Distinction between Color Photoreceptor Cell Fates Is Controlled by Prospero in *Drosophila*

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Summary

The *Drosophila* compound eye consists of ∼750 independently functioning ommatidia, each containing two photoreceptor subpopulations. The outer photoreceptors participate in motion detection, while the inner photoreceptors contribute to color vision. Although the inner photoreceptors, R7 and R8, terminally differentiate into functionally related cells, they differ in their molecular and morphological makeup. Our data indicates that several aspects of R7 versus R8 cell fate determination are regulated by the transcription factor Prospero (Pros). *pros* is specifically expressed in R7 cells, and R7 cells mutant for *pros* derepress R8 rhodopsins, lose R7 rhodopsins and acquire an R8-like morphology. This suggests that R7 inner photoreceptor cell fate is acquired from a default R8-like fate that is regulated, in part, via the direct transcriptional repression of R8 rhodopsins in R7 cells. Furthermore, this study provides transcriptional targets for *pros* that may lend insight into its role in regulating neuronal development in flies and vertebrates.

Introduction

The fly visual system provides a powerful paradigm for understanding cell type specification. In 5 days, a relatively flat epithelial disc develops into a complex compound eye comprised of ∼750 repeating ommatidial units containing cone, pigment, bristle, and photoreceptors cells (Wolff and Ready, 1993). The eight light-receiving photoreceptors (PRs), R1–R8, fall into two general subtypes: outer (R1–R6) and inner (R7 and R8) PRs (Figure 1A). Outer PRs, much like vertebrate rod PRs, participate in motion detection, while inner PRs are analogous to vertebrate cone PRs in that they are thought to function in color discrimination (Cook and Desplan, 2001). Several morphological characteristics help to distinguish between outer and inner subtypes, including the shape and position of their light-gathering apical membrane (the rhabdomere) and the location of their axonal terminations (Hardie, 1985). R1–R6 each possess a large diameter rhabdomere that spans the entire width of the retina, and together, form a trapezoidal array within an ommatidium; R7 and R8 are positioned at the center of the trapezoid, with their rhabdomeres spanning only half the thickness of the retina, and R7 sitting on top of R8. In addition, the axons of the outer PRs all terminate in the first optic tectum in the brain, the lamina, while both inner PR axons project to a deeper optic tectum, the medulla, with R7 cells projecting to a slightly deeper layer than R8 cells (Figure 1B).

Work on eye development in the larval imaginal disc has demonstrated that PRs are recruited from equipotent epithelial cells in a stereotyped order: R8 first, then R2/R5, R3/R4, R1/R6, and finally R7 (see Kumar and Moses, 1997 for review). Each recruitment event leads to a unique configuration of signaling and transcriptional activity that aids in specifying these cells and establishing their correct axonal projections to the optic lobes. During the next ∼100 hr, R1–R8 continue their differentiation program, acquiring the features described above that allow these cells to function properly. For instance, recent work on the spalt (sal) gene complex, spalt major and spalt-related, has revealed a role for these related zinc finger transcription factors in distinguishing between outer and inner PR fate decisions. The *sal* genes are specifically expressed in the inner PRs of the adult, and in the absence of the *sal* locus, R7 and R8 cells transform into outer PRs (Mollereau et al., 2001). These data revealed a new genetic switch between inner and outer PRs, and suggest that additional factors may be required downstream to further distinguish between R7 and R8 cells.

Photoreceptors absorb light with Rhodopsins (Rhs), the pigment proteins that are present in the stack of membranes that form the rhabdomeres. Several *rhodopsin* gene (*rh*) products exist with varying absorption spectra to ensure a broad range of light sensitivity. Outer PRs all express Rh1, while R7 cells express either Rh3 or Rh4 (UV-sensitive opsins), and R8 cells primarily express blue-sensitive Rh5 or green-sensitive Rh6 (Chou et al., 1996, 1999; Fortini and Rubin, 1990; Huber et al., 1997; Montell et al., 1987; Papatsenko et al., 1997) (Figures 1B–1E). Inner PR *rh* expression is coordinately regulated within an ommatidium (Figure 1B) with Rh3-expressing R7 cells associated with underlying Rh5-expressing R8 cells (termed “pale” ommatidia) (Figures 1B and 1C), and Rh4-expressing R7 cells associated with underlying Rh6-expressing R8 cells ("yellow" ommatidia) (Figures 1B and 1D) (Chou et al., 1996, 1999; Papatsenko et al., 1997). One exception to this is found in the two dorsal-most rows of ommatidia, the dorsal rim area (DRA), in which Rh3 is expressed in both R7 and R8 cells (Fortini and Rubin, 1990) (Figures 1B and 1C). The study of *rh* regulation has revealed that promoters of less than 300 bp can recapitulate endogenous *rh* expression in vivo (Fortini and Rubin, 1987, 1990; Mismer et al., 1988; Papatsenko et al., 2001) (Figures 1C–1E), and are organized in a simple bipartite structure: a conserved proximal element (RCSI, rhodopsin conserved sequence I) common to all *rh* promoters, and upstream *rh*-specific sequences (RUS, rhodopsin upstream sequences) unique to each *rh* promoter (Fortini and Rubin, 1990; Papatsenko et al., 2001). This organization predicts that common as well as unique factors function together to dictate *rh* gene expression, and consequently, to properly specify different ommatidial subtypes.

