The *Drosophila* homeobox gene *optix* is capable of inducing ectopic eyes by an eyeless-independent mechanism

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**SUMMARY**

*optix* is a new member of the Six/so gene family from *Drosophila* that contains both a six domain and a homeodomain. Because of its high amino acid sequence similarity with the mouse Six3 gene, *optix* is considered to be the orthologous gene from *Drosophila* rather than *sine oculis*, as previously believed. *optix* expression was detected in the eye, wing and haltere imaginal discs. Ectopic expression of *optix* leads to the formation of ectopic eyes suggesting that *optix* has important functions in eye development. Although *optix* and *sine oculis* belong to the same gene family (Six/so) and share a high degree of amino acid sequence identity, there are a number of factors which suggest that their developmental roles are different: (1) the expression patterns of *optix* and *sine oculis* are clearly distinct; (2) *sine oculis* acts downstream of eyeless, whereas *optix* is expressed independently of eyeless; (3) *sine oculis* functions synergistically with eyeless absent in eye development whereas *optix* does not; (4) ectopic expression of *optix* alone, but not of *sine oculis* can induce ectopic eyes in the antennal disc. These results suggest that *optix* is involved in eye morphogenesis by an eyeless-independent mechanism.

Key words: *Drosophila*, Eye development, *optix*, Six/so family, Imaginal disc

**INTRODUCTION**

In *Drosophila*, the development of the compound eye has been studied extensively and many of the genes regulating cell fate determination in the late larval eye disc have been identified. It has been shown that the *eyeless* (*ey*) gene plays an early and fundamental role during *Drosophila* eye development (Quiring et al., 1994). *ey* encodes a member of the Pax-6 family of transcription factors and contains two DNA binding domains, a homeodomain and a paired domain (Callaerts et al., 1997). In loss-of-function mutants of *ey*, flies have reduced eyes or are completely eyeless. Surprisingly the gain-of-function mutation of *ey* can generate ectopic eyes on wings, legs and antennae (Halder et al., 1995). These results show that *ey* acts high up in the genetic cascade regulating eye development. Although it is important to understand this genetic cascade, little is known about the identity of subordinate target genes of *ey*. Several genes, including *sine oculis* (*so*), *eyes absent* (*eya*) and *dachshund* (*dac*) have been implicated in the early steps of eye development. These are candidate *ey* target genes. The *so* gene encodes a homeodomain protein that is required for the development of the entire visual system, including the compound eye, the ocelli, the optic lobe and the larval photoreceptor organ known as Bolwig’s organ (Cheyette et al., 1994; Serikaku and O’Tousa, 1994; Pignoni et al., 1997). In loss-of-function mutants of *so* there is extensive cell death anterior to the morphogenetic furrow, and the adult flies have reduced or no eyes. Recently we have shown that *so* is transcriptionally regulated by *ey* through an eye-specific enhancer in *so* (Niimi et al., 1999), indicating that *so* is one of the direct target genes of *ey*.

