Development 127, 1879-1886 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
DEV2529

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

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Accepted 14 January; published on WWW 6 April 2000

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 *eyes 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.,

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 phylogenic 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*.

MATERIALS AND METHODS

Fly strains

Flies were reared on standard medium at 25°C. Flies carrying the *dpp^{blink}-Gal4* transgene (line C40.6; Staehling-Hampton et al., 1994) were a gift from Michael Hoffmann. *ey^{2}*, *so^{1}* and *eya^{1}* have been described by Quiring et al. (1994), Cheyette et al. (1994) and Bonini

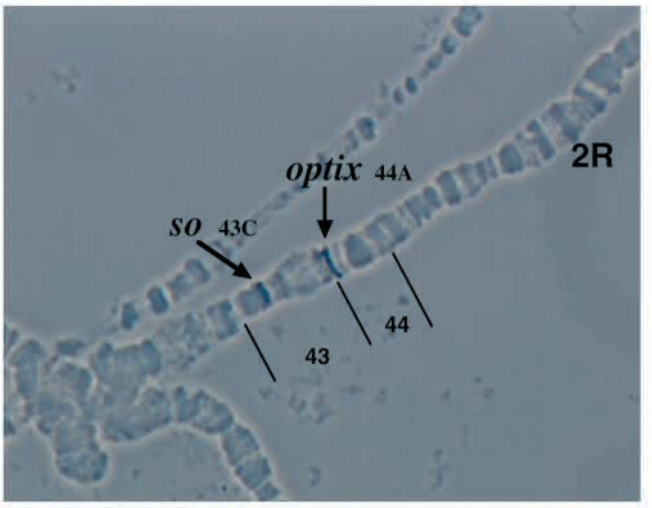

Fig. 1. In situ hybridization to polytene chromosomes. *optix* is located at 44A in 2R, very close to *so*, which is located at 43C.

A

optix (D)	DGEQKTHCFKERTRSLLREWYLQDPYPNPTKKRELAKATGLNPTQVGNWFKNRRQRDRAA	
Dsix3(D)	----------	100%
Six3(m)	----------S------Q----T----------	95%
Optx2/Six6 (m)	----------H----------S------Q----T----------	93%
SIX3 (h)	----------S------Q----T----------	95%
cSix3(c)	----------S------Q----T----------	95%
optx2 (c)	----------H----------S------Q----T----------	93%
six3 (zf)	----------S------Q----T----------	95%
six6 (zf)	----------G----------S------Q----T----------	93%
six7 (zf)	----------SR--H--Q----T----------	92%
six3 (mf)	----------G----------G------H----T----------	93%
ceh-32(e)	----------K------P--K---N----TQM----------	88%
so(D)	---ETSY----KS--V--D--SHN---S-RE--D--E----TT---S----------	68%
Six1(m)	---ETSY----KS-GV-----AHN---S-RE-----E----TT---S----------	70%
Six2(m)	---ETSY----KS--V-----AHN---S-RE-----E----TT---S----------	72%
SIX1(h)	---ETSY----KS-GV-----AHN---S-RE-----E----TT---S----------	70%
Six4(m)	---ETVY----KS-NA-K-L-K-NR--S-AE--H---I---SL---S----------NP	62%
Six5(m)	---ETVY-----S-AA-KAC-RGNR--T-DE--R--TL---SL---S----------TG	58%
SIX5(h)	---ETVY-------AA-KAC-RGNR--T-DE--R--TL---SL---S----------TG	60%
Dsix4(D)	---ETVY----KS-NA-KDC--TNR--T-DE-KT---K---TL---S----------TP	58%
six8(zf)	---ETVY-----S-NA-KDM-KRNR--S-AE--N---M---SL---S----------NP	60%

