Published in final edited form as:

Dev Dyn. 2009 October ; 238(10): 2530–2539. doi:10.1002/dvdy.22082.

The transcription factor D-Pax2 regulates Crystallin production during eye development in *Drosophila melanogaster*

Katharine Dziedzic¹, Julie Heaphy², Hallie Prescott², and Joshua Kavalier^{1,3}

¹Department of Biology, Colby College, Waterville, ME 04901, USA

²Department of Biology, Middlebury College, Middlebury, VT 05753, USA

Abstract

The generation of a functioning *Drosophila* eye requires the coordinated differentiation of multiple cell types and the morphogenesis of eye-specific structures. Here we show that D-Pax2 plays a significant role in lens development through regulation of the *Crystallin* gene and that Crystallin is also expressed in D-Pax2⁺ cells in the external sensory organs. Loss of D-Pax2 function leads to loss of *Crystallin* expression in both eyes and bristles. A 2.3 kilobase upstream region of the *Crystallin* gene can drive GFP expression in the eye and is dependent on D-Pax2. In addition, D-Pax2 binds to an evolutionarily conserved site in this region which, by itself, is sufficient to drive GFP expression in the eye. However, mutation of this site does not greatly affect the regulatory region's function. The data indicate that D-Pax2 acts to promote lens development by controlling the production of the major protein component of the lens. Whether this control is direct or indirect remains unresolved.

Keywords

D-Pax2; Crystallin; *Drosophila* eye development; transcriptional regulation; cone cells; external sensory organs

Introduction

The adult compound eye of *Drosophila melanogaster* is composed of an integrated array of approximately 800 ommatidia. Each ommatidium contains eight photoreceptors, four cone cells, and sets of primary (1°), secondary (2°), and tertiary (3°) pigment cells. These cell types are determined through an intricately orchestrated process of cell-cell communication and specification. Although the specification of the various cell types has been intensively studied, the subsequent events leading to cell differentiation and eye morphogenesis are less well understood. The development of the ommatidial lens provides an excellent model for these questions as it reflects the differentiation of a particular subset of cells in the eye and the generation of a distinct structure required for this sensory system to function properly.

Patterning of the eye begins in the posterior region of the larval third instar eye imaginal disc as Hedgehog signaling drives the morphogenetic furrow from posterior to anterior (Heberlein et al., 1993). This induces broad expression of the proneural gene *atonal* in the furrow which is eventually restricted to the R8 photoreceptor as sequential phases of cell specification occur (Jarman et al., 1994; Baonza and Freeman, 2001). Combinatorial signaling via the EGFR and Notch pathways eventually define the eight photoreceptors and

³Author for correspondence. Joshua Kavalier, Department of Biology, Colby College, 5734 Mayflower Hill, Waterville, ME 04901, USA, Phone: 207-859-5734, Fax: 207-859-5705, jkavalier@colby.edu.

the 15 non-neuronal cells (including the four cone cells and two 1° pigment cells) in each ommatidium (Flores et al., 2000; Nagaraj and Banerjee, 2007). The cone cells overlie the photoreceptors and, together with the surrounding 1° pigment cells, secrete Crystallin (Cry), the major protein component of the ommatidium's corneal lens (Janssens and Gehring, 1999).

The transcription factor D-Pax2 plays a central role in the development of the lens of the *Drosophila* eye (Fu and Noll, 1997). D-Pax2 expression is first seen in the late third instar larval eye in regions posterior to the morphogenetic furrow where cell fate specification has already occurred. The protein is confined to the cone and 1° pigment cells and this pattern is retained throughout pupal development. In addition, D-Pax2 has been shown to function in the development of the external sensory (es) organs of the adult fly (Fu et al., 1998; Kavalier et al., 1999). In the external sensory (es) organs, D-Pax2 is expressed early during the divisions of the sensory organ precursor (SOP) and its progeny. Later, D-Pax2 expression is confined to the trichogen cell that produces the bristle shaft and the thecogen cell the ensheaths the dendrite of the es organ's neuron. During this later expression period, D-Pax2 guides the differentiation of the trichogen and thecogen cells. The transcription factor presumably plays a similar role in the differentiation of the cone and 1° pigment cells.

D-Pax2 is the *Drosophila* homolog of the vertebrate Pax2/5/8 family. Although some transcriptional targets of vertebrate Pax2 orthologs are known, D-Pax2 targets remain unidentified. One potential target is the gene *Cry* as it is essential for the lens of the eye and is also expressed in the cone and 1° pigment cells. Among animals, crystallins comprise a large family of proteins that exhibit a surprisingly large amount of structural and functional diversity (Tomarev and Piatigorsky, 1996). It is likely that the crystallins arose during evolution via recruitment of several genes possessing functions unrelated to eye development (Piatigorsky, 1998). The transcriptional regulation of various vertebrate crystallin genes have been examined extensively and all require Pax6 for activation. Other transcriptional activators are often involved, although the exact combinations of transcription factor binding sites differ between the various crystallin promoter regions. Activation of the chicken δ 1-crystallin gene is dependent upon the physical interaction of Pax6 and Sox2 at the DC5 enhancer (Kamachi et al., 2001). The mouse α A-crystallin gene is regulated by a combination of Pax6, c-Maf and CREB (Yang et al., 2006). The mouse α B- and γ F-crystallin genes are also regulated by Pax6 and Maf but whereas α B-crystallin is activated by these factors, γ F crystallin is repressed (Yang et al., 2004). Vertebrate Pax6 and Pax2 binding sites are closely related and it has been demonstrated that the chicken DC5 enhancer can function in vivo in flies but is regulated by Pax2 instead of Pax6 (Blanco et al., 2005).