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We have taken advantage of the subtype-specific expression of the inner PR rh genes as a tool for understanding later events in PR development. Here, we report the identification of a conserved element that is specifically present in the R8 rh promoters, rh5 and rh6, which is responsible for repressing these genes in R7 cells. A yeast one-hybrid screen revealed that this element provides a binding site for Prospero (Pros), an atypical homeodomain transcription factor important for neuronal specification in the developing Drosophila embryo. We find that Pros is specifically expressed in R7 photoreceptors in the adult. Furthermore, genetic studies demonstrate that prospero (pros) is both necessary and sufficient for the repression of R8 rh genes in R7 cells. pros mutant R7 cells not only gain expression of R8 rhodopsins, but also acquire R8-like morphological features and lose R7-specific markers such as Rh3 and Rh4. These findings suggest that once inner PRs are coordinately distinguished from outer PRs through Sal, Pros then helps to further distinguish R7 from R8, with R8 being the ground state of differentiation for inner PRs. As the vertebrate homolog of prospero, prox1, is also expressed in subsets of retinal cell populations, we propose that a role for this transcription factor is evolutionarily conserved to provide cell type diversification in the eye.

Results

Identification of Seq56, an R7 Repression Element in R8 rhodopsin Promoters

We have found that, in addition to common RCSI sequences and unique RUS elements within the minimal rh promoters, some sequences are also shared between subsets of rh genes (Figure 2A) (A. Tahayato et al., submitted). Here, we describe the characterization of an 11 bp sequence that is shared between the R8-specific rh5 and rh6 promoters (Figure 2B), a sequence we refer to as seq56. Seq56 is evolutionarily conserved within the rh5 and rh6 promoters of D. melanogaster, D. pseudoobscura, and D. virilis, both in sequence and relative position to other conserved elements (Figure 2B) (A. Tahayato et al., submitted), but is absent in the rh1-rh4 promoters (see Experimental Procedures). Deletion mapping of the rh6 promoter revealed that while the promoter -246/-121 could drive wild-type expression (Figure 1E), a deletion construct that removed a portion of seq56 (-202/-121) led to inappropriate reporter expression in R7 cells (Figure 1F, arrowheads; Figure 2A). These data suggest that the presence of seq56 in both rh5 and rh6 is important for their correct expression.

Seq56 constitutes an imperfect palindrome of the pentameric sequence AG/C/GC, and several copies of this half-site are distributed throughout the rh5 and rh6 promoters (Figures 2A and 2B and http://homepages.nyu.edu/~dap5/rh/rhodopsins.html). To examine the in vivo function of seq56, we generated site-directed mutations that disrupted the A (5') or B (3') half site of the palindrome (a/B and A/b, Figure 2A) within the rh6 promoter. Neither mutation affected reporter expression in R8 cells, but led to strong derepression in R7 cells (compare Figure 2C with 2D and 2E). Derepression into R7 cells was determined based on lacZ reporter expression in rhabdomeres that spanned only the distal half of the retina (Figures 2C–2E, brackets) and projections that terminated in the medulla (data not shown). Similar mutagenesis studies within the context of the rh5 promoter did not affect reporter activity, even when combined with mutation of a perfect seq56 or site located...
Figure 2. Seq56 as an R7-Specific Repressor Element for R8 Rhodopsins

(A) Diagram of the minimal rh5 and rh6 promoters (A. Tahayato et al., submitted). ‘K’ boxes represent binding sites for K50 homeodomain proteins, and “A” and “B” boxes represent the seq56 half sites. Arrows represent the start of transcription.

(B) Alignment of seq56 sequences from Drosophila melanogaster (Dm), D. pseudoobscura (Dp), and D. virilis (Dv). K50 binding sites in close proximity to the seq56 palindrome in the rh5 promoters are underlined.

(C–H) X-gal staining of wild-type and site-directed mutants of the minimal rh6 promoter (A/B11002)246/H11001121) (C–E) or minimal rh3 promoter (A/B11002)159/H1100118) (F–H) driving Gal4 lines and a UAS-lacZ reporter gene. Wild-type seq56 sites and mutants in the “A” or “B” boxes are indicated, with mutated nucleotides underlined, bold, and lowercased. RUS3 represents an evolutionarily conserved element present within the rh3 promoter.

(C–E) Mutation of either side of the seq56 imperfect palindrome causes ectopic expression of lacZ in the R7 layer (D and E, arrowheads).

(F–H) Wild-type rh3 expression (F) is lost by the insertion of three wild-type seq56 sequences (A/B)3, but is maintained with the introduction of three mutant seq56 elements (A/b)3 (H).

Upstream of its RCS1 (see Figure 2A) (data not shown). However, both loss- and gain-of-function experiments indicate that rh5, like rh6, is regulated by a seq56-mediated process (see below), suggesting that other elements are also involved in rh5 repression.

