCAGGAAGCCGAAAGCTACTCCTTTTGTGCAACATGCCGTGGTG
 551 ACCTTTCCCGCGAACTCGTGACTGCAACGGCTCAGGCTGCCTAATAATTT
 601 GTATAAATATTTATTTTTGCTACCGATTTCTATCTGCTCGCTCAACGCCT
 651 CCGAGGGAGCTCTCGCTGGAAAAGGTGTGAAATTCGAAACCCCTGACAAT
 701 GATGATCTGTACCGGGAATCGGGGAATCAAGGGGAATCAGGACGGGGCTC
 751 GGTTTCGAATTC
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Fig. 5. Sequence of the *rhomboid* regulatory region conferring midline and tracheal expression. The 762 bp *Eco*RI fragment of the *rho* regulatory region conferring midline and tracheal expression (obtained from T. Ip and M. Levine) is shown. ST, consensus sequence for Sim/ARNT and Trh/ARNT heterodimers; D, consensus sequence for the POU-domain protein Drifter/Ventral veinless. Orientation of sites is shown by arrows.

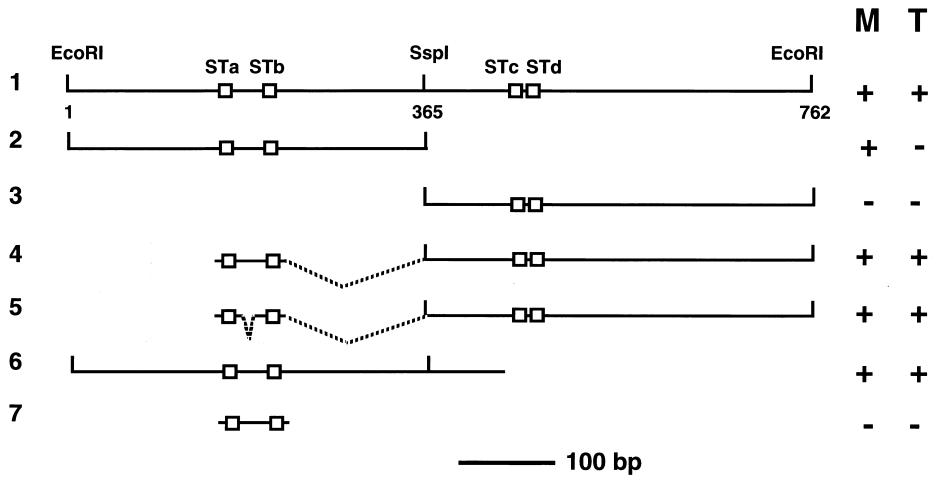

Fig. 6. Constructs used for dissection of the *rho* regulatory region. The 762 bp *rho*-regulatory region was dissected, and *lac*Z expression conferred by the smaller fragments was followed in embryos. The conclusions can be summarized as follows: STa and STb are necessary, but not sufficient, for midline and tracheal expression. Distinct additional cis elements are required. In the case of tracheal expression, these sequences can be narrowed down to a 110 bp fragment located 3′ of the *Ssp*I site. M, midline expression; T, tracheal expression.

same fragment as construct 6, but with four base changes in Db (5′-ATGGAGAA instead of ATCTATTA), retained midline and tracheal expression (not shown).

Several explanations may account for these results. The consensus for binding of POU proteins to DNA is extremely flexible, due to permutations in the order and direction of binding of the POU-specific and POU-homeo domains. Cryptic binding sites for Dfr in the 44 bp fragment may have remained after mutagenesis of the Db site. Alternatively, Drifter may bind the *rho* regulatory fragment at other sites (e.g. Da in Fig. 5) or associate with the Trh/ARNT heterodimer without binding DNA directly, and an additional unknown factor may bind the 44 bp fragment. Finally, a transcription factor induced by Dfr may bind the *rho* regulatory region.

