

Dual regulation and redundant function of two eye-specific enhancers of the *Drosophila* retinal determination gene *dachshund*

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Summary

Drosophila eye development is controlled by a conserved network of retinal determination (RD) genes. The RD genes encode nuclear proteins that form complexes and function in concert with extracellular signal-regulated transcription factors. Identification of the genomic regulatory elements that govern the eye-specific expression of the RD genes will allow us to better understand how spatial and temporal control of gene expression occurs during early eye development. We compared conserved non-coding sequences (CNCSs) between five *Drosophilids* along the ~40 kb genomic locus of the RD gene *dachshund* (*dac*). Our analysis uncovers two separate eye enhancers in intron eight and the 3' non-coding regions of the *dac* locus defined

by clusters of highly conserved sequences. Loss- and gain-of-function analyses suggest that the 3' eye enhancer is synergistically activated by a combination of *eya*, *so* and *dpp* signaling, and only indirectly activated by *ey*, whereas the 5' eye enhancer is primarily regulated by *ey*, acting in concert with *eya* and *so*. Disrupting conserved So-binding sites in the 3' eye enhancer prevents reporter expression *in vivo*. Our results suggest that the two eye enhancers act redundantly and in concert with each other to integrate distinct upstream inputs and direct the eye-specific expression of *dac*.

Key words: *dac*, Enhancer, Eye, *Drosophila*, Retina

Introduction

The compound eye of *Drosophila melanogaster* is composed of a regular hexagonal array of 750 to 800 individual light-sensing ommatidia (Wolff and Ready, 1993). The initiation of eye development in *Drosophila* is controlled by a set of conserved tissue-specific genes. These genes, *twin-of-eyeless* (*toy*), *eyeless* (*ey*), *eyes absent* (*eya*), *sine oculis* (*so*) and *dachshund* (*dac*), function in a complex genetic regulatory hierarchy called the retinal determination (RD) network (Bonini et al., 1993; Cheyette et al., 1994; Czerny et al., 1999; Mardon et al., 1994; Quiring et al., 1994). The precise regulation of RD gene expression is crucially dependent on the integration of extracellular signaling inputs with cell-autonomous cues. Transcriptionally, such integration is largely directed by non-coding DNA sequences that include promoters and enhancers (Arnosti, 2003; Kadonaga, 2004). Enhancers are usually non-coding DNA stretches within the genome that are bound directly by upstream transcription factors and can regulate gene expression from a distance (Blackwood and Kadonaga, 1998). Transcription factors that bind enhancers are regulated by either an extracellular signal or are signal independent.

The adult *Drosophila* eye develops from an epithelial

monolayer called the eye imaginal disc, which is derived from a group of about 20 cells set aside during embryonic development (Garcia-Bellido and Merriam, 1969). Photoreceptor differentiation begins at the posterior margin of the eye disc in third instar larvae and proceeds anteriorly following a dorsoventral groove termed the morphogenetic furrow (MF) (Ready et al., 1976). The RD network consists of a series of gene regulatory events, which are initially linear and then progress to include extensive cross and feedback regulation, resulting in the conversion of undifferentiated epithelial cells to retinal cells (Chen et al., 1997; Halder et al., 1998; Pignoni et al., 1997). In addition to the cell-autonomously acting RD genes, extracellular signaling molecules such as Hedgehog (Hh), Decapentaplegic (Dpp) and Wingless (Wg) are also required for coordinating growth, proliferation, patterning and cell fate specification during retinal morphogenesis in *Drosophila* (Baonza and Freeman, 2002; Borod and Heberlein, 1998; Chanut and Heberlein, 1997; Dominguez and Hafen, 1997; Heberlein et al., 1995; Heberlein et al., 1993; Pignoni and Zipursky, 1997b; Treisman and Rubin, 1995).

dac is the most downstream member of the RD network to be identified in *Drosophila* (Chen et al., 1997). *dac*-null mutants in *Drosophila* develop with severely truncated legs and

dramatically reduced or absent eyes (Mardon et al., 1994). In addition, *dac* mutants display defects in genital disc, mushroom body and antennal development (Dong et al., 2001; Dong et al., 2002; Kurusu et al., 2000; Martini et al., 2000; Noveen et al., 2000). Misexpression of *dac* is sufficient to induce ectopic eye development in non-retinal tissue (Shen and Mardon, 1997). *dac* encodes a nuclear protein that contains a conserved domain (Dachshund Domain 1 or DD1) which resembles DNA-binding motifs similar to those found in the winged helix/forkhead subfamily of helix turn helix proteins (Kim et al., 2002). In addition, a second conserved domain (Dachshund domain 2 or DD2) in *Dac* can form a complex with *Eya*, although recent studies have suggested that DD2 is largely dispensable for *Dac* protein function in vivo (Chen et al., 1997; Tavsanli et al., 2004). *Dac* is expressed in multiple tissues during *Drosophila* development, including the embryo, eye, leg, wing, antenna, male and female genital discs, and the mushroom bodies in the brain (Keisman and Baker, 2001; Kurusu et al., 2000; Mardon et al., 1994; Martini et al., 2000; Noveen et al., 2000). In the eye disc, *Dac* is expressed at the posterior margin prior to the initiation of the MF. After initiation of photoreceptor differentiation, *Dac* is expressed in the MF and its expression tapers both anterior and posterior to the furrow (Mardon et al., 1994).

Genetic analysis suggests that *Dac* expression in the eye is controlled by other members of the RD gene network. *Dac* expression is lost in *eya* or *so* mutant eye discs, and misexpression of *ey* or *eya*, but not *so* alone, leads to the inappropriate activation of *Dac* expression (Chen et al., 1997). Moreover, ectopic expression of a combination of *eya* and *so* leads to the synergistic activation of *Dac* (Chen et al., 1999). Furthermore, *dpp* signaling can strongly synergize with *eya* and *so* to dramatically activate the expression of *Dac* in an ectopic expression assay, and *dpp* is required for *dac* expression in the eye disc (Chen et al., 1999). Last, the ability of *ey* to activate *Dac* expression is highly reduced but not completely eliminated in *eya*² mutants (Chen et al., 1997). Taken together, these results suggest that *dac* regulation is under the control of *ey*, *eya* and *so* coupled with extracellular inputs from *Dpp* signaling. Despite a host of genetic data, the exact nature of the protein complexes that regulate *dac* expression in the eye are still unknown. It has been proposed that *So* acts as the DNA binding unit of a protein complex that includes *Eya*, which in turn is thought to act as a transactivator (Chen et al., 1997). Furthermore, the roles of *ey* and downstream effectors of *dpp* signaling in the regulation of *dac* expression in the eye remain to be characterized.