B

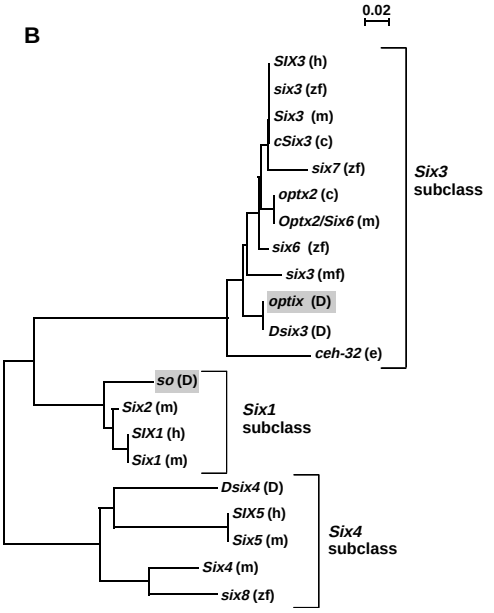

Fig. 2. (A) Comparison of *optix* homeodomain amino acid sequence with *Six*/*so* family genes. A dash indicates amino acid identity with *optix*. The genes can be divided into three groups from their sequence similarity. *optix* and *so* belong to different groups. D, *Drosophila*; m, mouse; h, human; 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, six6*(zf): Seo et al. (1998a), Kobayashi et al. (1998); *six7*(zf): Seo et al. (1998b); *six3*(mf): Loosli et al. (1998); *ceh-32*(e): Dozier 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); *SIX5*(h): Boucher et al. (1995); *six8*(zf): Seo et al. (1998c); *Dsix3, Dsix4*(D): Seo et al.(1999). *optix* and *Dsix3* 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 ey^2 mutant were described previously by Quiring et al. (1994). The so^1 and eya^1 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 $y\ ac\ w^{1118}$ 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, 5× 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, 1× Denhardt's solution, 50 mM $NaPO_4$ buffer pH 7.2, 5 mM $MgCl_2$, 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 λgt10 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

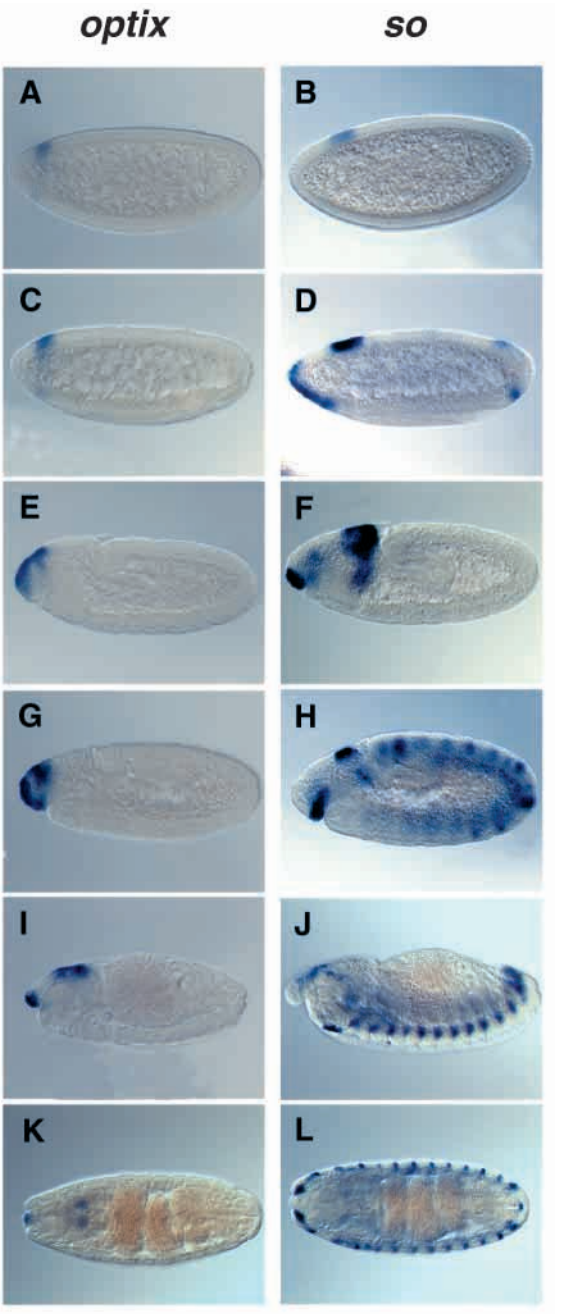

Fig. 3. Comparison of *optix* and *so* expression during embryogenesis. Transcripts of *optix* (left) and *so* (right) were detected by whole-mount in situ hybridization. All embryos are oriented with anterior to the left and are shown as lateral (A-J) or dorsal (K,L) views. (A,B) Cellular blastoderm (stage 5). (C,D) Early gastrula (stage 6). (E,F) Germ band elongation (stage 9). (G,H) Beginning of germ band retraction (stage 11). (I,J) Beginning of head involution (stage 14). (K,L) After head involution (stage 16).

