

Targeted expression of *teashirt* induces ectopic eyes in *Drosophila*

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Contributed by Gerald M. Rubin, October 28, 1998

ABSTRACT *teashirt* was initially identified as a gene required for the specification of the trunk segments in *Drosophila* embryogenesis and encodes a transcription factor with zinc finger motifs. We report here that targeted expression of *teashirt* in imaginal discs is sufficient to induce ectopic eye formation in non-eye tissues, a phenotype similar to that produced from targeted expression of *eyeless*, *dachshund*, and *eyes absent*. Furthermore, *teashirt* and *eyeless* induce the expression of each other, suggesting that *teashirt* is part of the gene network that functions to specify eye identity.

Although the *Drosophila* compound eye is anatomically distinct from the vertebrate eye, recent studies have revealed striking parallels in eye development between flies and vertebrates. Mutations in the *Pax-6* gene in *Drosophila* [*eyeless* (*ey*)], mice (*Small eye*), and humans (*Aniridia*) all lead to defects in eye development (1–4), whereas ectopic expression of the *Drosophila* or murine *Pax-6* gene can result in ectopic eye formation in other fly tissues (5). Additional genes, including *eyes absent* (*eya*), *sine oculis* (*so*), and *dachshund* (*dac*), have also been implicated in the early steps of eye development, because loss-of-function mutations in these genes lead to complete loss of eyes in *Drosophila* (6–8). Moreover, targeted expression of either *eya* or *dac* can result in ectopic eyes in other tissues, and *so* can potentiate the activity of *eya* and *dac* in such assays (9–12). More recent experiments have revealed aspects of the relationship between these genes and *ey* in *Drosophila* eye specification (5, 9–13). Although *ey* is required for the initial expression of *eya*, *so*, and *dac* in the eye primordium, the later genes are also involved in a positive feedback loop to activate the expression of *ey*. Therefore, *ey* does not function simply as a “master regulatory gene” to activate a linear pathway specifying the eye fate; rather, *ey*, *eya*, *so*, and *dac* form part of a regulatory network that together “locks in” the eye specification program. Apparent orthologs of *eya*, *so*, and *dac* have been isolated and are expressed in the developing eye primordium in vertebrates, suggesting that these genes are likely to be involved in a similar regulatory network in vertebrate eye development.

Although *eya* and *dac* can induce ectopic eyes, they are much less potent in doing so than *ey*, even when *eya* or *dac* is simultaneously expressed with *so* (5, 9–12), suggesting the existence of genes that can be induced by *ey* but not by *eya* (or *eya* plus *so*) and *dac* (or *dac* plus *so*). In addition, *ey* itself cannot induce ectopic eye formation in all fly tissues (5, 13). These observations suggest that additional genes are yet to be identified that in combination with *ey*, *eya*, *so*, and *dac* form a gene network that lies at the highest level of the eye specification hierarchy. A diagnostic feature of such genes is that ectopic expression of these genes either alone or in combina-

tion with other eye specification genes should induce ectopic eyes in noneye tissues.

We report here that *teashirt* (*tsh*) is part of this gene network that controls eye specification. *tsh* was initially identified as a homeotic gene required for specifying the trunk segments in *Drosophila* embryogenesis (14). Loss-of-function mutations in *tsh* leads to trunk-to-head transformation, whereas ectopic expression of *tsh* results in head-to-trunk transformation (14, 15). However, unlike genes in the *Antennapedia* (*Antp*) and bithorax complexes (HOM-C), which encode homeobox-containing proteins, *tsh* encodes a nuclear protein with zinc finger motifs (14). The function of *tsh* during imaginal disc development is less well understood. A potential role for *tsh* in eye development was suggested by the observation that flies transheterozygous for *tsh* and certain gain-of-function *Antp* mutations exhibit a reduced-eye phenotype that is not associated with either mutation alone (16). Such a phenotype was interpreted as an eye-to-cuticle transformation. Here we show that targeted expression of *tsh* is sufficient to induce ectopic eye development in the antennal disc. These ectopic eyes have ommatidia with properly differentiated photoreceptor neurons. In addition, we investigated the relationship between *tsh* and other eye specification genes. We show that *tsh* induces the expression of *ey*, *so*, and *dac*, whereas ectopic *ey* induces the expression of *tsh*. Moreover, ectopic eye formation induced by *tsh* depends on *eya* and *so* activity. Our results suggest that *tsh* is intimately linked to the other eye specification genes and that these genes function together to specify eye identity.