3. Discussion

Proteins belonging to the bHLH-PAS family represent a subgroup of bHLH proteins. This group plays central roles in key biological processes such as responses to hypoxia, detoxification of harmful substances, midline glial development and maintenance of circadian rhythms. In spite of these diverse and non-overlapping roles, the basis for the capacity to recognize distinct target genes by each member of the family has not been elucidated. Here, we describe the interaction between Trh and the POU-domain protein Dfr. This interaction is important to maintain the expression of these two genes and to co-regulate tracheal specific target genes.

3.1. Trachealess/Drifter interaction

POU-domain proteins comprise a large and highly conserved family of transcription factors which are grouped into six subclasses, based on their structure. They fulfill a wide range of functions including pituitary gland development (Pit-1), expression of immunogloblin genes (Oct-1) and lineage determination (Unc-86) (reviewed in Ryan and Rosenfeld, 1997). The POU domain is a bipartite DNA-binding domain consisting of two highly conserved regions: the POU-homeo domain and the POU-specific domain. These two domains are capable of binding a specific DNA sequence termed the octamer motif, which is a crucial component in many regulatory elements requiring activation by POU-domain proteins (reviewed in Ryan and Rosenfeld, 1997). While the consensus binding site for the POU-homeo domain is stringent, the binding site for the POU-specific domain is more relaxed.

Several POU-domain proteins have been identified in *Drosophila*, including Pdm-1 (Nubbin), Pdm-2, I-POU and Drifter/Ventral veinless (reviewed in Ryan and Rosenfeld, 1997). Dfr appeared to be a promising candidate for interactions with Trh for several reasons. First, *dfr* mutant embryos display a severe tracheal phenotype (Anderson et al., 1995; de Celis et al., 1995). Second, the ability of Trh to induce tracheal-target genes at ectopic sites (Wilk et al., 1996) coincides with the expression pattern of Dfr. Early in embryogenesis Dfr is expressed in the midline glia and in 13 tracheal placodes, i.e. also in three placodes in segments which normally do not produce tracheal cells. From stage 13/14 onwards, Dfr expression becomes ubiquitous in the ectoderm (Anderson et al., 1995; de Celis et al., 1995).

Among the bHLH-PAS proteins tested, including Trh, DARNT, Sima and HIF-1α and in particular Sim, only Trh was capable of interacting with Dfr. This interaction appears to be avid since it was maintained even at high salt concentrations. Sim is required for midline expression of rho (Nambu et al., 1990; Zhou et al., 1997). Sustained expression of rho in the midline of dfr mutant embryos (Fig.

