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RESEARCH ARTICLE

Fly SIX-Type Homeodomain Proteins Sine Oculis and Optix Partner With Different Cofactors During Eye Development

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Two members from the Six class of homeobox transcription factors, Sine oculis (SO) and Optix, function during development of the fly visual system. Differences in gain-of-function phenotypes and gene expression suggest that these related factors play distinct roles in the formation of the fly eye. However, the molecular nature of their functional differences remains unclear. In this study, we report the identification of two novel factors that participate in specific partnerships with Sine oculis and Optix during photoreceptor neurons formation and in eye progenitor cells. This work shows that different cofactors likely mediate unique functions of Sine oculis and Optix during the development of the fly eye and that the repeated requirement for SO function at multiple stages of eye development reflects the activity of different SO–cofactor complexes. ***Developmental Dynamics 234:497–504, 2005.*** **© 2005 Wiley-Liss, Inc.**

Key words: Gro; Eya; CG30443; Obp; CG8991; Sbp; Taf1; Six3; Six6; Six1

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INTRODUCTION

The Six class homeobox transcription factors Sine oculis (SO) and Optix play fundamental roles in the development of the *Drosophila* visual system (Cheyette et al., 1994; Serikaku and O'Tousa, 1994; Pignoni et al., 1997; Seo et al., 1999; Seimiya and Gehring, 2000). SO is required for the development of the larval photosensitive organ called Bolwigs and the entire adult visual system, including the photosensitive ocelli and eye organs, and the optic lobes of the brain (Cheyette et al., 1994). During eye development, SO function is required at multiple stages, including establishment of an eye primordium, morphogenesis of the photoreceptor array, and neuronal differentiation (Pignoni et al., 1997). Evidence that Optix also functions in eye formation is indirect, because loss-of-function alleles are not yet available. Nonetheless, Optix is expressed in the developing eye epithelium and it can induce eye formation when ectopically expressed (Seo et al., 1999; Seimiya and Gehring, 2000). SO and Optix are coexpressed through most of the eye disc before the start of neurogenesis; however, their expression domains diverge at later stages (Cheyette et al., 1994; Seo et al., 1999; Seimiya and Gehring, 2000; this work). As the morphogenetic furrow (MF) sweeps across the eye field leaving in its wake differentiating ommatidial clusters (see legend of Fig. 1 for a description of eye morphogenesis), SO continues to be expressed both anterior and posterior to the MF, whereas Optix is only expressed in progenitor cells anterior to the MF (Fig. 1; Cheyette et al., 1994; Seimiya and Gehring, 2000; this work).

As mentioned above, SO and Optix belong to the Six class of homeobox transcription factors. In addition to a homeobox type DNA-binding domain (HD), they bear a large (~115 amino acids [aa]) protein–protein binding domain called the Six domain (SD). Based on sequence conservation within HD and SD regions of multiple vertebrate and invertebrate family members, Six genes have been grouped into three subclasses named

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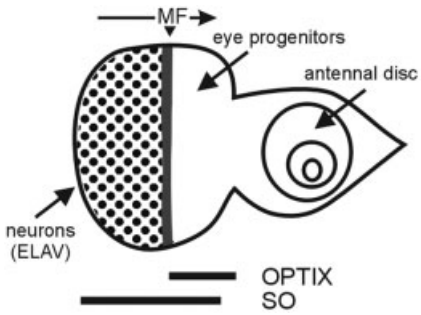

Fig. 1. Schematic representation of an eye disc at the time of eye morphogenesis (Wolff and Ready, 1993). The fly eye forms from an epithelium called eye imaginal disc. The eye disc remains undifferentiated and proliferates during the first and second larval stages of development. Early in the third and last larval period (L3), morphogenesis of the compound eye begins when neurons start differentiating along the posterior margin of the eye field. Thereafter, neurogenesis spreads across the epithelium like a wave (arrow) leaving in its wake clusters of differentiating photoreceptor neurons and accessory cells (lens and pigment producing cells). The front of this wave is marked by a visible indentation in the epithelium called morphogenetis furrow (MF). The distribution of Optix and Sine oculis (SO) within the eye disc is indicated by the bars (see also Fig. 4). The antennal disc is continuous with the eye disc, but its development is not addressed in this work.

Six1/2, Six3/6, and Six4/5 (Seo et al., 1999; Kawakami et al., 2000; nomenclature follows the class type of the murine Six1 through Six6 genes). *So* and *Optix* belong to the Six1/2 and Six3/6 classes, respectively. The one member of the Six4/5 gene subclass present in *Drosophila*, *dSix4*, does not function in the eye, but is required for gonads and muscle development (Kirby et al., 2001).

