

Regulation of bHLH-PAS protein subcellular localization during *Drosophila* embryogenesis

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SUMMARY

The *Drosophila* Single-minded and Tango basic-helix-loop-helix-PAS protein heterodimer controls transcription and embryonic development of the CNS midline cells, while the Trachealess and Tango heterodimer controls tracheal cell and salivary duct transcription and development. Expression of both *single-minded* and *trachealess* is highly restricted to their respective cell lineages, however *tango* is broadly expressed. The developmental control of subcellular localization of these proteins is investigated because of their similarity to the mammalian basic-helix-loop-helix-PAS Aromatic hydrocarbon receptor whose nuclear localization is dependent on ligand binding. Confocal imaging of Single-minded and Trachealess protein localization indicate that they accumulate in cell nuclei when initially synthesized in their respective cell lineages and remain nuclear throughout embryogenesis. Ectopic expression experiments show that Single-minded and Trachealess are localized to nuclei in cells throughout the ectoderm and mesoderm, indicating that nuclear accumulation is not regulated in a cell-specific fashion and unlikely to be ligand dependent. In contrast, nuclear localization of Tango is developmentally regulated; it is

localized to the cytoplasm in most cells except the CNS midline, salivary duct, and tracheal cells where it accumulates in nuclei. Genetic and ectopic expression experiments indicate that Tango nuclear localization is dependent on the presence of a basic-helix-loop-helix-PAS protein such as Single-minded or Trachealess. Conversely, *Drosophila* cell culture experiments show that Single-minded and Trachealess nuclear localization is dependent on Tango since they are cytoplasmic in the absence of Tango. These results suggest a model in which Single-minded and Trachealess dimerize with Tango in the cytoplasm of the CNS midline cells and trachea, respectively, and the dimeric complex accumulates in nuclei in a ligand-independent mode and regulates lineage-specific transcription. The lineage-specific action of Single-minded and Trachealess derives from transcriptional activation of their genes in their respective lineages, not from extracellular signaling.

Key words: Arnt, bHLH-PAS, Nuclear localization, Single-minded (Sim), Tango (Tgo), Trachealess (Trh)

INTRODUCTION

The Single-minded (Sim) and Trachealess (Trh) basic-helix-loop-helix-PAS (bHLH-PAS) proteins control gene transcription and development in the *Drosophila* central nervous system (CNS) midline cells and trachea, respectively (reviewed by Crews, 1998). Genetic and biochemical data demonstrated that both Sim and Trh dimerize in vivo with the Tango (Tgo) bHLH-PAS protein (Ohshiro and Saigo, 1997; Sonnenfeld et al., 1997). Tgo is the *Drosophila* orthologue of the mammalian Aromatic hydrocarbon nuclear translocator (Arnt) protein (Hoffman et al., 1991). The Sim::Tgo and Trh::Tgo protein complexes bind DNA and activate transcription. The binding site for both heterodimer complexes is the CNS midline element (CME) (Wharton et al., 1994; Ohshiro and Saigo, 1997; Sonnenfeld et al., 1997). This sequence is required for Sim and Trh transcriptional activation and when multimerized is sufficient for transcription in both midline cells and trachea. Work on *sim* and *trh* coupled with

research on related mammalian bHLH-PAS proteins reveal that the bHLH-PAS/Arnt/CME regulatory cassette has been highly conserved throughout animal development (Probst et al., 1997; Sonnenfeld et al., 1997). The major issue addressed in this paper is how the function of these proteins is controlled at the level of subcellular localization during embryonic development.

There exist numerous examples of how transcription factor function can be regulated at the level of nuclear localization (reviewed by Vandromme et al., 1996). The *Drosophila* Dorsal protein, which is related to the mammalian NF κ b, is uniformly distributed in the syncytial blastoderm embryo. In the absence of an extracellular ventralizing signaling, Dorsal protein exists as part of a multiprotein complex tethered to the plasma membrane (Edwards et al., 1997). In response to the ventralizing signal, Dorsal forms a gradient of nuclear localization in the ventral blastoderm (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989), where it functions to pattern the embryo along the dorsal/ventral axis (Rusch and

Levine, 1996). Another example is *Drosophila* Armadillo, related to mammalian β -catenin, that accumulates in nuclei in response to Wingless signaling (Orsulic and Peifer, 1996). In the bHLH protein family, nuclear localization of MyoD is developmentally controlled (Rupp et al., 1994), while nuclear localization of the Aromatic hydrocarbon receptor (Ahr; or the dioxin receptor) is regulated by ligand binding (Rowlands and Gustafsson, 1997).

The mammalian Ahr complex (AHRC) (Rowlands and Gustafsson, 1997) represents a paradigm for understanding how bHLH-PAS proteins function. The AHRC consists of a dimer between Ahr and Arnt. In cell culture, Arnt is found in the nucleus (Pollenz et al., 1994; Eguchi et al., 1997), although in embryos it is localized in either cytoplasm alone, cytoplasm plus nucleus, or nucleus alone (Abbott and Probst, 1995). Cell culture experiments show that in the absence of exogenously added ligand, Ahr is found exclusively in the cytoplasm, where it is complexed with accessory proteins including Hsp90 and Ahr-interacting protein (Denis et al., 1988; Perdew, 1988; Ma and Whitlock, 1997). Ligands such as dioxin diffuse through the membrane and bind Ahr. Ahr dissociates from the accessory proteins and binds Arnt. The complex is compartmentalized in nuclei where it binds DNA and activates transcription of genes involved in toxin metabolism. Thus, Ahr acts as a receptor for a small molecule signaling pathway that controls nuclear localization of AHRC. This raises the issue of whether the subcellular localization of other bHLH-PAS proteins, including those that control developmental processes, is regulated or unregulated.

In this paper, we investigate the *in vivo* regulation of Sim, Trh, and Tgo subcellular localization. We show that Tgo is cytoplasmic in most embryonic cells, but is strongly localized to nuclei in the CNS midline cells, trachea, and salivary duct. Since these are the cells in which Sim and Trh are functional, Tgo nuclear localization correlates with bHLH-PAS::Tgo function. Genetic, ectopic expression, and cell culture experiments indicate that Tgo nuclear localization is dependent on the presence of either Sim or Trh protein, and that nuclear entry of Sim and Trh requires interaction with Tgo. Both Sim and Trh are able to enter cell nuclei in many, if not all, ectodermal and mesodermal cells, and form transcriptionally competent complexes with Tgo. These results suggest that unlike Ahr, which is broadly expressed and whose nuclear transport and function is dependent on ligand binding, Sim and Trh control cell lineage development in a ligand-independent mode by being specifically expressed in their respective cell types.