To test whether seq56 was sufficient to repress R7-specific expression of a heterologous promoter, we placed wild-type or mutant versions of seq56 within the rh3 minimal promoter. The addition of wild-type seq56 elements (A/B)3 abolished rh3 reporter activity in R7 cells (compare Figures 2F and 2G), whereas the addition of a mutant seq56 element (A/b)3 at the same locations allowed normal rh3 expression (Figure 2H). Together, these data indicate that seq56 is both necessary and sufficient to repress rh expression in R7 photoreceptors.

Identification of Prospero as a Seq56-Interacting Protein

To identify potential seq56 trans-acting factors, we screened a Drosophila adult head cDNA library with this element using the yeast one-hybrid approach. The strongest his3/lacZ positives from this screen (five out of nine) encoded the DNA binding domain and C terminus of the homeodomain transcription factor, Pros. Immunostaining of head cryosections revealed nuclear expression of Pros in all adult R7 PRs (Figures 3A–3C). To test whether seq56 is both necessary and sufficient to repress rh expression in R7 photoreceptors.

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Figure 3. Prospero as an R7-Specific Factor that Binds seq56 In Vitro

(A–E) Expression pattern of Prospero in the adult retina (A–D) or pupal retina 72 hr APF (E) (dorsal up, distal right). Costaining of adult retinas with anti-Pros (red, [A]) or anti-Sal (green, [B]) antibodies (B). R7 nuclei are distally placed within the cells, whereas R8 nuclei are proximally located ([B], arrowheads). Overlapping these images revealed that Pros is present in all R7 nuclei (C). Also note that weak Pros immunoreactivity is present in the cone cells of the retina ([A and C], arrow “C”). (D) Costaining Pros (red) with a pros nuclear-lacZ enhancer trap, pros10419 (green) revealed that Pros expression in R7 cells is nuclear (arrowhead), whereas cone cell expression is cytoplasmic (arrow). (E) 72 hr pupal retinas were stained for the sal nuclear lacZ enhancer trap, salm03602 (green, sal-NLZ) and Pros (red) expression. Similar expression as that observed in adult retina (C) was observed.

(F) PCR amplification of pros-S (“S”) and pros-L (“L”). Lane 1, DNA from a pros-encoding one-hybrid clone; lane 2, adult Drosophila head cDNA library used for our screen; lane 3, 0–6 hr Drosophila embryonic cDNA library; lane 4, genomic DNA isolated from UAS-pros-L flies.

(G) Gel shift assays of GST (“G”), GST-ProsS (“S”), and GST-ProsL (“L”) fusion proteins purified from E. coli. Seq56-based oligonucleotides are aligned, with gray nucleotides representing those that differ from the control sequence. GST shows no specific interaction.

(H) Pros-S binding to seq56. Probes represent variations of the core sequence from the oligonucleotide, tcagggatcttcggTAAGACGtctctagatct.

Sheng et al., 1997), we analyzed the expression of Pros in retinas at this stage of development. Both at 72 and 96 hr APF, we detected Pros expression in a similar pattern as what we observed in the adult (Figure 3E and data not shown). These data, in conjunction with those of Kauffmann et al. (1996), suggest that pros is only very transiently turned off in R7 cells during midpupation, and its expression is reestablished in the same cell populations. The finding that Pros is expressed in R7 cells at the time when rh genes are expressed is consistent
with a role for Pros in mediating seq56-directed repression.

Binding Specificity of Prospero

The region of pros identified in our screen is alternatively spliced in Drosophila embryos, resulting in proteins that differ by the absence (Pros-S) or presence (Pros-L) of 27 aa directly upstream and including a portion of the homeodomain (Chu-Lagraff et al., 1991) (Figure 3F, lane 3). All five of the clones we identified represented pros-S (Figure 3F, lane 1). PCR from a 0–6 hr embryonic cDNA library, as well as the head cDNA library used in our screen, revealed that the pros-S isoform was more abundant than pros-L (Figure 3F, lanes 2 and 3); RT-PCR from head mRNA revealed similar findings (data not shown). Since these proteins differ within their DNA binding domains, we tested both Pros-S and Pros-L fusion proteins in DNA binding assays with seq56. Both Pros-S (S) and Pros-L (L) bound strongly to the wild-type imperfect palindromic seq56 (CGGCTAGACG) (Figure 3G). As both half sites of the palindrome were required for rh6 repression in R7 cells (Figures 2D and 2E), we also tested the ability of Pros to bind either the A or B site alone. While mutation of the B site (A/b) abolished Pros binding, mutation of the A site (a/B) had no effect (Figure 3G). Additionally, a perfect A/A palindrome provided no binding, indicating that Pros does not recognize seq56 as a palindromic, but instead, binds to the sequence present in the a/B sequence TAAGACG. Two complexes were observed in these experiments; however, the slower migrating band is likely to represent a GST-induced dimer, as identical complexes were formed with probes carrying A/B, B/B, or B sites alone (Figures 3G and 3H), and the slower migrating complex was not observed under different buffer conditions (data not shown). Regardless of the experimental conditions, however, no significant differences in binding strength or specificity were observed between Pros-S and Pros-L (Figure 3 and data not shown). We also created site-directed mutants within the minimal binding sequence, TAAGACG. While mutation in any position disrupted Pros binding to some extent, mutation of the T at position 1 resulted in the weakest disruption, while mutation at positions 3 and 4 drastically decreased binding (Figure 3H). Together, these data indicate that Prospero preferentially recognizes the seq56aT element, AAGACG.

