Identification of functional *sine oculis* motifs in the autoregulatory element of its own gene, in the *eyeless* enhancer and in the signalling gene *hedgehog*

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Summary

In *Drosophila*, the *sine oculis* (*so*) gene is important for the development of the entire visual system, including Bolwig's organ, compound eyes and ocelli. Together with *twin of eyeless*, *eyeless*, *eyes absent* and *dachshund*, *so* belongs to a network of genes that by complex interactions initiate eye development. Although much is known about the genetic interactions of the genes belonging to this retinal determination network, only a few such regulatory interactions have been analysed down to the level of DNA-protein interactions. Previous work in our laboratory identified an eye/ocellus specific enhancer of the *sine oculis* gene that is directly regulated by *eyeless* and *twin of eyeless*. We further characterized this regulatory element and identified a minimal enhancer fragment of *so* that sets up an autoregulatory feedback loop crucial for proper ocelli development. By systematic analysis of the DNA-binding specificity of *so* we identified the most important nucleotides for this interaction. Using the emerging consensus sequence for SO-DNA binding we performed a genome-wide search and have thereby been able to identify *eyeless* as well as the signalling gene *hedgehog* as putative targets of *so*. Our results strengthen the general assumption that feedback loops among the genes of the retinal determination network are crucial for proper development of eyes and ocelli.

Key words: *sine oculis*, *so*, *hedgehog*, *hh*, *eyes absent*, *eya*, *eyeless*, *ey*, Ocelli, *Drosophila*

Introduction

The *Drosophila* visual system consists of two compound eyes and three ocelli, which are simple eyes located on the adult vertex (Stark et al., 1989). Both types of optical organs develop from a small number of cells that are set aside during development in the early embryo. These cells form the eye part of the eye-antennal imaginal disc and proliferate during the larval stages. The compound eye emerges from the central part of the eye imaginal disc, whereas the ocelli develop from the anterior-medial region. The compound eye in *Drosophila* consists of a precisely organized array of approximately 750 ommatidia, each containing eight photoreceptor neurons and 12 accessory cells. The ommatidia begin to form in the early third instar larva, when the morphogenetic furrow (MF), a wave of pattern formation marked by an indentation, moves across the eye disc from posterior to anterior (reviewed by Wolff and Ready, 1993). Although committed to retinal fate, cells anterior to the furrow are still undifferentiated, whereas cells posterior to it are sequentially recruited into ommatidial clusters undergoing retinal differentiation (reviewed by Treisman and Heberlein, 1998).

Determination of the eye primordium requires several nuclear proteins that are known to act as transcriptional regulators. The *Drosophila Pax6* gene *eyeless* (*ey*) was the first gene shown to display the capacity to induce ectopic eye morphogenesis upon ectopic expression (Halder et al., 1995). A second *Drosophila Pax6* gene, *twin of eyeless* (*toy*), like *ey* encodes a protein with two DNA-binding domains (Czerny et al., 1999). Further genes involved in early eye determination are *eyegone* (*eyg*), which also shows some similarity to *Pax6* (Jun et al., 1998; Chao et al., 2004; Dominguez et al., 2004), *eyes absent* (*eya*) and *dachshund* (*dac*), both encoding nuclear proteins (Bonini et al., 1993; Mardon et al., 1994), and *sine oculis* (*so*) (Cheyette et al., 1994). Analyses of the expression patterns of these genes combined with genetic approaches have revealed a complex genetic regulation network during compound eye development. *toy* is the first of the mentioned genes to be expressed during embryogenesis and activates *ey* in the eye primordium (Czerny et al., 1999). *so* is required later for the development of the entire visual system, including the compound eyes, the ocelli, the optic lobe of the brain and the larval photoreceptors designated as Bolwig's organ (Cheyette et al., 1994; Serikaku and O'Tousa, 1994; Pignoni et al., 1997). *eya* expression comes up later in the compound eyes and can be found in the ocelli-specifying region in third instar eye imaginal discs. Recently, *eya* has been shown to have protein phosphatase activity (Li et al., 2003; Tootle et al., 2003). *so* and *eya* are both required for compound eye and ocellus formation, as the respective mutants lack both visual systems (Cheyette et al., 1994; Zimmerman et al., 2000). *so*, *eya* and *dac* have been shown to be regulated by *ey* (Halder et al., 1998; Niimi et al., 1999; Zimmerman et al., 2000). SO and DAC have

been proposed to function as co-factors for EYA, and genetic studies in *Drosophila* have demonstrated synergistic interactions between *so*, *eya* and *dac* during eye development (Chen et al., 1997; Pignoni et al., 1997). The respective protein complexes feed back on *ey* expression and *eya* and *dac*, like *ey* and *toy*, are capable of inducing ectopic eye morphogenesis (Bonini et al., 1993; Bonini et al., 1997; Pignoni et al., 1997).

Although much knowledge has been gathered during the last years about the complex genetic network that orchestrates eye development, only a small number of observed regulatory interactions have been analysed down to the level of DNA-protein interactions. Analysis of further components controlling expression patterns of genes involved in early eye development should therefore provide important details on the genetic hierarchy that mediates eye specification and may help to identify direct targets of the known eye specification genes.

Among the already described direct interactions, *toy* has been shown to induce *ey* expression by an eye-specific enhancer in embryonic eye precursor cells, but not during larval stages in the later emerging eye imaginal disc (Czerny et al., 1999). However, *ey*, together with *toy*, directly regulates *so* expression by an eye-specific enhancer that is deleted in the so^1 mutant allele (Niimi et al., 1999; Punzo et al., 2002). Furthermore, *ey*-regulated, eye-specific enhancers have been identified using deletions within the *eya* gene locus (Zimmerman et al., 2000).

In this study, we address the regulatory potential of a previously described *so*7 enhancer fragment during ocellar morphogenesis. So7 represents the DNA fragment that is deleted in the so^1 mutation and contains the *ey*- and *toy*-regulated enhancer element so10 (Punzo et al., 2002). We show that a 27 bp fragment within so7, soAE, is sufficient to expand expression of a reporter gene to the ocellar region when fused to the so10 enhancer and, consequently, this so10-soAE enhancer fragment is sufficient to rescue the eyeless and the ocelliless phenotype of so^1/so^1 flies when used as a driver for *so*. Furthermore, we show that soAE is a direct target of *so* in compound eye and ocellar development and that the autoregulatory feedback of *so* on its own expression is required for the ocellus-specific expression of *so*.

By analysing the DNA-binding specificity of SO in more detail, we were able to identify those nucleotides that are essential for SO-soAE interaction. Using the emerging *cis*-regulatory signature for *so*-dependent regulation, we performed a genome-wide search for additional putative *so*-target genes. Sequences that fit our selection criteria were identified in the *ey* and *hedgehog* (*hh*) loci. We show that both these genes contain eye-specific enhancers that are directly regulated by *so*. Our results emphasize the importance of autoregulatory feedback loops in morphogenesis and development.

Materials and methods

Fly strains and histology

Flies were reared on standard medium at 25°C. Lines used: UAS-*so* (Pignoni et al., 1997), UAS-*eya* (Bonini et al., 1997), so10-*lacZ* (Niimi et al., 1999), so7-*lacZ*, so9-*lacZ*, $so10^{EY+TOYmt}$-*lacZ* (Punzo et al., 2002), dpp^{blink}-GAL4 (Staehling-Hampton and Hoffmann, 1994), *ey*-GAL4 (Halder et al., 1998), *FRT42D*, so^3/CyO (Pignoni et al., 1997), *eyFLP* (Newsome et al., 2000), *FRT42D*, *ubiquitinGFP* (Duchek et al., 2001). so^2/so^2 (Bloomington Stock Center). Clones of homozygous so^3 mutant cells were generated by the expression of FLP recombinase under the control of an *ey* enhancer.

Specific genotypes generated: (1) *eyFLP*; *FRT42D*, *ubiquitinGFP*, (2) so^2/so^2; so10-soAE-*lacZ*, (3) so^2/so^2; so7-*lacZ*, (4) UAS-*so*/UAS-*so*;UAS-*eya*/UAS-*eya* and (5) so^1/so^1; so10-soAE-*so*.

lacZ reporter plasmids and rescue constructs were introduced into w^{1118} by standard P-element transformation procedures. Three to 10 independent transgenic lines were established for each construct and tested for expression or rescue potential.