The isolation of genomic elements that direct the eye-specific expression of the RD genes provide important tools for deciphering the molecular interactions that regulate early eye specification and determination. The eye enhancers of *ey*, *eya*, and *so* have been defined in some detail (Bui et al., 2000; Hauck et al., 1999; Niimi et al., 1999; Punzo et al., 2002; Zimmerman et al., 2000). These studies used eye-specific alleles of these genes to identify genomic lesions that disrupt regulatory elements that direct transcription in the eye. However, despite multiple attempts, no eye-specific alleles of *dac* have been isolated to date. Therefore, we turned to the use of functional genomics to identify the eye-specific regulatory elements of the *dac* gene in *Drosophila*. We hypothesized that crucial cis-regulatory non-coding sequences are highly

sensitive to mutational changes and remain largely unaltered over millions of years of evolution. Therefore, significant conservation in non-coding sequences among evolutionarily disparate species is a strong indicator of functional constraint and often uncovers cis-regulatory elements. We compared the sequences of the ~40 kb *dac* genomic region among five different species of Drosophilids to uncover highly conserved non-coding sequences (CNCSSs). Two such CNCSSs define eye-specific regulatory elements in the *dac* genomic locus. We demonstrate that one of these eye enhancers maps to the 3' non-coding region of the *dac* locus and is under the genetic control of *eya*, *so* and *dpp* signaling. Two potential *So*-binding sites are embedded within an ~40 bp conserved stretch in this 3' eye enhancer and disruption of these binding sites abolishes enhancer activity in vivo. Surprisingly, in spite of the 3' eye enhancer being completely deleted in *dac*⁷ homozygotes, these animals develop with only moderately disrupted eyes. Our genomic analysis identifies a second, independent 5' eye enhancer that maps to intron 8 of the *dac* locus and that acts redundantly and in concert with the 3' eye enhancer. This 5' eye enhancer is not deleted in *dac*⁷ mutants and is regulated by a combination of *ey*, *eya* and *so*. Our results highlight the power of functional genomics to uncover genomic regulatory elements, especially in the absence of tissue-specific genetic mutants and in cases with redundant enhancers.

Materials and methods

Comparative genomics

Drosophila erecta and *willistoni* clones were isolated from the BACPAC Resources 50 kb fosmid libraries. High-density filters were probed with a labeled fragment of exon 2 of *D. melanogaster dac* using standard hybridization techniques. For *D. virilis*, this probe was used on an amplified lambda library provided by Ron Blackman and Thomas Kaufman (Thummel, 1993). Positive clones were fingerprinted and end sequenced. Appropriate clones were shotgun sequenced to ~10× coverage. Conserved regions were identified using the BLASTZ program using the command line parameters H=2200 K=2200 to increase sensitivity (Schwartz et al., 2003). *D. pseudoobscura* sequence was obtained by BLAST searches at <http://www.hgsc.bcm.tmc.edu/projects/drosophila/>. A BioPerl script was used to mask coding regions, to find conserved non-coding sequences (CNCSSs) in all clones, to cluster nearby CNCSSs together and to perform T-COFFEE alignments of all CNCSS blocks (Notredame et al., 2000; Stajich et al., 2002). Graphic representations of enhancer conservation were generated using the AVID/mVISTA server at <http://www.gsd.lbl.gov/vista/> (Bray et al., 2003; Dubchak et al., 2000; Frazer et al., 2004; Mayor et al., 2000).

Drosophila genetics

All *Drosophila* crosses were carried out at 25°C on standard media. The *mad*¹⁻² *FRT40A* recombinant stock was provided by Marek Mlodzik (Curtiss and Mlodzik, 2000). The nature of the *dac*³ and *dac*⁷ mutant alleles were previously described (Tavsanli et al., 2004). The presence of intron 8 in *dac*⁷ mutants was confirmed by PCR on genomic DNA prepared from *dac*⁷ homozygotes with intron 8 specific primers. A similar assay was used to demonstrate the deletion of exon 9, placing the deletion in *dac*⁷ beyond intron 8 but including exon 9 (data not shown). The *30A-GALA*, *UAS-ey*, *UAS-eya* and *UAS-so* flies were previously described (Brand and Perrimon, 1993; Pignoni et al., 1997). *UAS-eya* and *UAS-so* stocks were provided by Francesca Pignoni and Larry Zipursky. All other stocks were obtained from the Bloomington stock center. Flies containing multiple transgenes were generated by meiotic recombination using eye color as an initial

selection. Polymerase chain reaction (PCR) with gene-specific primers was used to confirm genotypes. Ectopic expression followed by antibody staining (where possible) was used to confirm expression of individual genes from recombinant chromosomes.

P-element vectors and reporter transgene construction

Genomic fragments spanning the *dac* locus were subcloned into appropriate P-element reporter vectors using convenient restriction sites. Three different P-element reporter vectors were used in this study: *pCasper-hs43-AUG-βGal* (Thummel et al., 1988), *pH-Pelican* and *pH-Stinger* (Barolo et al., 2000). The reporters in *pH-Pelican* and *pH-Stinger* are β-galactosidase and nuclear GFP, respectively. To generate an HA-*dac* version of the enhancer-reporter construct, we deleted the entire GFP-coding region from the *pH-Stinger* vector and replaced it with an HA tag in frame with the *dac* cDNA. This vector still contains the 390 bp eye enhancer and a minimal hsp70 TATA promoter. Detailed information about this vector is available upon request.

Sub-fragments of 1 kb or less were obtained by PCR amplification using appropriate primers with artificial *EcoRI-BamHI* restriction site tails. PCR products were digested with *EcoRI* and *BamHI*, and ligated with similarly digested P-element vectors. Positive clones were sequenced to confirm sequence integrity and orientation. Fragments with mutated binding sites were obtained by overlap extension PCR as previously described (Ho et al., 1989). Subcloned PCR products were sequenced to confirm the sequence and orientation. Transgenic flies were obtained by standard transgenic injection techniques (Rubin and Spradling, 1982). A minimum of three independent transgenic lines were tested for reporter activity for each construct.

β-Galactosidase activity staining

Imaginal discs from second or third instar larvae were dissected into phosphate buffered saline [PBS; 0.1 M phosphate (pH 7.2), 150 mM NaCl], fixed for 20 minutes in 1% glutaraldehyde in PBS, and washed three times for 10 minutes each in PBS. The imaginal discs were then incubated in pre-warmed active staining solution (10 mM Na₂HPO₄, 10 mM NaH₂PO₄, 150 mM NaCl, 1 mM MgCl₂, 3 mM K₃[Fe(CN)₆], 3 mM K₄[Fe(CN)₆]) with 0.1% X-gal in N,N-dimethylformamide. The discs were allowed to stain for appropriate times up to 16 hours and then washed in PBS three times for 10 minutes each wash. The discs were allowed to equilibrate in 80% glycerol in PBS overnight before they were mounted on glass slides.

Immunohistochemistry and scanning electron microscopy

Primary antibodies used in this study were: monoclonal mouse anti-Dachshund (mAbdac2-3: 1:200, Developmental Studies Hybridoma Bank), rabbit anti-β-galactosidase (1:1000; Cappel), rabbit anti-GFP (Molecular Probes), chicken anti-GFP (Upstate) and mouse anti-HA (Covance). Conjugated goat anti-mouse, chicken and rabbit fluorescent secondary antibodies were ALEXA 488 (Molecular Probes), Cy3 (Jackson Immunochemicals) or Cy5 (Jackson Immunochemicals), all at 1:600 dilution. HRP-conjugated goat anti-mouse antibodies were used as previously described (Mardon et al., 1994). Discs were then processed as previously described (Frankfort et al., 2001). Fluorescent images were captured with a Zeiss LSM 510 confocal microscope. All other images were captured on a Zeiss Axioplan microscope with Nomarski optics. All images were processed with Adobe Photoshop software. Adult flies were prepared for electron microscopy as previously described (Kimmel et al., 1990).