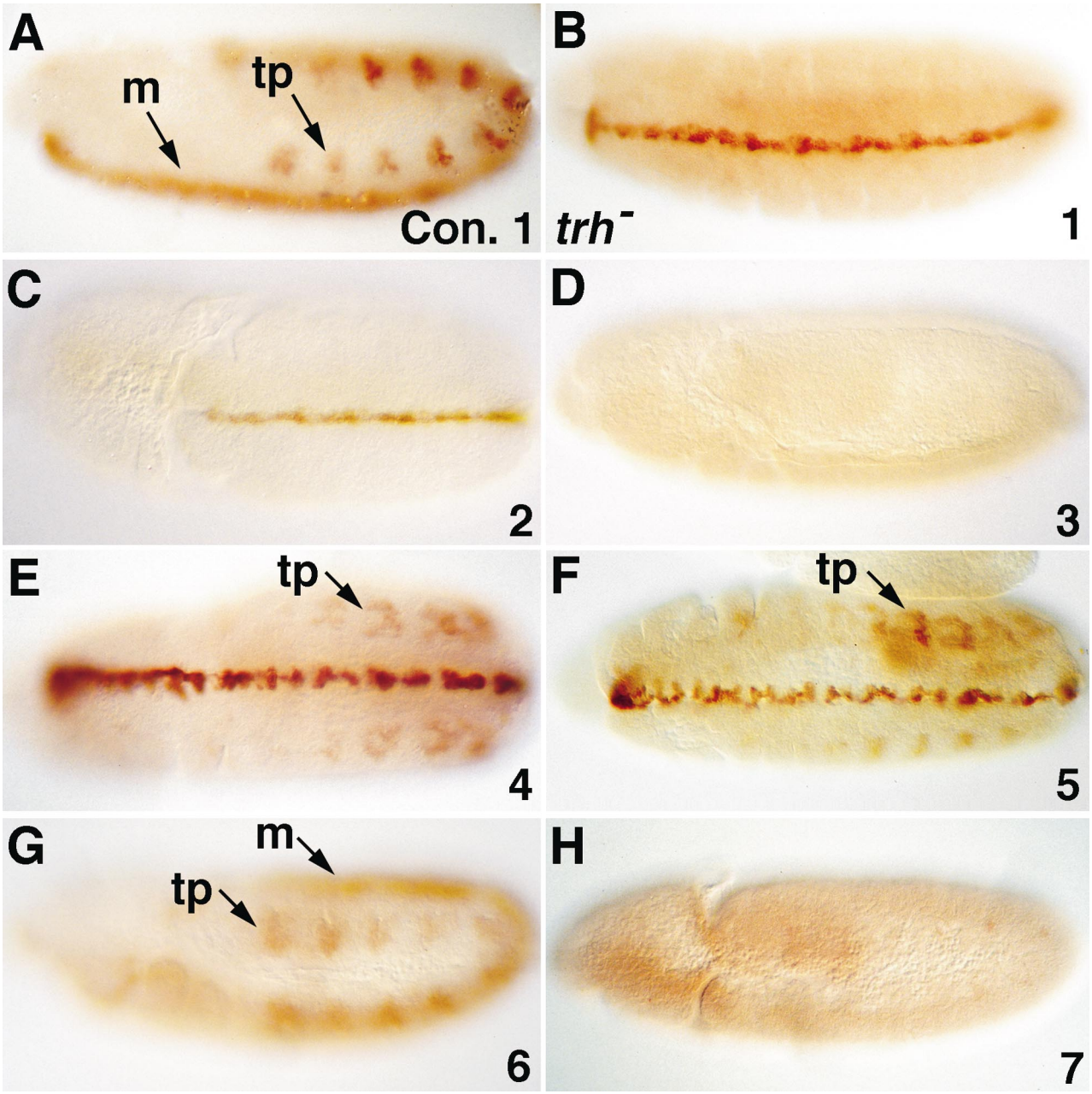

Fig. 7. Expression in embryos conferred by fragments of the *rho* regulatory region. The constructs used correspond to the ones shown in Fig. 7, construct numbers are shown at the bottom right. Expression was monitored by staining stage 11 embryos with anti-βGal antibodies. tp, tracheal pits; m, midline.

7C), suggests that Dfr is not required for Sim activity, in accordance with the lack of interaction between these two proteins (Fig. 3).

In order to test if this interaction between Trh and Dfr can account for the capacity of the PAS domain to confer target specificity, it was important to show not only that the interaction is specific to Trh, but also that it is mediated by the PAS domain. Dfr was capable of associating with the PAS domain alone. We suggest that the distinct target specificities of vertebrate bHLH-PAS proteins which have the same DNA binding sites, are also mediated by specific interactions of the PAS domain with other DNA-binding proteins.

We showed that Dfr interaction with Trh is mediated by the POU domain. This domain was shown to be essential not only for DNA binding, but also for the capacity of POU-domain proteins to interact with a variety of other proteins. For example, the B-cell specific co-activator OCA-B was shown to bind Oct-1 (Gstaiger et al., 1996). High mobility group (HMG) proteins interact with POU proteins, and bind specific DNA sequences, adjacent to the binding sites for the POU protein. In the regulatory region of the FGF4 gene,