In this report, we examine the ability of D-Pax2 to regulate the *Drosophila* *Cry* gene. We show that D-Pax2 is necessary for the expression of *Cry* in both the eye and the es organs. This expression is controlled in part by a 2.3 kilobase (KB) sequence located directly upstream from the *Cry* transcription start site. This enhancer region represents an extension of a previously identified 441 base pair proximal enhancer (Janssens and Gehring, 1999). A potential D-Pax2 binding site exists in the larger 2.3 KB region and D-Pax2 is able to interact with this site in vitro. Furthermore, a multimerized version of this site by itself is sufficient to drive gene expression in vivo in a D-Pax2-dependent manner. However, the D-Pax2 binding site is dispensable for the function of the *Cry* enhancer. Sequences that vary slightly from the consensus D-Pax2 site also exist within this region but the minimal sequence requirements for function of D-Pax2 sites are unknown. These results demonstrate that the *Cry* gene is under the control of D-Pax2 but whether this control is direct or indirect remains to be established.

Results

Loss of D-Pax2 function in the eye leads to loss of both Crystallin protein and mRNA

D-Pax2 functions in the differentiation of specific cell types in *Drosophila* photosensory and mechanosensory organs. Both D-Pax2 and Cry, the major lens protein in *Drosophila*, are expressed in the cone and 1° pigment cells of the eyes (Komori et al., 1992; Fu and Noll, 1997). D-Pax2 mutants can be divided into two classes. Whereas mutations that cause defects in es organ development (the *shaven* (*sv*) class) generate flies that lack es organ shafts, mutations disrupting eye development (the *sparkling* (*spa*) class) give rise to flies with malformed lenses (Fu et al., 1998). All identified D-Pax2 mutations appear to affect transcriptional regulation of the gene. The mutations found in *spa* alleles are localized to the fourth intron eye enhancer while the mutations found in *sv* alleles occur in an upstream bristle enhancer. The *spa* class of D-Pax2 mutants exclusively perturb eye development and show disrupted ommatidial structure and poor lens formation while the es organs remain unaffected. Conceivably, the eye defects in *spa* mutants may arise because D-Pax2 is necessary for Cry expression. To investigate this possibility, we compared developing pupal eyes from *w¹¹¹⁸* and *spa^{pol}* mutants for the presence of Cry protein. Cry is a secreted protein and is first observable in the pupal eye around two days after puparium formation (APF). Immunostaining with an anti-Cry antiserum at this time point shows a latticed array of Cry surrounding each ommatidium in *w¹¹¹⁸* eyes (Figure 1A). In contrast, pupal eyes from *spa^{pol}* flies stained very weakly and showed no obvious pattern (Figure 1C). We also examined *w¹¹¹⁸* and *spa^{pol}* pupal eye tissue for the presence of Cry mRNA by in situ hybridization. Whereas *w¹¹¹⁸* pupal eyes clearly show expression of Cry mRNA in the lattice-like structure reminiscent of Cry protein, pupal eyes from *spa^{pol}* flies show much less Cry mRNA and the Cry⁺ cells are disordered (Figure 1B, D). The Cry⁺ cells are likely to include the few remaining cone and 1° pigment cells but may also represent D-Pax2 expressing cells from the interommatidial bristles, which are not affected in *spa^{pol}* mutants.

Crystallin is also expressed by D-Pax2⁺ es organ cells

The residual expression of Cry mRNA in *spa^{pol}* mutants suggested that D-Pax2 might regulate Cry in the es organ cells as well, albeit there is no characterized role for Cry in the bristle. Although D-Pax2 protein is initially present in all four cells of the es organ during cell fate specification, it is lost from the socket cell and neuron as the es organ cells differentiate (Kavaler et al., 1999). Immunostaining of pupal nota at 32 hours APF with the anti-Cry antiserum reveals diffuse patches of Cry protein in a microchaete-like pattern (Figure 2A-C). The patches appear to include one large and one small region. The small region is approximately coincident with a Prospero⁺ cell, which is the sheath cell. In addition, Cry transcripts can be detected in the notum by in situ hybridization with an antisense Cry probe and also appear in a pattern consistent with developing microchaetes (Figure 2D). The in situ hybridizations reveal a single large cell. The identity of this cell as the shaft cell was confirmed by a co-immunostaining with an anti-D-Pax2 antiserum (Figure 2F,G). We also tested similarly aged nota from a strong D-Pax2 mutant of the *sv* class, *sv⁶*, for expression of Cry mRNA. In the *sv⁶* mutant, all four es cells are present but the shaft cell does not differentiate properly (Kavaler et al., 1999). Cry expression in these nota is absent or greatly diminished, demonstrating that it is also dependent upon D-Pax2 function (Figure 2E).

Identification of a Cry enhancer fragment that drives GFP expression in vivo in cone and 1° pigment cells

Janssens and Gehring (1999) identified a 441 base pair 5' region (-349 to +92) capable of driving *lacZ* expression in the eye. We extended this region to include a larger 2.3 KB sequence of DNA (-2271 to +58) into the GFP reporter vector pH-Stinger. Three

independent transgenic lines bearing this *Cry-GFP* construct were generated and each line exhibited fluorescent eyes and ocelli (Figure 3B). To determine the cell types expressing GFP, approximately two to three day old pupae were dissected and the eyes were immunostained for D-Pax2. Eyes from younger pupae (44-48 hours APF) tended show GFP expression exclusively in 1° pigment cells (data not shown) whereas eyes from older pupae (48 to 72 hours APF) also demonstrated expression in the cone cells as well (Figure 3C-E). We saw no GFP in any cells of the es organ lineage (data not shown). To determine if D-Pax2 is required for the GFP expression in the cone and 1° pigment cells, we introduced the *Cry-GFP* transgene into a *spa^{pol}* background. Adult and pupal eyes from *spa^{pol}* flies did exhibit some fluorescence, but the levels were much lower than those seen in a *w¹¹¹⁸* background (Figure 3B, F-H). Eye tissue from *Cry-GFP;spa^{pol}* pupae was stained for Cut protein, which is expressed in the ommatidium specifically in the cone cells. Cells exhibiting residual GFP expression also expressed Cut and were mostly disorganized, although arrangement of four cells in a diamond-like pattern was occasionally seen. In addition, many other GFP⁻ cells in the eye were Cut⁺. In the *spa^{pol}* mutant, Cut expression in cone cells is reduced during early pupal development (24 hours APF) but recovers by 48 hours APF (Fu and Noll, 1997). Some of the Cut expression that we see in GFP⁻ cells may also mark interommatidial bristles.