Results

The 3' non-coding region of the *dac* locus contains an eye-specific enhancer

To understand the molecular regulation of *dac* in various

tissues, we sought to uncover the genomic regulatory elements that control *dac* expression. The genomic organization of the *dac* locus is shown in Fig. 1A. *dac* comprises 12 exons and the last exon is separated from the 3' neighboring *Idgf* gene complex by ~13.3 kb of non-coding genomic DNA. The 5' neighbor, predicted gene CG4580, is separated by 2.2 kb from the first exon of *dac*. The 5' gene *tpr2* is 3.7 kb upstream of the first coding exon of *dac* (not shown). Prior to using a functional genomics approach to uncover novel enhancer elements in the *dac* locus, we generated transgenic flies that carry large genomic fragments spanning the entire *dac* locus cloned upstream of a minimal, *heat shock protein* (*hsp*) TATA promoter driving a β-galactosidase reporter (Thummel et al., 1988). Third instar imaginal discs from these transgenic lines were then tested for β-galactosidase activity. We found that a 16.6 kb *NotI-SpeI*, genomic fragment from the 3' end of the *dac* locus contained reporter activity in patterns reminiscent of endogenous *dac* transcript and protein expression in the eye, lamina, leg, antenna and wing (Fig. 1A and data not shown; see Materials and methods). Using restriction sub-fragments that span this 16.6 kb region, we were able to narrow the eye-lamina enhancer to a 1.9 kb fragment that contains eye-specific reporter activity posterior to the morphogenetic furrow (*3EE^{1.9 kb}*; see Fig. S1A in the supplementary material). All the 3' eye enhancer fragments are henceforth denoted by 3EE followed by their length in superscript. Six overlapping, PCR generated sub-fragments that span the 1.9 kb eye enhancer were then tested for reporter activity. A 390 bp sub-fragment (*3EE^{390 bp}*) within the 1.9 kb fragment contains eye enhancer activity. Further dissection of the *3EE^{390 bp}* fragment with smaller PCR fragments uncovered a 194 bp eye reporter fragment (*3EE^{194 bp}*; see Fig. S1A in the supplementary material). However, all the eye-specific enhancer fragments described above drive reporter expression only posterior to the MF in the eye, suggesting that these fragments lack important sequences that regulate *dac* expression anterior to the MF (Fig. 1D and data not shown).

We then used a functional genomics approach to uncover new genomic non-coding sequences across the entire the *dac* locus that are required for tissue specific enhancer activity (see Materials and methods). We hypothesized that non-coding regions that remain unaltered over the course of millions of years of evolutionary time are under functional constraint and define important regulatory protein binding targets. We compared the conservation of non-coding DNA across the ~40 kb *dac* genomic locus among five related species of Drosophilids, *D. melanogaster*, *D. pseudoobscura*, *D. erecta*, *D. willistoni*, and *D. virilis* that represent over 60 million years of evolutionary time (see Materials and methods). As we were primarily interested in uncovering eye enhancer fragments, we initially focused on sequences within *3EE^{1.9 kb}*. The VISTA output of pairwise comparisons to *D. melanogaster* along *3EE^{1.9 kb}* is shown in Fig. 1C (Mayor et al., 2000). Six conserved non-coding sequences (CNCSs) are present in *3EE^{1.9 kb}*. To test the correlation of CNCSs with enhancer activity, we cloned an 850 bp fragment (*3EE^{850 bp}*) that contains all six CNCS blocks upstream of a minimal promoter driving expression of a GFP or β-galactosidase reporter. Transgenic flies were then tested for reporter (GFP or β-galactosidase) expression in the eye. *3EE^{850 bp}*, like *3EE^{1.9 kb}*, is expressed only posterior to the furrow (Fig. 1D). However, a smaller 659