MATERIALS AND METHODS

Plasmid Construction, P Element Transformation, and Plasmid Rescue. To construct DMREP, the P element transposon used in our overexpression screen, an *EcoRI*–*XhoI* fragment that contains the *dpp* disc enhancer and the *hsp70* basal promoter was excised from pDMR (17) and blunt-ended with Klenow. This fragment was then cloned into the P element transformation vector PEG117 (18) that was treated with *NotI* and Klenow. Dysgenic males containing DMREP on the X chromosome and a source of transposase ($\Delta 2-3$) on the third chromosome were individually crossed to w^{1118} females. Insertions on the autosomes were identified as red-eyed males in the progeny and were screened for phenotypes in the eye, the wing, and the thorax.

Multiple *Four eyes* alleles were isolated in the screen. Some alleles are homozygous lethal, and others are homozygous viable. They all complement the lethality of *tsh*⁸, a null allele of *tsh*, indicating that they do not inactivate the *tsh* gene. To determine the location of the *Four eyes* insertions, DNA flanking the DMREP insertion was isolated from one *Four eyes* allele by plasmid rescue and sequenced. This analysis revealed that the P element is inserted within an intron in the

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Abbreviations: *tsh*, *teashirt*; *ey*, *eyeless*; *dac*, *dachshund*; *eya*, *eyes absent*; *Antp*, *Antennapedia*.

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5'-untranslated region of the *tsh* gene, with the *dpp* enhancer/*hsp70* promoter cassette reading in the same direction as the endogenous *tsh* gene. This intron is located between +270 and +271 of the published *tsh* cDNA sequence (14), and the predicted translation start site of *tsh* is located at +1009 of the *tsh* cDNA.

To confirm that the *Four eyes* mutations are caused by overexpression of *tsh*, a full-length *tsh* cDNA was cloned into pDMR (17) and introduced into *w*¹¹¹⁸ flies by standard procedures.

Genetics. All crosses were done at 25°C. *so* (8), *eya* (7), *dac* (6), and *tsh* (14) mutants used in this study have been described previously.

For the analysis of hypomorphic *tsh* mutants, *tsh*⁴/*Df*(2L)*TW161* and *tsh*¹/*Df*(2L)*TW161* were used. For the analysis of *tsh* mutant clones, *tsh*⁸ (14) was used. X-ray induction of somatic clones was carried out according to standard procedures (19). A P[w⁺] inserts 39E-F was used to mark the wild-type chromosome. An isogenized *w*¹¹¹⁸ stock was used as a wild-type control.

Histology and Microscopy. Enhancer trap lines *dac*^P, *so*⁷, and *tsh*¹ were used to monitor the expression of *dac*, *so*, and *tsh*, respectively. *ey-lacZ* (kindly provided by Walter Gehring, University of Basel, Basel) and *GMR-lacZ* were used to monitor the expression of *ey* and *glass*, respectively. β -Galactosidase activity staining and immunohistochemical staining of imaginal discs were carried out as described (6).

RESULTS

Isolation of *Four eyes*, a Gain-of-Function Mutation of *tsh*.

To isolate genes that may play important roles in imaginal disc development, we carried out a gain-of-function genetic screen with P element-mediated gene overexpression. In such screens, a P element transposon that contains an enhancer/promoter cassette reading off one P element end is mobilized. An overexpression phenotype may result if the enhancer/promoter cassette contained within the transposon drives the expression of a nearby endogenous gene (20, 21). In our screen, we used the *dpp* disc enhancer coupled with the *hsp70* basal promoter. The *dpp* disc enhancer was chosen because this element is known to drive gene expression in all imaginal discs (22), allowing for the examination of overexpression phenotypes in a variety of tissues. In addition, because this enhancer drives gene expression in only a subset of cells in each imaginal disc, the deleterious effects of gene overexpression on animal viability is minimized.