MATERIALS AND METHODS

Drosophila strains and transgenes

Drosophila mutant strains were: (1) *sim*^{H9}, a protein null allele of *sim*, and (2) *Df(3L)emc-E12* (61A-61D3), a deletion that removes the *trh* gene (Isaac and Andrew, 1996). Ectopic expression experiments involved crossing either *engrailed* (*en*)-Gal4 or *twist* (*twi*)-Gal4 flies to UAS-*sim*, UAS-*trh*, and UAS-*tgo* flies. The *en*-Gal4 line expresses Gal4 in *en* stripes, and *twi*-Gal4 expresses Gal4 in the mesoderm. UAS-*sim* and UAS-*trh* transgenic *Drosophila* strains were acquired from J. Nambu (U. Massachusetts, Amherst) and Benny Shilo (Weizmann Institute, Israel), respectively. The UAS-*tgo* transgenic strain was generated by injecting *w*¹¹¹⁸ flies with a P-element plasmid

that contains the complete *tgo* coding sequence (Sonnenfeld et al., 1997) fused to Gal4 UAS sequences in the pUAST vector (Brand and Perrimon, 1993). P[*w*⁺; 4x*CME-lacZ*] flies have a transgene in which four copies of the *Toll* site 4 *CME* are cloned into the C4PLZ *lacZ* enhancer tester vector (Wharton et al., 1994).

Generation of antibodies

Monoclonal antibodies against Tgo were previously described (Sonnenfeld et al., 1997). Polyclonal antibodies against Sim and Trh were generated against glutathione S-transferase (GST) fusion proteins. GST-Sim (aa413-650) was induced in *E. coli* and purified as a soluble protein using GST-agarose affinity chromatography. GST-Trh (aa307-596) was induced, prepared as inclusion bodies, solubilized in 10% SDS, dialyzed in 0.05% SDS, 1 mM PMSF, and stored in 0.01% SDS, 1 mM PMSF (Williams et al., 1995). The purified proteins were injected into rats as previously described (Sonnenfeld et al., 1997).

Immunostaining of embryos

Antibody staining of embryos was carried out according to standard protocols (Patel et al., 1987). Supernatant from the murine anti-Tgo monoclonal antibody, mAb-Tgo-3, was concentrated 10 fold using Pharmacia EZ-Sep, and used undiluted. Rat anti-Sim and anti-Trh polyclonal antisera were used at a 1:200 dilution. The anti- β -galactosidase antibody was a murine monoclonal antibody (Promega). Texas Red-conjugated anti-rat antibody (Molecular Probes) was used at a 1:200 dilution. Biotinylated anti-mouse secondary antibody was used at a 1:200 dilution followed by HRP-avidin (Vector Labs) and FITC conjugated tyramide (TSA Direct, NEN) used at a 1:50 dilution. Embryos were mounted in Aquapolymount (Polysciences, Inc.) and viewed on a Zeiss laser scanning confocal microscope. Data from double-labeled images were superimposed using LSM 3.8 software so that the green signals from FITC and red from Texas Red were yellow when merged. Bandpass filters were adjusted to eliminate bleed-through from the different emissions.

SL2 cell transient expression and immunostaining assays

SL2 cells were transiently transfected with expression plasmids, pAct-*sim*, pAct-*tgo*, and pAct-*trh*, and assayed for their ability to activate transcription from the P[6x*CME-lacZ*] reporter plasmid using a fluorescent substrate as described previously (Sonnenfeld et al., 1997). Tissue culture cells used for staining were fixed in 4% paraformaldehyde, washed in PBS, and incubated with anti-Sim (1:500) or anti-Trh (1:500) antibodies in 0.1% saponin, 1% normal goat serum, PBS for 1 hour. Following a wash in PBS, the cells were incubated with Texas Red conjugated anti-rat secondary antibody (1:500) for 1 hour. Cells were washed, mounted in Aquapolymount, and visualized by fluorescent imaging with either a Zeiss Axiophot microscope or Zeiss laser-scanning confocal microscope.

RESULTS

Sim accumulates rapidly in CNS midline cell nuclei during embryogenesis

Analysis of AHRC function indicates that Ahr acts as a receptor whose nuclear entry is regulated by small molecule binding. The similarities between Sim and Ahr have led to speculation that Sim may also function as a ligand-dependent receptor. Relevant to this issue is the subcellular distribution of Sim during embryogenesis. Previous studies using antibodies raised against Sim protein have shown that Sim is primarily localized to the nuclear compartment during development of the CNS midline cells (Crews et al., 1988). However, the

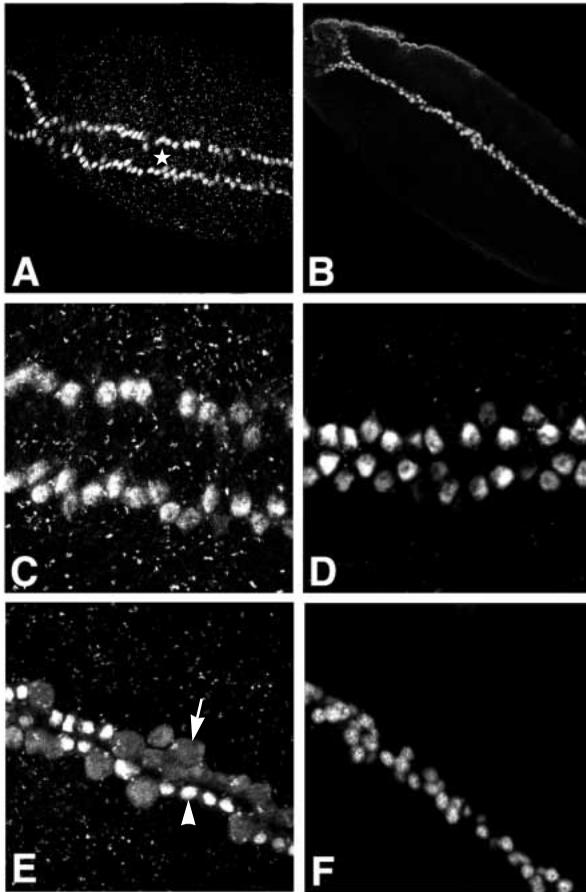


Fig. 1. Sim protein accumulates in cell nuclei at all stages of embryonic development (Campos-Ortega and Hartenstein, 1985). All images are taken from whole-mount embryos stained with anti-Sim, followed by FITC-conjugated 2° antibody, and observed by confocal imaging. Ventral views are shown with anterior to the left. (A) Stage 6 embryo near the end of gastrulation. Shown are the two rows of mesectodermal cells approaching the ventral midline (★). (B) Embryo at stage 9 showing midline precursor cells joined at the midline. (C) Higher magnification of same stage 6 embryo shown in A revealing concentrated nuclear Sim staining. (D) Midline precursor cells with Sim nuclear staining at stage 7 have joined together at the midline. (E) Stage 8 embryo showing some midline precursor cells undergoing mitosis with corresponding nuclear membrane breakdown (arrow), and other cells having completed mitosis with strong nuclear accumulation of Sim (arrowhead). (F) Higher magnification of stage 9 embryo in B showing midline precursor cells with nuclear Sim staining having completed mitosis.

dynamics of Sim subcellular distribution have not been carefully examined during embryogenesis. We have utilized new Sim antibodies and confocal imaging to describe in detail Sim subcellular distribution during development of the CNS midline cells, and provide a foundation for understanding the dynamics of Tgo subcellular distribution.