*so* homologues have been identified in human (Boucher et al., 1996; Granodino et al., 1999; Winchester et al., 1999), mouse (Oliver et al., 1995a,b; Kawakami et al., 1996a,b; Toy et al., 1998; Jean et al., 1999), chicken (Bovolenta et al., 1996, 1998, Toy et al., 1998), frog (Seo et al., 1999; Zuber et al., 1999), zebrafish (Seo et al., 1998a,b;c; Seo et al., 1999; Kobayashi et al., 1998), medaka fish (Loosli et al., 1998), shark (Seo et al., 1999), lamprey (Seo et al., 1999) and fruitfly (Toy et al., 1998; Seo et al., 1999). In all of them, a Six domain and a Six/so type homeodomain are conserved, therefore they are all included in the Six/so family. Both domains are necessary for sequence-specific DNA binding activity (Kawakami et al., 1996a,b). In mouse, 6 different genes have been found in the Six/so family so far. Interestingly, only Six3 and Optx2/Six6 are expressed in the eye primordia. The functional importance of Six3 in eye development is clearly illustrated by the experiments of Oliver et al. (1996) and Loosli et al. (1999) who have shown that the ectopic expression of the mouse Six3 gene in medaka fish embryos can induce ectopic lens and retinal tissue. In addition, three of the four zebrafish Six genes, *six3*, *six6* and *six7*, are expressed in the optic primordium (Seo et al., 1998a,b), the medaka fish *six3* gene is expressed in the developing eye (Loosli et al., 1999).
1998) and the chicken cSix3 gene is expressed in the optic vesicle and the ectoderm, and its expression becomes restricted to the prospective neural retina and to the lens placode (Bovolenta et al., 1998). These genes are the structural orthologues of mouse Six3, and it seems that their functions are also conserved. Previously it was believed that Drosophila so is an orthologue of mouse Six3, because they share important functions in eye development. However, the phylogenetic analysis of Six/so family genes seems to indicate that so belongs to a different group of genes than the mouse Six3. Recently another gene from the Six/so family, named optix, was isolated from Drosophila (Toy et al., 1998). optix also has a conserved Six domain and a homeodomain with a high degree of sequence similarity to Six3. Comparison of the amino acid sequence of optix with other Six/so genes places optix in the same group as Six3, and therefore, optix is considered to be the real orthologue of Six3. Because of the functional conservation of Six3 subclass genes, there is a possibility that optix has similar functions during eye development in Drosophila as in the mouse.

In this paper we report the expression pattern and discuss the possible functions of optix. Its capacity for inducing ectopic eyes strongly suggests that optix has an important role in eye development. The mechanism of ectopic eye induction appears to be independent of ey. Although the amino acid sequence of optix is similar to that of so, it has a clearly distinct expression pattern in embryos and imaginal discs, and exerts a different function during eye development in Drosophila.

![Fig. 1.](image)

**Fig. 1.** In situ hybridization to polytene chromosomes. optix is located at 44A in 2R, very close to so, which is located at 43C.

![Fig. 2.](image)

**Fig. 2.** (A) Comparison of optix homeodomain amino acid sequence with Six/so family genes. A dash indicates amino acid identity with optix. The genes can be divided into three groups from their sequence similarity. optix and so belong to different groups. D, Drosophila; m, mouse; h, human; c, chicken; mf, medaka fish; zf, zebrafish; e, C. elegans. Six3(m): Oliver et al. (1995a); Optx2/Six6(m): Toy et al. (1998); Jean et al. (1999); SIX3(h): Granadino et al. (1999); cSix3(c): Bovolenta et al. (1998); optx2(c): Toy et al. (1998); six3, six7(zf): Seo et al. (1998a), Kobayashi et al. (1998); six7(zf): Seo et al. (1998b); six3(mf): Loosli et al. (1998); ceh-32(e): Dobier and Bürglin personal communication; so(D): Cheyette et al. (1994); SIX1, SIX2(m): Oliver et al. (1995b); SIX1(h): Boucher et al. (1996); SIX4(m): Kawakami et al. (1996a); SIX5(m): Kawakami et al. (1996b); SIX3(h): Boucher et al. (1995); six8(zf): Seo et al. (1998c); SIX3, SIX4(D): Seo et al. (1999). optix and Dsx3 have the same amino acid sequence in and upstream of the homeodomain, but downstream the sequences are completely different. Since there still are no genomic sequence data available, we cannot decide whether these differences are due to differential splicing or to duplicated genes. (B) Phylogenetic tree of the Six/so family gene. The tree was constructed from the homeodomain sequences (A) using clustalw. This family can be divided into three subclasses; Six3, SIX1 and SIX4 subclass.
et al. (1993). The line carrying UAS-eya (Bonini et al., 1993) was kindly provided by Nancy Bonini. The molecular lesions of the ey2 mutant were described previously by Quiring et al. (1994). The sof and eya1 mutant stocks showed high penetrance and expressivity of the eyeless phenotype.

**Generation of UAS-optix transgenic flies**

The UAS-optix transgenics were made by subcloning the full-length Drosophila optix cDNA (a kind gift of Olof Sundin) into the pUAST vector (Brand and Perrimon, 1993). Embryos of the y ac w1118 strain were used as recipients for DNA injection to generate transgenic lines. Flies were transformed using standard transgenic techniques (Rubin and Spradling, 1982).