D-Pax2 binds specifically to a short DNA sequence located 5' to the Cry transcription start site

Although the binding specificity for D-Pax2 has not been established, a consensus binding site for the mouse Pax2 homolog has been determined (Epstein et al., 1994; Phelps and Dressler, 1996). The murine Pax2 binding site is GTCA(C/T)GC(G/A)TGA and is present in the 2.3 KB enhancer region used to drive GFP expression, located approximately 1.3 KB in front of the transcription start site of the *Drosophila Cry* gene. Comparison of the 5' upstream regions among related *Drosophila* species indicates that this region has been conserved during evolution (Figure 4A). To test the ability of D-Pax2 to bind to this sequence, the D-Pax2 coding region was cloned into a bacterial expression vector in frame with the maltose binding protein (MBP) gene and this construct was used to synthesize and purify a D-Pax2-MBP fusion protein. The purified fusion protein was tested in an electrophoretic gel mobility shift assay for its ability to recognize a 35 bp DNA fragment corresponding to the *Cry* Pax2 binding site and flanking nucleotides. When incubated with the labeled probe, the fusion protein generated a pronounced shift, demonstrating that it was capable of binding to this region of DNA (Figure 4B, lane 2). As expected, unlabeled DNA identical in sequence to the 35 bp probe was able to compete for binding in a dose-dependent manner (Figure 4B, lanes 3-5). In contrast, a similar 35 bp DNA fragment containing six nucleotide changes in the putative D-Pax2 binding site was unable to compete with the probe for binding to the fusion protein even at 80-fold excess (Figure 4B, lanes 6-8).

The D-Pax2 binding site by itself is capable of driving gene expression in most D-Pax2⁺ cells

To determine the functional potency of the D-Pax2 binding site in vivo, we synthesized a short fragment of DNA containing four copies of the site each separated by a few nucleotides and cloned this fragment into pH-Stinger (Px2BS-GFP). Flies bearing this construct exhibited strong GFP expression reminiscent of D-Pax2 expression patterns. In the eye imaginal discs, the leading edge of D-Pax2 expression is approximately ten rows posterior to the morphogenetic furrow. The initial expression of the Px2BS-GFP reporter was slightly more posterior but marked the D-Pax2⁺ cells (Figure 5A-C). This expression continued through pupal development and was restricted again to the cone and 1° pigment cells (Figure 5D-F).

GFP was also easily detectable in the wing discs in some of the SOPs for es and chordotonal organs (Figure 6A-C). Finally, in pupal nota, the Px2BS-GFP reporter was able to drive GFP expression in all four progeny cells of the microchaete SOPs at 20 hours APF (Figure 6D-G). An occasional fifth cell, representing the extra glial cell generated by division of the precursor pIIB cell (Gho et al., 1999; Reddy and Rodrigues, 1999), can occasionally be seen at this time point (data not shown). By 32 hours APF, GFP expression was predominantly restricted to the trichogen and tormogen cells, although a low level of GFP was detectable in all four cells at this time (Figure 6H-K).

Mutation of the D-Pax2 binding site does not disable the function of the Cry enhancer region

There is a single exact match to consensus Pax2 binding site in the 2.3 KB Cry enhancer region. We performed site-specific in vitro mutagenesis to alter this site in the Cry-GFP reporter and introduce the same nucleotide changes that abolished binding of D-Pax2 protein in the EMSA assay. Transgenic flies bearing the *Cry^{MUT}-GFP* construct still exhibited fluorescent eyes. At 48 hours APF, both cone and primary pigment cells express GFP and at levels that are not appreciably different from levels produced by the wild-type version of the enhancer (Figure 7). Therefore, this single D-Pax2 site is not required for the function of the enhancer, although it remains possible that other D-Pax2 binding sites exist within this region.

Discussion

Lens formation in the eye requires D-Pax2 via regulation of Crystallin

The transcription factor D-Pax2 plays a critical role in the development of the *Drosophila* eye. D-Pax2 in the eye is restricted to the cone and 1° pigment cells of the ommatidium (Fu and Noll, 1997). These cells are responsible for the generation of the lens of the ommatidium, which is composed predominantly of the secreted Cry protein. In the *spa* class of *D-Pax2* mutants, which specifically affect the eye, the ommatidia are disorganized and the lens is improperly formed. We have examined the relationship between D-Pax2 and *Cry* and have demonstrated that loss of D-Pax2 function results in loss of *Cry* mRNA and protein. In addition, we have identified an enhancer region for the *Cry* gene that can drive gene expression in the eye and is dependent upon D-Pax2 function. This enhancer region contains a conserved murine Pax2 site to which D-Pax2 protein is capable of binding; however, this site is dispensible for the function of this region. Even so, a multimerized version of the murine Pax2 site alone is capable of driving gene expression in cells of both the eye and es organs. We believe these data indicate that *Cry* is regulated by D-Pax2 although we have been unable to demonstrate that this regulation is direct transcriptional activation by D-Pax2.