Although several potential partners of Six-type proteins have been identified (Zhu et al., 2002; Tessmar et al., 2002; Giot et al., 2003), the interaction with only two types of nuclear factors, Eya and Grc family members, has been characterized thus far (Heanue et al., 1999; Ohto et al., 1999; Kobayashi et al., 2001; Zhu et al., 2002; Li et al., 2003; Lopez-Rios et al., 2003). Grc-type proteins appear to function as general cofactors, because they are able to interact with essentially all Six proteins (Zhu et al., 2002; Lopez-Rios et al., 2003). Interactions with Eya family members appear to be more selective. Binding to vertebrate Eya 1, Eya 2, or Eya 3 has been shown to occur with Six proteins of the Six1/2 and Six4/5 families but not of the Six3/6 type (Heanue et al., 1999; Ohto et al., 1999; Zhu et al., 2002). Because these nuclear factors lack DNA binding domains of their own, they are thought to rely on their Six partners for DNA binding and, thus, function as coactivators (Eya) or corepressors (Grc) (Paroush et al., 1994; Xu et al., 1997; Chen and Courey, 2000).

In *Drosophila*, the Grc and Eya factors are represented by single loci, *groucho* (*gro*) and *eyes absent* (*eya*), respectively (Hartley et al., 1988; Delidakis et al., 1992; Bonini et al., 1993). Gro is ubiquitously expressed and, therefore, is equally available for interaction with either SO or Optix. Recently, *Drosophila* Gro has been shown to interact with SO in biochemical assays (Silver et al., 2003), and as reported here, Gro interacts with both SO and Optix in yeast two-hybrid assays. Eya is quite broadly expressed in the eye epithelium and would be available for interaction with either SO or Optix in this tissue. Fly Eya was the first factor shown to directly interact with a Six domain protein, specifically with SO (Pignoni et al., 1997). However, consistent with the lack of interaction observed between vertebrate Eya proteins and Six3, fly Eya does not interact with Optix (Seimiya and Gehring, 2000). In this respect, SO and Optix display properties typical of their Six families, Six1/2 and Six3/6. The picture emerging from these studies is that SO and Optix likely bind to several cofactors, some of which may be specific to one or the other protein. To date, however, only one selective protein partner, Eya, has been recognized.

We report here the identification and initial characterization of two new partners of SO and Optix. These cofactors exemplify additional mechanisms involved in generating unique interactions between SO or Optix and their partners. One factor is coexpressed with both SO and Optix in progenitor cells; however, it behaves as a strong Optix interactor in yeast two-hybrid assays. The other interacts with both SO and Optix in yeast; however, its expression overlaps with SO and not Optix. Both factors are conserved and expressed in the developing eye epithelium in restricted patterns. These findings support the view that different Six-cofactors complexes mediate unique functions of SO or Optix during eye development.

RESULTS

Sbp and Obp Genes Encode Novel Partners of the Sine Oculis and Optix Transcription Factors

In a yeast two-hybrid screen (see Experimental Procedures section), we isolated multiple putative partners of the SO transcription factor including the previously described Eya protein, the TATA-box binding protein-associated factor 1 (Taf1) and two new potential interactors, CG8991 and CG30443. The interaction between these proteins and SO was also confirmed by glutathione-*S*-transferase (GST) -pulldown assays (see Experimental Procedures section). In yeast and in vitro, Eya, Taf1, and CG8991 displayed a clear interaction with the SD of SO (Fig. 2A,B). The CG30443 protein, however, appeared to interact weakly in both cases (Fig. 2A and not shown).

Because the Optix gene also bears a protein–protein interaction domain of the Six type and its expression in the eye epithelium overlaps with SO, CG8991and CG30443 were further tested for possible interactions with the SD of Optix. In yeast, CG8991 interacted with both SO and Optix, albeit somewhat more robustly with the SO-based bait (Fig. 2A). Surprisingly, we found that CG30443 interacted much more robustly with Optix than with SO. In fact, when using pGBKT7-based baits (see Experimental Procedures section for differences between pBTM and pGBKT7 baits), CG30443 interacted with Optix and not SO (Fig. 2B). Thus, although isolated through its weak interaction with SO, CG30443 displays a strong preference for Optix. For these reason, we named the CG8991 factor SO binding protein (Sbp) and CG30443 Optix binding protein (Obp).