Sim protein is first detected during gastrulation as the mesectodermal (CNS midline precursor) cells move towards the ventral midline of the embryo (Fig. 1A,C). Most of the protein is highly concentrated in cell nuclei (Fig. 1C). As the mesectodermal cells merge at the midline (Fig. 1B,D), Sim protein remains concentrated in cell nuclei (Fig. 1D). The first

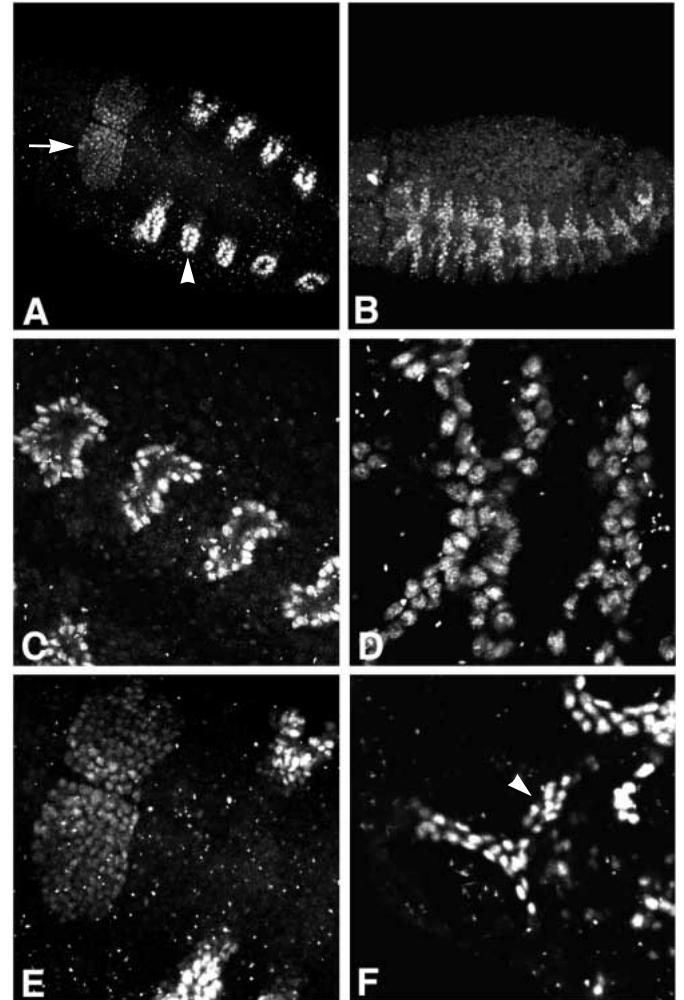
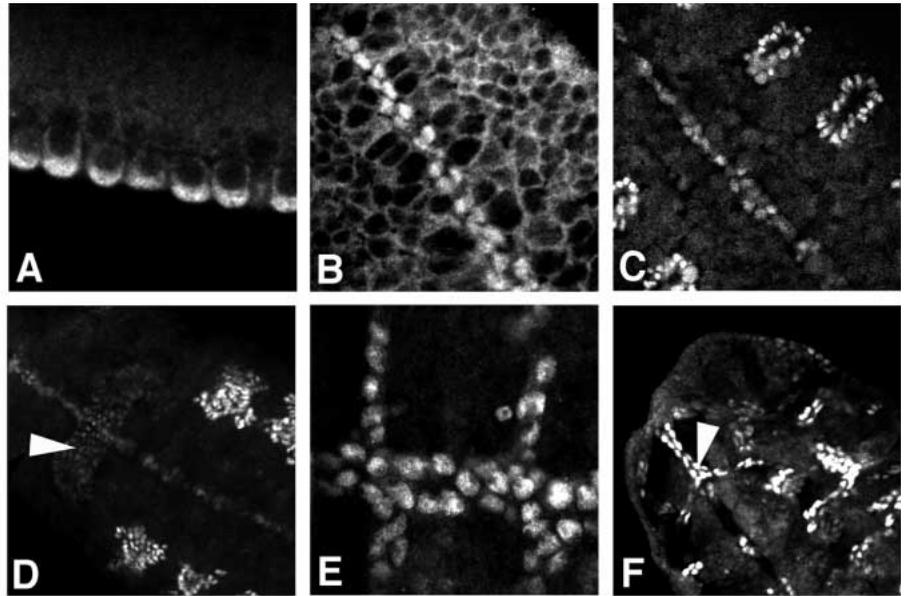


Fig. 2. Trh protein accumulates in tracheal and salivary duct cell nuclei throughout embryogenesis. All embryos are whole-mounts stained with anti-Trh antibody. Anterior is to the left. (A) Ventral view of a stage 11 embryo showing strong Trh staining in the tracheal pits (arrowhead). Weaker staining is observed in the salivary primordia (arrow). (B) Sagittal view of a stage 14 embryo showing localization of Trh in the tracheal branches. (C) Higher magnification view of a stage 11 embryo showing nuclear staining in the tracheal pits. (D) Higher magnification view of B showing nuclear staining in the tracheal branches. (E) Nuclear localization of Trh in the salivary primordia of the embryo shown in A. (F) The salivary duct (arrowhead) of a stage 15 embryo shows nuclear Trh staining. There is no Trh staining in the salivary gland.

developmental event that occurs in the CNS midline lineage is a synchronous cell division in which all midline precursor cells divide (Foe, 1989; Nambu et al., 1991). During mitosis, the nuclear membrane breaks down and Sim is uniformly distributed throughout the cell (Fig. 1E; arrow). However, quickly after mitosis is completed, Sim protein reaccumulates in cell nuclei (Fig. 1E; arrowhead), where it remains throughout embryogenesis (Fig. 1F). Thus, Sim protein is highly concentrated in cell nuclei at all stages of embryogenesis, and does not provide evidence for a prolonged cytoplasmic stage and a regulated cytoplasm-to-nucleus transition.

Fig. 3. Subcellular localization of Tgo protein is cell-type specific. All panels show whole-mount embryos stained with anti-Tgo antibody and visualized by confocal imaging.

(A) Sagittal view of a stage 5 syncytial blastoderm embryo showing cytoplasmic staining of Tgo. (B) Ventral view of a stage 9 embryo showing nuclear Tgo staining in the midline precursor cells and cytoplasmic Tgo in the adjacent ectoderm. (C) Ventral view of a stage 11 embryo showing nuclear Tgo staining in the CNS midline cells and tracheal pits. (D) Ventral view of a stage 11 embryo showing Tgo nuclear staining in the salivary primordia (arrowhead). (E) High magnification view of nuclear Tgo staining in the tracheal tubules of a stage 14 embryo. (F) Nuclear Tgo localization in the salivary duct (arrowhead) of a stage 15 embryo.



