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

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Accepted 13 April 2005
Development 132, 2895-2905
Published by The Company of Biologists 2005
doi:10.1242/dev.01869

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 (CNCs) 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 third 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 ofeya, 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′ 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*) 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 ey 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 eya2 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 (CNCSs). Two such CNCSs 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 dac7 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 dac7 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/drosophilal. A BioPerl script was used to mask coding regions, to find conserved non-coding sequences (CNCSs) in all clones, to cluster nearby CNCSs together and to perform T-COFFEE alignments of all CNCS blocks (Notredame et al., 2000; Stajich et al., 2002). Graphic representations of enhancer conservation were generated using the AVID/n/VISTA 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 mad1-2 FRT40A recombinant stock was provided by Marek Mlodzik (Curtiss and Mlodzik, 2000). The nature of the dac7 and dac9 mutant alleles were previously described (Tavsanli et al., 2004). The presence of intron 8 in dac7 mutants was confirmed by PCR on genomic DNA prepared from dac7 homozygotes with intron 8 specific primers. A similar assay was used to demonstrate the deletion of exon 9, placing the deletion in dac7 beyond intron 8 but including exon 9 (data not shown). The 30A-GAL4, 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 Lary 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-hs34-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.

Mouse antibodies were used as previously described (Mardon et al., 1989). Subcloned PCR products were sequenced to confirm sequence integrity 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.

### Immunohistochemistry and scanning electron microscopy

Primary antibodies used in this study were: monoclonal mouse anti-Dachshund (mAbd2c-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 *ldgf* 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 (*3EE1.9 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 (*3EE390 bp*) within the 1.9 kb fragment contains eye enhancer activity. Further dissection of the *3EE390 bp* fragment with smaller PCR fragments uncovered a 194 bp eye reporter fragment (*3EE194 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. viridis* 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 *3EE1.9 kb*. The VISTA output of pairwise comparisons to *D. melanogaster* along *3EE1.9 kb* is shown in Fig. 1C (Mayor et al., 2000). Six conserved non-coding sequences (CNCSs) are present in *3EE1.9 kb*. To test the correlation of CNCSs with enhancer activity, we cloned an 850 bp fragment (*3EE850 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. *3EE850 bp*, like *3EE1.9 kb*, is expressed only posterior to the furrow (Fig. 1D). However, a smaller 659
bp fragment (3EE659 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 fragment identified is 194 bp (3EE194 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 3EE650 bp-GFP (D) and 3EE659 bp-GFP (E) larvae triple labeled with GFP (green), Sens (magenta, D’E’), and Dac (blue, D’’E’’). GFP expression in 3EE650 bp eye discs is detected anterior to the earliest Sens expression and overlaps with anterior Dac expression (E’E’’). By contrast, GFP expression in 3EE650 bp eye discs is not detected anterior to the anterior-most Sens-expressing column (D’).

The dac7 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 (Tavsanli et al., 2004). One such allele, dac7, 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, dac7 mutants provide us with a tool to analyze the role of this 3′ eye enhancer in an in vivo context. We hypothesized that dac7 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 dac7 mutants, we predicted that any dac7 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 dac3 null mutants (Tavsanli et al., 2004).

**dac7 homozygotes develop with only moderately disrupted eyes**

Surprisingly, dac7 homozygotes develop with only moderately disrupted eyes compared with wild-type adults (compare Fig. 2C with 2B). By contrast, dac3 null mutants have no eyes, suggesting that the dac7 mutant is a hypomorph (Fig. 2D). We also examined the expression of Dac protein in the eye imaginal discs of dac7 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 dac7 transcript. Eye imaginal discs from dac7 larvae are almost identical to wild-type controls in their Dac protein expression profiles (compare Fig. 2F to 2E). dac3 null mutants display no detectable Dac protein (Fig. 2G). As the entire 16.6 kb 3′ enhancer is completely deleted in dac7 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 5EE enhancer or 5EE; Fig. 1B). Importantly, this 1.7 kb region is intact in the dac7 allele. 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 for other dac-specific cis-regulatory elements.
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 3EE659bp 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 activates 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 30A-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 3EE194 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 (Tkv123D), was just as effective as Dpp in synergistically activating GFP expression from the 3′ eye enhancer in the presence of Eya (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 eya2 and so1 eye-specific mutants (Fig. 4B,C). However, as has been shown previously, endogenous Dac protein expression is dramatically reduced but not completely eliminated in eya2 and so1 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 (mad12). We found that 3EE-GFP expression is drastically reduced or completely lost from posterior margin mad12 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. eya 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
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 eya2 and so1 mutant backgrounds in an ectopic expression assay. We used the dpp-Gal4 driver in this assay, which drives UAS-ey expression in 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 eya2 or so1...
Fig. 6. Mutating the putative So binding sites in 3EE abolishes enhancer activity in vivo. (A) A multiple sequence alignment of the 40 conserved bases in the 3′ eye enhancer. Mismatched bases are shown in grey and the two putative So-binding sites are shown in green. Mutated So-binding sites are shown in red. (B-E) Each panel shows a single eye disc co-stained with anti-Dac (blue) and anti-GFP (green). Mutating each putative So-binding site individually (C,D) results in the dramatic reduction of GFP reporter expression from 3EE-lacZ. Mutated So-binding sites are shown in red. (B-E) Each panel shows a single eye disc co-stained with anti-Dac (blue) and anti-GFP (green). Mutating each putative So-binding site individually (C,D) results in the dramatic reduction of GFP reporter expression from 3EE-lacZ. Mutated So-binding sites are shown in red. (B-E) Each panel shows a single eye disc co-stained with anti-Dac (blue) and anti-GFP (green). Mutating each putative So-binding site individually (C,D) results in the dramatic reduction of GFP reporter expression from 3EE-lacZ. Mutated So-binding sites are shown in red. (B-E) Each panel shows a single eye disc co-stained with anti-Dac (blue) and anti-GFP (green). Mutating each putative So-binding site individually (C,D) results in the dramatic reduction of GFP reporter expression from 3EE-lacZ. Mutated So-binding sites are shown in red.
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 Drosophila 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 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 and so mutant eye discs, and in posterior margin mad1-2 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 null mutant clones. However, dac3 mutants 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 homozygotes demonstrates that the 3′ eye enhancer is dispensable for MF initiation and progression. We propose that in dac 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, dac7 mutants have readily observable defects in ommatidial organization. Thus, we further propose that this lack of normal patterning in dac7 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.

We thank Marek Mlodzik, Gary Struhl, Francesca Pignoni, Nancy Bonini, Larry Zipursky, Hugo Bellen, the Bloomington stock center and the Developmental Studies Hybridoma Bank for providing fly stocks and antibodies. We thank Benjamin Frankfort and Richard Davis for discussions and comments on the manuscript. Research in the laboratory of G.M. is supported by funds from the National Eye Institute (RO1 EY11232), the Retina Research Foundation and an Ophthalmology Core Grant from the National Eye Institute (EY02520), M.R.A. is supported by NEI training grant T32 EY07102.

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

References
recognition by an isolated DNA-binding domain of the sine oculis protein. Biochemistry 36, 3680-3686.