In the course of this genetic screen, we isolated five lines that have ectopic eyes in the anterior region of the head, just ventral to the antenna. These mutations were named *Four eyes* because often two ectopic eyes were seen in each fly (in Fig. 1 compare *A* and *B*). The position of ectopic eye formation on the head corresponds to the ventral region of the antennal disc where the *dpp* disc enhancer is known to be active, suggesting that the *Four eyes* mutations are caused by overexpression of gene(s) mediated by the *dpp* disc enhancer. The frequency of flies

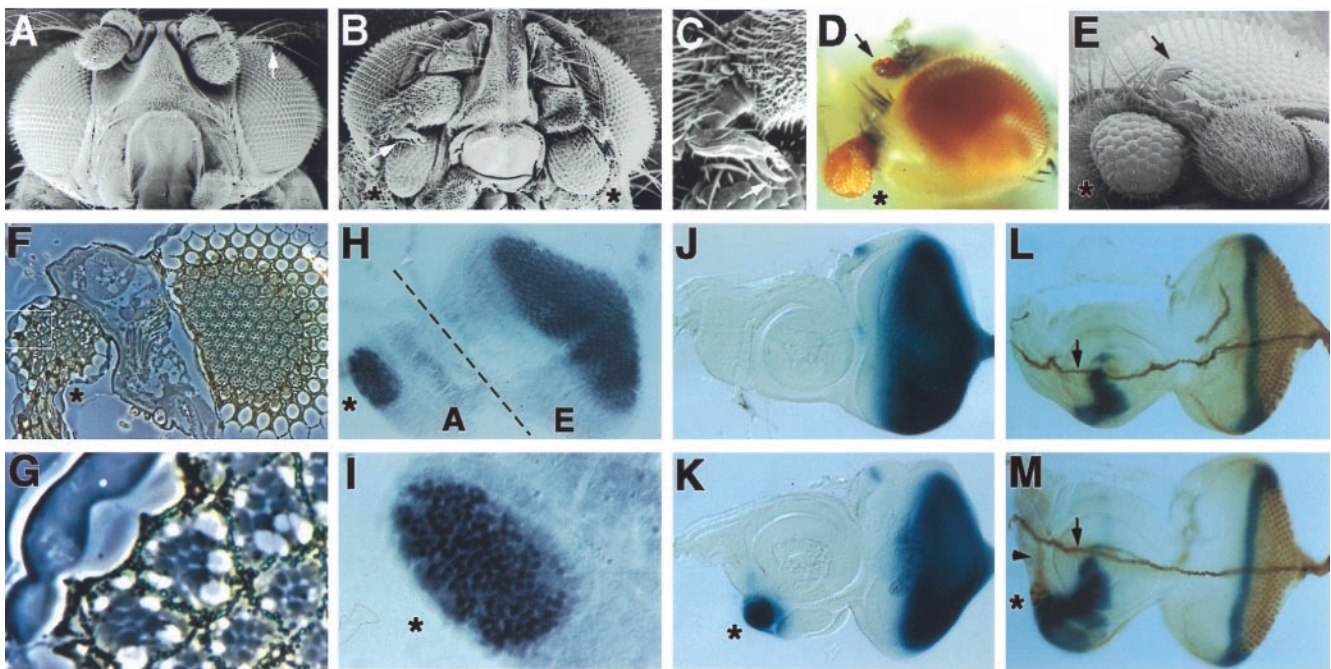


FIG. 1. Targeted expression of *tsh* induces ectopic retinal development. (*A* and *B*) Scanning electron microscopy pictures of wild-type and *Four eyes* head morphology, respectively. Note the presence of two ectopic eyes (asterisks) in the *Four eyes* mutant. Also note that the arista (arrow in *A*), a thin branched structure on the antenna, was replaced with a leg-like structure in *Four eyes* (arrow in *B*; shown at higher magnification in *C*). Like normal legs, a claw (arrow in *C*) is present at the distal tip of this leg-like structure. (*D*) A light microscope image of a *Four eyes* fly. One ectopic eye is present at the anterior ventral surface of the head (asterisk), and another ectopic eye is present on a proximal segment of the antenna (arrow). (*E*) Scanning electron microscopy image of a transgenic fly expressing a full-length *tsh* cDNA in the pDMR vector. Note the presence of the ectopic eye (asterisk) and the arista-to-leg transformation (arrow). (*F*) A section through an ectopic eye (marked by an asterisk, to the left) and the normal eye (to the right) in a *Four eyes* mutant fly. A higher magnification of the outlined area in the ectopic eye is shown in *G*. Note the presence of cone cells, pigment granules, and rhabdomeres in the ectopic eye and that some ommatidia have normal numbers of photoreceptors. (*H*) A *Four eyes* eye (E)-antenna (A) disc stained with the Elav antibody. Note the presence of ectopic Elav staining in the antennal disc (asterisk), which normally does not express Elav. (*I*) A higher magnification of a portion of the antennal disc where ectopic Elav staining is observed. (*J* and *K*) *glass* expression in wild-type and *Four eyes* eye-antenna discs, respectively. Note the presence of ectopic *glass* expression (asterisk in *K*) in the antennal disc in *Four eyes*. Anterior is to the left and dorsal is up. (*L* and *M*) Wild-type and *Four eyes* eye-antenna discs, respectively, stained for *dpp/lacZ* (blue) and 22C10 (brown). Note that ectopic 22C10 staining can be seen both in the cell bodies of the ectopic photoreceptors (asterisk) and on their axon tracts (slightly out of focus, arrowhead in *M*). On a different focal plane (data not shown), we observed that the axons projected medially and then stopped; they did not fasciculate with the Bolwig's nerve (arrows in *L* and *M*).