Sbp and Obp Encode Factors With Conserved Domains

The Sbp gene encodes a novel protein of 813 aa. BLAST analysis identifies

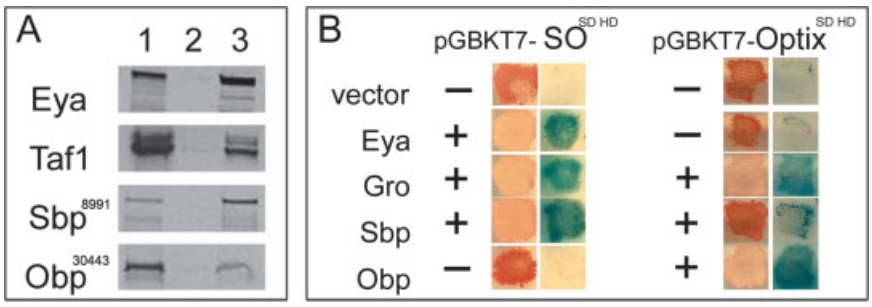

Fig. 2. **A.** Pull down experiments using a glutathione-*S*-transferase (GST) fusion protein containing the Six domain (SD) and homeobox domain (HD) of Sine oculis (SO). Lane 1, 10% of total ^{35}S-labeled protein input. Lane 2, control lane, pulled down fraction using GST alone. Lane 3, total ^{35}S-labeled protein pulled down using the GST–SO^{SD+HD} fusion protein. The TATA-box binding protein-associated factor 1 (Taf1) is a developmentally regulated component of the TFIID transcription factor complex (Pham and Sauer, 2000). Because Taf1 mutant flies display eye defects (Wassarman et al., 2000), the interaction we observed in vitro may reflect an in vivo function. However, as a rather general component of the basic transcription machinery, Taf1 likely interacts with multiple transcription factors involved in eye development. As our aim was to isolate more specific Six-protein partners, we did not investigate the SO–Taf1 interaction further. **B.** Testing interactions with SO and Optix in the yeast two-hybrid system (+, yes interaction; −, no interaction). Nonselective plates lacked Trp and Leu to ensure cells contained both prey and bait plasmids but did not select for prey–bait interactions. Selective plates in this experiment also lacked Ade and contained X-α-gal. Growth on Ade minus plates and galactosidase activity (blue) reflects binding between bait and prey proteins. Baits (SO^{SD+HD} and Optix^{SD+HD}) were in the pGBKT7 vector; preys were in the pAct2 (Eya, Sbp, Obp) or pGAD (Gro) vectors. Although the pACT2–Obp clone was obtained in the original screen due to a modest interaction with the pBTM–SO^{SD} and pBTM–SO^{SD+HD} baits, only a very weak interaction or no interaction at all (as in this experiment) was observed with the pGBKT7–SO^{SD} and pGBKT7–SO^{SD+HD} baits. Sbp, SO binding protein; Obp, Optix binding protein.

two highly conserved regions within the N-terminal half of the protein (Box 1 and Box 2; Fig. 3A,B). Following Box 2 lies a proline-rich region (Fig. 3A). In the *Drosophila* protein, 63 of a total 99 prolines fall between aa 361 and 604, rendering the composition of this portion of the protein 26% P (Fig. 3B). Proteins with Box 1 and Box 2 followed by a 210–240 aa P-rich region are also found in *pseudobscura*, bee, fish, chicken, and mouse (Fig. 3B). In all but the chicken protein, the conserved Box 3 motif is present within the P-rich region (Fig. 3B). In the proteins from fish and chicken, Box 2 is interrupted by a stretch of 32 residues somewhat conserved between these two species (I = 51% S = 63%). This intervening sequence is also found in a predicted Box 2 coding region from rat (XP_228309.2), and it is nearly identical between rat and chicken (I = 97%; not shown). This type of larger Box 2 sequence is not found in *D. melanogaster*, *D. pseudoobscura*, or *A. gambiae*, and it may define a larger Box 2-related domain found only in vertebrates.

The Obp gene encodes a protein with nine putative zinc-finger domains of the classic C2H2 type [zf-C2H2 pfam00096] (Fig. 3C,D). No other conserved regions are found in the protein. By BLAST, we identified two Obp-related proteins one from *D. pseudoobscura* and the other from *A. mellifera*. Whereas overall conservation is low (S = 66% for *D. pseudoobscura* and S = 57% for *A. mellifera*), the sequences encoding zinc-fingers 4 through 8 show higher conservation (S = 86% for *pseudoobscura*, S = 81% for *mellifera*) and the sequence alignment is shown in Figure 3E. Whether these genes are indeed Obp homologues remains to be determined.

Sbp and Obp Are Expressed in the Larval Eye Disc

To investigate whether these in vitro interactions may in fact be of significance during eye development, we examined the expression of *Sbp* and *Obp* in the eye–antennal disc by in situ hybridization. We found Sbp to be expressed posterior to the MF, i.e., across the differentiating eye field but not in progenitor cells (Fig. 4A). This expression effectively overlaps with SO but not with Optix, whose expression is restricted to progenitor cells (compare Fig. 4A with C and D). On the contrary, Obp is expressed anterior to the MF within progenitor cells and not in the developing neuronal field (Fig. 4B). Obp is coexpressed with both Optix and SO in progenitor cells (compare Figure 4B with C and D). Thus, both factors are expressed in developing eye tissue, and coexpression with SO and/or Optix suggests that the interaction observed in vitro are relevant to eye development.