Trh accumulates rapidly in tracheal cell nuclei during embryogenesis

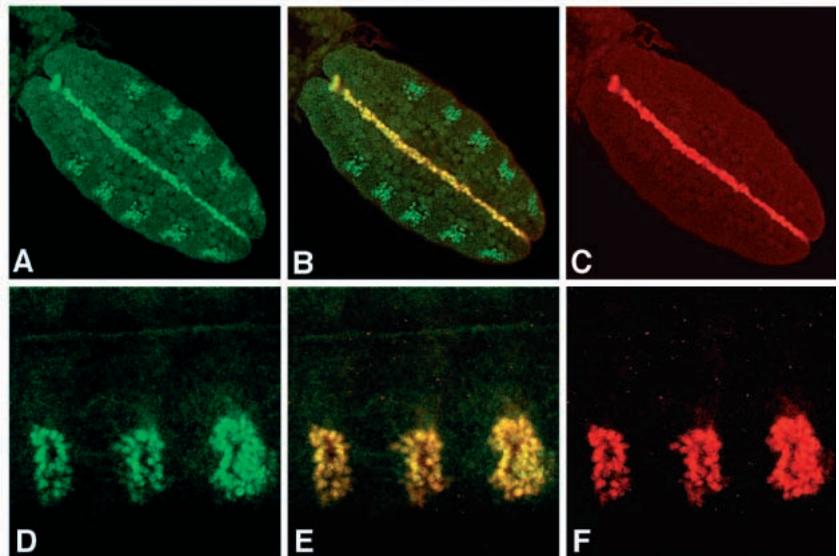
Early in embryonic development, the *trh* gene is expressed in tracheal and salivary primordia (Isaac and Andrew, 1996; Wilk et al., 1996). Later it is expressed in the tracheal network and in the salivary duct. Previous reports have detected Trh in cell nuclei (Wilk et al., 1996). We have utilized anti-Trh antibodies and confocal imaging to examine the dynamics of Trh nuclear accumulation. Trh protein is first observed in the tracheal pits, where it shows strong nuclear accumulation (Fig. 2A,C), except in dividing cells. During fusion and formation of the tracheal network, Trh remains nuclear (Fig. 2B,D). Trh protein is also found in nuclei of the salivary primordia, which have relatively low levels of Trh protein (Fig. 2E). Later, Trh protein is found in the nuclei of the salivary duct, but is undetectable in the salivary gland (Fig. 2F). These results demonstrate that Trh protein rapidly accumulates in cell nuclei and remains nuclear throughout embryogenesis. Like Sim, there is no extended cytoplasmic Trh interval, and no evidence for regulated Trh nuclear entry.

Nuclear accumulation of Tgo protein correlates with cellular sites of *tgo* function

Previous work has shown that the *tgo* gene is expressed in all embryonic cells (Ohshiro and Saigo, 1997; Sonnenfeld et al., 1997). Transcripts of *tgo* were found at similar levels in most embryonic cells, although enhanced levels were observed in the developing trachea and CNS. The protein distribution, determined using a Tgo antibody has been shown to be similar to the RNA distribution (Sonnenfeld et al., 1997), although in that study a detailed analysis was not performed. In this paper, we have used Tgo antibodies and confocal imaging to study the subcellular distribution of Tgo protein during embryogenesis.

Both *tgo* transcripts and protein are observed at the earliest stages of the syncytial blastoderm. It is likely that *tgo* transcripts are derived from both maternal and zygotic contributions. Confocal imaging of Tgo protein in the stage 5 syncytial blastoderm embryo revealed that the protein is found throughout the embryo and it is exclusively cytoplasmic,

Fig. 4. Tgo nuclear localization coincides with Sim and Trh nuclear localization. (A-C) Ventral views of a stage 11 whole-mount embryo double-stained with anti-Tgo (green) and anti-Sim (red). (A) Anti-Tgo staining showing nuclear localization of Tgo in the CNS midline cells and tracheal pits. (B) Merged images of anti-Tgo and anti-Sim double staining showing colocalization (yellow) in the CNS midline cells. (C) Anti-Sim staining showing nuclear localization in the CNS midline cells. (D-F) Sagittal views of a stage 11 whole-mount embryo double-stained with anti-Tgo (green) and anti-Trh (red). (D) anti-Tgo staining showing nuclear localization of Tgo in the tracheal pits. (E) Merged image of anti-Tgo and anti-Trh co-staining showing colocalization (yellow) in the tracheal pits. (F) Anti-Trh staining showing nuclear localization in the tracheal pits.



residing at the apical ends of the developing cells (Fig. 3A). During gastrulation Tgo protein accumulates in nuclei of the midline precursor cells (Fig. 3B), but remains predominantly cytoplasmic in other cells (Fig. 3B). Tgo protein remains nuclear in the midline cells throughout the rest of embryogenesis (Fig. 3C), whereas most other embryonic cells have a cytoplasmic subcellular distribution (Fig. 3C-F). Additional Tgo nuclear staining is observed in the tracheal pits beginning around stage 11 (Fig. 3C), and the salivary primordia beginning at stage 11 (Fig. 3D). Tgo remains nuclear in the trachea throughout their development (Fig. 3E). As the salivary glands develop, Tgo nuclear localization is restricted to the salivary ducts and is absent from the salivary glands (Fig. 3F). There are additional sites of Tgo nuclear localization in the cephalic region and in the post-stage 14 CNS.

Colocalization of nuclear Tgo with Sim and Trh

Comparison of Fig. 1-3 shows that the appearance of Tgo in the nuclei of the developing CNS midline, tracheal and salivary primordia and duct cells correlates with the nuclear localization of Sim and Trh in these cells. Confirmation of this was obtained by double-staining embryos with anti-Tgo and either anti-Sim or anti-Trh. Co-staining with anti-Sim and anti-Tgo shows that Tgo nuclear localization in the CNS midline precursor cells coincides exactly with Sim nuclear staining (Fig. 4A-C). Similar results are observed for anti-Tgo and anti-Trh co-staining. Fig. 4D-F shows that Tgo nuclear staining in the tracheal pits coincides exactly with Trh nuclear staining. Colocalization of nuclear Sim and nuclear Tgo continues throughout embryonic development, as is also the case for nuclear Trh and nuclear Tgo. Generally, it appears that more Tgo protein is present in cells with concentrated nuclear Tgo than the adjacent cells where it is cytoplasmic.

Embryonic nuclear localization of Tgo requires the presence of Sim and Trh

The results described above showed that Tgo nuclear localization correlates with Sim and Trh nuclear localization. Evidence that Tgo nuclear localization requires the presence of Sim or Trh was achieved by staining embryos mutant for either *sim* or *trh* with anti-Tgo. The *sim^{H9}* allele is a protein null mutant of *sim* and has a severe collapsed CNS phenotype (Thomas et al., 1988; Nambu et al., 1990). Stage 11 mutant embryos do not have nuclear accumulation of Tgo in cells at the midline, while Tgo continues to localize to nuclei in tracheal cells (Fig. 5A). This suggests that the presence of Sim nuclear protein is required for Tgo nuclear accumulation. *Df(3L)emc-E12* mutant embryos lack the *trh* gene, and thus lack Trh protein. Embryos mutant for *trh* fail to show nuclear concentration of Tgo in tracheal pits when stained with anti-Tgo (Fig. 5B). In addition, there is no nuclear accumulation of Tgo in the salivary primordia and salivary duct in *Df(3L)emc-E12* mutant embryos (data not shown). Tgo continues to localize to nuclei of the midline cells, however (Fig. 5B). These results indicate that nuclear accumulation of Tgo requires *sim* and *trh* gene function, most likely through direct protein interaction.