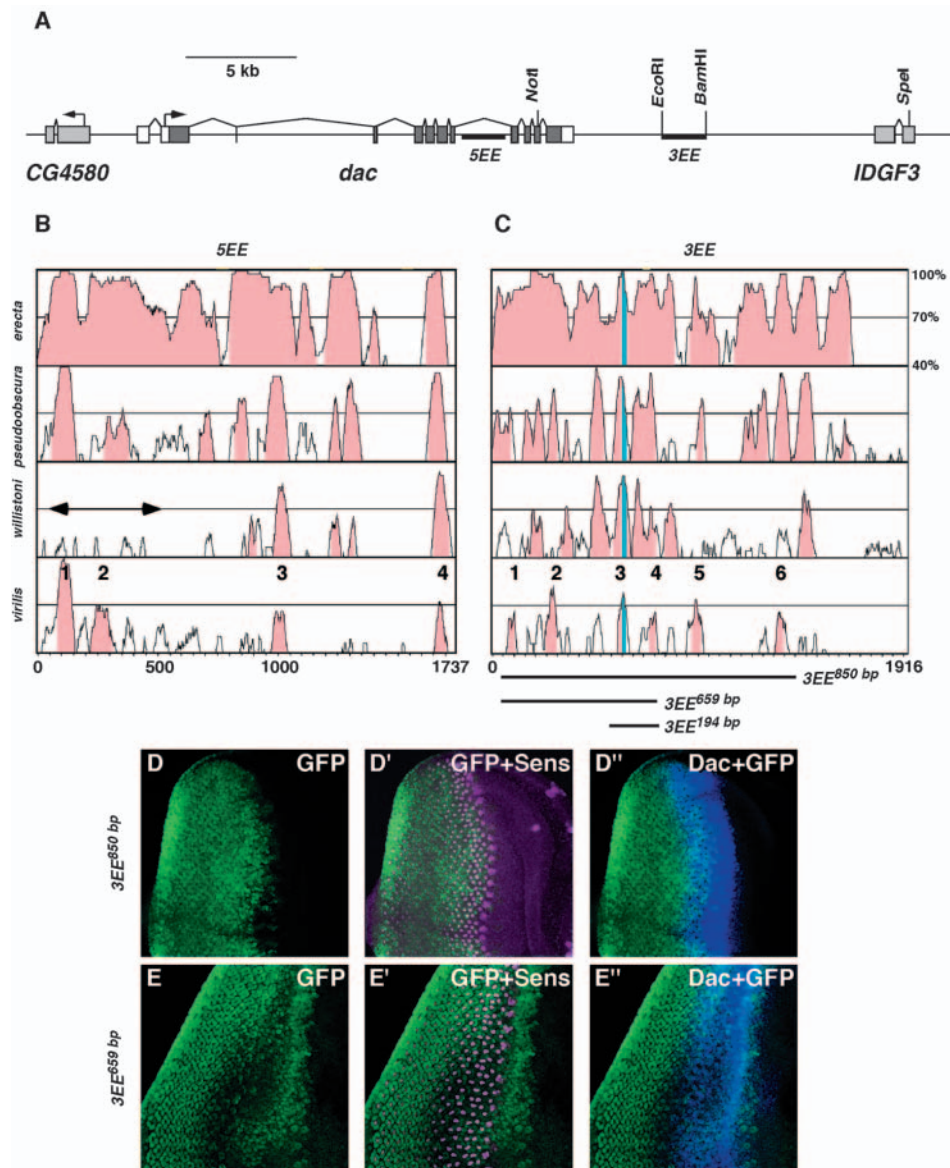


Fig. 1. Conserved non-coding sequences in the *dac* locus uncover two eye enhancers. (A) The *dachshund* genomic locus, with 5' and 3' eye enhancers indicated (see text for details). (B) AVID/mVISTA representation of the 5' eye enhancer (5EE). The numbered peaks indicate four areas of significant conservation. A small inversion in *D. willistoni* masks the first two peaks. (C) AVID/mVISTA representation of the 3' eye enhancer (3EE). The numbered peaks indicate six areas of significant conservation and two sub-fragments tested are shown. $3EE^{850 bp}$ contains all six CNCS blocks and is expressed only posterior to the MF. $3EE^{659 bp}$ contains the first four CNCS blocks and is expressed both anterior and posterior to the MF. The smallest active enhancer fragment identified is 194 bp ($3EE^{194 bp}$) and contains CNCS blocks 3 and 4. Regions of significant conservation are indicated in pink (B,C), and the predicted Sine oculis-binding site is highlighted in bright blue and is within CNCS block 3. (D,E) Representative third instar eye discs from $3EE^{850 bp}$ -GFP (D) and $3EE^{659 bp}$ -GFP (E) larvae triple labeled with GFP (green, D,E), Sens (magenta, D',E') and Dac (blue, D'',E''). GFP expression in $3EE^{659 bp}$ eye discs is detected anterior to the earliest Sens expression and overlaps with anterior Dac expression (E',E''). By contrast, GFP expression in $3EE^{850 bp}$ eye discs is not detected anterior to the anterior-most Sens-expressing column (D').

bp fragment ($3EE^{659 bp}$) that contains only the first four CNCS blocks drives strong expression of GFP in the eye disc both anterior and posterior to the furrow, similar to endogenous Dac protein expression (Fig. 1E). The smallest active enhancer

$3EE^{194 bp}$ contains only the third and fourth CNCS blocks, and is expressed only posterior to the MF in third instar eye discs (data not shown). These results suggest that $3EE^{659 bp}$ (expressed both anterior and posterior to the MF) lacks repressor binding sites contained in the 1.9 kb eye enhancer that normally inactivate reporter expression anterior to the furrow. In addition, these results suggest that $3EE^{194 bp}$ (expressed only posterior to the MF) further lacks positive regulatory sites normally present in $3EE^{659 bp}$ that are required for reporter expression anterior to the MF. Although the expression of endogenous Dac protein is decreased posterior to the furrow, GFP expression driven by $3EE^{659 bp}$ persists all the way to the posterior margin of the eye disc. To rule out the possibility that the 3' enhancer lacks repressive elements that normally downregulate *dac* expression posterior to the furrow, we generated transgenic flies in which the GFP reporter was replaced by an *HA-dac* reporter (see Materials and methods). HA-Dac reporter expression, visualized using an anti-HA antibody, reveals a rapid downregulation of HA staining posterior to the MF (see Fig. S1B-D in the supplementary material). Thus, we conclude that GFP expression far posterior to the MF in $3EE$ -GFP transgenic eye discs occurs because of the perdurance of GFP protein and/or transcript, and not because of the lack of negative regulatory elements in the 3' enhancer. We hypothesized that a deletion of the 3' eye enhancer would block *dac* expression in the eye, thereby causing eye-specific defects. We therefore examined known *dac* mutants to identify an allele that contains genomic lesions in this 3' eye enhancer but does not affect the function of the protein.

The *dac*⁷ mutant contains a large deletion in the 3' region of the *dac* genomic locus that includes the 3' enhancer

The eye enhancers of *ey*, *eya* and *so* have been defined through eye-specific alleles of these genes (Cheyette et al., 1994; Quiring et al., 1994; Zimmerman et al., 2000). Such eye-specific mutants often disrupt genomic regulatory sequences that direct expression of the transcript to the eye imaginal disc. Despite two large-scale F1 genetic

screens over deficiencies spanning the *dac* locus, we have been unable to isolate eye-specific alleles of *dac*. However, in a previous study aimed at analyzing the structure and function of the conserved domains of the Dac protein, we molecularly characterized several *dac* mutant alleles to identify coding region mutants that truncate the Dac protein prematurely (Tavsanlı et al., 2004). One such allele, *dac*⁷, is a large deletion in the *dac* locus that begins in exon 9 and extends beyond the neighboring *Idgf* genes (Fig. 2A; the distal extent of this deletion has not been mapped). In light of our finding that the 3' non-coding region of *dac* contains cis-regulatory elements, *dac*⁷ mutants provide us with a tool to analyze the role of this 3' eye enhancer in an in vivo context. We hypothesized that *dac*⁷ mutants should be severe hypomorphs or null mutants owing to the lack of cis-regulatory elements. Furthermore, as the first eight exons are intact in *dac*⁷ mutants, we predicted that any *dac*⁷ transcript would encode a protein with an intact N terminus, DD1 and middle region. Previous structure-function analyses suggest that such a truncated protein is functional in vivo and can completely rescue *dac*³ null mutants (Tavsanlı et al., 2004).

*dac*⁷ homozygotes develop with only moderately disrupted eyes

Surprisingly, *dac*⁷ homozygotes develop with only moderately disrupted eyes compared with wild-type adults (compare Fig. 2C with 2B). By contrast, *dac*³ null mutants have no eyes, suggesting that the *dac*⁷ mutant is a hypomorph (Fig. 2D). We also examined the expression of Dac protein in the eye imaginal discs of *dac*⁷ homozygous larvae. A monoclonal antibody to Dac (mabdac 2-3) recognizes an epitope predicted to be present within the potentially truncated protein encoded by the *dac*⁷ transcript. Eye imaginal discs from *dac*⁷ larvae are almost identical to wild-type controls in their Dac protein expression profiles (compare Fig. 2F to 2E). *dac*³-null mutants display no detectable Dac protein (Fig. 2G). As the entire 16.6 kb 3' enhancer is completely deleted in *dac*⁷ mutants, these results suggest that additional eye-specific enhancers exist in the genome, either within the *dac* locus or outside the genomic fragments we tested.

A second eye enhancer is present in intron 8 of the *dac* genomic locus

We next extended our pairwise sequence comparison to the entire *dac* genomic locus to identify additional functionally relevant CNCSs. Multiple regions of significant conservation were found, spread along the entire locus (data not shown). We used PCR amplification to clone these CNCS-containing fragments upstream of a β -galactosidase reporter. One such fragment contains four CNCS blocks in a 1.7 kb stretch within intron 8 of the *dac* locus (called 5' eye enhancer or 5EE; Fig. 1B). Importantly, this 1.7 kb region is intact in the *dac*⁷ allele.