Misexpression of Sbp but Not Obp Interferes With Eye Development

Because Sbp can interact with either SO or Optix in yeast, its expression specifically posterior to the MF, where SO but not Optix is present, may play a significant role in restricting protein–protein interactions to the Sbp–SO pair. If this is indeed the case, directed expression of Sbp in progenitor cells may be expected to disrupt eye development. To test this prediction, we ectopically expressed Sbp during eye development in such a way to generate an overlap in expression with Optix in progenitor cells. Flies carrying *UAS-Sbp* were crossed to the *ey-Gal4* line (Hazelett et al., 1998) to efficiently induce expression anterior to the MF. Ectopic expression of Sbp, in cells expressing normal levels of Optix, caused loss of eye structure in the adult (not shown). In late L3 discs, expression of the pan-neuronal marker Elav was either present in very few cells or completely absent (Fig. 5B, compare with wild-type in A). Expression of an eye specification marker, Dachshund, was present but reduced (Fig. 5D, compare with wild-type in C). Hence, misexpression of Sbp in progenitor cells interferes with eye development at an early stage (before neurogenesis). In the converse experiment, misexpression of Optix in cells that normally express Sbp also disrupted eye development. Flies carrying *UAS-Optix* were crossed to the *pGMR-gal4* line (Freeman, 1996) to induce strong expression in the differentiating epithelium posterior to the MF; this population normally expresses Sbp. In this case, we did not observe an arrest in neurogenesis but a disorganization of the ordered array of eye facets in the adult compound eye, an effect typically referred to as a