(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

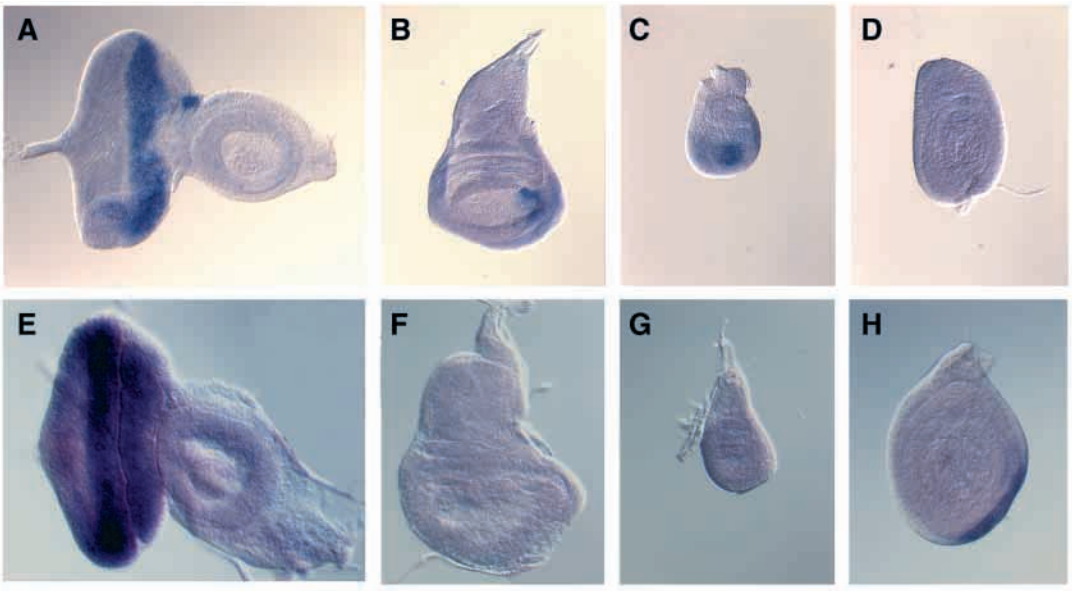

Fig. 4. Comparison of *optix* and *so* expression in the wild-type discs of late third instar larvae. Transcripts of *optix* (top, A-D) and *so* (bottom, E-H) were detected by in situ hybridization. All discs are oriented with posterior to the left and dorsal to the top. (A,E) eye-antennal disc. *optix* expression is detected in front of the MF, in the head vertex region and a part of the antennal disc medial region (A). *so* expression is detected just in front of the MF, within and behind the MF (E). (B,F) wing disc. (C,G) haltere disc. (D,H) leg disc. No *so* expression is detected in wing and haltere discs (F,G). Absence of *optix* expression in the leg disc (D).

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^2 mutant. In ey^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^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^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

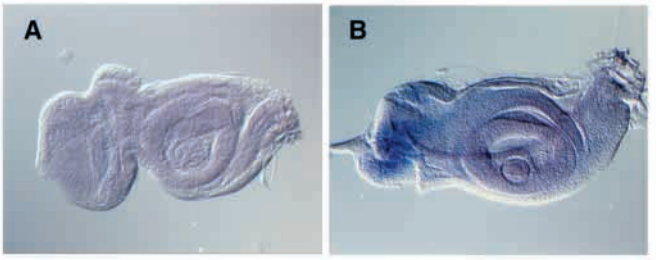

Fig. 5. Expression pattern of *ey* (A) and *optix* (B) transcripts in the eye discs of ey^2 mutants. Same orientations as in Fig. 4. No *ey* expression is detected (A). *optix* expression is still detected in the reduced eye disc of ey^2 mutant (B).

used the GAL4 system to target *optix* expression to various imaginal discs where *optix* is normally not expressed. We crossed UAS-*optix* to dpp^{blink}-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