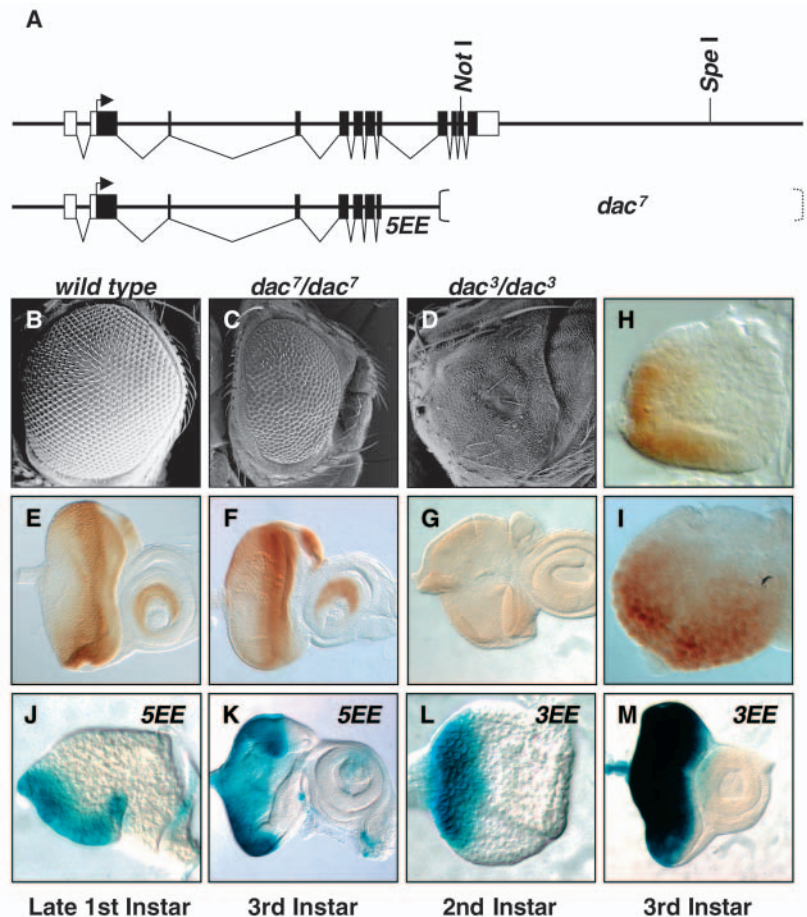


Fig. 2. The 3' eye enhancer is dispensable for *dac* activation in vivo. (A) *dac*⁷ is associated with a deletion, beginning at exon 9, that uncovers the entire 3' genomic region of *dac*. (B-D) Scanning electron micrographs (SEMs) of adult eyes from wild-type (B), *dac*⁷ (C) and *dac*³ (D) animals. (E-G) Third instar eye imaginal discs from wild type (E), *dac*⁷ (F) and *dac*³ (G) animals stained with a monoclonal Dac antibody, mab2-3. *dac*⁷ eye discs show relatively normal Dac protein expression when compared with wild-type eye discs (compare F with E). *dac*⁷ adult eyes are rough and disorganized but still contain ~50% of the normal number of ommatidia (compare C with B). By comparison, *dac*³ homozygotes express no Dac protein (G) and develop with no eyes (D). (H, I) A first instar (H) and second instar (I) eye disc of wild type shown for comparison with J and L. (J-M) Late first (J) and third (K) instar eye imaginal discs from 5EE-*lacZ* and second (L) and third (M) instar eye imaginal discs from 3EE-*lacZ* transgenic larvae. β -Galactosidase activity is detected in both first instar and third instar 5EE-*lacZ* eye discs, primarily at the posterior margin (J,K). The earliest 3EE-*lacZ* reporter expression is observed in second instar eye discs and this β -galactosidase activity persists in the third instar eye disc (L,M).

We found that third instar eye discs from 5EE transgenic larvae are positive for β -galactosidase activity, which appears to be highest at the posterior margin of the eye disc (Fig. 2M). Furthermore, late first instar and second instar 5EE transgenic eye discs also have β -galactosidase activity, suggesting that this enhancer is active prior to initiation of the MF (Fig. 2L; data not shown). A smaller fragment that contains only the first two CNCS blocks does not have eye enhancer activity (data not shown). Taken together, these results suggest that another eye enhancer exists in intron 8 of the *dac* locus that perhaps acts redundantly or in concert with the 3' enhancer. We next tested

the response of these putative eye enhancers to known upstream regulators of *dac* in the *Drosophila* eye.

The 3' *dac* eye-specific enhancer is regulated by *dpp*, *eya* and *so*

Many studies have shown that *dac* expression in the eye is regulated by upstream members of the RD network such as *ey*, *eya* and *so* (Chen et al., 1997; Chen et al., 1999; Shen and Mardon, 1997). We tested whether any of these upstream factors could activate the expression of either the 3' enhancer or the 5' (intron 8) enhancer in an ectopic expression assay. All 3' eye enhancer fragments tested respond identically in these ectopic assays and are described *3EE* for simplicity (data are shown only for the *3EE*^{659 bp} fragment). We used the previously described *30A-Gal4* line in this ectopic expression assay as it drives the expression of UAS-transgenes in a ring around the wing pouch (Chen et al., 1999; Pappu et al., 2003). *so* alone does not activate reporter expression or endogenous *Dac* in this assay (Chen et al., 1999) (data not shown). However, either *ey* or *eya* expressed alone can activate endogenous *Dac* and *3EE-GFP* in this assay, but only in regions of the wing that express *dpp* endogenously (Fig. 3A; data shown only for *ey* misexpression). Expression of a combination of *eya* and *so* induces synergistic expression of *3EE-GFP*, but this induction is also limited to regions that coincide with endogenous *dpp* expression (Fig. 3B). Thus, the 3' eye enhancer is activated similarly to endogenous *dac* in this ectopic expression assay.

Previous studies have shown that *dpp* signaling acts synergistically with *eya* and *so*, and strongly activate *dac* expression in the *30A-Gal4* ectopic expression assay (Chen et al., 1999). We tested if *3EE-GFP* is also synergistically activated by a combination of *eya*, *so* and *dpp* in the ectopic wing expression assay. As with endogenous *Dac* protein, the expression of *3EE-GFP* was strongly induced in a ring around the wing pouch upon expression of *dpp*, *eya* and *so* using the *30-Gal4* driver (Fig. 3C). These results suggest that the 3' *dac* eye-specific enhancer may be directly regulated by a combination of Dpp signaling effector molecules and upstream RD proteins. Furthermore, these results suggest that *3EE*^{194 bp} is sufficient to integrate the input from Dpp signaling with the tissue-specific factors Eya and So. Interestingly, the intracellular transducers of Dpp signaling, Mothers against Dpp (Mad) and Medea, do not bypass the requirement for Dpp in this assay (data not shown). However, a constitutively active form of the Dpp receptor, Thickveins (Tkv^{Q253D}), was just as effective as Dpp in synergistically activating GFP expression from the 3' eye enhancer in the presence of Ey (Lecuit et al., 1996) (data not shown). Therefore, we conclude that the ability of Dpp to synergize with Eya and So to activate *3EE* is dependent on downstream signaling events such as the phosphorylation of Mad. A less probable alternative is that non-canonical events downstream of Tkv mediate the synergy between Eya, So and Dpp signaling. We found no evidence for autoregulation of *3EE* by *Dac* itself, as ectopic expression of *Dac* with the *30-Gal4* driver does not activate reporter expression in the wing (data not shown).