Prospero Is Necessary and Sufficient to Repress R8 Rhodopsins In Vivo

To test whether Pros binds seq56 to repress R8 rhodopsins in R7 cells, we generated flies entirely null for pros function in the eye (see Experimental Procedures) (Stowers and Schwarz, 1999). Immunostaining of cryosections of pros[1] null mutant eyes demonstrated that both rh5 and rh6 expression was expanded into the R7 layer of the retina, while their expression in R8 cells was maintained (Figures 4A–4D). In contrast, the misexpression of pros-L in differentiated cells of the eye using the GMR-GAL4 driver significantly repressed both Rh5 and Rh6 expression in R8 cells (Figures 4E–4H), but had no effect on the expression of Rh1 in outer PRs, Rh3 and Rh4 in R7 cells, or Cut, a cone cell marker expressed in the adult (data not shown). These results support the idea that rh5 and rh6 expression is directly regulated by pros, and that Pros does not require an R7-specific factor for repression.

Derepression of R8 Rhodopsins Results in Loss of R7 Rhodopsins

Most sensory systems have developed a mechanism of mutual exclusion of their various receptor proteins to prevent the overlap of inputs into the brain (Pilpel et al., 1998). To test whether this occurs in the context of pros mutant eyes, where R8 rhodopsins are inappropriately present in R7 cells, we next examined the expression of Rh3 and Rh4 in pros[17] mutants. Rh3 expression was maintained in the dorsal rim area of the retina, where it is normally present in both R7 and R8 cells; however, in the remaining portion of the retina, Rh3 expression was significantly reduced or abolished (Figure 5A versus 5B). The concomitant gain of Rh5 and loss of Rh3 in pale mutant eyes demonstrates that both pros-S and pros-L prevent the overlap of inputs into the brain (Pilpel et al., 1998). To test whether this occurs in the context of pros mutant eyes, where R8 rhodopsins are inappropriately present in R7 cells, we next examined the expression of Rh3 and Rh4 in pros[17] mutants. Rh3 expression was maintained in the dorsal rim area of the retina, where it is normally present in both R7 and R8 cells; however, in the remaining portion of the retina, Rh3 expression was significantly reduced or abolished (Figure 5A versus 5B). The concomitant gain of Rh5 and loss of Rh3 in pale mutant eyes demonstrates that both pros-S and pros-L prevent the overlap of inputs into the brain (Pilpel et al., 1998).
Figure 4. Pros Is Necessary and Sufficient to Repress R8 rh Expression

Adult retinal cryosections from control heads (A, C, E, and G), eyes homozygous for the pros
t null allele (B and D), or eyes misexpressing pros-L with the GMR-GAL4 driver (F and H). All images are oriented with dorsal left, and distal up, and R7 and R8 layers are separated by a dotted line.

(A–D) Wild-type expression of Rh5 (A) and rh6-lacZ (C) in heterozygous pros
t retina (A and C) and derepression of Rh5 (B) and rh6-lacZ (D) in homozygous pros
t mutant eyes.

(E–H) Misexpression of pros-L results in significant repression of Rh5 (F) and Rh6 (H) expression in R8 cells, while flies carrying only UAS-pros-L or GMR-GAL4 appear wild-type (data not shown). The eye is slightly disrupted by the misexpression of Pros, indicated by the misplaced photoreceptor nuclei and rhabdomeres (F and H).

consistent results, independent of the presence or absence of the rh6
t allele.

As mentioned earlier, R7 and R8 cells both project to the medulla lobe, with R7 cells projecting slightly deeper than R8 cells. Labeling whole-mounted brain preparations with the PR-specific chaoptin antibody 24B10, we found that although the retinotopic map was slightly disturbed (data not shown), R7 and R8 terminations formed two obvious layers in the medulla of pros mutant eyes (Figures 6A and 6B). Staining for the rh6-lacZ reporter also revealed that, while rh6 expression was restricted to the R8 layer in controls, pros mutants led to lacZ-positive terminations in both the R7 and R8 layer (Figures 6C and 6D). While the results shown were acquired from pros
t, rh6
t double mutants, rh6-lacZ-positive R7 terminations were also observed with pros alleles carrying a wild-type rh6 gene, albeit fewer in number. R7- and R8-positive terminations were also observed in pros mutants carrying the rh5-lacZ reporter (data not shown). These data are consistent with findings by Kauffmann et al. (1996) who detected no changes in early PR differentiation in pros mutant cells, and indicate that neuronal specification and terminal PR differentiation can be uncoupled in R7 cells lacking pros.