Antibody staining on discs was performed according to Halder et al. (Halder et al., 1998). Primary antibodies were anti-EyaMab10H6, 1:10 (Bonini et al., 1997), Rabbit anti-β-galactosidase, 1:500 (Promega). Secondary antibodies used were from Jackson ImmunoResearch Laboratories: Cy5 α-rabbit (1:400), Alexa586 α-mouse (1:400).

To detect β-galactosidase activity, third instar larval imaginal discs were fixed and subjected to a standard X-gal colour reaction for 2 hours at 37°C.

Reporter transgenes and rescue construct

Inserts of the reporter constructs were obtained by PCR, using so7 as a template, and subcloned into the *lacZ* pCβ vector (Niimi et al., 1999). For the rescue construct a modified pUAST vector (Brand and Perrimon, 1993) was used. The 5× UAS sequence was replaced by so10-soAE. *so* cDNA was placed downstream of hsp70 within the polylinker resulting in so10-soAE–hsp70–*so* in the pUAST backbone (so10-soAE-*so*).

For the constructs: ey enhancer, B4M and B4M SOmut, the sequences given in Fig. 5 were used. A *Bam*HI and a *Kpn*I site was added at the 5′ and 3′-end, respectively, and used for subcloning into the *lacZ* pCβ vector.

The hh^1 (*bar-3*) sequence was obtained by PCR on genomic DNA of wild-type (wt) flies by using the following primer set: 5′-CTGTGCGCTCGAGTGGGCCACACAGGGTGGG-3′; rightward orientation, 5′-CGGCCCGTCTCAGATCTCGGATCTGAGATC-3′ leftward orientation. Mutations were introduced by PCR. For the deletion construct hh^1 Δ5′, 5′-GGGGTACCCAAGACAAGTAA-TCCCCCACCCTCGC-3′ was used as rightward oriented primer (the SO site is mutated by changing GAG to CCC).

so² mutant

Genomic DNA was amplified by PCR from so^2/so^2 flies and sequenced. The sequences were confirmed on independent amplification events. Genomic DNA isolation was performed according to Bui et al. (Bui et al., 2000a). Primers used for mapping the so^2 deletion were: 5′-GAAGGGCACTGCTTACTGAGAGCT-CG-3′, 5′-GCCCATCGAATCCGCATCTCCCCCAG-3′ rightward orientation; 5′-GCGCACACTCGACAAATTTGCGATCTGGC-3′ leftward orientation. Primers are located at positions 2355, 3116 and 6218, respectively, within the last intron. Nucleotides 3983-5181 are deleted in so^2 (the first nucleotide of the last intron is set as 1).

Southern blotting was performed according to Sambrook and Russel (Sambrook and Russel, 2001). Genomic DNA was digested using *Cla*I, *Eco*RV and *Xho*I. As probes, DIG-labelled PCR products of so10 and so9 were used. so10 and so9 are described previously (Punzo et al., 2002).

Transfections and reporter gene assays

Drosophila S2 cells were maintained in Schneider's insect medium (Invitrogen) supplemented with 10% fetal calf serum and were transfected with the Effectene Transfection Reagent (Qiagen). For reporter gene assays 2×10^6 cells were transfected with a total of 200 ng plasmid DNA (20 ng reporter plasmid, 5 ng of a plasmid constitutively expressing firefly luciferase, the indicated amounts of expression plasmids and the parental vector pAc5.1B/V5His, to bring the total amount of DNA to 200 ng). Cells were lysated 48 hours after transfection and lysates were assayed for

β-galactosidase and luciferase activity as described previously (Muller et al., 2003).

Electrophoretic mobility shift assays

Radioactively labelled probes were generated by annealing and filling in partially overlapping oligonucleotides in the presence of (α-^{32}P)ATP. Binding reactions were carried out in 20 µl of 100 mmol/l KCl, 20 mmol/l HEPES pH 7.9, 20% glycerol, 1 mmol/l DTT, 0.3% BSA, 0.01% NP40 containing 10,000 cpm probe and 1 µg dIdC. As a protein source, full-length SO protein was synthesized in reticulocyte lysates using the T7 promotor according to the manufacturer's specification (Promega). For the binding reaction, 1 µl of a standard 50 µl reaction was used. After incubation for 30 minutes at 4°C, the reactions were analysed by non-denaturing 6% polyacrylamide gel electrophoresis followed by autoradiography. For the cold competition experiments, the proteins were first incubated with a 100× molar excess of unlabeled double-strand oligonucleotides for 10 minutes at RT, followed by incubation with the radiolabelled probe at 4°C for 30 minutes.

Computer-assisted search for SO-binding sites

Putative *so* target genes were identified by screening the entire *Drosophila* genomic sequence with the consensus GTAANYNGANAYS using the program FLY ENHANCER [freely available at http://flyenhancer.org (Markstein et al., 2002)].

Alignments of different *Drosophila* species were obtained from http://hanuman.math.berkeley.edu/genomes/drosophila.html.

Results

Defining a minimal eye/ocellus-specific enhancer of the *so* gene

A 1.6 kb enhancer fragment (so7, Fig. 1A) spanning the genomic region deleted in *so*^{1} is able to recapitulate the