having at least one ectopic eye varied from 20% to 80% depending on the line. Ectopic eyes were also seen on the antenna (Fig. 1D), although they are relatively smaller and occurred at a lower frequency. Ectopic eyes were never observed in any other adult tissue. Another phenotype associated with the *Four eyes* mutations is the transformation of the arista of the antenna into a leg-like structure (Fig. 1B and C).

We mapped the P elements associated with three *Four eyes* lines to the 40A region of the second chromosome. Plasmid rescue and sequencing of the genomic DNA flanking the P element in one of these *Four eyes* alleles revealed that it was inserted into the 5'-untranslated region of the *tsh* gene, with the *dpp* enhancer/*hsp70* promoter cassette reading into *tsh*. To confirm that the *Four eyes* mutations are caused by overexpression of *tsh*, we generated transgenic flies that expressed a full-length *tsh* cDNA under the direct control of the *dpp* enhancer/*hsp70* promoter cassette with the pDMR vector (17). Consistent with *tsh* being responsible for the *Four eyes* mutations, such transgenic flies exhibited ectopic eyes in the head (Fig. 1E). Moreover, these transgenic flies showed an arista-to-leg transformation similar to that observed in the *Four eyes* mutants (Fig. 1E). The arista-to-leg transformation is also consistent with the known role of *tsh* in specifying trunk versus head identities (15). Taken together, these data demonstrate that the *Four eyes* mutations are gain-of-function alleles of *tsh* caused by its ectopic expression under the control of the *dpp* enhancer.

Normal Retinal Development in the Ectopic Eyes. Scanning electron microscopy revealed that *tsh*-induced ectopic eyes contain nearly normal facets with interommatidial bristles (Fig. 1B and E). Sections through such eyes indicate that the

ectopic ommatidia consist of various cell types found in normal ommatidia, such as the cone cells, pigment cells, and photoreceptors with rhabdomeres (Fig. 1F and G). To follow the development of the ectopic eyes, we analyzed several markers that are normally expressed in the developing retina. The nuclear protein Elav is normally expressed in all neurons, including the photoreceptors, and is not expressed in the antennal disc in third-instar larvae (23). In *Four eyes* mutants, ectopic Elav staining was observed in the antennal discs (Fig. 1H and I). *Glass*, a gene that is only expressed in the visual system in *Drosophila* (ref. 24; Fig. 1J) was also induced in the antennal discs in the *Four eyes* mutants (Fig. 1K), suggesting that *tsh* not only induced neuronal differentiation but more specifically conferred retinal cell fate to these antennal disc cells. On the fate map of the antennal disc (25), the location of the ectopic retina development corresponds to the site where the ectopic eyes are formed on the adult head. As seen in normal photoreceptors, the ectopic photoreceptors elaborate axons that fasciculate with each other and project medially (Fig. 1L and M). Taken together, these data indicate that *tsh* has the capacity to turn on a complete program of eye specification in the antennal disc.