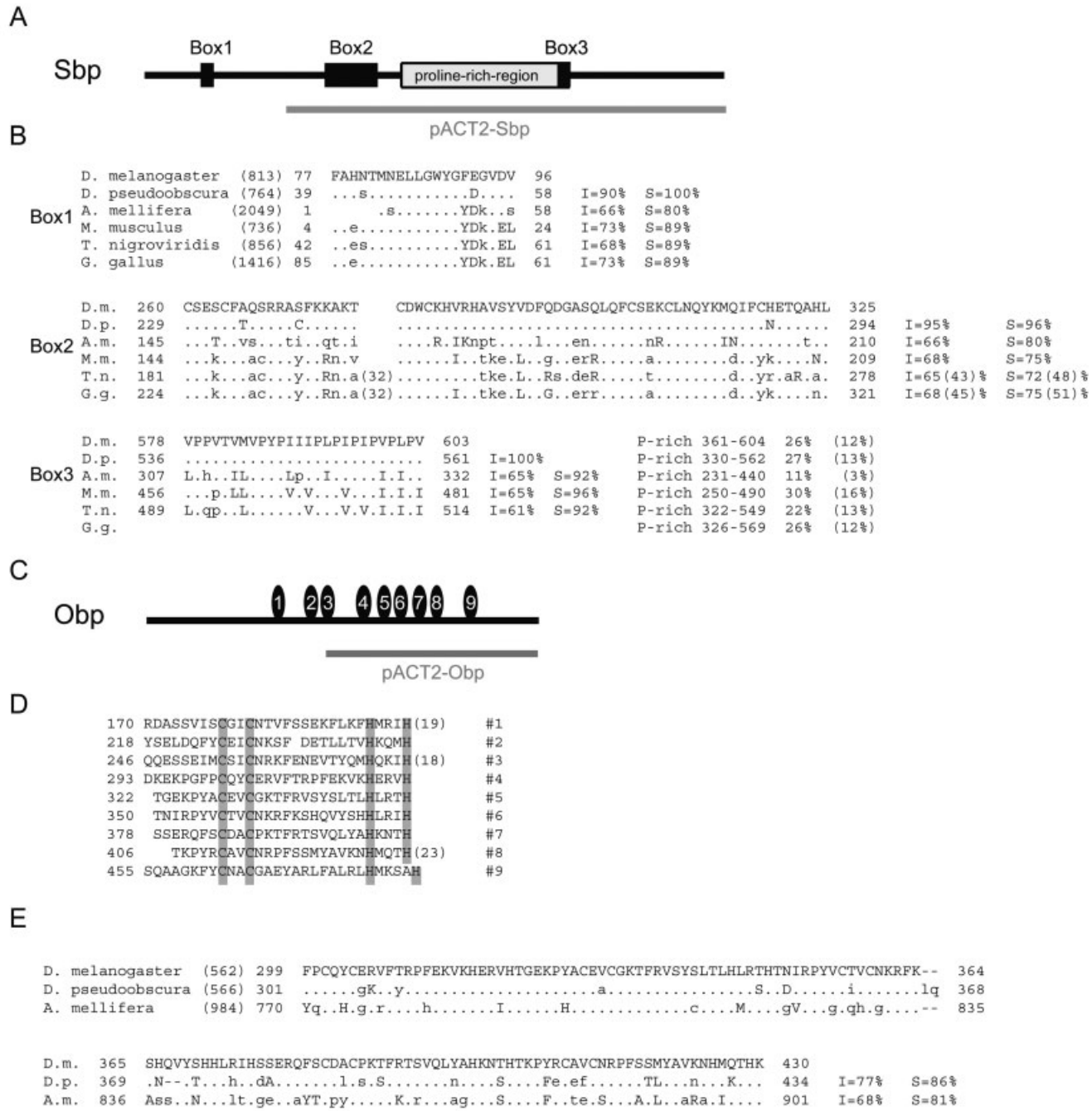

Fig. 3. **A:** Schematic representation of the Sine oculis binding protein (Sbp) showing distribution of conserved and proline-rich regions within the protein; the gray bar below indicates the portion of the protein encoded in the yeast two-hybrid pAct2 clone. **B:** Sequence alignment of conserved regions of Sbp (NP_610703.3) and related proteins from fly (*Drosophila peudoobscura*, EAL26394.1), bee (*Apis mellifera*, XP_395078.1), mouse (*Mus musculus*, NP_780616.2), fish (*T. nigroviridis*, CAG03656.1), and chicken (*Gallus gallus*, XP_419803.1). Alignment is provided for Box 1, Box 2, and Box 3; for the P-rich region the %P content and amino acid (aa) coordinates are provided to the left of the to Box 3 alignment, overall P content is shown in parenthesis. Size of the full-length proteins follows (in parentheses) the species name in the Box 1 alignment. Sequence identities relative to *D. melanogaster* Sbp are indicated by dots, gaps required for optimal alignment by hyphens. Changes are shown in upper case (strongly or weakly similar residues) and lower case (nonsimilar). I (identity) and S (similarity) values relative to the *D. melanogaster* Sbp protein are listed next to the sequence; values in parenthesis (for fish and chicken) reflect the 32-aa gap introduced to optimize alignment. Several other proteins also showed highly conserved Box 1, 2, or 3 sequences; however, these sequences were excluded from the alignment because they either contained only one of the three regions or appeared to be significantly incomplete. In particular, a predicted protein fragment containing Box 2 and Box 3 motifs that are nearly identical to the *D. melanogaster* sequence is present in *A. gambiae* (XP_320700; Box 2 I = 88%, S = 98%, Box 3 I = 81%, S = 96%). Because this protein sequence lacks the N-terminus, the presence of the Box 1motif could not be determined. **C:** Schematic representation of the Optix binding protein (Obp) showing distribution of the C2H2 zinc-finger domains within the protein; the gray bar indicates the protein region encoded in the yeast two-hybrid pAct2 clone. **D:** Sequence of the nine zinc-finger domains of Obp. Additional sequence is present between zinc-fingers 1 and 2, 3 and 4, 8 and 9; number of residues is reported in between parentheses. **E.** Alignment of the protein region containing zinc-fingers 4 through 8 with related proteins from *D. pseudoobscura* (EAL26432.1) and *A. mellifera* (XP_397350.1). Size of the full-length proteins follows (in parentheses) the species name in the Box 1 alignment. Sequence identities relative to *D. melanogaster* Obp are indicated by dots, gaps required for optimal alignment by hyphens. Changes are shown in upper case (strongly or weakly similar residues) and lower case (nonsimilar). I and S values relative to the *D. melanogaster* Obp fragment are reported next to the sequence. Overall conservation between full-length *D. melanogaster* and *D. pseudoobscura* proteins is I = 285/558 (51%), S = 370/558 (66%), Gaps = 29/558 (5%); overall conservation between *D. melanogaster* and *A. mellifera* proteins is I = 138/313 (44%), S = 180/313 (57%), Gaps = 38/313 (12%).

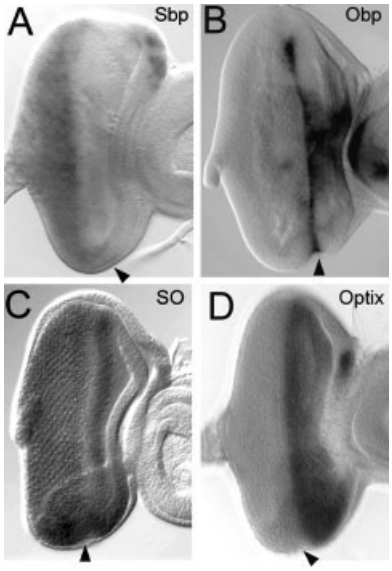

Fig. 4. Expression patterns in the L3 eye disc. In all panels, arrowheads mark the position of the morphogenetic furrow (MF). **A:** Sine oculis binding protein (Sbp) mRNA distribution detected by in situ hybridization. **B:** Optix binding protein (Obp) mRNA distribution detected by in situ hybridization; expression appears to be higher along the MF and the disc midline within the anterior region. **C:** Anti-SO antibody staining; SO is expressed anterior as well as posterior to the MF. **D:** Anti-Optix antibody staining; Optix is expressed exclusively anterior to the MF.