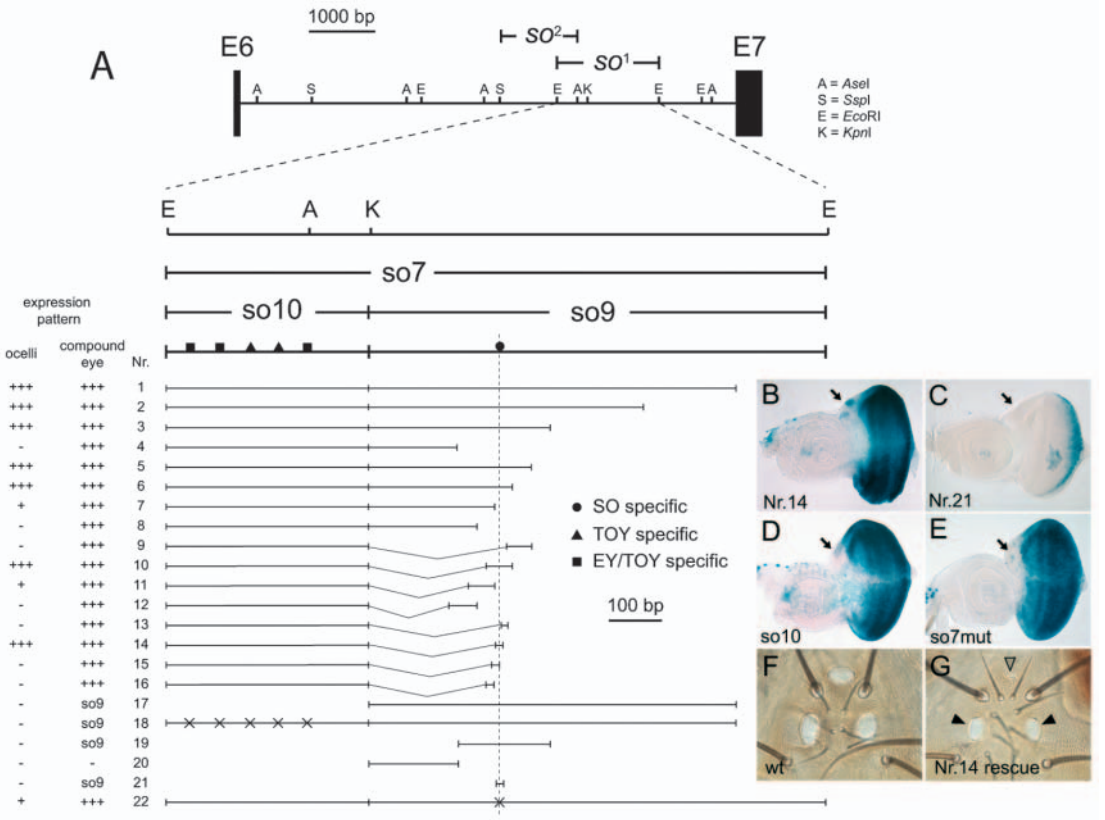

Fig. 1. Defining a minimal version of so7 important for ocellus development. (A) Genomic map of the last intron of the *so* locus between exon6 (E6) and exon7 (E7) (black boxes). The physical mapping is indicated as: A, *Ase*I; S, *Ssp*I; E, *Eco*RI; K, *Kpn*I. An enlargement of the region deleted in *so*^{1} shows relative positions of the enhancer fragments so7, so10 and so9. so10 contains EY/TOY- and TOY-specific binding sites (black boxes and black triangles, respectively). So9 harbours the SO-binding site. The deletion map illustrates the constructs that were tested for the expression pattern they mediate (X indicates introduced mutations). Nr, number. Twenty-seven bp fused to so10 (number 14) are sufficient to resemble the so7-mediated pattern including ocellus-expression (B, referred to as +++ expression in ocellus and +++ expression in compound eye). All constructs including so10 but missing the 27 bp (represented by number 21) just resemble the so10-pattern and show no expression in the ocellar region (indistinguishable from D). Constructs that are devoid of functional EY and TOY sites (number 17, 18, 19 and 21) but include the 27 bp of number 21, show an expression pattern identical to so9 (C, illustrated by the pattern mediated by number 21). Fragment number 21 is sufficient to recapitulate the expression pattern of so9; it is referred to as soAE in the article. Construct number 20 (no so10 and no number 21) does not mediate any expression at all (–). (B-E) Arrows indicate the ocellar region. (B) Expression pattern mediated by number 14, similar to wt *so* expression pattern and the so7-mediated pattern. (C) Number 21-*lacZ*: expressing only along the posterior margin of the eye disc similar to so9-lacZ. (D) so10-lacZ: resembling wt-expression despite the ocellar signal. (E) So7mut: so7 where the SO-binding site is mutated mediates only weak expression to the ocellar region (fragment number 22). (F) Vertex region of a wt fly showing three normal ocelli. (G) *so* driven by so10-soAE (number 14) rescues ocellus development of *so*^{1} mutant flies. The lateral ocelli appear almost normal (arrowhead). The size of the anterior ocellus is reduced (empty arrowhead).

expression pattern of *so* in third instar eye imaginal discs when driving a *lacZ* reporter gene (Punzo et al., 2002). Furthermore, so7 is able to completely rescue the eyeless, and partially the ocelliless, phenotype of so^1 mutant flies when driving the *so* gene (Punzo et al., 2002).

So10 (400 bp) and so9 (1.2 kb) (Fig. 1A) are subfragments of so7. so10 mediates expression in the compound eye part of third instar eye-antennal imaginal discs and contains the previously described *ey*- and *toy*-specific binding sites (Fig. 1D). These include five binding sites bound by *toy*. Three of these are also binding sites for *ey* and are important for compound eye development, whereas the two *toy*-specific sites are required for ocellar development (Niimi et al., 1999; Punzo et al., 2002). Consistent with its expression pattern, so10 is able to rescue the eyeless phenotype but not the ocelliless phenotype of so^1 mutant flies (Punzo et al., 2002).

so9-mediated expression appears at the posterior margin of the eye disc (similar to Fig. 1C). When combined with so10, so9 provides additional transcriptional input to expand the expression to the ocellar region.

Trans-acting factors that bind the *cis*-regulatory so9 element and cooperate with *toy* to confer expression in the ocellar region were unknown when this work was started. In order to locate the binding sites of such additional transcription factors we first aimed at the isolation of the smallest version of so9 that still would be able to drive expression of a *lacZ* reporter to the ocellar region of eye imaginal discs when combined with so10 (Fig. 1A). Our search resulted in the identification of a fragment as small as 27 bp (Fig. 1A, number 21), which in the following text will be referred to as soAE (*sine oculis* autoregulatory element).

The expression pattern mediated by a combined so10-soAE-element (Fig. 1A, number 14 and Fig. 1B) was indistinguishable from expression mediated by so7, whereas soAE alone resembled the expression pattern of so9 (Fig. 1A, number 21 and Fig. 1C). In addition, so10-soAE driving *so* is sufficient to rescue both the eyeless and ocelliless phenotype of so^1 mutant flies (Fig. 1G). Therefore, soAE contains all regulatory elements that are sufficient for so9-mediated expression. Further evidence for the functional relevance of this sequence came from the comparison of *D. melanogaster* and the genomes of six other *Drosophila* species in which the soAE sequence shows a high degree of conservation (see Materials and methods).

sine oculis is able to recognize its own enhancer

In soAE three sequence motifs can be found that are reminiscent of well-known transcription-factor-binding sites. These are a motif related to the *Pax6*-consensus-binding site (Epstein et al., 1994), a TAAT-motif that is a hallmark of most homeodomain recognition sequences and a GATA-motif. We mutated these sites, and tested the respective fragments (so10-mutPAX, so10-mutHD, so10-mutGATA) for the resulting expression patterns.

so10-mutPAX-mediated expression was indistinguishable from the so10-soAE expression pattern (Fig. 2C). Conversely, mutating the putative homeodomain-binding site (so10-mutHD) or the GATA sequence (so10-mutGATA) resulted in loss of reporter gene expression in the ocellar region (Fig. 2A,B).

We then oligomerized soAE four times, to boost its expression. As a result, an expression signal became apparent posterior and slightly in front of the MF (Fig. 2E) as well as in the optic lobe (data not shown). However, 4xsoAE was not able to drive expression in the ocellar region. Additional copies of soAE did not lead to a further strengthened expression. Expression of 10xsoAE, for example, appears blotchy and weaker in the eye disc than expression of 4xsoAE (Fig. 2F).

As the expression pattern of 4xsoAE is reminiscent of *so*-expression in the eye disc, we hypothesized that *so* itself might be the soAE regulating factor. Both expression patterns show a signal in the optic lobe as well as posterior to, within and in a few cells in front of the MF. The only difference is the ocellar expression of *so*, which cannot be seen using the 4xsoAE reporter construct.

The idea that *so* itself is the soAE-binding factor was further supported by previous work in which Hazbun et al. showed that SO binds in vitro to (C/T)GATA (Hazbun et al., 1997), a motif that is present in soAE (Fig. 4C nt. 7-11).

To determine experimentally whether the expression pattern of the mutated fragments correlates with the ability of these fragments to bind SO in vitro

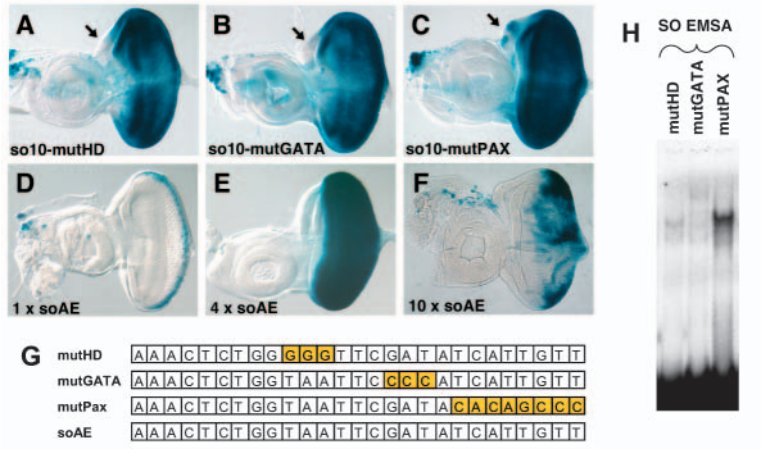

Fig. 2. soAE is a direct target of *so*. soAE (*sine oculis* autoregulatory element) corresponds to fragment number 21. Arrows indicate the ocellar region. (A-C) soAE harbours three putative transcription-factor-binding sites, which have been mutated. (A) Mutating the core of the HD recognition sequence (mutHD) or (B) mutating the GATA sequence (mutGATA) abolishes expression of the *lacZ* reporter in the ocellar region. (C) so10-mutPAX in which the putative *Pax6*-binding site is mutated, mediates expression indistinguishable from wt/so10-soAE-mediated expression. (D-F) Oligomerization of soAE boosts its expression. By contrast to soAE alone, which only mediates expression in the posterior margin of the eye disc (D), 4xsoAE drives expression posterior of the MF, within the MF and in some cells in front of the MF, but not in the ocellar region (E). This expression resembles wt *so*-expression despite the ocellar region. (F) 10xsoAE does not further amplify expression intensity but results in a more blotchy type of expression pattern. (G) Sequences of soAE and the mutated versions of it. Sequences TAA and GAT of soAE are important for the ocellus-specific expression of so10-soAE-*lacZ*. (F) Radiolabelled probes of mutHD and mutGATA are not shifted by SO in EMSA. By contrast, mutPAX is bound by SO and therefore shifted in EMSA.

we performed electrophoretic mobility shift assays (EMSAs). SO protein was able to shift radiolabelled mutPAX but failed to bind to mutHD and mutGATA DNA fragments (Fig. 2H; see also Fig. 4A).