***tsh* Plays a Redundant Role During Normal Eye Development.** Although the data presented so far indicate that *tsh* can induce eye cell fate in overexpression studies, they do not prove that *tsh* does play a role in specifying the eye identity during normal development. To address this issue, we first examined whether *tsh* is expressed at the right time and the right place to have a role in specifying the eye identity. Indeed, *tsh* mRNA is expressed in the eye disc, with the strongest expression anterior to the morphogenetic furrow (Fig. 2I). This pattern of

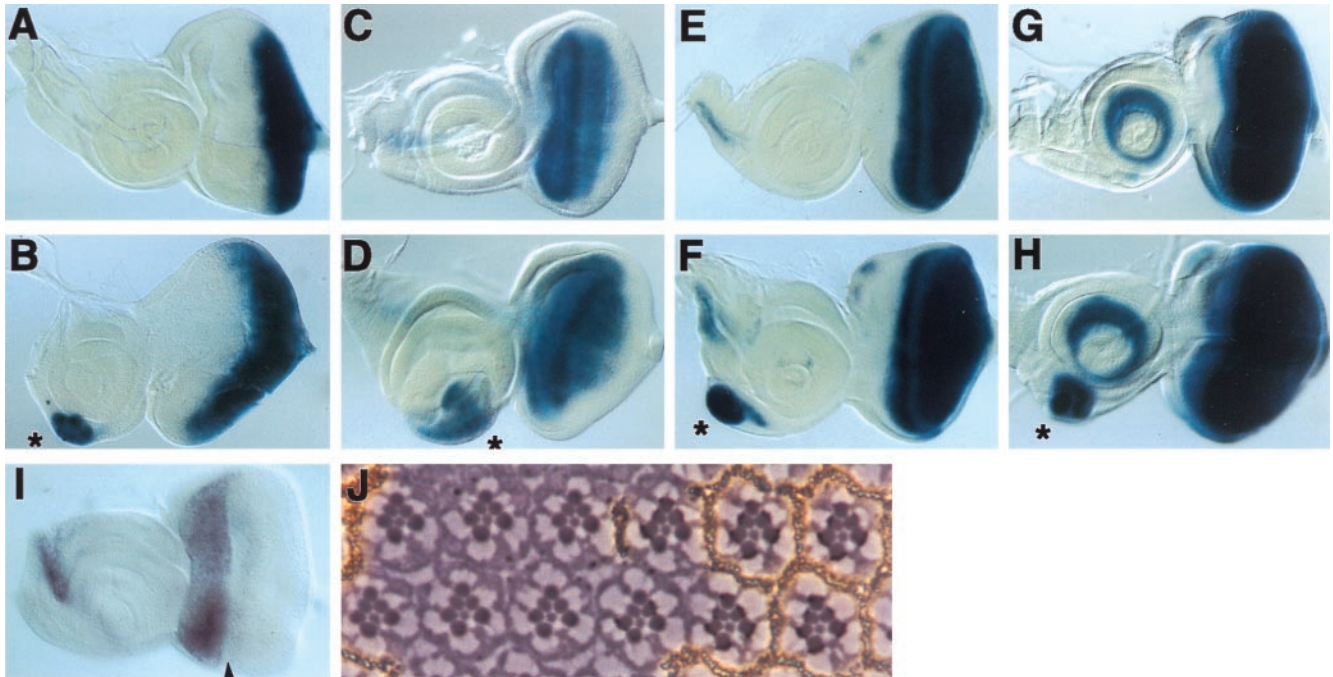


FIG. 2. Relationship among *tsh*, *ey*, *so*, and *dac*. (A and B) Expression of *ey/lacZ* in wild-type and *Four eyes* eye-antenna discs, respectively. Ectopic *ey* expression is observed at the ventral region of the antennal disc in *Four eyes* (marked with asterisk). (C and D) Expression of *tsh* in wild-type flies and flies of genotype *UAS:ey* \times *dpp* GAL4, respectively. *tsh* expression was monitored with a P element enhancer trap *tsh* allele, *tsh*¹. *tsh* is normally expressed only in the eye portion of the eye-antenna disc. Its expression is strongly induced in the ventral antennal region in flies bearing *UAS:ey* and *dpp*-GAL4 (marked with asterisk). (E and F) Expression of *so* in wild-type and *Four eyes* eye-antenna discs, respectively. *so* expression was monitored using a P element enhancer trap allele, *so*⁷. Ectopic *so* expression is observed at the ventral region of the antennal disc in *Four eyes* (marked with asterisk). (G and H) Expression of *dac* in wild-type and *Four eyes* eye-antenna discs, respectively. *dac* expression was monitored using a P element enhancer trap *dac* allele, *dac*^P. Ectopic *dac* expression is observed at the ventral region of the antennal disc in *Four eyes* (marked with asterisk). (I) Expression pattern of *tsh* in the eye antenna disc as revealed by RNA *in situ* hybridization. Note that *tsh* is most strongly expressed anteriorly to the morphogenetic furrow (arrowhead). The *tsh/lacZ* expression in G and H extends more posteriorly, presumably because of the perdurance of β -galactosidase. (J) Normal ommatidial development in *tsh* mutant clones. Shown is a section through an adult eye containing a *w*⁻ clone that is homozygous for a null *tsh* allele, *tsh*⁸. The mutant clone, which is located to the left, is recognizable by the lack of pigment granules. Note that the mutant ommatidia show normal number and organization of photoreceptors and accessory cells.