ey acts through *eya* and *so* to regulate the 3' *dac* eye enhancer

Results from our ectopic expression analysis suggested that the 3' *dac* eye enhancer is regulated by a combination of *eya* and

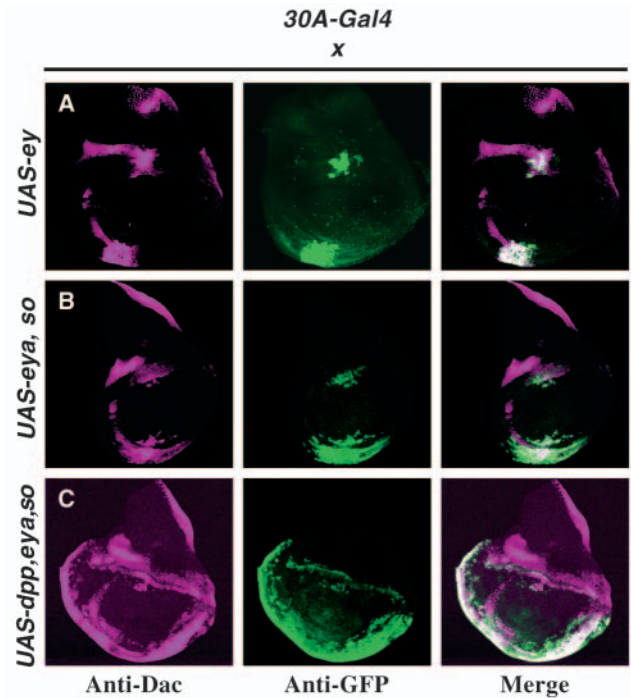


Fig. 3. Synergistic activation of *3EE-GFP* by *eya*, *so* and *dpp*. (A-C) Each set of three panels shows the same wing disc stained with anti-*Dac* (magenta) and anti-GFP (green) or a merge of the two channels. (A) Wing imaginal discs in which the expression of *UAS-ey* is driven by *30A-Gal4* show ectopic expression of *3EE-GFP* and *Dac* in two regions at the anteroposterior (AP) compartment boundary. (B) Wing imaginal discs expressing a combination of *eya* and *so* driven by *30A-Gal4* can strongly induce *3EE-GFP* and *Dac* at the AP compartment boundary where their expression coincides with endogenous *dpp* (white). (C) A combination of *dpp*, *eya* and *so* driven by *30A-Gal4* synergistically induces the expression of *3EE-GFP* and *Dac* in the entire ring around the wing pouch.

so. Consistent with this prediction, *3EE-GFP* expression is completely lost in *eya*² and *so*¹ eye-specific mutants (Fig. 4B,C). However, as has been shown previously, endogenous *Dac* protein expression is dramatically reduced but not completely eliminated in *eya*² and *so*¹ mutants (Fig. 4A-C). As our ectopic expression data suggest that *dpp* signaling acts in concert with *eya* and *so* to activate *3EE*, we tested the expression of *3EE-GFP* in eye imaginal discs cells that have lost the ability to signal downstream of the *dpp* receptor *tkv*. To disrupt *dpp* signaling, we induced *mad* mutant mitotic clones in the eye disc using a strong hypomorphic allele of *mad* (*mad*^{l-2}). We found that *3EE-GFP* expression is drastically reduced or completely lost from posterior margin *mad*^{l-2} clones (Fig. 4D). These loss- and gain-of-function experiments suggest that *3EE* is regulated by a combination of *eya*, *so* and *dpp*. Coupled with the ectopic expression data, we conclude that *3EE* activation is dependent on the canonical *dpp* signaling pathway acting synergistically with *eya* and *so*.

ey can also activate endogenous *Dac* protein and *3EE-GFP* in an ectopic expression assay using the *30A-Gal4* driver. As ectopic *ey* expression activates *eya* and *so* expression in regions where *dpp* signaling is present (Chen et al., 1999) and *ey* directly activates *so* expression (Niimi et al., 1999; Punzo et