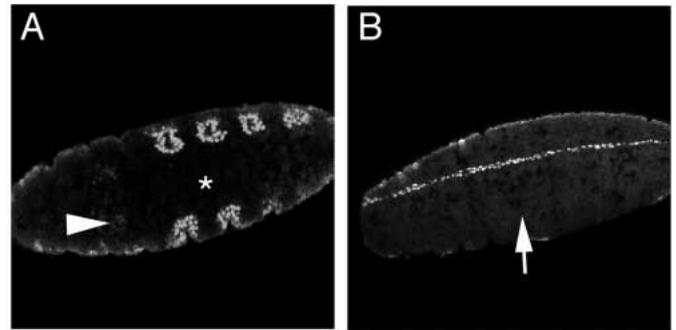


Fig. 5. Tgo nuclear localization requires *sim* and *trh* function. (A) Ventral view of a stage 11 whole-mount *sim^{H9}* mutant embryo stained with anti-Tgo. There is an absence of nuclear Tgo staining in the vicinity of the CNS midline cells (★). Nuclear Tgo staining in the tracheal pits and salivary primordia (arrowhead) is unaffected. (B) Ventral view of a stage 11 whole-mount *Df(3L)emc-E12* (which deletes *trh*) mutant embryo stained with anti-Tgo. There is an absence of nuclear Tgo in the area around the tracheal pits (arrow), although nuclear Tgo is present in the CNS midline cells.

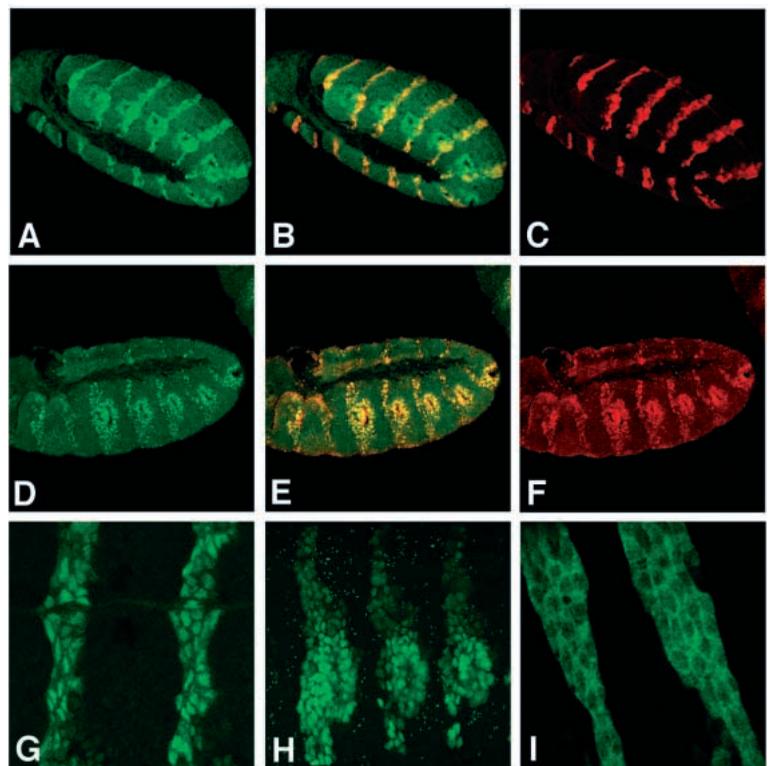


Fig. 6. Ectopic expression of *sim* and *trh* result in ectopic nuclear accumulation of Sim, Trh and Tgo proteins. Sagittal views of whole-mount stage 11 embryos. Anterior is to the left and dorsal is up. (A-C) Embryo from *en-Gal4* × *UAS-sim* flies double-stained with anti-Sim (C; red) and anti-Tgo (A; green) showing nuclear accumulation of both proteins in *en-sim* stripes. (B) Merged image showing colocalization of Sim and Tgo in *en-sim* stripe nuclei (yellow). (D-F) Embryo from *en-Gal4* × *UAS-trh* flies double-stained with anti-Trh (F; red) and anti-Tgo (D; green) showing nuclear accumulation of both proteins in *en-trh* stripes. (E) Merged image showing colocalization of Trh and Tgo in *en-trh* stripe nuclei (yellow). (G-I) High magnification views of embryos stained with anti-Tgo. (G) Nuclear Tgo staining in *en-Gal4* × *UAS-sim* stripes. (H) Nuclear Tgo staining in *en-Gal4* × *UAS-trh* stripes. (I) Cytoplasmic Tgo staining in *en-Gal4* × *UAS-tgo* stripes.

Ectopic expression of *sim* and *trh* result in ectopic nuclear localization of Sim, Trh and Tgo

The results described above indicate that Sim and Trh enter cell nuclei when expressed in their correct cell types, and are required for nuclear accumulation of Tgo in the same cells. However, those experiments do not directly address whether there are developmentally relevant spatial or temporal signals that control Sim, Trh, or Tgo nuclear localization. This can be addressed by ectopically expressing these genes and examining, by antibody staining, whether nuclear localization occurs. These experiments were performed using the bipartite UAS-Gal4 system of Brand and Perrimon (1993). Existing transgenic *Drosophila* strains were used in which the *sim* and *trh* coding sequences were fused to Gal4-UAS. We also created a *Drosophila* strain in which the *tgo* coding sequence was fused to Gal4-UAS. These lines were crossed to transgenic *Drosophila* strains in which the *en* regulatory region drives expression of Gal4 in ectodermal stripes and the *twi* regulatory region drives Gal4 in the mesoderm. The resulting progeny express *sim*, *trh*, or *tgo* either in *en* circumferential stripes or the mesoderm.

Examination of *en*-Gal4 × UAS-*sim* embryos stained with anti-Sim indicate that Sim accumulates in nuclei throughout the *en* stripe (Fig. 6C). Double staining with anti-Tgo shows that Tgo also accumulates in cell nuclei in the *en*-*sim* stripes (Fig. 6A,B,G). Analysis of *en*-Gal4 × UAS-*trh* shows similar results. Trh nuclear staining is observed throughout the *en*-*trh* stripes (Fig. 6F), and Tgo is also nuclear in the stripes (Fig. 6D,E,H). In contrast, when *tgo* is ectopically expressed in *en*-Gal4 × UAS-*tgo* embryos, Tgo protein is predominantly cytoplasmic (Fig. 6I). These experiments indicate that both Sim::Tgo and Trh::Tgo are able to enter cell nuclei even when expressed in cells spatially distinct from their normal sites of expression, and that Tgo nuclear accumulation is dependent on the presence of a partner protein such as Sim or Trh.

The ectopic expression experiments described above show that Sim, Trh, and Tgo nuclear localization can occur throughout the ectoderm. However, the *sim* and *trh* genes normally function in ectodermal tissues, and factors controlling nuclear localization in the ectoderm may be absent in other non-ectodermal cell types such as mesoderm. This issue was addressed by ectopically expressing *sim* and *trh* in mesodermal cells using *twi*-Gal4. Fig. 7A-C shows that *twi*-Gal4 × UAS-*sim* embryos have strong nuclear Sim and nuclear Tgo protein throughout the mesoderm. Similar results were obtained from *twi*-Gal4 × UAS-*trh* embryos (Fig. 7D-F). These results, along with those using *en*-Gal4, demonstrate that Sim and Trh are localized to nuclei throughout the ectoderm and mesoderm. This reinforces the idea that there is no spatially or temporally restricted signal that is required for nuclear localization of Sim and Trh. Furthermore, Tgo can enter nuclei in the presence of Sim and Trh in multiple cell types showing that there is no cell-specific control of Sim::Tgo or Trh::Tgo dimerization and nuclear entry.