expression is similar to that of *ey* (13), a gene that is known to play an essential role in specifying eye identity. Next we examined whether loss-of-function mutations of *tsh* affect eye development. We first examined several weak loss-of-function *tsh* alleles (14) and did not find any eye defects (see *Materials and Methods* for alleles used). Making mosaic clones of *tsh* null alleles with the FRT/FLP system was troublesome because *tsh* is located at 40A, exactly where the most proximal FRT insert on the left arm of the second chromosome is located (26). Therefore, we used x-ray-induced mitotic recombination to generate mutant clones of a null *tsh* allele, *tsh*⁸ (14). *tsh* mutant clones were recovered at a frequency similar to the wild-type control, and sections through the mutant clones revealed a normal ommatidial organization (Fig. 2J). These data suggest that *tsh* may play a redundant role during normal eye development, and the requirement for *tsh* may be masked by other factor(s) that play a role similar to *tsh*.

***tsh* and *ey* Can Turn on the Expression of Each Other.** Ectopic eye formation induced by *tsh* overexpression is remarkably similar to that resulting from the expression of eye specification genes, including *ey*, *eya* (or *eya* + *so*), and *dac* (or *dac* + *so*). Therefore, we examined the relationship between *tsh* and these eye specification genes. We first examined the relationship between *tsh* and *ey*, because during normal development *ey* activates the initial expression of the other eye specification genes and appears to play the most critical role in specifying the eye identity (13). We asked whether *ey* expression was induced during ectopic eye formation directed by the *tsh* gene. Normally, *ey* is expressed in the eye but not the antennal disc (Fig. 2A). In the *Four eyes* mutants, ectopic *ey* expression was detected in the ventral part of the antennal disc, where ectopic retinal development occurs (Fig. 2B). Although this result may be interpreted as *tsh* acting upstream of *ey*, we also observed that *tsh* expression is induced by *ey* (Fig. 2C and D), suggesting that *tsh* may function both upstream and downstream of *ey* during retinal development.

***tsh* Acts Upstream of *eya*, *so*, and *dac* in Ectopic Eye Development.** We next examined the relationship between *tsh* and several other eye specification genes, including *eya*, *so*, and *dac*. We found that the expression of *so* (in Fig. 2, compare E and F) and *dac* (in Fig. 2, compare G and H) are induced in the antennal disc by the ectopic expression of *tsh*, suggesting that *tsh* may act upstream of these genes in eye development. We further examined the functional requirement of *eya* and *so* in *tsh*-induced ectopic eye formation. We found that the eye-specific *eya*¹, *eya*², and *so*¹ mutations suppressed completely ectopic eye formation in *Four eyes* mutants (data not shown). These results suggest that *tsh* may function upstream of *eya*, *so*, and *dac* in ectopic eye development.