These results, in combination with our in-vivo data, strongly suggest that *so* itself is responsible for the ocellus-specific expression of so10-soAE.

so10-soAE-*lacZ* and so7-*lacZ* are not expressed in the ocellar region of *so*^{2} mutant flies

To further test this hypothesis, we moved on to a genetic approach. *so*^{2} is a hypomorphic allele that originated as a spontaneous partial reversion of *so*^{1} (Lindsley and Zimm, 1992). Different from *so*^{1} adult flies, which completely lack compound eyes and ocelli, *so*^{2} flies develop compound eyes that range from normal appearance to slightly reduced shapes but still lack ocelli completely. In *so*^{2}/*so*^{1} flies, eyes are of intermediate size (Heitzler et al., 1993). Because of the common origin and the genetic interaction of these two alleles, we tested if there is a mutation in *so*^{2} flies that affects the genomic so9/so10 sequences. Using PCR on genomic *so*^{2} DNA, we found a deletion of 1.2 kb that indeed affected so7. We further confirmed this result by Southern blotting (data not shown). The deletions of *so*^{1} and *so*^{2} partially overlapped (Fig. 1A) and in *so*^{2}, four of the five previously described *Pax6*-binding sites (Punzo et al., 2002) were missing. In fact, both binding sites exclusively recognized by TOY were deleted. According to Punzo et al. (Punzo et al., 2002), these *toy*-specific binding sites within the so10 enhancer fragment are required for ocellus development. The sequence representing so9, which contains the soAE fragment, appeared not to be affected by the *so*^{2} deletion.

Next we took advantage of *so*^{2} mutant flies to test whether the *cis*-regulatory potential of soAE depends on SO protein in vivo. Therefore we analysed so7- and so10-soAE-mediated expression in the ocellar region in *so*^{2} mutant flies. As expected, so7-*lacZ* and so10-soAE-*lacZ* expression was lost in the ocellar region of *so*^{2} mutant flies (Fig. 3D), supporting the idea of *so* being required for the ocellus-specific expression of so7 and so10-soAE further. The absence of reporter gene expression cannot be explained by a loss of ocellus-specific precursor cells, as *eya* expression, which represents a marker for this specified cell population, was detectable in *so*^{2} mutant flies in the prospective ocellar region (Fig. 3E).

Taken together, *toy* and *so* binding to so10 and soAE, respectively, seem to cooperatively drive *so*-expression in the ocellar region of third instar eye discs.

To further examine the hypothesis that *so* autoregulation is important for ocellus development, we did the reverse experiment by mutating the SO-binding site of so7 (so7mut). So7mut-*lacZ* expression was hardly detectable in the ocellar region in a wt background (Fig. 1E) and resembled expression of fragments number7 and number11 (Fig. 1A).

These data strongly suggest that feedback of *so* on its own enhancer is needed for ocellus development.

4xsoAE is not expressed in *so*^{3} clones

To assess whether soAE is a target of *so* also in the compound eye part of the eye disc, we tested the expression of the 4xsoAE reporter construct in cells homozygous for *so*^{3}, a null allele of *so* (Cheyette et al., 1994). *so*^{3} mutant cells, however, tend to overproliferate, fail to differentiate into neurons and subsequently die (Pignoni et al., 1997). Hence, to be able to analyse reporter gene activity in living cells within *so*^{3} clones we tested them for *eya* expression. *Eya* is a suitable marker for viable cells in *so*^{3} mutant clones for the following reasons. First, *so* and *eya* are both targets of *ey* and show the same expression pattern in third instar eye discs (Halder et al., 1998;

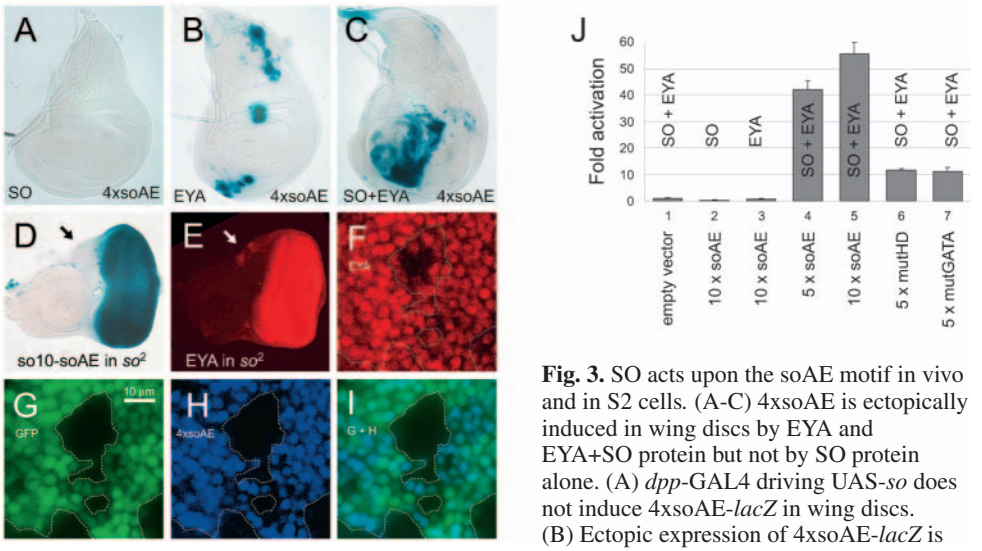

Fig. 3. SO acts upon the soAE motif in vivo and in S2 cells. (A-C) 4xsoAE is ectopically induced in wing discs by EYA and EYA+SO protein but not by SO protein alone. (A) *dpp*-GAL4 driving UAS-*so* does not induce 4xsoAE-*lacZ* in wing discs. (B) Ectopic expression of 4xsoAE-*lacZ* is induced in spots along the AP boundary by *dpp*-GAL4:UAS-*eya*. (C) Co-expression of *so* enhances *eya*-mediated reporter gene activity. (D) Ocellus-specific expression of so10-soAE is lost in *so*^{2} mutant flies (arrow). *so*^{2} is a regulatory mutant that displays an ocelliless phenotype. (E) EYA protein is detectable in the ocellar region of *so*^{2} mutant flies (arrow). (F-I) *so* is necessary for soAE activation: in *so*^{3} clones, 4xsoAE-*lacZ* expression is lost although EYA is present in the cells. Clones are delimited by a dashed line. (F) EYA expression is shown in red and marks the non-dying cells within the clone. (G) *so*^{3} clones are negatively marked by the absence of GFP expression (in green). (H) 4xsoAE-*lacZ* reporter gene expression (blue) is lost in *so*$^{-/-}$cells. (I) overlay of G and H. (J) β-Galactosidase reporter assays in *Drosophila* S2 cells. Reporter plasmids containing the *lacZ* gene under the control of different enhancer fragments were transfected into the cells. Co-transfection of *so* or *eya* alone together with the 10xsoAE-*lacZ* reporter plasmid does not exceed basal activity (lanes 2, 3). By contrast, co-expression of *so+eya* with the 5xsoAE or 10xsoAE reporter leads to a strong induction of β-galactosidase (lanes 4, 5). The mutated versions of the 5xsoAE reporter (5xmutHD, 5xmutGATA) still show some amount of induction when co-transfected with *so+eya* (lanes 6, 7). β-Galactosidase values were normalized by co-transfecting 5 ng of plasmid expressing luciferase as an internal standard. The results represent an average β-galactosidase activity taken from transfections done in triplicates (±s.d.) and are illustrated as the X-fold activation over the basal activity found for the reporter plasmid alone.