The *spa* class of *D-Pax2* mutations all affect the eye-specific enhancer located in the fourth intron of the *D-Pax2* gene. These mutations severely depress the expression of *D-Pax2*. In *spa^{pol}* mutants, D-Pax2 protein is virtually missing from the cone and 1° pigment cells but at normal levels in bristle cells (Fu and Noll, 1997). The eyes of *spa^{pol}* flies exhibit poor lens formation and the eye appears sunken relative to *wild-type* eyes. Interommatidial bristles are present but disordered as is the general ommatidial structure. The lens phenotype in *spa* mutants is consistent with the hypothesis that D-Pax2 regulates *Cry* gene expression and is integral in the proper differentiation of the cone and 1° pigment cells. Another possible explanation for this phenotype could be the apoptotic death of these cell types in the absence of D-Pax2. However, although the ommatidia are severely affected and 2° and 3° pigment cells do undergo apoptosis, cone and 1° pigment cells remain present (Siddall et al., 2003). Therefore, the cone and 1° pigment cells do not die in *spa^{pol}* mutants and yet these cells are

not capable of producing *Cry* mRNA or *Cry* protein (Figure 1). This differentiative failure is similar to the effect of loss of D-Pax2 function in es organ cells. In strong *sv* mutants, the trichogen cell of the bristle remains but either produces a small shaft or fails to produce one entirely (Kavaler et al., 1999). At least in these instances, D-Pax2 does not contribute to the specification of cell fate nor regulate cell survival but acts as a differentiation factor for cells whose fate has already been established.

D-Pax2 regulates *Cry* in cells of the es organs

Analysis of *Cry* expression in the es cells of the notum demonstrated that both *Cry* mRNA and protein are produced at a time point when D-Pax2 is driving the differentiation of the trichogen and thecogen cells (Figure 2). The trichogen cell is clearly expressing *Cry* mRNA although it is unclear if the thecogen cell also does so; in situ hybridization with a *Cry* probe showed no discernable staining of the thecogen cell. However, by this time point, the smaller thecogen cell has flattened out and its cytoplasm may not be easily distinguishable from the large trichogen cell adjacent to it. *Cry* expression in the es organs also requires D-Pax2, as *sv*⁶ mutants lack *Cry* expression in the notum.

Why should *Cry*, a protein associated with the lens of the eye, be found in other tissues of the fly? Interestingly, Janssens and Gehring (1999) noted that *Drosophila* *Cry* bears sequence similarity with various insect cuticle proteins and is most closely related to the *Drosophila* protein EDG-84, which is present in the pupal cuticle. Vertebrate crystallins have been best studied and encompass a diverse family of proteins which possess other functions outside of the lens. Invertebrate crystallins also exhibit unusual diversity in both form and function (Tomarev and Piatigorsky, 1996). Both vertebrate and invertebrate crystallins often exhibit stress-protective enzymatic functions and crystallin genes may have arisen via a gene sharing strategy (Piatigorsky, 1998). Indeed, *Drosophila* *Cry* was originally identified as a 52 kDa protein which acted as a substrate for bacterial toxin-dependent ADP-ribosylation and may have been recruited from an ancestral G protein (Komori et al., 1992). Furthermore, a more general arthropod crystallin is identified by the monoclonal antibody 3G6 which was originally used to detect an antigen present in neuropil glial cells (and sensory nerves to a lesser extent) in crickets (Meyer et al., 1987; Edwards and Meyer, 1990). Whether *Drosophila* *Cry* serves dual functions in the lens and in other sensory systems is not known but its expression in both eyes and bristles is consistent with the multifunctional nature of crystallins from many organisms.

D-Pax2 acts through a conserved binding site present in the *Cry* enhancer region

We have identified an eye-specific enhancer region directly upstream of the *Cry* gene. This region contains the 11 base pair sequence that matches the binding site for the mouse Pax2 homolog – GTCA(C/T)GC(A/G)TGA. This sequence can be found within 4 KB of the *Cry* gene in all *Drosophila* species that we have examined, although there are single nucleotide differences in the *D. virilis*, *D. grimshawi*, and *D. erecta* sites. This enhancer is capable of driving GFP expression in both the cone and 1° pigment cells in the eye but shows no activity in es organs. Whereas the 2.3 KB enhancer region guides expression that is restricted to D-Pax2⁺ cells in mid-stage pupal eyes, the isolated Pax2 binding site guides expression in both eyes and es organs.

If D-Pax2 activates *Cry* by binding to the Pax2 site located in the 2.3 KB enhancer, then mutation of this site should decrease expression. We introduced six base pair changes that were sufficient to abolish binding of D-Pax2 to the site in vitro (Figure 4) but these same changes did not significantly affect reporter expression in vivo (Figure 7). Also, Janssens and Gehring (1999) have already noted that a shorter 441 base pair reporter construct is capable of driving *lacZ* expression in the eye. It is possible that the D-Pax2 site embodies a

redundant or complementary mechanism for *Cry* expression. In this case, D-Pax2 might regulate *Cry* indirectly through factors that act upon the proximal 441 base pair enhancer region as well as directly through the more distal D-Pax2 site. Alternatively, there may be other D-Pax2 sites that vary from the consensus sequence. If we assume that a functional site may diverge to some degree from the consensus sequence, there are a number of other potential D-Pax2 sites in the 2.3 KB enhancer. The closest matches to the consensus sequence are two sites that differ by three nucleotides, at -693 and -113. The latter site is within the previously defined 441 base pair enhancer region. Conceivably, D-Pax2 directly regulates *Cry* via interactions with a combination of these sites.

The results presented here are consistent with the hypothesis that D-Pax2 activates the *Cry* gene but they also raise two questions. First, D-Pax2 is initially expressed in the cone and 1° pigment cells soon after they are specified during the late third instar larval stage but both normal *Cry* expression and the *Cry-GFP* reporter gene are activated much later during pupal development. Why does this lag exist? Most likely, *Cry* is regulated by one or more other transcription factors. Therefore, although D-Pax2 is required, *Cry* expression may also be dependent upon other factors which become active later in the pupal eye. For example, if the 2.3 KB fragment contains a site for a transcriptional repressor which is expressed in the developing eye up until two days APF, the presence of D-Pax2 at earlier time points might not be sufficient to overcome the repression. However, we have so far been unable to identify any other potential regulatory factors. Secondly, we have demonstrated that *Cry* expression also occurs in the es organs during their differentiation but the *Cry-GFP* reporter gene shows no evidence of being active in any es organ cells. If D-Pax2 regulates *Cry*, why isn't the reporter active in the es organs as well? The D-Pax2 binding site upstream of *Cry* may be important for expression in the es organs but not sufficient. Perhaps other cis-acting elements may exist beyond the borders of the 2.3 KB region that we used as our enhancer region. In such a model, D-Pax2 may act as a general activating factor for *Cry* expression, while additional regulatory factors act specifically in the eyes or in the es organs to refine expression spatially and temporally.