DISCUSSION

In this paper, we provide several lines of evidence suggesting that *tsh* is part of an interactive network of genes that function together at the highest level of the eye specification hierarchy. First, *tsh* is normally expressed in the eye imaginal disc, mostly anterior to the morphogenetic furrow. This pattern of expression is similar to that of *ey* (13), a gene that is known to play a critical role in eye specification. We noticed that unlike *ey*, whose expression in the eye primordium can be detected as early as in embryos, *tsh* is not expressed in the embryonic eye primordium (14). This feature of *tsh* expression is similar to that of *eya*, which is not expressed in the eye primordium until the second-instar larval stage (7). We suggest that like *eya* the initial expression of *tsh* in the eye proper is likely to be induced by *ey* at larval stages. Second, targeted expression of *tsh* is sufficient to induce ectopic retinal development in the antennal disc, a property shared by *ey*, *eya*, and *dac*, genes that are known to be part of the network that functions at the highest level of the eye specification hierarchy. Third, the reciprocal

control of expression between *ey* and *tsh* is similar to that observed between *ey* and *eya* or between *ey* and *dac*, suggesting that the functions of all these genes are likely to be closely related.

Our studies lend further support to the view that eye development is executed by an interactive network of genes rather than a linear hierarchy dictated by *ey*. During normal development, *ey* is the first gene to be expressed in the eye primordium, and its expression leads to the initial expression of the other eye specification genes, including *eya*, *so*, *dac*, and *tsh*. However, positive feedback loops exist such that the later genes, when turned on by *ey*, may also positively regulate the expression of *ey*. It should be noted that *ey* is a much more potent inducer of ectopic eyes than any single gene in the later group, suggesting that none of these genes represents the sole direct transcriptional target of *ey* and that no single gene can recapitulate the full spectrum of *ey* activity. Previous studies suggested that *eya* and *so* may be direct targets of *ey* (9, 11, 13). Our studies suggest that *tsh* could also be a direct target of *ey*. It will be interesting to determine whether coexpression of *eya*, *so*, and *tsh* can recapitulate the full activity of *ey*. If so, these three genes may represent the only direct targets of *ey*.

The cross-regulatory relationship between *tsh* and *ey*, a homeobox-containing protein, is especially interesting in light of a similar relationship between *tsh* and HOM-C proteins in embryonic development. Previous studies of *tsh* in embryogenesis demonstrated that *tsh* functions as a transcription factor that may directly control the transcription of its downstream target genes. These studies further indicated that *tsh* function is closely linked to the HOM-C proteins and that *tsh* may function upstream, downstream, or at the same level as HOM-C proteins, depending on the developmental context. *tsh* plays at least two essential roles during embryogenesis. First, *tsh* is required for the subdivision of the midgut mesoderm. In this process, *tsh* simply acts downstream of HOM-C proteins such as ANTP and UBX (27, 28). Second, combinatorial action of TSH, ANTP, and bithorax complex proteins is required for the thoracic and abdominal (trunk) identities, through repression of the head homeotic gene *labial* (29). Because the initial expression of *tsh* is independent of *Antp* and bithorax complex genes and vice versa (29), *tsh* likely functions in parallel to *Antp* and bithorax complex genes. Clearly, in this process, *tsh* functions both in parallel with (*Antp* and bithorax complex) and upstream of (*labial*) HOM-C genes. The cross-regulatory relationship between *tsh* and homeobox-containing transcription factors in both eye development and embryonic development may reflect a similar molecular mechanism of *tsh* function in both processes.

Our studies also underscore the importance of gain-of-function genetics. Although *ey*, *eya*, *so*, and *dac* were identified as eye specification genes based on their loss-of-function phenotypes, *tsh* does not have a visible mutant eye phenotype under normal conditions. Its role in eye development could be revealed only under certain sensitized genetic backgrounds such as in the presence of *Antp* mutations, or as in our study, through overexpression. Indeed the majority of the genes in eukaryotes do not have easily assayable loss-of-function phenotypes (30). Therefore, gain-of-function genetics through gene overexpression is an important alternative approach to probe gene-regulatory networks and will be useful in elucidating many complex genetic pathways.

We thank Walter Gehring, Graeme Mardon, Matthew Scott, and Larry Zipursky for fly strains and reagents; Tom Serano for providing Fig. 2I; and Miro Pastrnak for help with plasmid rescue. Brian Avery and Tom Serano provided useful comments concerning the manuscript.

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