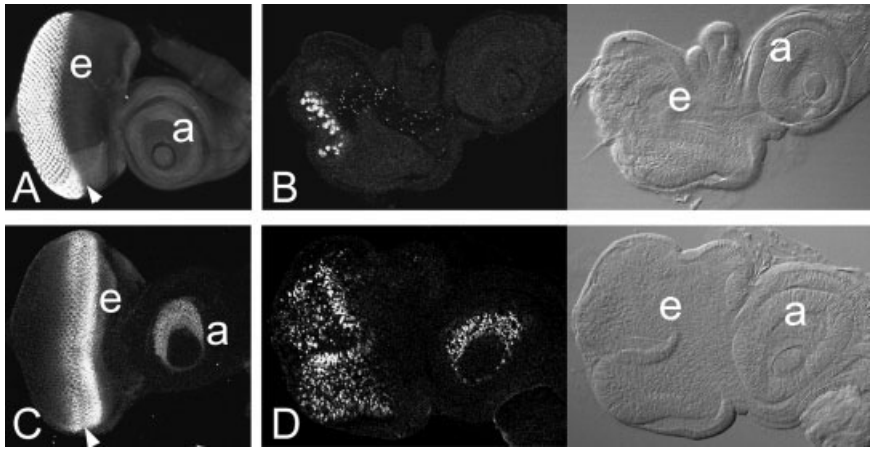

Fig. 5. Effect of Sine oculis binding protein (Sbp) misexpression on eye development. A–D: Neurogenesis of the photoreceptor array was visualized by staining for the pan-neural marker Elav (A,B), eye primordium formation by staining for the eye specification marker Dachshund (C,D). Eye (e) and antennal (a) discs are marked. **A:** Expression of Elav in the wild-type L3 eye–antennal disc. Elav is expressed in the developing neuronal cell clusters posterior to the MF (arrowhead). **B:** *ey-Gal4 UAS-Sbp* disc. Left panel, Elav expression; right panel, differential interference contrast microscopy (DIC) image of the same disc. Elav was always strongly reduced; discs displayed few groups of neuronal clusters or no expression at all. **C:** Expression of Dachshund in the wild-type L3 eye–antennal disc. Dachshund is expressed anterior and posterior to the MF (arrowhead). **D:** *ey-Gal4 UAS-Sbp* disc. Left panel, Dachshund expression; right panel, DIC image of the same disc. Dachshund was always present but strongly reduced.

rough eye (not shown). In summary, misexpression of Sbp in progenitor cells (expressing Optix) resulted in a loss of neurogenesis, whereas misexpression of Optix in cells posterior to the furrow (expressing Sbp) interfered with differentiation.

Contrary to the relationship between Sbp and Optix, the specificity of interaction between Obp and Optix appears to be controlled at the level of protein–protein binding and not at the level of gene expression. In fact, Obp is coexpressed with both SO and Optix in eye progenitor cells, but it interacts preferentially with Optix and not SO in yeast two-hybrid tests. Nonetheless, we generated *UAS-Obp* transgenic flies and crossed them to Gal4 drivers to generate different overlaps in Obp and SO expression. In particular, flies carrying *UAS-Obp* were crossed to the *pGMR-gal4* line to induce strong expression in the differentiating epithelium posterior to the MF where only SO protein is present. This ectopic expression of Obp did not cause any defects in eye formation. Viable adults were recovered in crosses with four different *UAS-Obp* lines and all flies looked normal (not shown). This result is consistent with an inability of Obp to partner with SO even when Optix is not available for interaction.

In conclusion, eye development was significantly altered when Sbp and Optix (which show a protein–protein interaction in yeast but are not normally coexpressed in vivo) were present in the same population of cells within the eye disc; whereas, eye development was not affected when Obp and SO (which interact weakly or not at all in yeast) were expressed in overlapping patterns.

DISCUSSION

The fly SIX-HD transcription factors SO and Optix function during eye development and are expressed in different but overlapping patterns within the eye epithelium (Cheyette et al., 1994; Seimiya and Gehring, 2000; this work). Targeted expression of SO or Optix results in distinct phenotypes. Optix is able to induce eye development when ectopically expressed in the antenna and blocks eye morphogenesis when overexpressed within the eye epithelium (Seimiya and Gehring, 2000; Pignoni, unpublished observations). On the contrary, SO does not induce ectopic eye formation nor does it block neuronal differentiation within eye tissue (Pignoni et al., 1997; Pignoni, unpublished observations). These observations strongly suggest that SO and Optix fulfill different roles during eye development. The identification of multiple unique partners for these Six factors clearly supports a model in which differences in cofactor recruitment contribute significantly to their specific function. In this study, we describe the identification of two novel potential partners of SO and Optix: one that is SO-specific by virtue of its expression pattern, and another likely to be Optix-specific by virtue of its protein binding profile.

The *Sbp* gene (*Flybase ID FBgn0033654*) encodes a novel factor of unknown function. As shown in this report, it does, however, contain three highly conserved motifs and a proline-rich region (Fig. 3A). The high level of similarity from fly to vertebrates suggests that these sequences represent novel protein motifs or domains with specific functions. As they do not appear related to previously characterized domains, little can be concluded about their poten-

tial function. However, Box 1 is not required for binding to the SD of SO, because the clone isolated through the yeast two-hybrid screen does not include this sequence (Fig. 3A). Its interaction with SO suggests that Sbp may function as a transcriptional regulator. The presence of a proline-rich region supports this hypothesis because proline-rich domains have been implicated in both transcriptional activation and repression (Mitchell and Tjian, 1989; Han and Manley, 1993). In a manner similar to Gro and unlike Eya, Sbp can interact in yeast with both SO and Optix. However, Sbp is specifically coexpressed with SO and not Optix within the eye epithelium. Hence, its interaction in vivo is restricted to SO by virtue of its expression posterior to the MF. This hypothesis is consistent with the observation that ectopic expression of Sbp in progenitor cells, where Optix is also expressed, interferes with normal eye development. However, alternative explanations are also possible. The effect of Sbp misexpression may be due to its abnormal association with SO itself, or other nuclear factors, in progenitor cells. Similarly, the disruption of eye development due to the misexpression of Optix in the developing neuronal field may result from abnormal interactions with other yet unidentified factors. Nonetheless, restricted expression of Sbp posterior to the MF and Optix anterior to the MF is obviously critical for normal eye development and effectively excludes binding between Optix and Sbp.