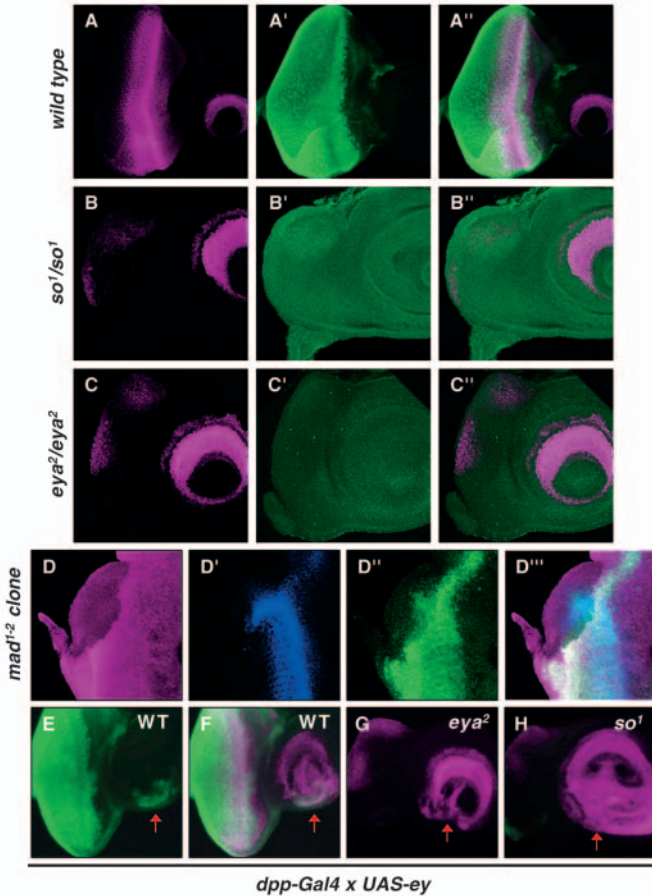


Fig. 4. *eya*, *so* and *dpp* signaling are required for regulation of *3EE-GFP*. (A-C) Each set of three panels shows the same eye disc stained with anti-Dac (magenta in A-C) and anti-GFP (green in A'-C') or a merge of the two channels (A''-C''). (A-A'') Wild-type eye imaginal discs stained with GFP and Dac reveal the normal expression of endogenous Dac (A) and *3EE-GFP* (A'). (B-C') *so*¹ (B,B') and *eya*² (C,C') eye imaginal discs have drastically reduced levels of Dac (B,C) and completely lack *3EE-GFP* (B',C'). (D-D''') Each set of four panels shows the same eye disc stained with anti- β -galactosidase (magenta in D), anti-Dac (green in D'), anti-GFP (green in D''), or a merge of the three channels (D'''). Posterior margin *mad* mutant clones, negatively marked by the lack of β -galactosidase, block Dac (D') and GFP (D'') expression. (E,F) An eye-antennal disc from a *w*; *UAS-ey*, *3EE-GFP*; *dpp-Gal4* third instar larva stained with an antibody against GFP alone (E) or GFP and Dac (F). Ectopic *ey* expression in the antenna driven by *dpp-Gal4* can strongly induce *3EE-GFP* (E) and Dac (F) in the ventral antenna (arrows). (G,H) Eye-antennal discs from *w*; *eya*²; *dpp-Gal4*, *3EE-GFP/UAS-ey* (G) and *w*; *so*¹; *dpp-Gal4*, *3EE-GFP/UAS-ey* (H) larvae co-stained with antibodies against GFP and Dac (both panels show a merge of the two channels). Ectopic *ey* expression in the antenna driven by *dpp-Gal4* cannot induce *3EE-GFP* expression (green in G and H) but retains the ability to induce Dac expression in the ventral antenna (magenta in G and H).

al., 2002), we predicted that *3EE-GFP* is indirectly activated by *ey* in ectopic expression assays via the induction of *eya* and *so*. We tested this hypothesis by determining if ectopic *ey* expression could activate *3EE-GFP* in *eya*² and *so*¹ mutant backgrounds in an ectopic expression assay. We used the *dpp-Gal4* driver in this assay, which drives *UAS-ey* expression in

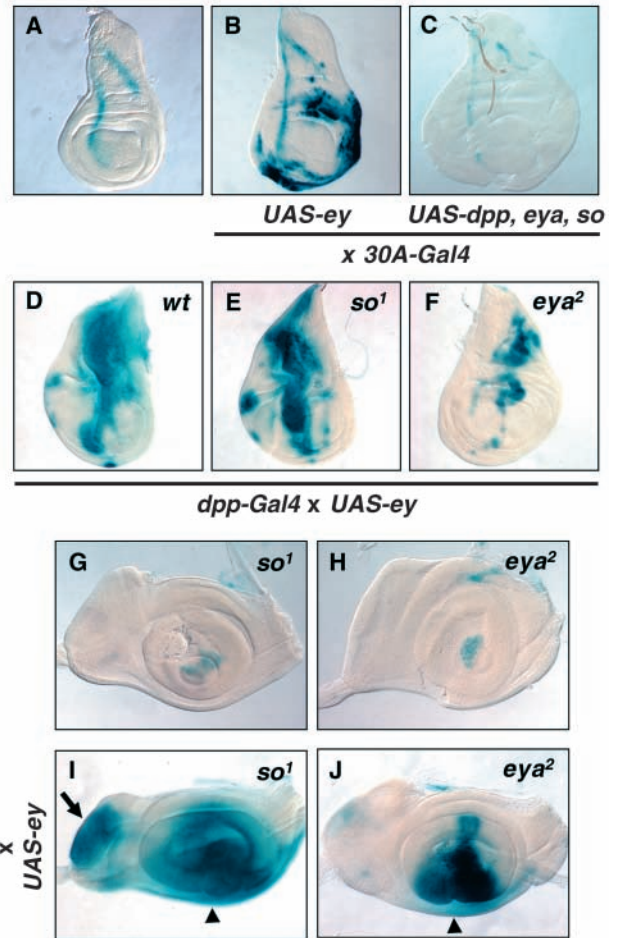


Fig. 5. *5EE-lacZ* is regulated by *ey*, *eya* and *so*. (A-J) All panels show wing (A-F) or eye-antennal (G-J) discs from *5EE-lacZ* transgenic lines stained to reveal β -galactosidase reporter activity. (A) A wing imaginal disc from a *5EE-lacZ* third instar larva shows weak enhancer activity (present in multiple transgenic lines). (B,C) Wing imaginal discs from *5EE-lacZ* third instar larvae in which the expression of *UAS-ey* alone (B) or a combination of *UAS-dpp*, *UAS-eya* and *UAS-so* (C) is driven by *30A-Gal4*. (B) *ey* alone, but not (C) a combination of *UAS-dpp*, *UAS-eya* and *UAS-so*, is capable of inducing *5EE-lacZ* in the ring around the wing disc. (D-F) Wing imaginal discs from *5EE-lacZ* third instar larvae in which the expression of *UAS-ey* is driven by *dpp-Gal4* in wild-type (D), *so*¹ (E), *eya*² (F) or mutant backgrounds. Ectopic *ey* expression is able to induce β -galactosidase reporter expression via *5EE* at the AP boundary in all three cases. The activity is stronger in *so*¹ mutant wing discs than *eya*² mutant wing discs. (G,H) Eye-antennal imaginal discs from *so*¹ (G) or *eya*² (H) mutant *5EE-lacZ* third instar larvae stained for β -galactosidase activity. No reporter activity is detected in these mutant eye discs. (I,J) Eye-antennal imaginal discs from *w*; *so*¹; *dpp-Gal4*, *5EE-lacZ/UAS-ey* and *w*; *eya*²; *dpp-Gal4*, *5EE-lacZ/UAS-ey* third instar larvae. Strong induction of *5EE-lacZ* is seen in the ventral antenna in both *so*¹ and *eya*² mutants (arrowheads in I and J, respectively). In addition, β -galactosidase activity is restored at the posterior margin of *so*¹ mutant eye discs (arrow in I).

all imaginal discs including the ventral antennal disc (Shen and Mardon, 1997). *dpp-Gal4* driven *ey* expression can induce endogenous Dac protein expression and *3EE-GFP* expression in the ventral antenna (Fig. 4E,F). However, in *eya*² or *so*¹

is often governed by coupling tissue-specific inputs with signaling from extracellular growth and patterning factors such as Dpp, Hh and Wg. In particular, it has been proposed that the sequential induction and repression of a small subset of genes allows the formation of specialized protein complexes that in turn activate progressively refined gene expression programs. *dac* is the most downstream member of the RD network identified so far. Isolation of the genomic regulatory elements of *dac* provide an opportunity to study the interplay between intracellular transcription factors and downstream effectors of signaling pathways to control RD gene expression. In this study, we use functional genomics to uncover two independent genomic regulatory sequences that direct the expression of *dac* to the eye. Both eye enhancers are located within non-coding genomic regions that exhibit significant sequence conservation among five species of Drosophilids separated by ~65 million years of evolutionary time. Our results suggest that significant conservation in non-coding genomic regions is a strong predictor of regulatory function. In the absence of easily available genetic reagents, in silico functional genomics approaches provide efficient tools to uncover the complexity of gene regulation across phylogeny.

Dual regulation of *dac* expression: the roles of the 5' and 3' eye enhancers

Loss- and gain-of-function analyses with the two eye enhancers suggest that each enhancer is regulated by a distinct set of protein complexes. The 5' eye enhancer is activated by a combination of *ey*, *eya* and *so*, but is not activated by Dpp signaling. *5EE* is activated by ectopic *ey* expression even in *eya* and *so* mutants, suggesting that it is regulated exclusively by *ey*. However, somewhat paradoxically, *5EE* expression is lost in *eya* and *so* mutants even though ectopic expression of a combination of *dpp*, *eya* and *so* does not activate this enhancer. Furthermore, driving high levels of *ey* in *so*¹ mutant eye discs restores *5EE-lacZ* expression. Coupled together, these results suggest that *5EE* is primarily regulated by *ey* but that the regulation of *5EE* by *ey* also requires *eya* and *so*.