**In situ hybridization**

Digoxigenin-labeled DNA probes were prepared from full length optix cDNA and hybridized to polytene chromosomes, whole-mount embryo and whole-mount imaginal discs. For embryos and imaginal discs, hybridization was carried out in 50% formamide, 5x SSC, 100 μg/ml of sonicated salmon sperm DNA, 100 μg/ml of tRNA, 50 μg/ml of heparin, and 0.1% Tween 20 overnight at 48°C. After washing several times with PBS-0.1% Tween 20, embryos and discs were incubated with an anti-digoxigenin antibody coupled with alkaline phosphatase (1:2000; Boehringer Mannheim) and the staining reaction allowed to proceed for 2 hours. For polytene chromosomes double staining, in addition to digoxigenin-labeled probes, FITC labeled so-cDNA was used as a probe. Hybridization was carried out in 0.6 M NaCl, 1x Denhardt’s solution, 50 mM NaPO4 buffer pH 7.2, 5 mM MgCl2, for 12-16 hours at 58°C in a moist chamber. After washing several times with PBS-0.1% Tween 20, chromosomes were incubated with a sheep α-FITC antibody coupled with alkaline phosphatase (1:2000 Boehringer), washed and stained with Fast Red solution (Boehringer Mannheim). After removing excess antibody with glycine pH 2.2, the second antibody, AP-conjugated sheep α-Dig was applied and detected with NBT/BCIP (Boehringer). Embryonic stages are according to Campos-Ortega.

**RESULTS**

**optix is a member of the Six/so gene family and orthologous to Six3**

The optix cDNA clone was isolated from a 3-12 hour Drosophila embryo Agt10 cDNA library compiled by Jeffrey Toy (Toy et al., 1998) and kindly made available to us for further analysis. Using double labelling in situ hybridization to polytene chromosomes, optix was mapped to position 44A on chromosome 2, a position relatively close to the so locus (43C) (Fig. 1). This suggests that optix and so arose by a tandem gene duplication event. Similarly, the mouse Six2 and Six3 genes are closely linked on chromosome 17 (Oliver et al., 1995a), as are the human SIX1 and OPTX2 genes on chromosome 14 (Toy et al., 1998). Alignment of the optix and other Six/so family homeodomain amino acids sequences revealed that optix is the putative orthologue of mouse Six3 (Fig. 2), rather than so as had been previously assumed.

**Expression pattern of optix as compared to sine oculis**

The expression pattern of optix during embryonic and larval stages was examined by whole-mount in situ hybridization and compared to the pattern of so expression. optix is first expressed in a ring at the anterior end of the blastoderm embryo (Fig. 3A,C). This expression is similar to the so expression (Fig. 3B), but lies more anteriorly. During germ band extension, optix is restricted to the anterior and in contrast to so, is not expressed in the optic lobe primordia (Fig. 3E,F). At st.11, optix expression is detected in the clypeolabrum and remains limited to the anterior region, whereas so expression is also detected bilaterally at the segmental boundaries in a set
of unidentified epidermal cells, and in the optic lobe primordium (Fig. 3G,H). At st.14 optix is expressed in the ectoderm covering the supraesophageal ganglion which will give rise to parts of the brain, but it is not expressed in Bolwig’s organ (Fig. 3I,K).

In wild-type eye imaginal discs, optix RNA is detected in front of the morphogenetic furrow (MF), in the head region and just anterior to the vertex region (Fig. 4A). In late second instar larvae, before the MF forms, optix expression covers the entire eye disc but later the expression becomes restricted to a region anterior to the MF. This expression pattern is similar to the pattern of ey and twin of eyeless (toy) (Quiring et al., 1994; Czerny et al., 1999) and it suggests that optix may play an important role in early eye disc development as do ey and toy. In contrast, so starts to be expressed at early third instar just before MF initiation (Cheyette et al., 1994; Halder et al., 1998) and later becomes restricted to a zone just anterior, inside and posterior to the MF. In addition, optix is expressed in wing and haltere discs, but not in leg discs (Fig. 4B-D). This expression continues throughout the third instar larval stage, whereas so is not expressed in wing and haltere discs, but in the leg discs (Fig. 4F-H).