Sbp expression within the developing neuronal array, and not in progenitor cells, indicates that this gene is not required at an early stage but functions at the time of neuronal differentiation. On the contrary, SO function is required at multiple stages of eye development, including during specification of eye progenitor cells, at the time of patterning (in the MF) and during neuronal development (Pignoni et al., 1997). The Eya cofactor has also been implicated in these steps (Pignoni et al., 1997), and both proteins are continuously expressed in eye tissue through these developmental stages (Bonini et al., 1993; Cheyette et al., 1994). These studies do not provide clues regarding the function of SO at each developmental stage. Based on these data, SO could function simply by driving expression of a few genes required at all times to maintain eye identity. The identification of an SO partner specifically expressed in the differentiating epithelium contradicts this hypothesis and shows that there are significant differences in at least some of the SO-based complexes that function at distinct developmental stages. Thus, posterior to the MF, Sbp may associate with the SO–Eya complex and modify its activity turning it from an activator into a repressor. Evidence of complex interactions between the mouse Six1, Dac, and Eya proteins has been described (Li et al., 2003). Alternatively, Sbp may form a distinct SO–Sbp complex. In either case, however, SO function at various times during eye development would be modulated by its interaction with specific partners.

The *Obp* gene (*Flybase ID FBgn0050443*) encodes a protein characterized by nine putative zinc-finger domains of the C2H2 type. Although most members of this family are thought to regulate transcription through DNA binding, recent reports have also implicated C2H2-type zinc-fingers in protein–protein interactions (Fox et al., 1999; Polekhina et al., 2002). Because zinc-fingers 4 through 9 as well as the C-terminal sequence of Obp are present in the fragment isolated in our screen, we cannot exclude that one or more of these zinc-finger domains mediate the interaction with the Optix transcription factor. Obp is coexpressed with both SO and Optix in eye progenitor cells. However, it behaves as an Optix-specific interactor in yeast two-hybrid tests and does not impair eye development when ectopically expressed in the differentiating neuronal field (where only SO is present). Hence, we believe this factor to be a specific partner of Optix in vivo.

In this work, we have not been able to identify clear homologues of this factor. This finding is not entirely unexpected, because zinc-finger domains display only low-level conservation at positions other than the C and H residues that bind zinc. In the related proteins from *D. pseudoobscura* and *A. mellifera*, conservation is restricted to the region encoding zinc-fingers 4 through 8. Hence, additional criteria (such as expression in eye progenitor cells, interaction with Optix, and/or mutant rescue) are required to assess whether they are indeed homologues. *Obp* is reported in *Flybase* to encode a product putatively involved in cell proliferation based on an application of the PANTHER protocol (Mi et al., 2003; Thomas et al., 2003). In fact, the fourth and sizth zinc-fingers show similarity to zinc-fingers present in the Sfp1 transcription factor (Blumberg and Silver, 1991). In yeast, Sfp1 functions in cellular growth and proliferation by regulating ribosomal proteins expression (Xu and Norris, 1998; Fingerman et al., 2003; Jorgensen et al., 2004; Marion et al., 2004). *Obp* expression, specifically anterior to the MF where undifferentiated progenitors proliferate and then arrest just before the start of eye morphogenesis, is consistent with a potential role in regulating cell proliferation. However, the alignment between Obp and Sfp1 is limited to less than 20% of what is currently defined as the Sfp1 domain (*NCBI CD COG5189.1*). Given the limited conservation and the lack of functional data, a role for Obp in proliferation control is speculative at this time. Loss-of-function analyses of Obp and Optix mutant alleles, once available, will address this issue.

In conclusion, we have added two more factors to the list of the previously characterized partners of SO and Optix in the eye disc. That these factors behave differently in their association with SO or Optix adds further complexity to the study of Six proteins function during eye development. Based on what is known about binding specificity and expression patterns, we summarize in Figure 6 the spatial distribution of potential interactions between SO or Optix and their binding partners within the eye epithelium. To what extent this "interaction map" reflects the in vivo activity of SO- and Optix-based complexes remains the focus of future studies.