Evolutionary relationships between Pax2 and Pax6 in the regulation of crystallins

The *Pax2* gene has been well conserved during evolution and *Pax2* homologs identified in wide range of organisms have been demonstrated to play roles in eye development (Pichaud and Desplan, 2002). A *Pax2/5/8*-like *Pax* gene from *Tripedalia cystophora* regulates jellyfish crystallin and is also able to induce small ectopic eyes in *Drosophila* (Kozmik et al., 2003). Perhaps surprisingly, the vertebrate *Pax2* homologs have not been implicated in the regulation of crystallins. Instead, another Pax family member, Pax6, has been demonstrated to be the key factor in crystallin expression. In chickens, the *δ1 crystallin* gene has been shown to be regulated by Pax6 (Cvekl et al., 1995). Interestingly, Blanco et al. (2005) noted overlapping functions acquired by Pax2 and Pax6 during evolution. They cloned a multimerized 30 bp fragment of the chicken *δ1 crystallin* enhancer into a GFP reporter plasmid and introduced this construct into flies. This fragment (labeled the DC5 enhancer) contains a Pax6 and a Sox2 binding site and the multimerized version demonstrated activity in the cone cells of the adult eye. In addition, GFP expression was evident in the antenna, maxillary and labial palps, and larval eye disc – all regions that exhibit D-Pax2 expression. As with the multimerized version of the D-Pax2 binding site, the DC5 enhancer activity was dependent upon D-Pax2 function. The identified vertebrate Pax2 and Pax6 binding sites are very similar, differing primarily in the requirement of a flanking 5' G residue in the Pax2 site that corresponds to a T in the Pax6 site. However, the Pax6 binding site in the DC5 enhancer is a relatively poor site and differs from the Pax6 and Pax2 consensus sites by four and five nucleotides, respectively (Kamachi et al., 2001). In addition, the DC5 enhancer works in vivo in *Drosophila* only when multimerized. It has

been suggested that the similarity of the Pax2 and Pax6 binding sites and the close evolutionary relationship between Pax2 and Pax6 may allow for some functional overlap.

Relevance of Pax2 binding site to D-Pax2 control of gene transcription

Our multimerized version of the D-Pax2 binding site contained no known binding sites for other transcription factors. This construct was able to produce robust GFP expression in a pattern coincident with D-Pax2 expression (Figure 6). Whereas the *Cry* enhancer fragment drove expression relatively late and only in cone and 1° pigment cells, the multimerized D-Pax2 site drove expression in late third instar imaginal discs and expression was present not only in the eye but also in the es organs. The difference in expression may be the result of the multimerized nature of the D-Pax2 site reporter. Alternatively, the difference may be the natural context of the D-Pax2 site in the *Cry* enhancer region, such that expression of the *Cry-GFP* reporter is constrained by more complex transcriptional control mechanisms which limit *Cry* expression in the eye to mid to late pupal development even though D-Pax2 itself is present in the eye as early as late larval development. The consensus D-Pax2 site can be identified at 64 different locations in the *Drosophila melanogaster* genome using the fly enhancer search engine (<http://opengenomics.org>, (Markstein et al., 2002)). We have noted that *hibris*, another gene expressed in the cone and primary pigment cells in the eye, has two potential D-Pax2 sites within 600 bp of the transcription start site. Hibris protein plays a role in eye morphogenesis by interacting with Roughest to establish proper lattice formation in the eye (Bao and Cagan, 2005). One of the putative sites, located at -299, differs from the consensus sequence by three base pairs while the other, located in the first intron, differs by two base pairs. In addition, a perfect consensus sequence is located at the 3' of the gene, albeit 30 kilobase pairs away from the transcription start site. Whether any of these sites serve a regulatory function is unknown. However, the identification of a conserved D-Pax2 binding site upstream of *Cry* and its efficacy in driving gene expression in vivo suggests that such sites might play a broad role in the activation of a set of D-Pax2-responsive genes and may constitute a central cis-acting component allowing D-Pax2 to drive the differentiation of the specific cell types in which it is expressed.

Experimental Procedures

Drosophila stocks and crosses

Flies were cultured on standard yeast-cornmeal-molasses-agar medium at 25°C in a humidified chamber. The following lines used in this study include *w¹¹¹⁸* and *spa^{pol}*, obtained from the Bloomington Stock Center, *w; sv⁶/Tp(2;4) ey^D*, previously generated by J. Kavalier (Kavalier et al., 1999), and three new transgenic strains, *w; Cry-GFP*, *w; Cry^{MUT}-GFP*, and *w; Pax2BS-GFP*. Transgenic strains were made by BestGene Inc. (Chino Hills, CA) according to standard methods. For each transgenic strain, at least three independent isolates were generated and each isolate exhibited similar GFP expression patterns.

Plasmid constructs

Cry-GFP—The 2.3 KB region of the 5' upstream region of the *Cry* gene was amplified by PCR from *w¹¹¹⁸* genomic DNA using Phusion DNA polymerase (New England Biolabs) and the following primers: 5'- GCATTCAGGATCCAGCTATCTATG (located at and 5'- TATACGACTAGTTGGATCCTGCTT. The PCR product was cloned into the BamHI site of pBluescript KS+ (Stratagene) and subcloned into pH-Stinger (Barolo et al., 2004). The cloned fragment encompasses the genomic region extending from 2271 base pairs before the transcription start site to 58 base pairs into the first exon (5' untranslated region).