Niimi et al., 1999; Bui et al., 2000b). Both are expressed in a few cells anterior to the MF, within the MF, and in the differentiating photoreceptors posterior to the MF (Curtiss and Mlodzik, 2000). Second, SO and EYA proteins form a complex that works as a transcriptional activator when the proteins are co-expressed (Pignoni et al., 1997; Silver et al., 2003). Third, so^1 mutant eye discs still express *eya*, whereas in eya^1 mutants, expression of *so* is lost (Halder et al., 1998). Finally, *so* can be induced by *eya* in third instar eye imaginal discs (Curtiss and Mlodzik, 2000). For these reasons we assume *eya*-positive-cells of third instar eye discs also express *so* during normal development. Therefore, only *eya*-expressing cells within so^3 clones were examined in our assay. In fact, in *eya*-expressing cells within so^3 clones, expression of the 4xsoAE reporter construct was lost (Fig. 3F-I, the clones are negatively marked by the absence of ubiquitin-GFP expression; Fig. 3G). This strongly suggests that SO protein in general is required for activation of the soAE element in the eye field.

4xsoAE-*lacZ* is induced in ectopic eyes and in cell culture

To further analyse whether soAE is a general in-vivo target of SO we tested reporter gene activity as a result of ectopic eye induction. *so* on its own is not able to induce ectopic eyes. By contrast, *eya* alone, synergistically strengthened by *so*, is sufficient to induce ectopic eye development on antennae, wings and legs (Pignoni et al., 1997).

We induced ectopic eye development by combining a *dpp*-GAL4 driver with UAS:*so*, UAS:*eya* or both of them and tested whether the reporter construct 4xsoAE-*lacZ* was induced ectopically. As expected, ectopic *so* alone did not result in reporter gene activity, whereas *eya* alone or *eya* combined with *so* in a synergistic manner was able to activate the reporter construct in wing discs (Fig. 3A-C).

In another in-vitro approach, we took advantage of *Drosophila* S2 cells to address whether SO and EYA proteins work cooperatively as a complex on soAE DNA to induce

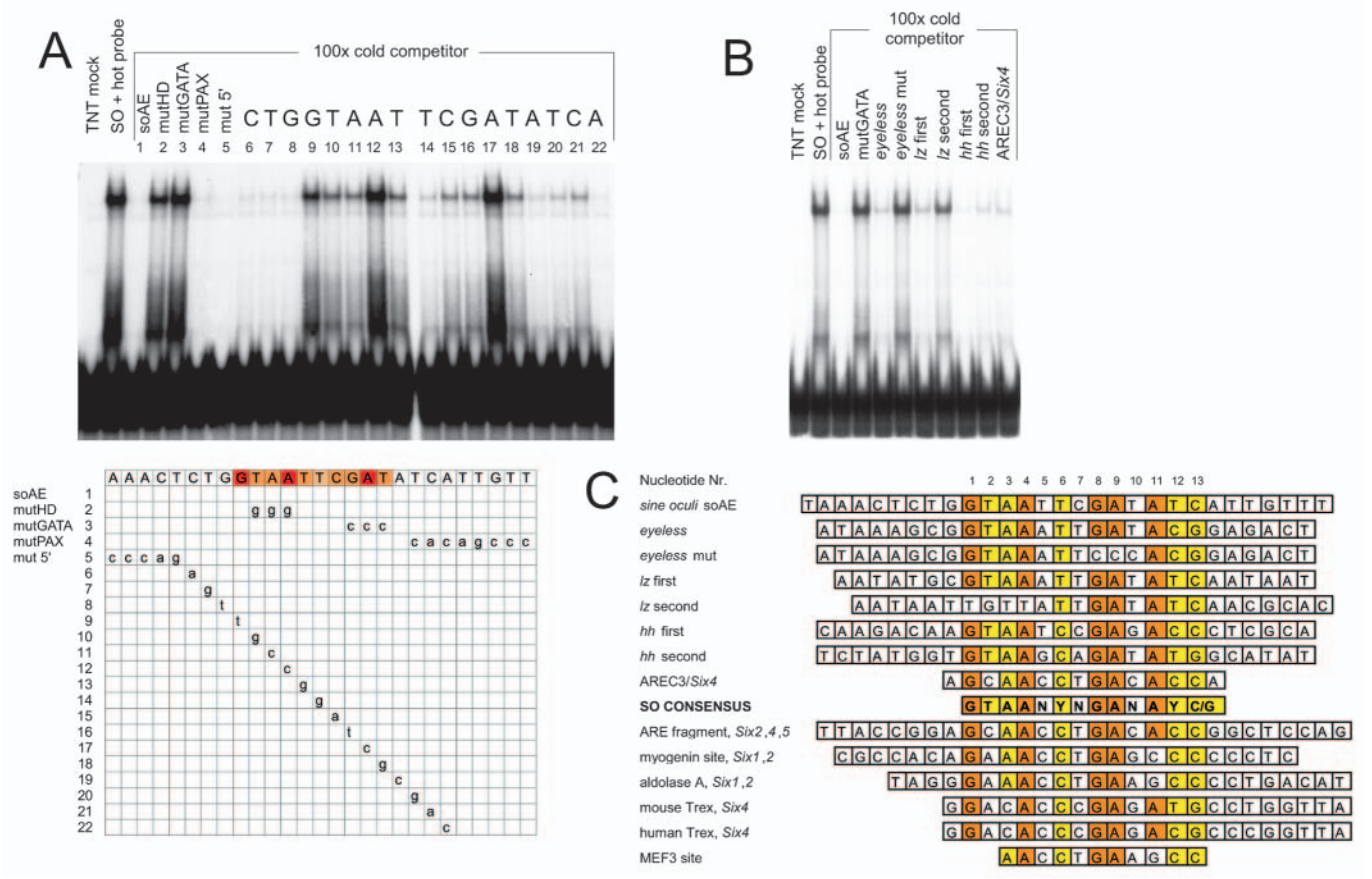

Fig. 4. Identification of nucleotides important for SO-DNA (soAE) interaction. (A) SO protein is shifted by soAE (SO + hot probe) in EMSA. Double-stranded probes bearing a single point mutation (6-22) or a stretch of mutations (2-5) were used as cold competitors (100× molar excess) and compared to soAE (1) for their ability to compete for SO-binding. Nucleotides important for protein-DNA interaction are highlighted in red (very important) and orange (important) according to their competing potential. As a control, mock transfected reticulocyte lysate was incubated with p^{32} marked soAE (TNT mock). (B) DNA probes of sequences resembling soAE taken from other genes were used as cold competitors as well. A sequence out of the eye-specific enhancer of the *ey* gene is a strong competitor. The fragment loses its binding property when the GAT sequence is mutated to CCC (*eyeless*, *eyeless* mut). Out of the *lozenge* gene, only one of the previously described SO-binding sites shows a strong competition potential in EMSA (*lz* first, *lz* second). Strong competing sequences are also found in the first intron of the *hh* gene (*hh* first, *hh* second). SO binding is furthermore strongly competed by the well-described AREC3/*Six4*-binding site. (C) Upper half: sequences of the probes that were used as cold competitors in Fig. 4B. Based on these sequences and the results shown in A, a consensus binding sequence for SO was proposed. Lower half: previously described binding sites for the vertebrate *Six1*,*2*,*4*,*5*. These sequences appear to be related to the SO-binding sequence.

transcription. Consistent with the in-vivo data, our in-vitro results using S2-cells showed that SO, which has DNA-binding properties but lacks a transactivation domain, on its own was not able to activate soAE-mediated *lacZ* expression (Fig. 3J). Likewise, EYA, which contains a transactivation domain but lacks DNA-binding properties, also failed to induce transcription in S2 cells when expressed alone (Fig. 3J). Only when co-expressed, SO and EYA cooperatively worked as transcriptional activators on soAE (Fig. 3J). Interestingly, both SO and EYA mediated weak transactivation when the oligomerized mutated sites mutHD and mutGATA were used (Fig. 3J), despite the fact that these sites do not mediate transgene expression in vivo in the developing ocellus (Fig. 2A,B).