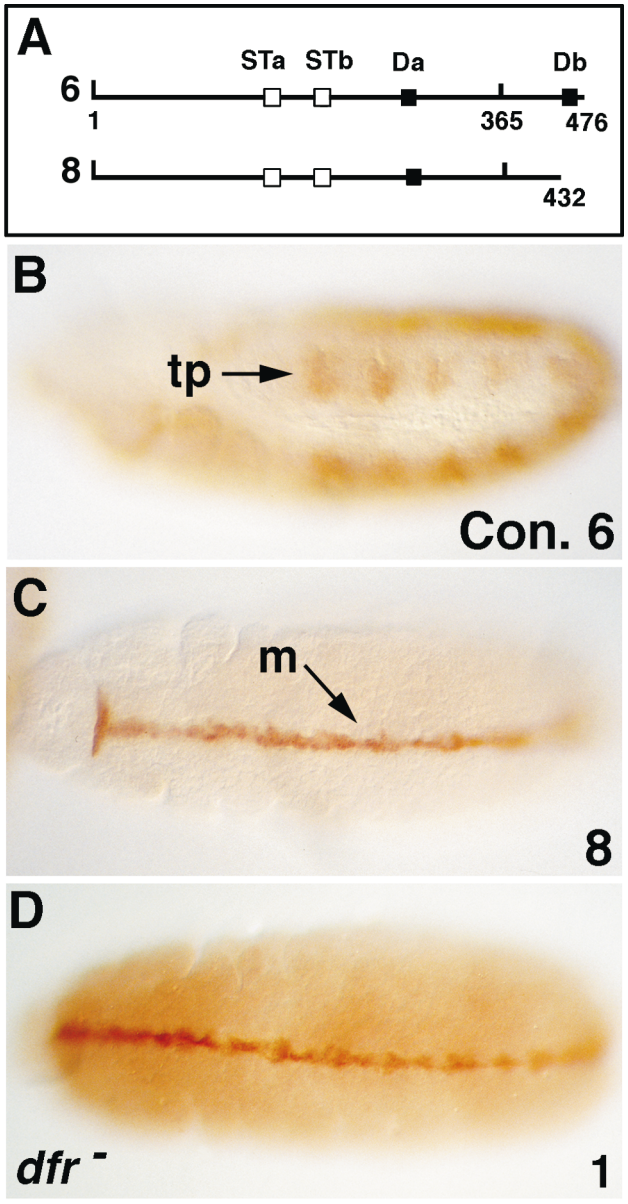

Fig. 8. Dissection of the cis elements mediating *rho* tracheal expression. (A) Constructs which contain deletions in the 3′ fragment conferring tracheal expression were generated. (B) Construct 6 containing Db, as well as STa and STb, confers both midline and tracheal expression. (C) Deletion of 44 bp containing Db (construct 8) abolished tracheal, but not midline expression. (D) In *dfr* mutant embryos tracheal expression of the 762 bp *rho* reporter is abolished, but midline expression is retained. This verifies the requirement of Dfr for *rho* tracheal expression, and demonstrates that Dfr is not required for the capacity of Sim to promote midline expression. Similarly, in *dfr* mutant embryos, construct 6 retained only midline expression.

synergistic activity of Oct-3 and Sox-11 was demonstrated (Ambrosetti et al., 1997). It is interesting to note that the conformation of POU proteins can be altered according to the relative positions on the DNA for binding the POU-specific and homeo domains. The spectrum of proteins recognized by POU proteins can be modified accordingly (reviewed in Ryan and Rosenfeld, 1997).

3.2. Induction of tracheal cell fates

The assignment and differentiation of cells that will give rise to different tissues during embryonic development of *Drosophila* can be roughly divided into two distinct phases. Once the anterior–posterior and dorso–ventral coordinates have been defined, the ectoderm can be viewed as a grid. It has the capacity to provide positional information for tissues that will be generated from the ectoderm, such as the peripheral nervous system, salivary glands and trachea, or for tissues that are in contact with the ectoderm such as the mesoderm. This positional information is manifested by a highly localized expression of genes that will play a central role in defining the fate of a given tissue. In many, cases these genes encode transcription factors. In the second phase, the identity of a tissue must be maintained, in spite of the fact that the original cues that led to specification of the cells have already faded. Expression of these early tissue-specific genes is maintained by their capacity to autoregulate their own expression. From this stage onwards, the general identity of the tissue is independent of external inputs, although signals from neighboring cells can still induce specific fates within the tissue.