Cry^{MUT}-GFP—Mutation of the D-Pax2 binding site were introduced into the pBluescript clone bearing the 2.3 KB *Cry* enhancer using the Phusion in vitro mutagenesis kit (New

England Biolabs) according to the manufacturer's instructions. Primers for amplification and introduction of mutations were 5'-CTCTTGATTTTAGGAATCTACCACAATTTTTTCC and 5'-TAAGAGCACAAAGTAGCACTGCACAGTG where underlined nucleotides represent the introduced mutations. The resulting mutated insert was verified by sequence analysis and subcloned into pH-Stinger.

Px2BS-GFP—Four identical D-Pax2 binding sites (GTCATGCATGA) separated by four to six nucleotide spacers were synthesized and cloned into the BamHI site of pH-Stinger.

All construct inserts were confirmed by DNA sequence analysis.

Immunohistochemistry

Eye or notum tissue was dissected from fly pupae in PBS and fixed for 30 minutes in 4% paraformaldehyde and washed extensively in PBS with 0.1% Triton-X 100. The tissue was then incubated with the primary antibody for 3 hours at room temperature or overnight at 4°C, washed, and incubated with the appropriate secondary reagent in the same manner. Samples were then washed, mounted on slides in Biomedica Gel/Mount (Electron Microscopy Sciences) and images were taken using an Axiovision D1 fluorescence microscope or a Leica SM6000 confocal microscope. The following primary antibodies were used: anti-D-Pax2 antiserum diluted 1:20 (Fu and Noll, 1997), anti-Cry antiserum diluted 1:400 (Komori et al., 1992), anti-Senseless antiserum diluted 1:5000 (Nolo et al., 2000). MR1A anti-Prospero and 2B10 anti-Cut and 9F8A9 anti-Elav culture fluids (Developmental Studies Hybridoma Bank, University of Iowa) were diluted 1:500. Secondary reagents included goat anti rabbit Ig–Alexafluor 488, goat anti rabbit Ig–Alexafluor 568, goat anti mouse Ig–Alexafluor 568, and goat anti mouse Ig–Alexafluor 647 all used at 1:1000 dilutions (Invitrogen). All antibody dilutions were made with PBS with 0.1% Triton-X 100 and 2% bovine serum albumin.

In situ hybridization

Eye or notum tissue was dissected from fly pupae in PBS, fixed for 30 minutes in 4% paraformaldehyde and washed extensively in PBS with 0.1% Tween20. Hybridization with digoxigenin-labeled antisense RNA probes was performed as previously described (Tautz and Pfeifle, 1989). The plasmid used for the generation of the Cry probe was constructed by PCR amplification of an 1142 base pair fragment of the Cry coding region (+193 to +1334) from *w¹¹¹⁸* genomic DNA. The PCR fragment was cloned into pBluescript II KS+ (Stratagene) between the HindIII and BamHI sites. For probe synthesis, T7 RNA polymerase was used after linearization of the plasmid with HindIII.

DNA binding assays

The D-Pax2 coding region was amplified by PCR from the full-length cDNA clone, cpx1 (Fu and Noll, 1997) and cloned into the expression vector pMal-c2X (New England Biolabs) and the cloned region was confirmed by sequence analysis. BL21-CodonPlus(DE3)-RIPL cells (Stratagene) were transformed with the resulting plasmid, grown in SOC medium at 37°C to 0.6 A⁶⁰⁰ U and induced with 0.5 mM IPTG for 2 hours. The cells were pelleted at 4000 × g for 20 minutes and resuspended in lysis buffer (20 mM Tris pH 7.5, 0.2 M NaCl, 1 mM EDTA, 0.5 mM DTT) and sonicated for 2 minutes with 15 second pulses at 50% power (Fisher Dismembrator model 300). The lysate was cleared by centrifugation at 9000 × g for 30 minutes and the supernatant was diluted 5-fold with lysis buffer. Cleared lysate was applied to an amylose resin column and bound fusion protein was eluted with lysis buffer with 10 mM maltose. Electrophoretic mobility shift assays were performed using the DIG gel shift kit (Roche Biochemicals). Briefly, double-stranded oligonucleotides were labeled with 32P-digoxigenin-ddUTP by terminal transferase. DNA binding reactions used 1.5 ng of

labeled oligonucleotide and 50 ng of D-Pax2-MBP fusion protein in TEN buffer (10 mM Tris pH 8.0, 1 mM EDTA, 0.1 M NaCl) with or without the addition of competing unlabeled oligonucleotides. Reactions were incubated for 15 minutes and separated on 6% Novex DNA retardation gels (Invitrogen). Gels were electroblotted to Hybond N+ membranes (GE Healthcare Life Sciences) and detection of labeled nucleotides was performed as described in the Roche gel shift kit manual.

Acknowledgments

We wish to thank Naoka Komori and Marcus Noll for the anti-Cry and anti-D-Pax2 antisera, respectively. Monoclonal antibodies MR1A, 2B10, and 9F8A9 – developed by Chris Doe and Gerald Rubin, respectively – were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. We would also like to thank Eric Lai and Katharine Harmon for critical reading of the manuscript. Fluorescent and DIC imaging data were recorded in part in the Advanced Imaging Facility at Middlebury College funded by a National Science Foundation CCLI grant NSF-0088412. This publication was made possible by NIH Grant Number R15-HD04369-01A1 awarded to J.K. and NIH Grant Number P20 RR-016463 from the INBRE Program of the National Center for Research Resources.