Two additional characteristics that distinguish R7 from R8 cells are the distal versus proximal positions of their rhabdomeres and nuclei. As indicated in Figures 4 and 5, distal rhabdomere staining for Rh5 and Rh6 was observed in pros mutant eyes. These are not R8 rhabdomeres that have escaped into the R7 layer, a feature observed with certain R7-less mutants (e.g., sev, boss) (Chou et al., 1999), as we could observe both R7 and R8 rhabdomeres positive for Rh5 or Rh6 within a single ommatidium (Figures 6G–6J). Staining for rh6-lacZ and the DNA marker, Hoechst 33258, however, revealed that the position of the R7 nuclei most often adopted a proximal localization (Figures 6L and 6N), while wild-type R7 nuclei were distal (Figures 6K and 6M). These results indicate that while the rhabdomeres of pros mutant R7 cells are able to properly assemble within an ommatidium, their nuclei remain proximal, giving the impression that two “R8-like” inner PRs are present on top of each other. This phenotype was observed even in cells which maintained Rh4 expression (data not shown), indicating that this event is due to the loss of pros function and not from the acquisition of R8 rhodopsins. Because nuclear position is associated with the developmental state of the cell (Tomlinson, 1985), these data indicate that pros mutant R7 cells not only acquire final R8-specific differentiation markers, but also additional R8-specific features.

We next examined non-rh molecular markers that are expressed in adult inner PRs to ascertain the degree of R7-to-R8 transformation that occurs in pros mutant R7 cells. Two such markers, Spalt and Senseless (Sens), are specifically expressed in inner PRs (Mollereau et al., 2001) and R8 cells (Figure 6Q) (Frankfort et al., 2001), respectively, in the adult retina. Spalt is expressed in R7 and R8 cells from larval to adult stages and is concomitant with Pros activation in the imaginal disc (Wernet, F.P. and C.D., unpublished data). Previous experiments aimed at characterizing the sal mutant phenotype indicated that pros expression remained in R7 cells lacking sal (Mollereau et al., 2001), despite the fact that
Prospero Regulation of R7 Photoreceptors

Figure 5. R8 versus R7 rh Expression in pros Mutant Eyes

Adult cryosections from control heterozygous (A, C, E, G, and I), pros17 rh6[1] homozygous (B, D, and F), or prosJ01 homozygous (H and J) eyes immunostained with antibodies to Rh3 (A and B), Rh4 (C–F, I, and J), Rh5 (E and F), or Rh6 (G–J). pros17 mutant flies also carried a 55D/121 rh6-lacZ reporter detected using anti-LacZ (C and D). Rh3 expression is lost in the majority of the retina in pros17 mutant eyes (A versus B), although its expression in the DRA is maintained ([B], arrowhead). (C and D) Rh4 expression (green) remains essentially unchanged and overlaps significantly with rh6-lacZ (red) in pros17 mutant eyes. (E and F) Costaining for Rh4 (green) and Rh5 (red) demonstrates that, in pros17 mutants (F), Rh5 is expressed in R7 cells not expressing Rh4. (G and H) Homozygous prosJ01 eyes exhibit derepression of Rh6 (H) compared to control flies (G), but the extent of derepression is less than that observed with rh6-lacZ with the pros17 allele (compare H and D) due to the presence of the rh6[1] mutation on the pros17 chromosome (see text for details). (I and J) Costaining for Rh4 (green) and Rh6 (red) reveals little to no Rh6/Rh4 overlap in FRT-prosJ01 mutant eyes.

R7 cells resembled outer PRs in all other later aspects of development. Contrary to this earlier report, we observed that eyes homozygous mutant for both sal genes no longer expressed pros (Figures 6Q and 6P). Furthermore, sal expression was maintained in pros mutant eyes (data not shown). These data strongly suggest that pros functions downstream of sal-mediated inner PR specification. We have recently found that Senseless (Sens), a transcription factor important for R8 specification in the imaginal disc (Frankfort et al., 2001), remains restricted to R8 cells in the adult retina (Figure 6Q). Sens was not expanded in pros mutants (Figure 6R) and was maintained in eyes misexpressing pros with GMR-GAL4 (data not shown). These data confirm our earlier observations that pros is necessary for preventing R7 cells from adopting many, but not all, R8 characteristics and suggest that inner PRs develop through several genetically distinct stages (see Figure 7).