The hierarchy of genes regulating the fate of a given tissue is critical for correct and reproducible patterning. If a single gene with an autoregulatory capacity would be sufficient for inducing tissue identity, fortuitous induction of the gene may give rise to the formation of ectopic tissue. It is thus reasonable to assume that more complicated regulatory circuits would be required for the induction of tissue fate. For example, it was shown that induction of eye fates requires a network of interactions between the genes *eyeless*, *eyes absent* and *sine oculis* (Bonini et al., 1997; Halder et al., 1998). A similar scenario appears to be taking place in tracheal development. The initial expression of Trh and Dfr, driven by anterior–posterior and dorso–ventral cues, is independent of each other (Llimargas and Casanova, 1997; and Fig. 2A,G). However, in the second phase, the two factors are simultaneously required for the autoregula-

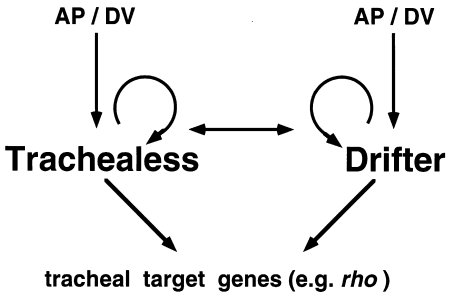

Fig. 9. Induction of tracheal cell fates by Trh and Dfr. Initially, expression of Trh and Dfr in the tracheal placodes depends only on exogenous AP and DV cues, defining the position of the tracheal placodes. However, once autoregulation ensues, both proteins are simultaneously required for autoregulation. Moreover, tracheal expression of at least some target genes also requires both Trh and Dfr. Thus, a more robust mechanism for inducing and maintaining tracheal cell fates is achieved.

tory circuits of their own expression. This strict interdependence for autoregulation provides a robust system for the induction of tracheal tissue identity. Future studies will determine if the dual requirement of the two proteins for autoregulation is based on direct binding to the respective autoregulatory regions. A scheme for the regulatory interactions between Trh and Dfr is presented in Fig. 9.

The paradigm that Trh or Dfr alone are not sufficient to induce their target genes or autoregulation, broadens the scope of activities of the two proteins. Trh is required not only for the induction of tracheal fates, but also for patterning the salivary ducts and posterior spiracles (Isaac and Andrew, 1996). It is possible that in these tissues, Trh associates with other proteins and induces a different set of tissue-specific target genes. Similarly, Dfr is also expressed in the midline cells. Dfr is not necessary for the induction of Sim-target genes, as can be deduced from the normal expression of *rho* in the midline of *dfr*-mutant embryos. However, Dfr could be functioning in conjunction with other midline proteins such as the Sox-domain protein Diachaete (Soriano and Russell, 1998).

In conclusion, the interactions between Trh and Dfr, which are necessary for autoregulation and induction of tracheal-target genes, provide a robust mechanism for establishment and maintenance of tissue identity. The association between the two proteins, mediated by the PAS and POU domains respectively, generates a transcription complex which has a distinct target-gene specificity. Such interactions are likely to be general for defining the diverse target specificities of proteins from the bHLH-PAS and POU families.

4. Experimental procedures

4.1. Fly lines

The following mutant lines were used: *trh* allele *l(3)10512*, *dfr/vvl*^{6A3} (provided by J. Casanova, Barcelona), and the *514dfr-lacZ* strain, carrying the autoregulatory *dfr* fragment (provided by W. Johnson, University of Iowa). For misexpression studies, the following lines were used: *HS-dfr* (provided by W. Johnson), *UAS-trh 14*, *K25 sev hs-Gal4*, and *1-eve-1*. A 20-min heat shock at 37°C was provided to synchronized embryos at 2.7 h after egg lay, and embryos were fixed 7 h after egg lay.