Funded by:

National Institutes of Health: Grant numbers R15-HD04369-01A1, P20 RR-016463

National Science Foundation: Grant number NSF-0088412

References

- Bao S, Cagan R. Preferential adhesion mediated by Hibris and Roughest regulates morphogenesis and patterning in the *Drosophila* eye. *Dev Cell*. 2005; 8:925–935. [PubMed: 15935781]
- Baonza A, Freeman M. Notch signalling and the initiation of neural development in the *Drosophila* eye. *Development*. 2001; 128:3889–3898. [PubMed: 11641214]
- Blanco J, Girard F, Kamachi Y, Kondoh H, Gehring WJ. Functional analysis of the chicken delta1-crystallin enhancer activity in *Drosophila* reveals remarkable evolutionary conservation between chicken and fly. *Development*. 2005; 132:1895–1905. [PubMed: 15790965]
- Crosby MA, Goodman JL, Strelets VB, Zhang P, Gelbart WM. FlyBase: genomes by the dozen. *Nucleic Acids Res*. 2007; 35:D486–491. [PubMed: 17099233]
- Cvekl A, Sax CM, Li X, McDermott JB, Piatigorsky J. Pax-6 and lens-specific transcription of the chicken delta 1-crystallin gene. *Proc Natl Acad Sci U S A*. 1995; 92:4681–4685. [PubMed: 7753864]
- Edwards JS, Meyer MR. Conservation of antigen 3G6: a crystalline cone constituent in the compound eye of arthropods. *J Neurobiol*. 1990; 21:441–452. [PubMed: 2351963]
- Epstein J, Cai J, Glaser T, Jepeal L, Maas R. Identification of a Pax paired domain recognition sequence and evidence for DNA-dependent conformational changes. *J Biol Chem*. 1994; 269:8355–8361. [PubMed: 8132558]
- Flores GV, Duan H, Yan H, Nagaraj R, Fu W, Zou Y, Noll M, Banerjee U. Combinatorial signaling in the specification of unique cell fates. *Cell*. 2000; 103:75–85. [PubMed: 11051549]
- Fu W, Duan H, Frei E, Noll M. shaven and sparkling are mutations in separate enhancers of the *Drosophila* Pax2 homolog. *Development*. 1998; 125:2943–2950. [PubMed: 9655816]
- Fu W, Noll M. The Pax2 homolog sparkling is required for development of cone and pigment cells in the *Drosophila* eye. *Genes Dev*. 1997; 11:2066–2078. [PubMed: 9284046]
- Gho M, Bellaiche Y, Schweisguth F. Revisiting the *Drosophila* microchaete lineage: a novel intrinsically asymmetric cell division generates a glial cell. *Development*. 1999; 126:3573–3584. [PubMed: 10409503]
- Heberlein U, Wolff T, Rubin GM. The TGF beta homolog dpp and the segment polarity gene hedgehog are required for propagation of a morphogenetic wave in the *Drosophila* retina. *Cell*. 1993; 75:913–926. [PubMed: 8252627]

- Janssens H, Gehring WJ. Isolation and characterization of drosocrystallin, a lens crystallin gene of *Drosophila melanogaster*. *Dev Biol*. 1999; 207:204–214. [PubMed: 10049575]
- Jarman AP, Grell EH, Ackerman L, Jan LY, Jan YN. Atonal is the proneural gene for *Drosophila* photoreceptors. *Nature*. 1994; 369:398–400. [PubMed: 8196767]
- Kamachi Y, Uchikawa M, Tanouchi A, Sekido R, Kondoh H. Pax6 and SOX2 form a co-DNA-binding partner complex that regulates initiation of lens development. *Genes Dev*. 2001; 15:1272–1286. [PubMed: 11358870]
- Kavaler J, Fu W, Duan H, Noll M, Posakony JW. An essential role for the *Drosophila* Pax2 homolog in the differentiation of adult sensory organs. *Development*. 1999; 126:2261–2272. [PubMed: 10207150]
- Kent WJ, Sugnet CW, Furey TS, Roskin KM, Pringle TH, Zahler AM, Haussler D. The human genome browser at UCSC. *Genome Res*. 2002; 12:996–1006. [PubMed: 12045153]
- Komori N, Usukura J, Matsumoto H. Drosocrystallin, a major 52 kDa glycoprotein of the *Drosophila melanogaster* corneal lens. Purification, biochemical characterization, and subcellular localization. *J Cell Sci*. 1992; 102(Pt 2):191–201. [PubMed: 1400628]
- Kozmik Z, Daube M, Frei E, Norman B, Kos L, Dishaw LJ, Noll M, Piatigorsky J. Role of Pax genes in eye evolution: a cnidarian PaxB gene uniting Pax2 and Pax6 functions. *Dev Cell*. 2003; 5:773–785. [PubMed: 14602077]
- Markstein M, Markstein P, Markstein V, Levine MS. Genome-wide analysis of clustered Dorsal binding sites identifies putative target genes in the *Drosophila* embryo. *Proc Natl Acad Sci U S A*. 2002; 99:763–768. [PubMed: 11752406]
- Meyer MR, Reddy GR, Edwards JS. Immunological probes reveal spatial and developmental diversity in insect neuroglia. *J Neurosci*. 1987; 7:512–521. [PubMed: 3546622]
- Nagaraj R, Banerjee U. Combinatorial signaling in the specification of primary pigment cells in the *Drosophila* eye. *Development*. 2007; 134:825–831. [PubMed: 17251265]
- Nolo R, Abbott LA, Bellen HJ. Senseless, a Zn finger transcription factor, is necessary and sufficient for sensory organ development in *Drosophila*. *Cell*. 2000; 102:349–362. [PubMed: 10975525]
- Phelps DE, Dressler GR. Identification of novel Pax-2 binding sites by chromatin precipitation. *J Biol Chem*. 1996; 271:7978–7985. [PubMed: 8626478]
- Piatigorsky J. Multifunctional lens crystallins and corneal enzymes. More than meets the eye. *Ann N Y Acad Sci*. 1998; 842:7–15. [PubMed: 9599288]
- Pichaud F, Desplan C. Pax genes and eye organogenesis. *Curr Opin Genet Dev*. 2002; 12:430–434. [PubMed: 12100888]
- Reddy GV, Rodrigues V. A glial cell arises from an additional division within the mechanosensory lineage during development of the microchaete on the *Drosophila* notum. *Development*. 1999; 126:4617–4622. [PubMed: 10498695]
- Siddall NA, Behan KJ, Crew JR, Cheung TL, Fair JA, Batterham P, Pollock JA. Mutations in *lozenge* and *D-Pax2* invoke ectopic patterned cell death in the developing *Drosophila* eye using distinct mechanisms. *Dev Genes Evol*. 2003; 213:107–119. [PubMed: 12690448]
- Tautz D, Pfeifle C. A non-radioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. *Chromosoma*. 1989; 98:81–85. [PubMed: 2476281]
- Tomarev SI, Piatigorsky J. Lens crystallins of invertebrates--diversity and recruitment from detoxification enzymes and novel proteins. *Eur J Biochem*. 1996; 235:449–465. [PubMed: 8654388]
- Yang Y, Chauhan BK, Cveklava K, Cvekl A. Transcriptional regulation of mouse alphaB- and gammaF-crystallin genes in lens: opposite promoter-specific interactions between Pax6 and large Maf transcription factors. *J Mol Biol*. 2004; 344:351–368. [PubMed: 15522290]
- Yang Y, Stopka T, Golestaneh N, Wang Y, Wu K, Li A, Chauhan BK, Gao CY, Cveklava K, Duncan MK, Pestell RG, Chepelinsky AB, Skoultchi AI, Cvekl A. Regulation of alphaA-crystallin via Pax6, c-Maf, CREB and a broad domain of lens-specific chromatin. *Embo J*. 2006; 25:2107–2118. [PubMed: 16675956]