Defining a consensus sequence for SO-DNA interaction

To date, there is only one direct target of *so* described in *Drosophila*, which is the Runx class transcription factor *lozenge* (*lz*) (Yan et al., 2003). Consistent with a previous in-vitro study that addressed the DNA specificity of the SO homeodomain (Hazbun et al., 1997), the authors show that the sequence (C/T)GATA plays a crucial role in SO-DNA interaction. Another study reports that SO together with EYA is able to transactivate by binding to an AREC3/Six4-binding site in cell culture. This motif, however, diverges to some extent from the C/TGATA-motif (Fig. 4C) (Silver et al., 2003).

Our soAE fragment harbours a CGATA motif, which is consistent with the SO-binding consensus of the *lz* promotor. In our experiments, however, also mutations upstream of this GATA core motif (Fig. 4C nt. 8-11) were able to abolish expression of the reporter construct in vivo and also impaired the capability of SO to shift DNA fragments in the EMSA. This observation suggested that additional sequences upstream of the GATA motif are also necessary for SO binding to its target site.

Therefore we decided to elucidate the sequence specificity of SO-DNA binding by analysing a systematic series of point mutations for their competitive effect on protein-DNA complex formation (Fig. 4A).

These in vitro experiments revealed a stretch of 13 nucleotides to be important for protein-DNA interaction of SO. There are three nucleotides, G, A, A at positions 1, 4, 9, respectively (Fig. 4A lanes 9, 12, 17 and Fig. 4C nt. 1, 4, 9), that appear to be most important for the interaction. These nucleotides, which show the strongest effects upon mutation, are found in the AREC3/*Six4*-binding site and are also substituted in the constructs so10-mutHD and so10-mutGATA. This provides strong evidence that these nucleotides are also important for soAE-mediated reporter gene expression in vivo (Fig. 2A,B).

Genome-wide search for potential *sine oculis* target genes

Combining our in-vitro data on the autoregulatory element with the known *so* target sequence of *lz* and the AREC3/*Six4*-binding site, we defined the consensus sequence GTAANYNGANAYC/G as necessary for SO binding to DNA. This consensus sequence was taken as a basis for scanning the *Drosophila* genome for similar sites (see Materials and methods). In total, 1632 putative *so* targets emerged from this survey. Out of the affected genes several candidates are already known to be involved in eye development. In the following we will describe two of the genes that we picked for further analysis: *ey* and *hh*.

eyeless is a direct target of *so*

The first soAE similar element that caught our attention was located within the previously described eye-specific enhancer of the *ey* gene (Czerny et al., 1999; Hauck et al., 1999). A

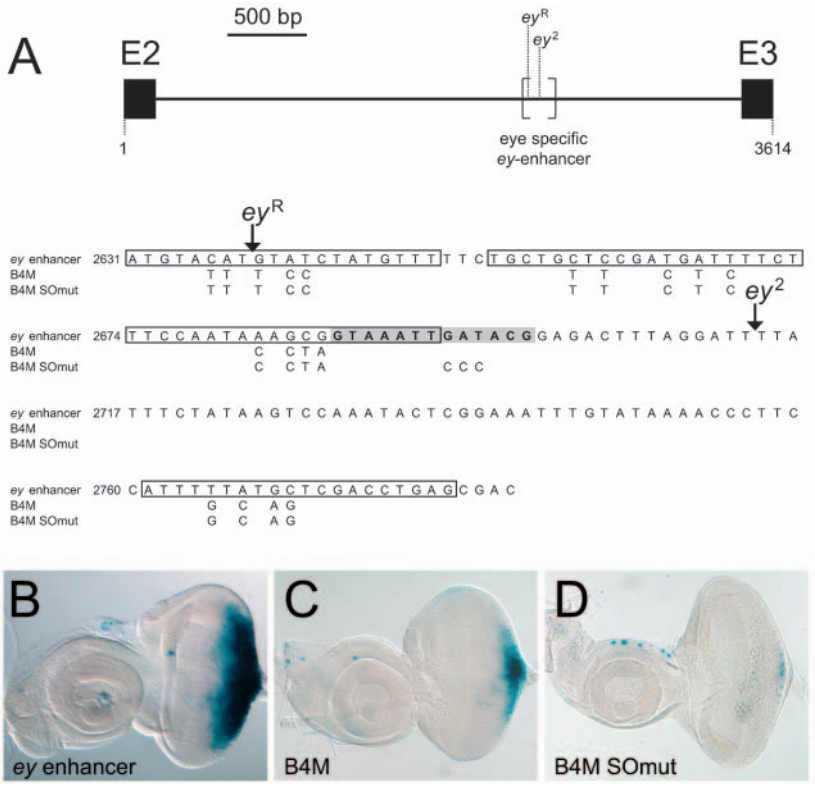

Fig. 5. The eye-specific enhancer of *ey* contains a functional SO-binding site. (A) Genomic map of the *ey* locus between exon 2 (E2) and exon 3 (E3) (black boxes). The previously described eye-specific *ey*-enhancer is indicated by brackets. Relative positions of transposable elements that interfere with eye development are indicated by their allelic name (ey^R, ey^2). The sequence of the eye-specific *ey* enhancer is given below (*ey* enhancer). B4M: the putative *Pax6*-binding sites (boxed) were mutated accordingly to Hauck et al. (Hauck et al., 1999) to get rid of *toy*-mediated signal. B4M SOmut: the putative *Pax6*-binding sites and additionally the putative SO-binding site (grey shaded) were mutated for comparison to B4M. (B) *lacZ* expression mediated by the wt *ey*-enhancer fragment in a third instar eye imaginal disc. (C) B4M-*lacZ* expression: without an influence of TOY protein, due to the mutated sites, the *so*-mediated expression is restricted to a portion of the posterior margin (similar to so9/Nr21 in Fig. 1C). (D) B4M SOmut-*lacZ*: mutating the *Pax6* sites and the *so* site, expression is reduced to a weak spot in the centre of the posterior margin.

positive feedback loop already has been postulated on the basis of the fact that *ey* is induced in ectopic eye development upon co-expression of *so* and *eya* (Pignoni et al., 1997). Furthermore, the ability of *so* and *eya* to induce ectopic eyes is lost in ey^2 mutants (Pignoni et al., 1997). In ey^2 mutant flies, the previously mentioned eye-specific enhancer of *ey* is disrupted by insertion of a transposable element (Quiring et al., 1994) (see also Fig. 5A). These experiments genetically show that *so* and *eya* are able to feedback on *ey* and that this feedback loop relies on the eye-specific enhancer of the *ey* gene. However, a direct interaction between SO, EYA and the *ey*-enhancer has not been previously demonstrated.

The fact that the potential *so* target site within the eye-specific enhancer is perfectly conserved between *D. melanogaster*, *D. pseudoobscura* and two other *Drosophila* species (see Materials and methods), encouraged us to perform additional assays to obtain molecular evidence for a direct interaction.