Discussion

Regulation of R7 Photoreceptor Differentiation

During eye disc development, R7 cells are specified by three different signaling cascades: Sevenless (Sev), EGFR, and Notch (Cooper and Bray, 2000; Freeman, 1996; Hafen and Basler, 1991; Tomlinson and Struhl, 2001). Previous studies have demonstrated that normal levels of pros expression in R7 cells require Ras pathway activation via EGFR and Sev signaling (Kauffmann et al., 1996; Xu et al., 2000), as well as Notch activation (R. Carthew, personal communication). These data suggest that pros could be a critical target for inducing R7 differentiation. However, no changes in early R7-specific markers in pros mutant eye discs could be detected (Kauffmann et al., 1996), and morphological characteristics such as correct projections to the medulla and rhabdomere positioning on top of the R8 rhabdomere within the ommatidial center were largely unaffected even in the adult retina (Kauffmann et al., 1996) (Figure 6). The findings reported here, however, indicate that while pros mutant R7 cells maintain some R7-specific gene products (e.g., Rh4) and lack R8-specific markers such as Sens, other aspects of R7/R8 differentiation involving R7 nuclear positioning and correct rh gene expression are dramatically affected (Figure 7). We also found that pros expression was lost in sal mutants, while sal expression remained in pros mutants. These findings suggest that R7 cells acquire their functional identity through several distinct stages (Figure 7A). Initially, all
eight PRs are recruited at distinct times within the imaginal disc, allowing each to be influenced by a unique cell signaling environment. This recruitment/specification results in the proper establishment of projections to the optic lobe. At some point afterwards, a common pathway involving sal converges onto both the R7 and R8 cells that allows them to continue to develop as inner PRs, rather than adopting an outer PR state. These cells,
expression of cell-specific markers such as Pros and Sens. (A) Diagram representing inner PR characteristics in wild-type (WT) or pros mutant eyes. These cells differ by their nuclear position in the cell, as well as the expression of the transcription factors Pros and Sens. Under wild-type conditions, Rh3 in R7 cells is coupled with Rh5 in R8 cells in pale ommatidia, whereas Rh4 in R7 cells is coupled with Rh6 in R8 cells in yellow ommatidia. In the absence of pros, the nuclei of R7 cells become distally localized in the cell, pale R7 cells express Rh5 and lose Rh3 expression, and yellow R7 cells express either Rh4 or Rh6, depending on the presence or absence of a functional Rh6 molecule.

(B) Model for the regulation of rh genes in the adult retina. Pros represses the expression of R8 rhodopsins, rh5 and rh6, in R7 cells. In pale ommatidia, Otd, probably in conjunction with a subset-specific factor (X), activates both rh3 and rh5 in both R7 and R8 cells (T.C. and C.D., unpublished data). We also have genetic evidence that factors Y and Z help to establish the expression of the yellow rhodopsins rh6 and rh4, respectively. In the absence of Pros in yellow R7 cells, rh6 expression is derepressed. The ectopic presence of the Rh6 protein induces an exclusion pathway that leads to the elimination of either rh4 or rh6. By extrapolation, we propose that in pale ommatidia, Rh5 is capable of excluding Rh3 expression.

Based on our pros loss-of-function experiments, are likely to adopt an R8-like morphology in the absence of additional signals, but must also remain distinct in their expression of cell-specific markers such as pros and sens. In R7 cells, pros helps to promote additional R7-specific characteristics, including the direct repression of R8 rhodopsins and nuclear positioning. Other factors must participate in events leading to their distal positioning in the retina as well as the subtype-specific expression of rh3 and rh4 (see Figure 7B). Similarly, additional factors such as Sens are likely to contribute to equivalent aspects of R8 development. The findings reported here for pros, however, are particularly exciting as they provide a genetic inroad for blocking PR differentiation at an intermediate step. Future studies aimed at investigating these later events should be useful for understanding the pathways that transform eight unique cell types into the two functional visual systems in the adult.

Prospero and the Regulation of Gene Expression

Propero is critical for neuronal cell specification in the developing central and peripheral nervous system, and is transiently expressed in the nucleus of neuronal precursor cells (Knoblich et al., 1985; Reddy and Rodrigues, 1999; Spana and Doe, 1996). Despite extensive work aimed at understanding Pros function, little is known regarding its direct molecular targets. As exit from the cell cycle often precedes terminal differentiation, several studies have correlated the expression of cell-cycle regulators with Pros function. Indeed, the expression of genes such as decapo, string, cyclin E, E2F, and cyclin A is reduced during pros-mediated differentiation, and increased in the absence of pros (Li and Vaessin, 2000; Liu et al., 2002). However, it has been difficult to assess which genes are directly controlled by Pros, and those whose expression changes as an indirect requirement to exit the cell cycle, as the Pros target sites within these genes are not known (Myster and Duronio, 2000).

Here, we have identified a functional Prospero target sequence, (T)AAGAGC. The only other known Pros binding site, C^Y/C^Y/NNCC^Y, was identified with a site selection assay (SELEX) (Hassan et al., 1997). We could not detect significant similarity between the minimal seq56A and the SELEX consensus, but did find a loose SELEX consensus within the full seq56 element, CGGCTAAGAC (underlined, reverse complement of C^Y/C^Y/NNCC^Y).

However, gel shifts with the GGCTAAG sequence showed no binding to Pros-S or Pros-L, consistent with our findings that the 3' end of seq56 is critical for Pros binding; additional gel shifts revealed that both Pros-S and Pros-L could bind weakly to individual SELEX sites, but only when these sites were multimerized was binding similar to that observed with seq56 (data not shown). Thus, it is likely that Pros exhibits some flexibility in its binding specificity, and as additional Pros target sites are identified, a clearer consensus will develop.

