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Otd/Crx, a Dual Regulator for the Specification of Ommatidia Subtypes in the *Drosophila* Retina

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Summary

Comparison between the inputs of photoreceptors with different spectral sensitivities is required for color vision. In *Drosophila*, this is achieved in each ommatidium by the inner photoreceptors R7 and R8. Two classes of ommatidia are distributed stochastically in the retina: 30% contain UV-Rh3 in R7 and blue-Rh5 in R8, while the remaining 70% contain UV-Rh4 in R7 and green-Rh6 in R8. We show here that the distinction between the *rhodopsins* expressed in the two classes of ommatidia depends on a series of highly conserved homeodomain binding sites present in the *rhodopsin* promoters. The homeoprotein Orthodenticle acts through these sites to activate *rh3* and *rh5* in their specific ommatidial subclass and through the same sites to prevent *rh6* expression in outer photoreceptors. Therefore, Otd is a key player in the terminal differentiation of subtypes of photoreceptors by regulating *rhodopsin* expression, a function reminiscent of the role of one of its mammalian homologs, Crx, in eye development.

Introduction

The adult fly compound eye is composed of approximately 800 ommatidia, each formed by 20 cells, including eight photoreceptors (PRs), R1–R8. Final differentiation of the various PRs occurs fairly late in retinal development and is achieved when specific *rhodopsin* genes (*rhs*) are activated, thus allowing detection of various wavelengths of light (Cook and Desplan, 2001). Each PR gathers light through the rhabdomere, a packed stack of microvillar membranes filled with rhodopsin molecules (Rh). The rhabdomeres of the PRs R1–R6 form a trapezoid whose center is occupied by those of the R7 and R8 PRs, with R7 sitting on top of R8 (Hardie, 1985). Thus, the PRs fall into two classes based on their position within the ommatidia: outer (R1–R6) and inner (R7 and R8). These two classes represent two visual systems with different functions: the outer PRs, the equivalent of the vertebrate rods, are involved in motion detection and project to the lamina part of the optic lobe; the inner PRs, which have been proposed to be the equivalent of the vertebrate cones, appear to be involved in color discrimination (Kirschfeld and Franceschini, 1968). The rhabdomeres of R7 and R8 are in the same optic path and have different spectral sensitivities. Their inputs are compared in the medulla part of the optic lobe where R7 and R8 both project (for a review, see Hardie, 1985).

Six *rhs* are expressed in the adult fly visual systems. R1–R6 contain the wide-spectrum Rh1, encoded by *rh1/ninaE*, while the ocelli contain the related Rh2 (Pollock and Benzer, 1988). Based on the Rh content of the inner PRs, three main classes of ommatidia can be distinguished (Chou et al., 1996; Fryxell and Meyerowitz, 1987; Huber et al., 1997; Montell et al., 1987; O'Tousa et al., 1985; Papatsenko et al., 1997; Zuker et al., 1985). In the dorsal rim area, both R7 and R8 contain the UV-Rh3. These ommatidia form a polarizing filter that detects the polarization vector of UV light reflected by the sky (Hardie, 1985). The two other classes are distributed stochastically in the rest of the retina (Kirschfeld and Franceschini, 1977; Kirschfeld et al., 1978; Pichaud and Desplan, 2001) and exhibit differences in the fluorescence of their inner PRs, appearing either yellow (y; ~70% of ommatidia) or pale (p; the remaining 30%; Franceschini et al., 1981). The y ommatidia express UV-*rh4* in R7 and green-*rh6* in R8, whereas p ommatidia express UV-*rh3* in R7 and blue-*rh5* in R8 (for a review, see Cook and Desplan, 2001). The biological significance of these two subtypes of ommatidia is not clear but presumably allows discrimination of a broader range of wavelengths, with p ommatidia better discriminating among short wavelengths, and y ommatidia discriminating colors extending to the green.