First we showed that oligonucleotides containing this sequence were strong competitors for the binding of SO to soAE in EMSA, whereas this competing potential was lost when the GAT core (Fig. 4C nt. 8-10) of the sequence was mutated (Fig. 4B, *eyeless* and *eyeless* mut). We then compared the expression pattern of different mutated versions of a 160 bp fragment, comprising the eye-specific *ey*-enhancer, driving a *lacZ* reporter (sequences shown in Fig. 5A). The wt enhancer mediated expression posterior to the MF (Fig. 5B) (see also Hauck et al., 1999) (Fig. 4D). By mutating the *Pax6* sites, expression in the eye disc was reduced to the posterior margin (Fig. 5C, observed in all four transgenic lines that were tested) (see also Hauck et al., 1999) (Fig. 4F). Mutating the *so* site and the *Pax6* sites further reduced expression in the eye disc (one transgenic line showed no pattern at all, five independent transgenic lines showed weak activity similar to Fig. 5D). These data indicate that *so* directly regulates *ey* expression through the eye-specific enhancer of the *ey* gene.

hh is a direct target of *so*

hh encodes a secreted signalling protein that plays an important role in patterning the *Drosophila* eye field. Many lines of evidence suggest that *hh* signalling is required for the initiation and the propagation of the MF. Accordingly, *hh* is expressed at the posterior margin of the eye imaginal disc prior to photoreceptor differentiation and in cells posterior to the MF during its progression (Borod and Heberlein, 1998). Loss of *hh* function blocks initiation of the MF and impedes its progression (Borod and Heberlein, 1998). Posterior margin clones of a null allele of *smoothened* (*smo*), the cell-autonomous receptor of *hh* signalling, lack differentiated photoreceptors (Greenwood and Struhl, 1999; Curtiss and Mlodzik, 2000). Conversely, ectopic *hh* expression anterior to the MF gives rise to a progressing ectopic MF (Heberlein et al., 1995; Pignoni and Zipursky, 1997).

so and *eya* also have been shown to be required for initiation and propagation of the MF (Pignoni et al., 1997), and both are expressed at the posterior margin before initiation and later in front of the MF (Bonini et al., 1993; Serikaku and O'Tousa, 1994). Furthermore, ectopic MFs are found in ectopic eyes induced by *so* together with *eya* (Pignoni et al., 1997). These data suggest that a feedback loop between *hh* and *so*/*eya* might influence the proper initiation and propagation of the MF. Consistent with that, *hh* fulfilled our criteria to be a putative SO target. Both sites found within the *hh* locus showed almost perfect conservation among seven *Drosophila* species (see Materials and methods) and were able to compete for SO binding in EMSA (Fig. 4B,C, *hh* first, *hh* second). In addition, we found these sites to be located within an area that is deleted in the hh^1 (*bar-3*) mutant allele, a weak *hh* allele affecting adult flies. The corresponding deletion can be found in the first intron of the *hh* gene (Mohler, 1988; Lee et al., 1992). The predominant phenotype of hh^1 is a reduction of eye facets. Therefore, the deletion leading to the hh^1 allele may affect an eye-specific enhancer of *hh* (Renfranz and Benzer, 1989).

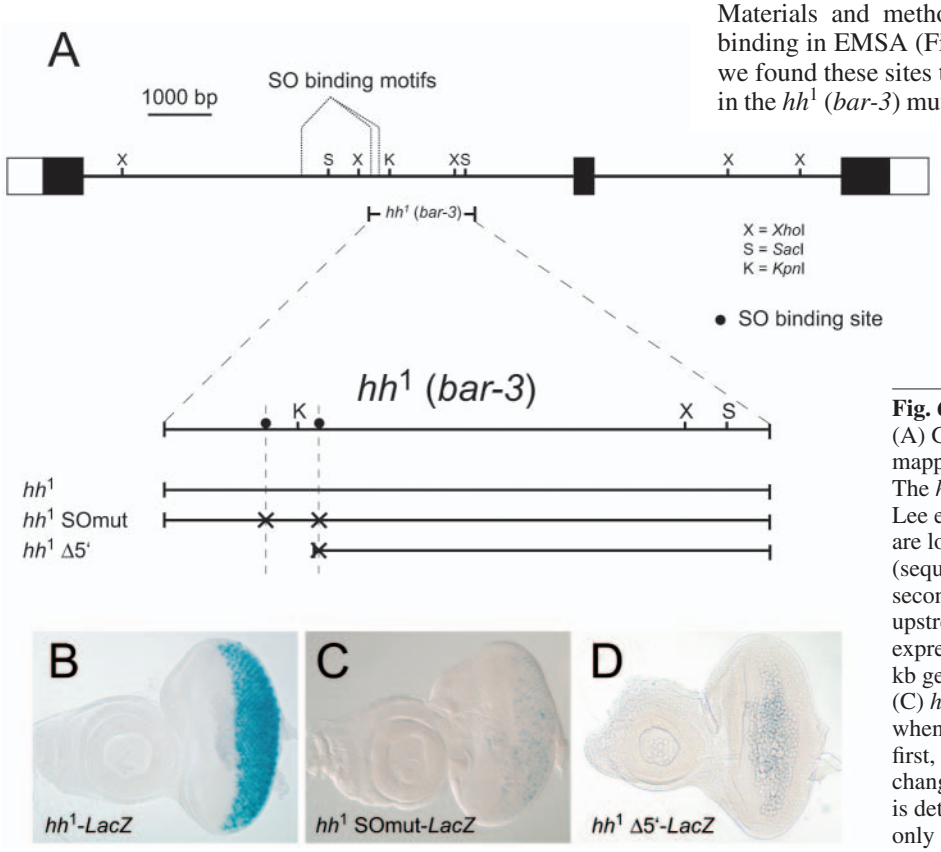

Fig. 6. *hh* contains functional SO-binding sites. (A) Genomic map of the *hh* locus. The physical mapping is indicated as: X, *Xho*I; S, *Sac*I; K, *Kpn*I. The hh^1 (*bar-3*) deletion is mapped according to Lee et al. (Lee et al., 1992). Two SO-binding sites are located within the region deleted in hh^1 (sequences are given in Fig. 4C: *hh* first, *hh* second). One additional SO-binding site is found upstream of the hh^1 deletion. (B) hh^1-*lacZ*: *lacZ* expression posterior to the MF is mediated by 1.4 kb genomic DNA from the region deleted in hh^1. (C) hh^1 SOmut: expression is hardly detectable when the two SO-binding sites are mutated. (*hh* first, GAG is changed to CCC; *hh* second, GAT is changed to CCC). (D) hh^1 Δ5′- *lacZ*: no expression is detected from a reporter construct containing only one mutated SO-binding site

This idea is supported by the observation of Kango-Singh et al. that in hh^1 mutant flies targeted expression of *ey* fails to induce ectopic eyes (Kango-Singh et al., 2003).

We chose to clone 1.4 kb out of the hh^1 deletion encompassing the two *so* sites and ligated this fragment to the *lacZ* reporter gene. Expression of the resulting hh^1-*lacZ* construct was found exclusively in the eye disc in cells posterior to the MF (Fig. 6B), in perfect agreement with the observation of Lee et al. that *hh* is expressed in differentiating photoreceptor cells (Lee et al., 1992).

Next we mutated the two SO-binding sites within the hh^1-*lacZ* construct by replacement of GAG by CCC (*hh* first, nt. 8-10 in Fig. 4C) and GAT by CCC (*hh* second, nt. 8-10 in Fig. 4C), resulting in the mutated construct hh^1 SOmut-*lacZ* (Fig. 6A hh^1 SOmut). In four out of 10 transgenic lines, the resulting construct had lost its capability to induce *lacZ* expression. In six out of 10 transgenic lines, weak expression in the same pattern as the wt construct was detectable (Fig. 6C). This residual activity is probably due to a weak interaction of SO with the mutated binding sites similar to that seen in our cell culture assays (Fig. 3J). When we tested a construct in which the first SO-binding site was deleted and the second was mutated, this residual expression was lost completely (Fig. 6A hh^1 $\Delta 5'$ and Fig. 6D).

These results show that the two SO-binding sites within the first intron of the *hh* gene are functional in vivo and sufficient to mediate expression, reflecting the known *hh* expression pattern in the eye part of late third instar eye imaginal discs. This strongly suggests that *hh* is directly regulated by *so*.

Discussion

so autoregulation is essential for ocellus development

so gene activity is crucial for proper development of the entire visual system of *Drosophila melanogaster*, including the larval visual system (Bolwig's organ), the optic lobe, the compound eye and the ocellus. Previous work from our laboratory identified an eye-specific enhancer of *so*, so10, that is regulated by *ey* and *toy* (Niimi et al., 1999; Punzo et al., 2002). When used as a driver for *so*, so10 is only sufficient to rescue eye development of so^1 mutant flies but not ocellus development. Here we show that a fragment of 27 bp, soAE, found downstream of so10, was sufficient to rescue the entire mutant phenotype of so^1 mutant flies when combined with so10. We show that the SO protein itself bound to soAE and, in cooperation with EYA, formed an autoregulatory feedback loop that is essential for ocellus development.