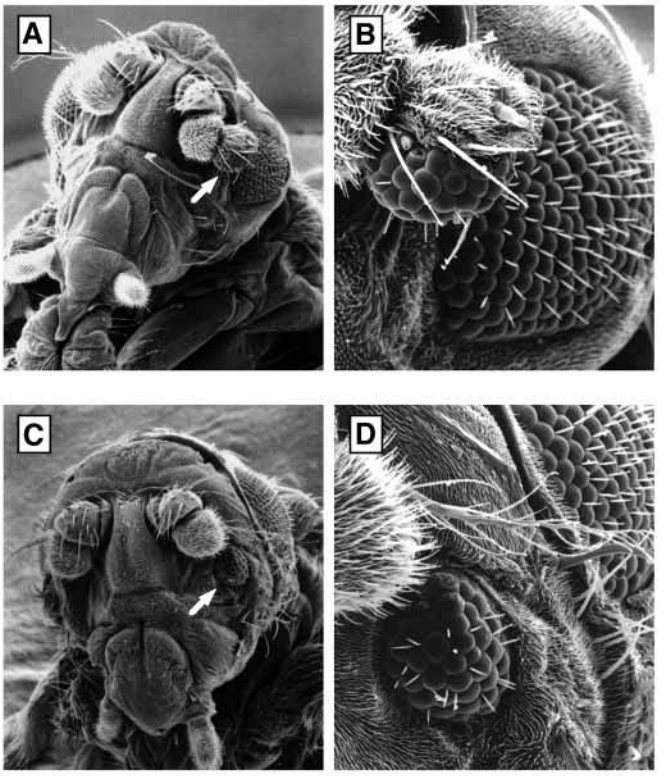

Fig. 6. Scanning electron micrograph of ectopic eyes. Misexpression of *optix* can induce ectopic eye formation. (A-D) Adult phenotypes due to *dpp*^{blink}-GAL4 induced UAS-*optix* expression. Targeted expression of *optix* induced the formation of ectopic eye structures on the antenna (A arrow) and the anterior medial region of the head (C). The ectopic eye contains hexagonal ommatidia and interommatidial bristles. (B) Higher magnification of A. The extra part of the antenna together with the ectopic eye originate from the position where the arista is usually located (A,B). (D) Higher magnification of C.

whether *ey* expression is also induced during ectopic eye formation by *optix*. However, in the eye discs of UAS-*optix* × *dpp*-GAL4 flies, no ectopic *ey* expression was detected (Fig. 7B). Therefore we attempted to induce ectopic eye formation with *optix* in an *ey*^{2} mutant background. Targeted expression of the *optix* gene in an *ey*^{2} 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 *ey* 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

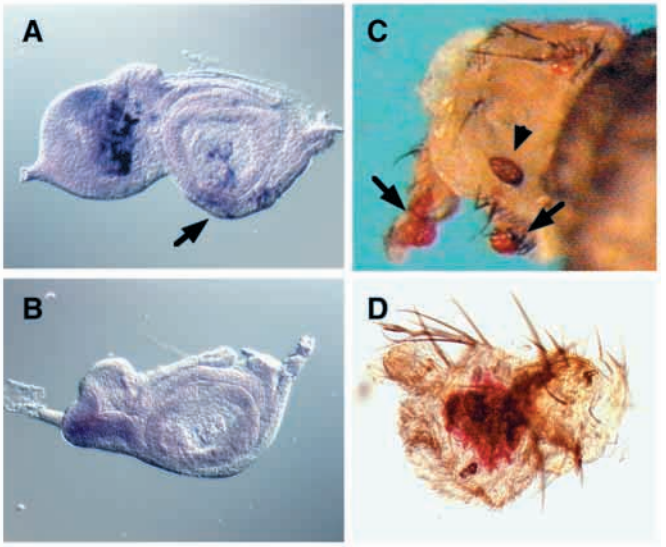

Fig. 7. Induction of *optix* and *ey* transcription by ectopic *optix* expression. By whole-mount in situ hybridization, *optix* transcripts were detected in the antennal disc (A, arrow) while *ey* transcripts were not (B). *optix* does not require *ey* expression for induction of ectopic eyes. This is demonstrated by the finding that ectopic *optix* expression in *ey*^{2} 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.

Table 1. The efficiency of the ocurrence of ectopic eyes

UAS construct (driven by *dpp*-GAL4)	Ectopic eye formation
UAS-*optix*	22%
UAS-*so*	0%
UAS-*eya*	10%
UAS-*optix* + UAS-*eya*	20%
UAS-*so* + UAS-*eya*	60%
UAS-*ey*	100%

60-85 animals scored for each data point; ectopic eye development is scored by the presence of ommatidia with pigment.

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 *dpp*^{blink}-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.

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 ey^2 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 ey^2. 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 ey^2 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 anntennal 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 so^1 or eya^1 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 *Optx2*, 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 (Ohto 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 *eya*, whereas *optix* does not.

We thank Olof Sundin for providing *optix* cDNA and letting us analyze it. We are grateful to Michael Hoffmann and Nancy Bonini for fly stocks. M. S. is grateful to Urs Kloter for help with the chromosome in situ hybridization, Andreas Hefti for assistance with electron microscopy, Susanne Flister for many helpful discussions and critical reading of the manuscript, Giuseppe Cassata for making the phylogenic tree figure, Christine Dozier for critical reading of the manuscript, and also all other members of the Gehring laboratory for helpful discussions. This work was supported by the Biotechnology Programme of the European Commission, the Swiss national Science Foundation and the Kantons of Basel and Basel-Landschaft for which we are grateful.

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