By contrast, the 3' *dac* eye enhancer is regulated by a combination of *eya*, *so* and *dpp* signaling, but is not directly dependent on *ey*. *3EE-GFP* expression is lost in *eya*² and *so*¹ mutant eye discs, and in posterior margin *mad*¹⁻² mutant clones. Furthermore, *ey* cannot bypass the requirement for *eya* and *so* to activate *3EE*. Conversely, *3EE* is strongly induced by co-expression of *eya* and *so*. Moreover, *dpp* signaling via the *tkv* receptor can synergize with *eya* and *so* to induce *3EE* in ectopic expression assays. Furthermore, we find that neither Mad nor Medea, the intracellular transducers of Dpp signaling, is sufficient to bypass the requirement for activation of the Dpp receptor Tkv in these assays (data not shown). Thus, we conclude that events downstream of Dpp-Tkv signaling, such as the phosphorylation of Mad, are essential for the synergistic activation of the 3' *dac* eye enhancer by *eya* and *so*. Taken together, these results suggest that there are distinct requirements for the activation of the 5' and 3' *dac* eye enhancers. However, the exact nature of the protein complexes that regulate *5EE* and *3EE* remain to be determined.

Initiation versus maintenance of *dac* expression: the roles of the 5' and 3' eye enhancers

MF initiation is completely blocked in posterior margin *dac*³-

null mutant clones. However, *dac*³ clones that do not include any part of the posterior margin develop do not prevent MF progression, but cause defects in ommatidial cell number and organization (Mardon et al., 1994). This dichotomy in *dac* function is reflected in the two eye enhancers we have characterized in this study. Our analysis of *dac*⁷ homozygotes demonstrates that the 3' eye enhancer is dispensable for MF initiation and progression. We propose that in *dac*⁷ mutants, the intact *5EE* enhancer is sufficiently activated by *ey* to drive high enough levels of *dac* expression to initiate and complete retinal morphogenesis. However, *dac*⁷ mutants have readily observable defects in ommatidial organization. Thus, we further propose that this lack of normal patterning in *dac*⁷ mutants is most likely due to the loss of *3EE*, which normally acts in concert with *5EE* after MF initiation, to integrate patterning inputs from extracellular signaling molecules such as Dpp with tissue-specific upstream regulators such as *ey*, *eya* and *so*. However, we do not know if the 3' eye enhancer is sufficient to initiate *dac* expression in the absence of the 5' eye enhancer.

Based on our results, we propose a two-step model for the regulation of *dac* expression in the eye. First, the initiation of *dac* expression in the eye disc is dependent on Ey binding to *5EE*. However, Ey is fully functional only when So and Eya are present. It is possible that Ey recruits So and Eya to *5EE*, but we favor a model in which Ey bound to *5EE* cooperates with an So/Eya complex bound to *3EE* to initiate *dac* expression in the eye. After initiation of the MF, *dac* expression is maintained by an Eya and So complex bound to *3EE*. In addition, *3EE* can integrate patterning information received via *dpp* signaling, thereby allowing the precise spatial and temporal expression of *dac* in the eye. This two part retinal enhancer ensures that *dac* expression is initiated only after *ey* activates *eya* and *so* expression. Thus, the *dac* eye enhancers provide a unique model with which the sequential activation of RD proteins allows the progressive formation of specialized protein complexes that can activate retinal specific genes.

The redundancy in *dac* enhancer activity also explains our inability to isolate eye-specific alleles of *dac*, despite multiple genetic screens (K.S.P., E.J.O. and G.M., unpublished). The modular nature of the two enhancers and their potential ability to act independently or in concert suggest that both enhancers must be disrupted to block high levels of transcription of *dac*. Thus, two independent hits in the same generation, a phenomenon that occurs infrequently in genetic screens, would be required to obtain an eye-specific allele in *dac*.

The *dac* eye enhancers provide powerful tools with which to study RD protein function

Despite much investigation, very few direct targets of RD proteins, especially for Eya and So, have been identified. One study suggests that So can bind to and regulate an eye-specific enhancer of the *lz* gene (Yan et al., 2003). However, *lz* is not expressed early during eye development and is required only for differentiation of individual cell types (Daga et al., 1996). Our results suggest that regulation of *dac* expression occurs via the interaction of two independent eye enhancers that are likely to be bound by Ey, Eya and So, and respond to *dpp* signaling. Our analysis of the 3' eye enhancer suggests that two putative conserved So-binding sites are essential for *3EE* activity in vivo. Mutation of individual So-binding sites dramatically

reduces, but does not completely eliminate, reporter expression in the eye. Mutating both predicted So-binding sites completely blocks enhancer activity in vivo. Thus, we conclude that So binds to *3EE* via these conserved binding sites. However, we have not been able to demonstrate a direct specific interaction of either So alone or a combination of Eya and So with oligos that contain these putative So-binding sites in vitro. It is possible that other unidentified proteins are required for stabilizing the Eya and So complex. Furthermore, the 194 bp fragment that responds to ectopic expression of *dpp*, *eya*, and *so* contains no conserved or predicted Mad-binding sites. This raises the intriguing possibility that *dpp* signaling activates other genes, which then directly act with *eya* and *so* to regulate the 3' eye enhancer. Alternatively, a large complex that includes Eya, So and the intracellular transducers of *dpp* signaling, such as Mad and Medea, may be responsible for activation of *3EE*. Similarly, our results suggest that the 5' eye enhancer is regulated primarily by *ey*. However, it is unclear whether Ey directly binds *5EE*. Furthermore, Ey is fully functional only in the presence of Eya and So. Thus, Ey either independently recruits Eya and So into a 5' complex or is activated by virtue of its proximity to the So/Eya complex bound to the 3' enhancer or both.

The exact order and dynamics of protein complex assembly at *5EE* and *3EE* requires further investigation. However, the two *dac* eye enhancers are extremely useful tools with which to investigate fundamental issues about the mechanism of RD protein action. One significant issue concerns the mechanism of Eya function during eye development. Eya consists of two major conserved domains, an N-terminal domain that has phosphatase activity in vitro and a C-terminal domain that can function as a transactivator in cell culture assays (Rayapureddi et al., 2003; Silver et al., 2003; Tootle et al., 2003). So contains a conserved Six domain and a DNA binding homeodomain (Cheyette et al., 1994; Kawakami et al., 2000). However, it is unclear if Eya provides phosphatase activity, transactivator function, or both, in this complex. Characterization of the components of the protein complexes that regulates *dac* expression may uncover the targets of Eya phosphatase activity during eye development. Thus, the isolation of two eye enhancers with distinct regulation provides very useful tools with which to study protein complex formation and function during *Drosophila* retinal specification and determination.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/132/12/2895/DC1>

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