Figure 1. Absence of Cry expression in D-Pax2 mutant pupal eyes

Eyes were dissected from *w¹¹¹⁸* (A, B) and *spa^{pol}* (C, D) pupae at 48 hours APF and used for immunohistochemistry staining with an anti-Cry antiserum (A, C) or for in situ hybridization with an antisense *Cry* mRNA probe (B, D). Microphotographs in A and C, as well as B and D, had identical exposure times. Scale bar represents 20 μ m.

Figure 2. Cry expression in es organs

Nota from *w¹¹¹⁸* pupae were dissected at 32 hour APF and used for immunohistochemistry staining with anti-Cry (A) and anti-Prospero (B). Merged image is shown in C. Similar nota were used for in situ hybridization with an antisense *Cry* mRNA probe (D) and were also stained with an anti-D-Pax2 antiserum (F). Merged image is shown in G. In situ hybridization with the *Cry* probe was performed in parallel on *sv⁶* nota (E). Scale bar represents 20 μ m.

Figure 3. Cry-GFP reporter expression in *w¹¹¹⁸* and *spa^{pol}* genetic backgrounds

Panels A and B: Adult *w¹¹¹⁸; Cry-GFP* (left) and *w¹¹¹⁸; Cry-GFP; spa^{pol}* (right) flies. Eyes were dissected from *w¹¹¹⁸; Cry-GFP* (C-E) and *w¹¹¹⁸; Cry-GFP; spa^{pol}* (F-H) pupae at 48 hours APF examined for GFP expression (C and F). In addition, *w¹¹¹⁸; Cry-GFP* eyes were stained with anti-D-Pax2 (D) while *w¹¹¹⁸; Cry-GFP; spa^{pol}* eyes were stained with anti-Cut (G). Merged images are shown in E and H. Scale bar (for E-H) represents 20 μm.

Figure 4. A murine Pax2 site is conserved in Cry enhancer and recognized by D-Pax2
Panel A: Sequence comparison of the D-Pax2 binding site among the related species. The position of the site with respect to the putative transcription start sites is shown in parentheses. The Pax2 binding sequence is highlighted in grey (single nucleotide differences occur in the ninth, tenth, and first positions in *D. erecta*, *D. virilis*, and *D. grimshawi*, respectively). Compilation and analysis of sequence data were performed using Flybase (Crosby et al., 2007) and the USCS Genome Browser (Kent et al., 2002). Panel B: Electrophoretic mobility shift assay was performed with purified D-Pax2-MBP fusion protein and a digoxigenin-labeled double stranded DNA probe of the following sequence: 5'-TCTTGTATTTTAGTCATGCATGACAATTTTTTC-3'. Lanes 1 and 2 contain free probe and probe with the fusion protein, respectively. Lanes 3-5 contain probe and fusion protein with increasing amounts of competing unlabeled wild-type probe (5×, 20×, 80× molar excess). Lanes 6-8 contain a similar competition but with an unlabeled mutated probe of the following sequence: 5'-TCTTGTATTTTAGGAATCTACCACAATTTTTTC-3' where the underlined nucleotides represent changes in the presumptive D-Pax2 binding site.

Figure 5. D-Pax2 binding site alone can drive expression in eyes

GFP reporter expression driven by a multimerized D-Pax2 binding site in a late third instar eye disc (A) and 48 hour APF pupal eye (D). The larval eye disc was stained with anti-D-Pax2 (B). The pupal eye was stained with anti-Cut (E). Overlaid images are presented in C and F. Scale bars for A-C and D-F both represent 20 μ m.

Figure 6. D-Pax2 binding site alone also drives expression in es organ cells

GFP reporter expression driven by a multimerized D-Pax2 binding site in late third instar wing disc (A), 20 hour APF pupal notum (D) and 32 hour APF pupal notum (H). The wing disc and nota were stained with anti-D-Pax2 (B, E, I, respectively) and nota were also stained with anti-Elav (F and J, respectively) to label the neuron. Overlaid images are presented in C, G, K. Scale bar for A-C represents 50 μ m. Scale bar for D-H represents 20 μ m.

Figure 7. Mutation of D-Pax2 binding site in Cry-GFP reporter does not abolish expression *Cry^{MUT}-GFP* reporter expression in 48 hour APF pupal eye (A), stained with anti-D-Pax2 (B). Overlaid image is presented in C. Scale bar represents 20 μ m.