In order to determine a possible epistatic relationship between optix and ey we examined optix expression in the ey\textsuperscript{2} mutant. In ey\textsuperscript{2} no ey transcripts can be detected, either in the embryonic eye primordia or in the larval eye disc (Fig. 5A; Quiring et al., 1994). In ey\textsuperscript{2} eye discs, optix expression was not affected (Fig. 5B). In contrast, so expression was no longer observed in the early third instar eye discs of ey\textsuperscript{2} mutants (Halder et al., 1998).

**Ectopic expression of optix can induce eye formation**

The expression pattern of optix in the eye imaginal discs strongly suggests that optix may play an important role in early eye disc development. Since we could not identify a mutant for optix so far, we have studied the potential of optix to induce the formation of ectopic eyes by a gain-of-function strategy. We used the GAL4 system to target optix expression to various imaginal discs where optix is normally not expressed. We crossed UAS-optix to dpp\textsuperscript{link}-GAL4 that expresses GAL4 along the anteroposterior compartment boundary in leg, wing and antennal imaginal discs. Targeted expression of optix cDNA induced ectopic eye structures just in the antenna (Fig. 6A,B) and the anterior medial region of the head (Fig. 6C,D), but neither in the legs nor in the wings. The normal eyes are reduced in size and rarely extra ocelli and interocellar bristles are detected around the vertex region. The efficiency of induction of ectopic eyes is relatively low (i.e. 20% as compared to 100% in ey). In contrast to optix, ectopic expression of so cannot induce ectopic eyes by itself. We also crossed UAS-optix to E132-GAL4 which can induce ectopic eyes in combination with UAS-ey (Halder et al., 1995). However, the UAS-optix × E132-GAL4 flies died as embryos, whereas the UAS-ey × E132-GAL4 controls survived and formed ectopic eyes.

**optix does not require eyeless for induction of ectopic eyes**

Since eya, dac, eya-so and eya-dac require ey to form ectopic eyes (Bonini et al., 1997; Shen and Mardon 1997; Pignoni et al., 1997; Chen et al., 1997; Halder et al., 1998), we examined...
whether eye expression is also induced during ectopic eye formation by optix. However, in the eye disc of UAS-optix × dppblink-GAL4 flies, no ectopic eye expression was detected (Fig. 7B). Therefore we attempted to induce ectopic eye formation with optix in an eye2 mutant background. Targeted expression of the optix gene in an eye2 background resulted in ectopic eye formation (Fig. 7C,D). The efficiency of occurrence of ectopic eyes did not change from the wild-type background situation, but extra ocelli were induced more often than in a wild-type background. From these results, we conclude that optix does not require eye expression for the induction of ectopic eyes.

**optix and sine oculis differ in their interaction with eyes absent**

Pignoni et al. (1997) reported that a SO/EYA complex regulates multiple steps in eye development and functions within the context of a network of genes to specify eye tissue identity. Ectopic expression of so alone did not induce ectopic eyes, and ectopic expression of eya alone induced ectopic eyes just in the antenna at low frequency (10%) (Bonini et al., 1997); but coexpression of so and eya led to an increase in the induction of ectopic eyes in the antenna both in frequency (76%) and size. This synergistic effect is probably due to the capability of SO and EYA to form a protein complex. The domains required for complex formation are the evolutionarily conserved Six and Eya domains. Since OPTIX has a Six domain as well, we therefore tested whether OPTIX and EYA also synergize and enhance ectopic eye induction. We crossed UAS-eya; UAS-optix to dppblink-GAL4 and examined the frequency of induction of ectopic eyes (Table 1). optix can induce ectopic eyes (22%) but so cannot (0%); so has a synergistic function with eya (0% and 10% individually to 60% when coexpressed), but coexpression of optix and eya did not lead to an increase in frequency (20%) nor in size of ectopic eyes. Therefore, although OPTIX has a Six domain, no synergistic interaction with EYA can be demonstrated.