As SO binds to its own enhancer and autoregulation cannot initiate expression of a gene, the initiation of *so* expression in the ocellar region must be triggered by other means. We propose the following model. Initiation of *so* expression in early third instar eye discs is mediated by *ey* and *toy* throughout the eye disc, including the ocellar precursors. Later, after this first induction, *so* cooperatively with *eya* can maintain its own expression in the ocellar region by a positive autoregulatory feedback. Thus, the initiation of *so* expression is mediated by so10, whereas for the maintenance of *so*, soAE is required. This is supported by the observation of Punzo et al. that so10, which is activated by *ey* and *toy* mediates expression in early third instar larvae all over the eye disc and only later gets restricted to the compound eye part (Punzo et al., 2002).

In this model the specificity of *so* expression for ocellar precursor cells is provided by the expression pattern of *eya*; EYA protein can be found only in the ocellar region itself, where it specifically interacts with SO, and no EYA is present in the proximity of these cells. The importance of *eya* is further strengthened by the fact that eya^4 mutants show an eyeless and ocelliless phenotype (Zimmerman et al., 2000). Therefore, to elucidate the mechanisms that control gene expression specifically in ocellar precursor cells, additional studies on *eya* are required.

Direct feedback regulation of *eyeless* by *sine oculis* in eye development

Positioned at the top of the hierarchy of the retinal determination network, *ey* is a potent inducer of ectopic eyes and is able to directly induce *so* and *eya*. Like *ey*, *so* and *eya* are able to induce ectopic eyes but only when co-expressed; *so* alone fails to do so.

To accomplish this induction, *eya* and *so* need to feed back on *ey*, obviously by binding to the eye-specific enhancer of *ey*. In an ectopic situation, the feedback of *so*/*eya* on *ey* is strong enough to induce *ey* for ectopic eye formation.

The function of this feedback loop in normal eye development remains to be elucidated. *so* and *eya* are both expressed posterior to the furrow and are important for neuronal development (Pignoni et al., 1997). Nevertheless, *ey* is tuned down posterior to the MF. The activity of the *so*-binding site in the *ey* gene might, therefore, be suppressed by other factors or by *so* itself during cellular differentiation posterior to the furrow. As co-expression of *ey*, *so* and *eya* is elevated only in a few cells in front of the MF and within the MF, a possible role for this feedback loop might be to boost *ey* expression in front of and within the furrow, which leads to a strengthening of *so* and *eya* expression in just a few cell rows.

For proper eye development, a well-balanced expression level of the genes belonging to the retinal determination network is crucial. Loss-of-function mutations, as well as overexpression of the eye specification genes *ey*, *eya*, *so* or *dac* during eye development, impede proper determination of the organ and result in a reduction in eye size (Halder et al., 1998; Curtiss and Mlodzik, 2000). Therefore, we hypothesize that a feedback loop of *so* on *ey* is also important for the fine-tuning of *ey* expression during normal eye development. Due to its previously proposed ability to activate as well as to repress the expression of genes (Silver et al., 2003), *so* is a potent regulator in this context.

Linking the transcriptional cascade to signal transduction by *hedgehog*

decapentaplegic (*dpp*) signalling plays an important role in the complex regulatory network of eye development. In *dpp* mutant eye discs, *so*, *eya* and *dac* are not expressed (Chen et al., 1999), whereas *dpp* is able to initiate ectopic expression of *so* and *dac* when expressed at the anterior margin of the eye disc (Chanut and Heberlein, 1997; Pignoni and Zipursky, 1997). Conversely, *dpp* expression is patchy in eye discs of *eya* and *so* loss-of-function mutants, suggesting that *eya* and *so* are required for either initiation or maintenance of *dpp* at the

posterior disc margin before MF initiation (Pignoni et al., 1997; Hazelett et al., 1998).

hh is required for *dpp* expression at the posterior margin before MF initiation (Borod and Heberlein, 1998), and *dpp* expression is induced by *hh* in the MF (Heberlein et al., 1993), supporting the assumption that *dpp* is downstream of *hh* signalling. As *dpp* alone is not able to rescue posterior margin clones of *hh*, there have to be more eye-relevant target genes of *hh* signalling during third instar larval development. *dpp* in combination with *eya* can restore photoreceptor differentiation in posterior margin clones lacking *smoothened* (*smo*) expression (*smo* is a cell-autonomous receptor of *hh* signalling). This shows that *dpp*, in combination with *eya*, is able to bypass the requirement of *hh* during eye development (Pappu et al., 2003). Taken together, it is evident that *hh* is necessary for proper *eya* and *dpp* expression, both of which can induce *so*, and it contains two *so* target sites. We therefore hypothesized that the transcriptional complex consisting of EYA and SO, as with *ey* might also feed back on *hh* in order to drive the furrow during late eye development. In this model the genetic cascade starts with *hh*, which induces *dpp* and *eya*, moves on to *so* and through the SO/EYA complex feeds back to *hh* in order to maintain *hh* expression as a driving force of the MF.

The impact of these *so*-binding sites in the *hh* enhancer on eye development becomes evident from the fact that hh^1 (*bar-3*) mutant flies have smaller eyes. The severity of the hh^1 mutant phenotype is probably diminished by an additional putative SO-binding site that resides outside the area covered by the hh^1 deletion (Fig. 6A, SO-binding motifs). If functional, this region (5′ to the hh^1 deletion) might mediate a residual *hh*-expression that overcomes the loss of the other sites to some extent. Another possible explanation for the rather weak hh^1 phenotype might be that the feedback of *so* on *hh* is not crucial for MF initiation but still might be of importance for the well-balanced expression of *hh* during MF propagation.

A general theme of Six-gene target sites

so belongs to the Six gene family. All Six proteins are characterized by a Six domain and a Six-type homeodomain, both of which are essential for specific DNA binding and protein-protein interaction. Based on the amino acid sequence of their homeodomain and Six domain, the Six genes were divided into three subgroups. Each of the three *Drosophila* homologues can be assigned to one of these subgroups: *so* is mostly related to *Six1/2*, *optix* to *Six3/6* and *DSix4* to *Six4/5* (reviewed by Kawakami and Kobayashi, 1998).

Promoter analyses of the mouse Six genes (*Six1*/2, *Six4*/5) revealed similar target sequence specificities for these mammalian counterparts of *so*. *Six2*, *Six4/AREC3* and *Six5* effectively bind to the same target sequence in a DNA fragment called ARE (Atpla1 regulatory element) that can be found in the Na,K-ATPase α1 subunit gene (Fig. 4C, ARE fragment) (Suzuki-Yagawa et al., 1992; Kawakami et al., 1996a; Kawakami et al., 1996b; Harris et al., 2000). *Six1* and *Six4* have been shown to bind to MEF3 sites in the myogenin and in the aldolase A muscle-specific (pM) promoters (Fig. 4C, MEF3 site) (Spitz et al., 1998). Recently, mammalian *Six4* has been shown to bind additionally to the transcriptional regulatory element X (TreX) within the muscle creatine kinase (MCK) enhancer (Fig. 4C, Trex) (Himeda et al., 2004).

Comparison of all these sites confirmed that the three nucleotides we suggest are the most important for SO-DNA interaction are present and conserved within these motifs (nt. 1, 4 and 9 in Fig. 4C). In the case of the MEF3 site, which comprises seven nucleotides that include only two of the nucleotides important for SO-DNA interaction (nt. 4 and 9 in Fig. 4C), we looked up the original publications to check if the third conserved nucleotide is also present, and in most of the cases were able to verify its conservation (Hidaka et al., 1993; Spitz et al., 1998; Himeda et al., 2004). In fact, there is only one exception published in a study that describes two *Six2* target sites (Brodbeck et al., 2004).

Nevertheless, by combining the vast majority of previous studies describing protein-DNA interaction of *Six* genes and our study of SO-DNA interaction, we infer that SO, *Six1*, *Six2*, *Six4* and *Six5* have very similar DNA-binding properties. In the case of *so*, we propose that the consensus sequence GTAANYNGANAY(C/G) marks a good starting point for the identification of additional targets of SO, thereby helping to unravel the complex genetic interactions that orchestrate the development of the visual systems of *Drosophila*.

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