Ectopically localized Sim forms active complexes with endogenous Tgo

To explore whether ectopically expressed nuclear Sim::Tgo complexes expressed in *en* stripes and in the mesoderm were functional, we examined whether an *in vivo* target sequence

of Sim::Tgo was ectopically activated. This was expected for some ectodermal cell types since previous experiments using a heat shock *sim* transgene resulted in ectopic expression of midline-activated genes (Nambu et al., 1991). The assayed gene was P[4xCME-lacZ], which contains four copies of the *Toll* site 4 CME fused to a lacZ enhancer tester vector (Wharton et al., 1994). Embryos with P[4xCME-lacZ] transgenes have high levels of lacZ expression in CNS midline cells (Wharton et al., 1994) and lower levels in trachea (Sonnenfeld et al., 1997; Zelzer et al., 1997). Expression of this element in ectodermal cells was examined by staining embryos with anti-β-galactosidase antibody in a genetic background including *en*-Gal4 and UAS-*sim*. Fig. 8A shows that P[4xCME-lacZ] was expressed strongly in stripes corresponding to the *en* stripes of expression. At the times examined (stage 12 or later), expression of P[4xCME-lacZ] was strongest in the dorsal-lateral ectoderm, and considerably weaker in the ventral ectoderm. Expression of P[4xCME-lacZ] was also examined in embryos in which *sim* was expressed in the mesoderm using *twi*-Gal4 and UAS-*sim*. Fig. 8B shows that P[4xCME-lacZ] was expressed in many mesodermal cells. These results show that ectopic expression of Sim results in the formation of functional, transcriptionally active Sim::Tgo complexes in both ectodermal and mesodermal tissues.

Transient expression studies in *Drosophila* cell culture indicate that Sim and Trh are localized in the cytoplasm in the absence of Tango

Both Sim and Trh rapidly enter the cell nucleus when expressed in embryonic cells and also direct Tgo to the nucleus. These observations are consistent with two models of Sim::Tgo and Trh::Tgo nuclear localization. In one model, Sim (or Trh) enters nuclei independent of Tgo. In the second model, Sim is unable to enter nuclei by itself, but does so only after dimerization with Tgo. Test of this model requires assay of the subcellular localization of Sim or Trh in the absence of Tgo. Currently, null mutations of *tgo* do not exist. Instead, this issue was addressed using transient transfection of *Drosophila* SL2 cultured cells. Previous work established that this was a useful system for assaying the transcriptional capability of Sim::Tgo and Trh::Tgo heterodimers (Sonnenfeld et al., 1997).

SL2 cells were transfected with varying amounts of expression vectors driving either *sim* (pAct-*sim*) or *trh* (pAct-*trh*) transcription. Staining of cells with anti-Sim or anti-Trh antibodies indicated that Sim and Trh were exclusively cytoplasmic in >70% of transfected cells in the absence of Tgo (Fig. 9). When a constant amount of pAct-*sim* or pAct-*trh* was cotransfected with increasing amounts of pAct-*tgo*, which drives *tgo* expression, the fraction of cells with cytoplasmic Sim or Trh protein decreased with a corresponding increase in nuclear stained cells (Fig. 9). This correlated with increased transcriptional activity as assayed from the P[6xCME-lacZ] reporter gene that contains multiple Sim::Tgo and Trh::Tgo binding sites (Fig. 9; see also Sonnenfeld et al., 1997). These results show that in SL2 cells, Sim and Trh are unable to enter cell nuclei by themselves, but do so in the presence of Tgo.

DISCUSSION

Subcellular localization of Tgo is developmentally regulated

Staining of *Drosophila* embryos with an antibody directed against Tgo indicates that Tgo is present in all embryonic cells. Tgo is localized predominantly to the cytoplasm of most cells, but strongly accumulates in nuclei of others, specifically the CNS midline cells, tracheal cells, salivary primordia, and salivary ducts (Fig. 3). These sites correspond to the cells in which Sim or Trh, dimerization partners of Tgo, are present. This provides *in vivo* evidence, in addition to that previously reported (Ohshiro and Saigo, 1997; Sonnenfeld et al., 1997), that Sim and Tgo, and Trh and Tgo form transcriptionally competent heterodimers. These results lead to the model that Tgo is localized to the cytoplasm in cells devoid of other bHLH-PAS dimerization partners, and upon their appearance dimerization occurs, and the complex translocates to and accumulates in the nucleus. Since nuclear Tgo correlates with the presence of bHLH-PAS::Tgo heterodimers, it will be interesting to discover additional sites of embryonic and postembryonic nuclear Tgo and identify relevant bHLH-PAS protein partners.

Confirmation of the model was obtained by using ectopic expression experiments that employ the Gal4-UAS system. Both *sim* and *trh* were mislocalized in ectodermal stripes in *en-Gal4* × UAS-*sim* or *en-Gal4* × UAS-*trh* embryos, or in the mesoderm using *twi-Gal4* × UAS-*sim* and *twi-Gal4* × UAS-*trh* embryos. Ectopic expression of *sim* and *trh* resulted in ectopic nuclear accumulation of Sim::Tgo and Trh::Tgo. In addition, the nuclear Sim::Tgo complexes observed in both tissues are able to bind DNA and activate transcription of the P[4xCME-lacZ] reporter gene. This indicates that Tgo nuclear localization is dependent on the presence of dimerization with another bHLH-PAS protein, and that Sim::Tgo heterodimers are competent for nuclear localization and transcriptional activity throughout the ectoderm and mesoderm. Experiments performed in *Drosophila* cell culture further suggest that Sim and Trh are not able to localize to cell nuclei by themselves, but first require dimerization with Tgo. When either *sim* or *trh* is expressed in SL2 cells in the absence of exogenously added *tgo*, both proteins are predominantly localized to the cytoplasm. When cotransfected with *tgo*, both Sim and Trh enter cell nuclei and activate transcription. Although SL2 cells support transcriptional activation by Sim::Tgo and Trh::Tgo, it is important to repeat this experiment *in vivo* when *tgo* null mutants are available since SL2 cell transfection experiments may not exactly reflect *in vivo* conditions.

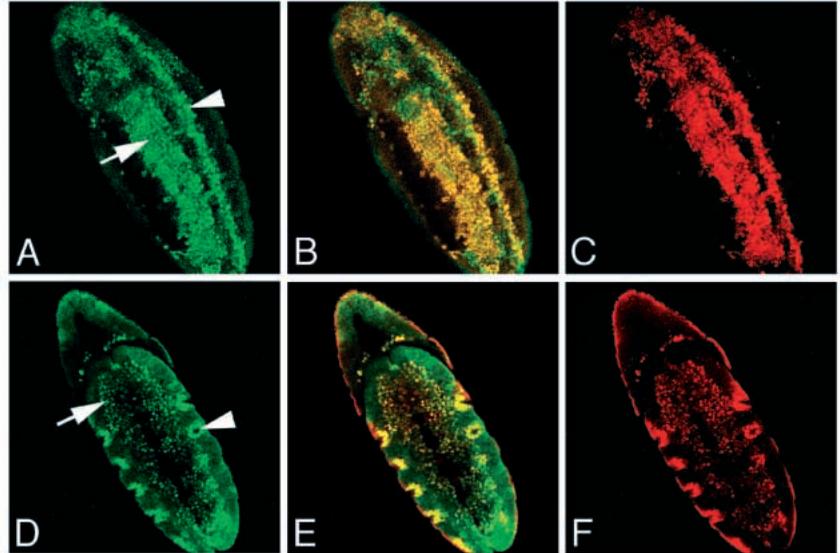
Comparison of Tgo protein levels in different cells suggests that there is significantly more Tgo protein in the midline and tracheal cells than in the adjacent cells. Tgo protein levels are also higher in locations in which *sim* or *trh* have been ectopically expressed. There are several possible explanations for higher Tgo levels in cells containing Sim and Trh. (1) Tgo protein could be stabilized by interacting with Sim or Trh. (2) Tgo protein could be stabilized by nuclear compartmentalization of the Sim::Tgo or Trh::Tgo complexes. (3) Tgo protein may be higher due to elevated amounts of *tgo* transcript in those cells. RNA concentration differences could be due to transcriptional autoregulation, transcriptional activation by other factors, or posttranscriptional control. The