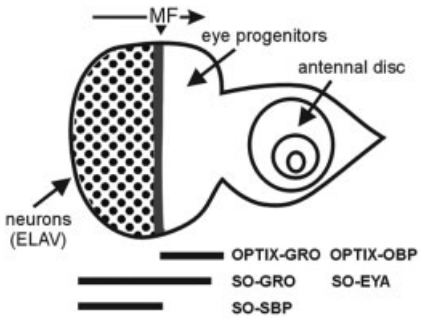

Fig. 6. Drawing showing the spatial distribution of potential interactions between Sine oculis (SO) or Optix and their binding partners within the eye epithelium. MF, morphogenetic furrow; SBP, SO binding protein; OBP, Optix binding protein; GRO, Groucho; EYA, eyes absent.

EXPERIMENTAL PROCEDURES

Yeast Two-Hybrid Screen and Gene Identification

A bait containing the SD of SO fused to the lexA DNA binding domain (pBTM116 vector, Bartel and Fields, 1995) was used to screen 5.5×10^6 clones of the Clonetech *Drosophila* embryonic library (pACT2 vector) in the yeast strain L40. Interactions were identified by a nutritional marker (HIS3) and by an enzymatic marker (β-galactosidase). All positives were further tested with a pBTM bait, including the HD in addition to the SD of SO to confirm the interaction and with the bait vector alone to eliminate false-positives. Twenty-one clones reproducibly showed weak to strong interactions with both pBTM–So baits. Additional testing was carried out using pGBKT7 baits (fusion to Gal4 DNA binding domain, Clonetech) in the yeast strain AH109. Two constructs were made: one contained the Six domain alone (pGBKT7-So^{SD}), the other contained the Six domain plus the homeodomain (pGBKT7-$So^{SD\text{-}SH}$). Both pGBKT7-based baits were found to be more selective and interactions already weak with pBTM-based baits generally failed this second test (e.g., the one positive Obp clone showed a modest interaction with BTM bait and a very weak to no interaction with the pGBKT7 baits). Clones were tested for the ability to interact with both baits (pGBKT7-So^{SD} and pGBKT7-$So^{SD\text{-}SH}$) and the pGBKT7 vector alone as a negative control. As an additional test for nonspecific interactions, we also used an unrelated bait containing the N-terminal domain of the mouse Tubby protein (pGBKT7-tubby, gift of Tiasen Li). Of the 21 positives, 18 were confirmed in this second round by nutritional selection (markers HIS3 and ADE2) and by a colorimetric assay for an enzymatic marker (MEL1/α-gal). All 18 clones were sequenced, and genetic loci were identified by BLAST (NCBI). In the final count, Eya was isolated 14 times, Taf1 twice, Sbp twice. Conserved regions were identified by BLAST search (NCBI). Composition analysis of the protein sequences was performed using SAPS (Brendel et al., 1992). The Groucho prey clone in Figure 2 is from Brugmann et al. (2004). Interactions with Optix were tested against a bait containing the Six and HD domains (pGBKT7-$Optix^{SD+HD}$).

GST-Pulldown Assay

Constructs (pGEX1 vector) encoding either GST alone or the fusion protein GST–SO^{SD+HD} were expressed in BL21 cells. GST proteins were purified using glutathione-beaded agarose as described in Smith (1983). PCR amplification was used to create fragments of each positive library clone that also contained a T7 polymerase site. S35-labeled proteins were produced from these fragments using an in vitro transcription/translation kit (Promega). For the binding assay, 10–15 μl of reticulocyte translate was added to 20 μl of glutathione-agarose containing approximately 2μg of bound GST or $GST^{SD\text{-}HD}$ in 500 μl of binding buffer. Proteins were incubated for 2 hr at 4°C. After binding, beads were washed 5 times in PBS before electrophoresis and autoradiography.

Histology

Anti-Optix antibodies were raised in rabbit and immunoaffinity purified over a column using the N-terminal peptide MAVGPTEGKQPPSESFSC. Digoxigenin-labeled full-length antisense RNA probes corresponding to the two novel genes were synthesized and used in in situ hybridization experiments following standard protocols. Immunostaining of larval eye–antennal disc was carried out by following standard protocols. Antibodies were as follows: mouse monoclonal anti-Elav (Robinow and White, 1991), mouse monoclonal anti-Dachshund (Mardon et al., 1994), rabbit polyclonal anti-Optix (this work), mouse polyclonal anti-SO (Cheyette et al., 1994), horseradish peroxidase (HRP) -conjugated anti-rabbit (Bio-Rad), HRP-conjugated anti-mouse (Bio-Rad), Cy2-conjugated anti-mouse (Jackson lab). Confocal laser scanning microscopy was performed at the MEEI Ophthalmology Core Microscope Facility supported by Core Grant for Vision Research P30EY14104.

Genetics

UAS-Optix (cDNA clone LD05472), *UAS-Sbp* (cDNA clone GH22790), and *UAS-Obp* (cDNA clone RE37461) were constructed by cloning full open reading frames into pUAST (Brand and Perrimon, 1993). Additional lines used are as follows: *UAS-So* (Pignoni et al., 1997), *ey-Gal4* (Hazelett et al., 1998), *pGMR-Gal4* (Freeman, 1996).

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