One observation that is supported by both this study and that of Hassan et al. (1997) is that Pros is likely to regulate transcription in combination with other factors. For instance, our findings that seq56 is a conserved imperfect palindrome, and that mutations that do not disrupt Pros binding still lead to R7 derepression (e.g., seq56A mutants), imply that the entire seq56 element is necessary to mediate repression. Indeed, although a perfect seq56A binding site was found within the rh4 promoter, no significant changes in Rh4 expression were observed in pros^{T17}, rh6^{S10} mutant eyes (Figure 5), nor in flies misexpressing pros-L in all photoreceptors (data not shown); furthermore, mutation of this site within the rh4 minimal promoter did not abolish reporter expression in R7 cells (data not shown). Thus, these data suggest an important role for the 5’ end of the seq56 element in mediating repression.
Potential for an Evolutionarily Conserved Role of Pros in Rhodopsin Repression and Photoreceptor Development

Similar to the Drosophila visual system, fate mapping in vertebrates has revealed that all retinal cell types are derived from the same precursor cell population, and that differentiation of these cell types occurs in a stereotyped order (Cepko et al., 1996). For instance, cone, horizontal, and amacrine cell genesis occurs prior to rod photoreceptor formation, followed by bipolar and Müller glia genesis. Recent findings have demonstrated that Prox1, the vertebrate counterpart of Pros, is embryonically expressed in dividing retinal progenitor cells, and postnatally expressed in differentiating horizontal cells, Aβ amacrine cells, and at lower levels in bipolar cells (Belecky-Adams et al., 1997; Tomarev et al., 1996; 1998; Dyer et al., 2003). Thus, Prox1 expression in horizontal/amacrine precursors may serve to drive these cells toward terminal differentiation and prevent them from dividing to become later cell types such as PRs. Indeed, in the prox1 knockout mouse, horizontal cells fail to form, while the rod PR number is increased 50%–70% (Dyer et al., 2003). Therefore, it is likely that Prox1/Pros plays an evolutionarily conserved role in specifying unique neuronal cell types in the eye. While a more precise comparison between vertebrate and invertebrate PRs is difficult, opsin gene expression represents a late differentiation step in all PR development. Thus, it will be interesting to explore the possibility that Prox1/Pros regulate opsin expression throughout evolution. Indeed, preliminary analyses have revealed the presence of putative Pros binding sites within the minimal promoter of the Xenopus rhodopsin promoter (T.C., unpublished data; data not shown). Furthermore, these sites are located precisely in a region demonstrated to function as a repressor element both in cultured cells and in vivo (Mani et al., 2001). Thus, we believe that Pros/Prox1 is a key regulator of both early and late stages of PR development, not only in insects but also in vertebrates.

Experimental Procedures

Identification of Common Promoter Elements in the Rhodopsin Genes

The six rh gene promoters from several Drosophila species was aligned using Megalign software (DNASTar, Madison, WI). Evolutionarily conserved elements (strings) were extracted and analyzed further using motif extraction software (e.g., Gibbs sampler). This revealed the specific presence of the motif CGGCTAAGACG in the two R8 rh promoters, rh5 and rh6 (data available from our web resource http://homepages.nyu.edu/~dap5/rh/rhodopsins.html).

One-Hybrid Screen

Bait plasmids carrying four concatamerized copies of 5′-gtacgtcg CGTGAAGACGttg CGGCTAAGACGttgtctagatct-3′ (capitalized letters representing seq56) were cloned into pHisi-1 and pLacZi (Clontech) and stably transformed into the yeast strain YPH499 (MATa ade2-101  lys2-801 ura3-52 trp1-526 his3-1200 leu2-3,1, kindly provided by Isabelle Brun). These yeast were then transformed with a transactivation fused Drosophila head-specific cDNA library (kindly provided by Michael Rosbash, Brandeis University). ~5 × 10^9 CFU were amplified on SD/His-/Ura-/Trp- media, and selected on the same media containing 40 mM 3-amino-1,2,4-triazole (Sigma), 2% galactose, 1% raffinose. Of ~5 × 10^9 amplified CFU, 9 HIS3/lacZ clones were identified: four that weakly activated lacZ expression, and five that strongly activated. The cDNAs from all positives were PCR-amplified and sequenced. The weak positives represented CG1883 twice (ribosomal protein S7), CG7726 (ribosomal protein D11), and CG8621 (function unknown). All five strong lacZ+ colonies corresponded to aa 1112–1371 of Pros-S (GenBank #P29617).

Characterization of Pros-S and Pros-L

0.5 μg DNA from a 0-6 hr Drosophila embryonic cDNA library (kindly provided by Isabelle Brun), the adult Drosophila head cDNA library used for the screen, one-hybrid positives, or genomic DNA from UAS-pros-L flies was used to amplify the homeodomain and C terminus of Pros using the primers tacgacgcgtCTCGAGCGCCATCGCC GGACTAC (5′) and cgtaactcctgTCCGCGCCCACTCTCCTC ACT (3′) (capital letters representing pros, restriction sites underlined). Both isoforms from the embryonic cDNA library were purified, digested with BamH1/XbaI (sites included in the primers), cloned into the GST fusion vector pGEX-SX-1 (Pharmacia), and verified by sequencing. GST protein purification and gel shift assays were performed as described (Cook et al., 1998). Gel shift probes were comprised of the sequences tgcagggatcctgCGGCTAAGACGtatctagatct (for B box variants) or tcagacgcgtCTCGACGTCCAACTCGCTCCTC (for seq56-derived sequence in capitals).