results of *in situ* hybridization experiments indicate that there is enhanced *tgo* expression in the trachea, but only weakly elevated or normal levels in the midline cells (Ohshiro and Saigo, 1997; Sonnenfeld et al., 1997; M. Sonnenfeld, and S. T. Crews, unpublished). However, the differences in Tgo protein levels still appear greater than the differences in transcript levels. In addition, the higher amounts of Tgo protein in cells that express ectopic *sim* or *trh* cannot be explained by transcriptional activation models unless they are autoregulatory. Control of Tgo protein levels may represent another mode of controlling Tgo heterodimer function.

The results described in this paper likely have implications for Arnt function in mammals, where Arnt-related physiological processes have been actively studied, but Arnt-related developmental processes less so. *Drosophila* Tgo and mammalian Arnt are highly conserved both structurally and functionally (Sonnenfeld et al., 1997). Just as *Drosophila* Tgo can dimerize with Sim, Trh, and Similar (Sima) (Sonnenfeld et al., 1997), mammalian Arnt can dimerize with the two mammalian Sim orthologues (Ema et al., 1997; Probst et al., 1997), hypoxia-inducible factor-1 α (closely related to Sima; Wang et al., 1995; Nambu et al., 1996), and Ahr (Burbach et al., 1992; Ema et al., 1992). Staining of mouse embryos with antibodies raised against Arnt indicate that, similar to the case in *Drosophila*, different cell types show Arnt localization in the cytoplasm, nucleus, or both compartments (Abbott and Probst, 1995). The importance of the *Drosophila* results is they demonstrate that sites of nuclear Tgo localization correlate with: (1) the presence of dimerization-competent bHLH-PAS proteins such as Sim and Trh, and (2) cells in which *tgo* is functional. This suggests that sites of Arnt nuclear localization in the mammalian embryo, such as the brain (Abbott and Probst, 1995), will be cell types in which bHLH-PAS::Arnt heterodimers control transcription. Proof of this awaits detailed *in vivo* analysis of bHLH-PAS::Arnt interactions, in particular, the relationship between the mammalian Sim proteins (Chen et al., 1995; Dahmane et al., 1995; Fan et al., 1996; Ema et al., 1997) and Arnt. However, the situation in mammals may be more complicated than in *Drosophila* since Arnt has a nuclear localization sequence, absent in Tgo, that can localize Arnt into nuclei in cultured cells in the absence of any known bHLH-PAS protein (Pollenz et al., 1994; Eguchi et al., 1997).

One implication of the *Drosophila* studies is that Tgo does not form transcriptionally active homodimers during embryogenesis. Studies with mammalian Arnt have shown that Arnt can homodimerize *in vitro* and in cell culture (Antonsson et al., 1995; Sogawa et al., 1995; Swanson et al., 1995; Sonnenfeld et al., 1997). The Arnt homodimer complex binds DNA and activates transcription, although the physiological significance, if any, of this is unknown. Biochemical studies have not been carried out to determine whether *Drosophila* Tgo can homodimerize. However, the Tgo immunostaining studies described here show that Tgo is concentrated in embryonic nuclei only in those cells in which it is part of a heterodimeric complex. This implies that in the embryo, Tgo does not act as a homodimeric complex that binds DNA and controls transcription. Conversely, it does not rule out the possibility that Tgo homodimers form in the cytoplasm and carry out other regulatory or functional roles. What is the role of cytoplasmic Tgo? Genetic analysis of *tgo* mutations is incomplete and potential phenotypes that might correlate with cytoplasmic Tgo

Fig. 7. Ectopic mesodermal *sim* and *trh* expression result in ectopic nuclear localization of Sim, Trh and Tgo in mesodermal cells. (A-C) Parasagittal view of a *twi*-Gal4 × UAS-*sim* whole-mount stage 12 embryo double-stained with anti-Sim (C; red) and anti-Tgo (A; green) showing nuclear accumulation of both proteins in the mesoderm (arrow). CNS midline nuclear staining is also observed (arrowhead). Anterior is at the top. (B) Merged image showing colocalization of Sim and Tgo in mesodermal nuclei (yellow). (D-F) Frontal view of a stage 11 *twi*-Gal4 × UAS-*trh* embryo double-stained with anti-Trh (F; red) and anti-Tgo (D; green) showing nuclear accumulation of both proteins in the mesoderm (arrow). Tracheal staining (arrowhead) is also observed. Anterior is up. (E) Merged image showing colocalization of Trh and Tgo in mesodermal nuclei (yellow).



are unknown, if they exist. However, one hypothesis is that cytoplasmic Tgo is not functional in itself, but dimerizes with developmental bHLH-PAS proteins or physiological bHLH-PAS proteins.

Nuclear localization of Sim and Trh during embryogenesis is not ligand-dependent

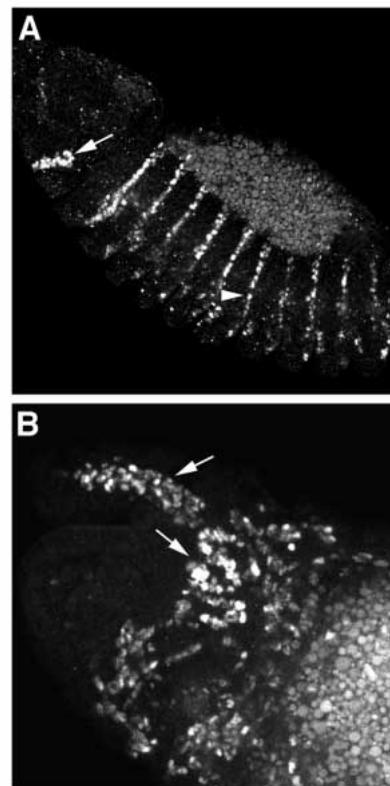
Careful examination of Sim and Trh subcellular localization during normal embryogenesis shows that both proteins enter cell nuclei as soon as protein appears within the cell, and that it persists in nuclei. Thus, analysis of normal embryos does not provide positive evidence that nuclear localization of Sim and Trh is controlled by ligand-driven reactions, in the manner in which Ahr nuclear localization is controlled by aryl hydrocarbons. However, analysis of Sim and Trh subcellular localization in wild-type embryos cannot directly demonstrate that Sim and Trh nuclear localization is unregulated by ligand binding. This issue has been addressed by ectopically expressing *sim* and *trh* in cells in which these genes are not normally expressed, and assaying embryos for nuclear localization at these novel sites. If nuclear localization is controlled by a diffusible ligand or by cell-cell interactions, it is predicted that these factors would not be present at all embryonic sites, and Sim and Trh would not accumulate in nuclei at some locations. The results showed that Sim and Trh enter nuclei efficiently at all ectodermal and mesodermal locations assayed. This suggests that Sim and Trh nuclear localization is not controlled by external factors. While it

cannot be ruled-out that Sim and Trh act as receptors for ligands that control nuclear localization, these ligands would not be temporally or spatially restricted.