**Table 1. The efficiency of the occurrence of ectopic eyes**

<table>
<thead>
<tr>
<th>UAS construct (driven by dpp-GAL4)</th>
<th>Ectopic eye formation</th>
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<tr>
<td>UAS-optix</td>
<td>22%</td>
</tr>
<tr>
<td>UAS-so</td>
<td>0%</td>
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<tr>
<td>UAS-eya</td>
<td>10%</td>
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<tr>
<td>UAS-optix + UAS-eya</td>
<td>20%</td>
</tr>
<tr>
<td>UAS-so + UAS-eya</td>
<td>60%</td>
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<tr>
<td>UAS-eya</td>
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60-85 animals scored for each data point; ectopic eye development is scored by the presence of ommatidia with pigment.

**Fig. 7. Induction of optix and ey transcription by ectopic optix expression.** By whole-mount in situ hybridization, optix transcripts were detected in the antennal disc (A, arrow) while ey transcripts were not (B). optix does not require ey expression for induction of ectopic eyes. This is demonstrated by the finding that ectopic optix expression in eye2 mutants can still induce ectopic eyes (C; arrows show ectopic eye, arrowhead indicates the original eye). (D) Higher magnification of the ectopic eye on the antenna.
DISCUSSION

We have determined that optix, a second gene from the Six/so family in Drosophila, functions during compound eye development. Although optix and so share highly related amino acid sequences they show a different expression pattern, suggesting that they serve different functions. Moreover, we can demonstrate that optix is sufficient to induce properly formed ectopic eye structures in the antennal disc of Drosophila, independent of ey. This result suggests that optix is high up in the regulatory hierarchy directing eye development.

The role of optix in eye development

We examined optix expression in the embryo and in the larval imaginal discs. optix starts to be expressed at the blastoderm stage and continues through all the embryonic stages. In contrast to so, which is expressed in the entire visual system including the optic lobe, Bolwig’s organ and the eye disc (Cheyette et al., 1994), optix is expressed only in the eye imaginal discs and apparently has no function in the embryonic and larval visual systems.

Even though both so and optix are expressed in the eye discs, their patterns differ from each other. The pattern of optix expression resembles the patterns of ey and toy, which are required at early developmental stages for the initiation of photoreceptor development (Halder et al., 1995; Czerny et al., 1999). Although ey and toy start to be expressed in the eye anlagen at embryonic stage 14, optix only becomes detectable at the second larval instar. Nevertheless, its expression pattern suggests that optix is required early in eye disc development.

Since no loss-of-function mutants for optix are available, the functional role of optix in the eye disc is uncertain, but ectopic expression of optix can lead to ectopic eye formation, indicating an important role in eye morphogenesis.

optix and sine oculis have different functions in eye development

Since both optix and so belong to the Six/so family, the functional differences between optix and so are of particular interest. Our results indicate that optix and so contribute to eye development by different mechanisms. Both so and optix have an important function in early eye morphogenesis. Although so expression is regulated by ey (Halder et al., 1998; Niimi et al., 1999), optix expression does not appear to be affected by ey since optix expression in ey2 mutant eye discs is normal. Recently we have isolated a new mutant allele of ey, which produces no detectable transcripts either in the embryo or in the larval eye disc (S. Flister, U. Kloter and W. J. G., unpublished data). This new mutant provided us with the opportunity to analyze optix in an ey null mutant rather than a hypomorphic mutation like ey2. Even in this null mutant optix transcription in the eye discs appears normal. These results show that the optix expression is independent of ey.

Furthermore, optix can induce ectopic eye formation, whereas so cannot induce ectopic eyes by itself. In addition, so has a synergistic function with eya (Pignoni et al., 1997) in contrast to optix. Therefore, although optix and so belong to the same gene family and share a conserved Six domain and so type homeodomain, they function differently in eye development.