Transgenic Constructs and Genetic Crosses

rh promoter mutants were generated by PCR and verified by sequencing. A pCasper GAL4 driver was generated by replacing the BamHI-XbaI lacZ gene fragment in pChAb3Sal (Wimmer et al., 1997) with a BamHI-SpeI GAL4 coding sequence from pGaTB. Transforms were generated in a yw– background as previously described (Wimmer et al., 1997), and at least five different lines were generated for each construct. Whole mutant pros eyes were generated using the EGUF system, a method which kills wild-type homozgyous and heterozygous eye tissue by virtue of a GMR-Hid transgene and a cell-internal mutation (I13CL-R) on the FRT82B chromosome (Stowers and Schwarz, 1999). FRT-induced mitotic recombination was catalyzed by ey-flp, an eye-specific source of the FLIP recombinase. ey-FLP; Sp/Cyo; FRT82B-GMR-Hid/TM6b flies were crossed to yw–;FRT82B-pros+/TM6b or yw–;FRT82B-pros+/O; TM6b. ey-FLP; +/Sp/Cyo; FRT82B-GMR-Hid/FRT82B-pros flies were analyzed as pros–, and ey-FLP; +/Sp/Cyo; FRT82B-pros/TM6b were used as controls. ey-FLP, GMR-GAL4, and UAS-lacZ flies were kindly provided from J. Treisman (Skirball, NYU), UAS-prosL and FRT82B-pros+ flies from C. Doe (University of Oregon), and FRT82B-pros– flies from R. Carthew (Northwestern University). pf35+1m1020 pros+1m1042, FRT82B-GMR-Hid, I13CL-R and FRT40-GMR-Hid, I12CL-L/Cyo flies were supplied by the Bloomington Stock Center. Flies carrying the FRT82B-pros– allele also carried the rh6– mutation (see below). Thus, we recombined wild-type rh6 from yw– flies onto the FRT82B-pros– chromosome. Wild-type rh6 and null pros expression of newly established FRT82B-pros– alleles were analyzed by immunostaining. Two stocks, named pros–1m10 and pros–1m10, were retained. Minimal rh–lacZ transgenes were as described (Papatsenko et al., 2001) and introduced on the second chromosome. Flies were raised at 25°C, and pupal staging was according to Bainbridge and Bowes (1981).

β-Galactosidase Stainings and Immunostainings

X-gal staining of adult head cryosections was performed as previously described (Papatsenko et al., 2001). Immunostaining of adult head cryosections was as follows: 8–10 um sections were fixed in 4% paraformaldehyde for 15 min, washed three times with PBS (0.3% Triton-X100), and incubated with primary antibodies diluted in BNT (PBS, 0.1% BSA, 0.1% Tween 20, 250 mM NaCl). The antibodies and dilutions used were: rabbit anti-Rh4 (gift from C. Züker, University of California at San Diego) 1:300, chicken anti-Rh3 (generated against the peptide sequence LALNEKAPSESSA) 1:20, mouse anti-Rh4 1:10 and mouse anti-Rh5 1:50 (both gifts from S. Britt, University of Colorado), rabbit anti-Rh6 (A. Tahayato et al., submitted) 1:5000, rabbit anti-β2-galactosidase (Cappell) 1:5000, mouse anti-β2-galactosidase (Promega) 1:500; guinea pig anti-Sens (H. Bellen, Baylor College of Medicine) 1:750, mouse anti-Pros (C. Doe, University of Oregon) 1:10, rabbit anti-Sal (B. Mollière, Rockefeller University) 1:100, and mouse anti-chaopticin, 2B10 (Developmental Cell, 862).
Studies Hybridoma Bank, University of Iowa) 1:10. Samples were then washed with PBX and incubated with appropriate secondary antibodies (AlexaFluor488, Cy3 or Cy5 conjugated) from Molecular Probes (Portland, OR) and Jackson ImmunoResearch Laboratories (West Grove, PA) at manufacturer’s suggestions. Nuclei were stained 2 min with 1 μg/ml Hoechst 33258 (Sigma) prior to mounting. Samples were mounted with Aquamount (Lerner Laboratories) and analyzed with a Leica TCS S2 confocal system or a Nikon Microphot-SA microscope. Digital images were obtained using SPOT software.

Rh6 Mutant Analysis

The 3R chromosomal arm carrying the FRT82B-pros17 allele contained a naturally occurring 19 bp deletion in the rh6 gene (the existence of this mutation in common stocks was originally pointed out to us by S. Britt). This mutation, rh6509, includes a stop codon that leads to truncation of the Rh6 protein within the 5th transmembrane domain and thus is not recognized by anti-Rh6 antibodies. It should be noted that this mutant sequence, and not that originally described (Huber et al., 1997), is currently deposited in the Berkeley Drosophila Genome Project, and thus is likely to be present in a large number of available fly stocks. Specifically, it is present in w1118 flies from the Bloomington Stock Center.

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