Ligand-dependent and transcriptional activation mechanisms can control bHLH-PAS protein function

bHLH-PAS proteins control a number of developmental and physiological events including neurogenesis, tubulogenesis, circadian rhythms, responsiveness to hypoxia, and toxin metabolism. The basic machinery of the bHLH-PAS::Arnt regulatory cassette is well-conserved throughout phylogeny. Control of hypoxia by HIF-1 α ::Arnt and toxin metabolism by

Fig. 8. Ectopically generated Sim::Tgo complexes induce transcription of a target gene in both ectodermal and mesodermal tissues. Whole-mount stage 14 *en*-Gal4 × UAS-*sim* embryos containing P[4xME-lacZ] were stained with anti- β -galactosidase. Anterior is to the left and dorsal is up. (A) Sagittal view showing P[4xME-lacZ] expression in *en* stripes. Ectopic expression is strong in the dorsal-lateral ectoderm (arrowhead) and ventral maxillary segment (arrow). Weak expression can be seen in the trachea as previously noted (Sonnenfeld et al., 1997; Zelzer et al., 1997). (B) Sagittal view of a stage 14 embryo showing ectopic P[4xME-lacZ] expression in the mesoderm. Shown is anterior mesoderm (arrows) including head mesoderm.



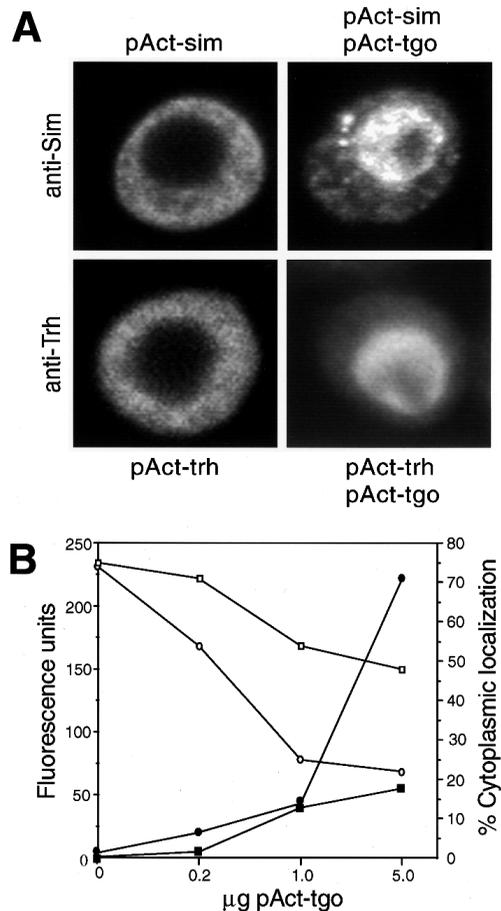


Fig. 9. Transient transfection experiments in *Drosophila* SL2 cultured cells indicate that Sim and Trh are cytoplasmic in the absence of Tgo. (A) Cells were transfected with 5 μ g of each of the indicated expression plasmids, stained with anti-Sim or anti-Trh antibodies followed by reaction with Texas Red conjugated secondary antibody, and analyzed by confocal microscopy. Shown are individual cells transfected with: pAct-sim alone stained with anti-Sim, revealing cytoplasmic Sim; pAct-sim and pAct-tgo stained with anti-Sim, revealing predominantly nuclear Sim; pAct-trh alone stained with anti-Trh, revealing cytoplasmic Trh; and pAct-trh and pAct-tgo stained with anti-Trh, revealing nuclear Trh. (B) Cells were transiently transfected with 5 μ g of pAct-sim or pAct-trh; increasing amounts of pAct-tgo; and the P[6x CME-lacZ] reporter, which contains multiple Sim::Tgo and Trh::Tgo binding sites. Cells were assayed for subcellular localization of Sim or Trh by staining with anti-Sim or anti-Trh and counting the number of transfected cells with exclusively cytoplasmic localization: (□) pAct-sim with pAct-tgo; (○) pAct-trh with pAct-tgo. Transcriptional activity was measured by quantitating β -galactosidase expression from P[6x CME-lacZ] using a fluorescent substrate and normalizing each sample by measuring luciferase activity from a control copia-LTR-luciferase plasmid included in each transfection. Normalized β -galactosidase activity is expressed in arbitrary fluorescence units: (■) pAct-sim with pAct-tgo; (●) pAct-trh with pAct-tgo. The results shown are from one representative set of experiments.

Ahr::Arnt are examples of inducible, physiological responses regulated by bHLH-PAS::Arnt heterodimers. Control of transcription in the CNS midline lineage by Sim::Tgo and tracheal lineage by Trh::Tgo are examples of developmental control by bHLH-PAS proteins. In addition to the distinction

between developmental regulation and inducible, physiological regulation, the results described here indicate that there is a distinction between these different functions and control of nuclear localization.

In one mode, utilized by AHRC, Ahr is localized to the cytoplasm in the absence of ligand, but dimerizes with Arnt and translocates to the nucleus in the presence of ligand. This represents a ligand-dependent, regulated bHLH-PAS-mediated signaling system. Another example of an inducible bHLH-PAS::Arnt response is the induction of HIF-1 α ::Arnt function by hypoxia. Consistent with an inducible response, both Ahr and HIF-1 α are broadly expressed (Abbott et al., 1995; Wiener et al., 1996). The second mode, utilized by Sim and Trh, represents regulation of bHLH-PAS::Arnt function by specific temporal and spatial localization of Arnt's bHLH-PAS partner protein. The *sim* gene is activated specifically in CNS midline precursor cells by transcription factors that control dorsal/ventral patterning (Rusch and Levine, 1996; Y. Kasai, S. Stahl, and S. T. Crews, unpublished). The *trh* gene is activated specifically in tracheal precursor cells by spatial cues that control anterior/posterior and dorsal/ventral patterning (Isaac and Andrew, 1996). In both cases, maintenance of transcription in these lineages is due to positive autoregulation (Nambu et al., 1991; Wilk et al., 1996). Once activated transcriptionally in their respective cell lineages, *sim* and *trh* mRNAs are translated, the Sim and Trh proteins dimerize with Tgo, and the complex translocates to the nucleus. Sim and Trh do not act as receptors for developmentally relevant molecules that trigger translocation to nuclei upon binding; instead their presence in cells is the developmental signal itself. The bHLH-PAS developmental regulatory proteins described here are controlled by transcriptional activation and not ligand-binding; it will be interesting to see if this correlation is a general feature as other bHLH-PAS proteins of developmental significance are analyzed.

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