optix has the potential to induce ectopic eye formation independent of eyeless

Ectopic optix expression can lead to ectopic eye formation. This capability has already been reported for ey (Halder et al., 1995) and for eya, dac, eya-so eya-dac and teashirt (tsh) (Bonini et al., 1997; Shen and Mardon 1997; Pignoni et al., 1997; Chen et al., 1997; Pan and Rubin, 1998). To induce ectopic eyes, eya, dac and tsh have to ectopically activate ey (Bonini et al., 1997; Shen and Mardon 1997; Pan and Rubin, 1998; Halder et al., 1998). so can only induce ectopic eyes in combination with eya, producing a synergistic effect compared to eya alone (Pignoni et al., 1997). A similar synergism was observed for eya-dac (Chen et al., 1997). Ectopic eye formation driven by eya, eya-so and eya-dac was shown to be blocked in ey2 mutant background (Bonini et al., 1997; Pignoni et al., 1997; Chen et al., 1997). These data demonstrate a dependence on ey gene activity for eya, dac, tsh, eya-so and eya-dac to be able to direct eye development. In contrast, optix does not require ey expression to form ectopic eyes. optix can induce ectopic eyes in ey mutant background. These results suggest that, with regard to ectopic eye induction, optix acts in an at least partially different pathway from the one regulated by ey. The same observation was reported for the corresponding mouse genes Optx2/Six6 and PAX6 (Jean et al., 1999).

We find that optix can induce ectopic eyes independently of ey. However, the induction is confined to the antennal disc; in contrast to ey, optix does not induce ectopic eyes on wings or legs. Since ey is expressed much earlier in the eye anlagen than optix, this difference suggests that ey induces a larger set of target genes than optix, and that the activity of some of those genes are required for eye induction by optix. This interpretation is supported by the observation that optix cannot induce ectopic eyes in a so1 or eya1 mutant background (unpublished observations).

Furthermore, the ectopic expression of ey is sufficient to induce ectopic optix expression (data not shown), although in normal eye development optix transcription is not regulated by ey. Since all these results came from an ectopic situation it will be necessary to analyze the relationship of optix and ey in an optix mutant background.

optix is the putative orthologue of mouse Six3

The isolation and functional analysis of optix provides new insights into the evolution of the Six/so gene family. optix belongs to the Six3 subclass, whereas so was assigned to the same subclass as Six1, and finally Six4 and Six5 form a third subclass. The mouse genes, Six3 and Optix2, which are in the Six3 subclass, the same as optix, are expressed in the optic vesicles and the lens, i.e. in eye morphogenesis (Oliver et al., 1995a; Toy et al., 1998). In contrast Six1 and Six2, members of the Six1 subclass, are expressed in phalangeal tendons, skeletal and smooth muscle, i.e. primarily in myogenesis (Oliver et al., 1995b). Although Six1, Six2 and Six4, Six5 are assigned to different subclasses on the basis of their amino acid sequences, both Six1 and Six5 seem to control early steps of myogenesis, and Six1 and Six4 are able to transactivate a reporter gene containing a myogenin promoter fragment (Spitz et al., 1998). Spitz et al. (1998) reported that these Six genes seem to act at a high level in the hierarchical cascade controlling myogenesis. Based on these reports, it is conceivable that genes in subclasses Six1 and Six4 share the
same functions and are controlling muscle formation. In contrast, Six3 subclass genes have an important function in eye development. Therefore, it seems that these two groups of Six genes might have diverged to serve different functions. This also applies to the interactions with Eya genes. In the mouse, Six2, Six4 and Six5 induce nuclear translocation of Eya1, Eya2 and Eya3 which are localized in the cytoplasm, but Six3 does not (Ohno et al., 1999). Furthermore Six1/Eya2 and Six2/Eya1 genes are widely coexpressed in many tissues during organogenesis (Xu et al., 1997). Moreover the Pax3 gene is also required for the same steps (Tajbakhsh et al., 1996; Maroto et al., 1997). These findings suggest the possibility that Pax, Six and Eya proteins, all of which are coexpressed during vertebrate somitogenesis, cooperate during vertebrate muscle development. Besides their major role in myogenesis, Six2, Six4 and Six5 are expressed in the retina (Kawakami et al., 1996b; Niiya et al., 1998), but the gene which plays a major role in eye development is Six3. For this reason, it had been thought that so is the Drosophila orthologue of Six3, but this assignment has to be revised. optix is the putative Six3 orthologue, and so clearly belongs to the Six1 subclass. This phylogenetic relationship is also supported by the fact that so interacts with eyr, whereas optix does not.

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