4.2. Antibodies

The following antibodies were used: rabbit polyclonal anti-βGal (Cappel), rat polyclonal anti-Trh (Wappner et al., 1997), and rat polyclonal anti-Dfr (provided by W. Johnson, University of Iowa).

4.3. DNA constructs

Fragments of the 762 bp *Eco*RI *rho* regulatory region were cloned into the *Eco*RI–*Bam*HI sites of PCaSpeR AUG βGal (provided by C. Thummel, University of Utah, Salt Lake City). The fragments were generated by PCR, where the oligonucleotides contained also the *Eco*RI or *Bam*HI restriction sites. The position of the fragments on the EcoRI *rho* fragment (Fig. 5) are listed below. Construct 1: 1–762; construct 2: 1–367; construct 3: 349–762; construct 4: 135–246, and 349–762; construct 5: 153–172, 197–224, and 349–762; construct 6: 1–476; construct 7: 135–246; construct 8: 1–432. In cases where several fragments were included, nested PCR reactions were carried out, adding the more distal sequences at subsequent stages. All constructs were injected into embryos, and transgenic lines generated by standard methods. At least two independent transgenic lines were monitored for *lac*Z expression of each construct.

To generate the GST fusions, the coding sequence of Dfr containing nucleotides 682–1960 was inserted, in frame, into the pGEX-2T expression plasmid (Pharmacia). We also used the Dfr POU domain region inserted into pGEX-KG (Turner, 1996) (provided by E. Turner, UC San-Diego). Both plasmids were transformed into *E. coli* strain BL21. For in vitro transcription and translation, the entire coding sequence of Trh, DARNT, Trh-Sim PAS, Sima and HIF-1α were cloned into the Bluescript vector. Sim was cloned in pNB40 plasmid. The Trh-PAS (nucleotides 1, 187–2, 111) was cloned into pcDNA3.1/HIS A (Invitrogen).

4.4. GST pull down

In vitro transcription and translation reactions were performed by incubating 1 microgram of each plasmid in a 50 μl TNT-coupled reticulocyte lysate system (Promega). Transcription of *trh-PAS* was carried out using T7 RNA polymerase, transcription of *trh*, *DARNT*, *sima*, and *HIF-1α* was carried out with T3 RNA polymerase, and transcription of *sim* with SP6 RNA polymerase. [^{35}S]methionine was added according to the TNT kit protocol.

Expression of GST fusion proteins was induced by addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C for 2 h. Cells were then sonicated in buffer A [20 mM Tris–HCl (pH 7.9), 0.2 mM EDTA, 0.1 M NaCl, 1 mM DTT, 1 mM PMSF, 0.2% Nonidet P-40]. Total lysates containing the GST, GST-DFR or GST-DFR-POU proteins were immobilized onto glutathione-agarose beads (Pharmacia) in buffer A, by shaking for 2 h at 4°C. Binding was carried out by shaking 5 μl of the in vitro translation lysate with 4 μg of each GST protein, for 1 h at 4°C. GST proteins were spun down and washed 3 times with buffer A containing 0.4% of NP-40. In one of the GST-Dfr samples that was incubated with Trh, 0.3 M NaCl was used. ^{35}S-labeled proteins were analyzed using 10% SDS-PAGE, and visualized by autoradiography.

Acknowledgements

We are grateful to J. Casanova (Barcelona), S. Crews (University of North Carolina, Chapel Hill), T. Ip (University of Massachusetts, Worcester), W. Johnson (University of Iowa), M. Levine (UC Berkeley), C. Thummel (University of Utah, Salt Lake City) and E. Turner (UC San Diego) for providing plasmids, fly lines and antibodies. We thank Ruchama Leiserowitz for excellent technical help, Batya Cohen, Ari Elson, Yosef Shaul, Hila Toledano-Kachalski and members of the Shilo lab for advice and discussions, and Batya Cohen, Ze'ev Paroush and Talila Volk for critical reading of the manuscript. This work was supported by grants from the Israel Science Foundation and by the Minerva Foundation, Germany to B-Z. S.

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