The expression pattern of *rhs* is controlled at the transcriptional level. *rh* promoters have a bipartite organization consisting of a conserved proximal domain and unique upstream sequences. The proximal domain (−60 bp) provides PR identity through an RCSI/P3 element present in all known insect *rhs* (Fortini and Rubin, 1990; Mismer et al., 1988; Mismer and Rubin, 1989; Pichaud and Desplan, 2001; Papatsenko et al., 2001; Sheng et al., 1997b). This element is a target for the homeodomain of Pax6 (Sheng et al., 1997b; Zuker, 1994), the "master regulator" for eye development (Desplan, 1997; Gehring and Ikeo, 1999; Pichaud et al., 2001). Although RCSI/P3 alone is not sufficient to drive *rh* expression, its multimerization allows expression of a reporter gene in all PRs (Kokoza et al., 2001; Sheng et al., 1997b). Thus, RCSI/P3 must confer general PR specificity to *rhs*, while interaction of this subthreshold element with upstream elements (RUS; rhodopsin upstream sequences) specific to each *rh* is required to achieve correct subtype expression.

The molecular players responsible for the specific expression of *rhs* in different inner PRs are not yet known.

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However, genetic experiments indicate that at least two mechanisms regulate the coordinate expression of R7/R8 *rhs*. For instance, in *sevenless* or *boss* mutants, both of which result in the absence of R7 cells, *rh5* is drastically reduced and *rh6* is expanded to almost all R8 cells (Chou et al., 1996; Papatsenko et al., 1997). In contrast, the specific loss of R8 cells does not affect the mutually exclusive expression of *rh3* and *rh4* in R7 (Chou et al., 1999). Thus, a stochastic choice is made between p and y subtypes in R7, which is then communicated to R8. This suggests that a "horizontal" pathway sets up exclusion between *rh3* and *rh4* in R7 cells, and a "vertical" mechanism coordinates the expression between R7 and R8 *rhs*, leading to expression of *rh4* and *rh6* in y ommatidia, and *rh3* and *rh5* in p ommatidia.

To explore the molecular mechanisms of p/y specification, we undertook an analysis of the promoters of R7 and R8 *rhs*. Following the detailed analysis of Fortini and Rubin (1990) on the *rh3* and *rh4* promoters, we found that short promoters (between 137 bp and 276 bp) can mimic the complex expression pattern of all four of the inner *rhs*. Although most of the upstream RUS elements are unique to each *rh* promoter, we focused our attention on a series of highly conserved binding sites for homeoproteins that bear a lysine at position 50 of their homeodomain, a residue that specifies DNA binding (Treisman et al., 1989). These sites (TAATCC) are present in the *rh3*, *rh5, and rh6* promoters, but absent in *rh1*, *rh2*, and *rh4*. Mutations of the K_{50} binding sites in *rh3* and *rh5* completely abolish their expression, while mutation of the same sites in the *rh6* promoter results in the expansion of its expression to the R1–R6 outer PRs. We also show that the K_{50} homeoprotein Orthodenticle (Otd) acts through these sites, as *rh3* and *rh5* are lost in *otd* mutants, while *rh6* is expanded to PRs. Thus, Otd plays a dual role: it is essential for establishing the expression of *rh* genes in the p subset as well as for repressing *rh6* in outer PRs. This function of Otd is reminiscent of that of one of its vertebrate orthologs, Crx, which also plays a critical role in late aspects of PR differentiation, including the regulation of PR-specific genes (Chen et al., 1997; Furukawa et al., 1997; Swain et al., 1997).

Results

Regulatory Elements Common to Subsets of Rhodopsin Promoters

We analyzed the inner PR *rh* promoters by comparing their sequences from different species. We refined the analysis of the *rh3* and *rh4* promoters performed by Fortini and Rubin (1990) and achieved a similar comparison for *rh5* and *rh6* (Figure 1). Using searches with position-weighted matrices for known transcription factors, we identified a set of conserved binding sites (named K_{50}) present in the *rh3*, *rh5*, and *rh6* promoters. This analysis also revealed the RCSI/P3 Pax6 site common to all *rhs* genes (Papatsenko et al., 2002), and a sequence (Seq56) present in *rh5* and *rh6* (Cook at al., 2003). Most other upstream RUS elements are unique to each promoter, reflecting their absolute restriction to subtypes of PRs.

Truncation of the *rh3* promoter (−2300, −343, −245, −160, and −137) did not affect its expression in pR7 and dorsal rim area. However, most of the lines analyzed also exhibited a differential dorsoventral expression, which was also observed with an anti-Rh3 antibody (data not shown): the transgenes were expressed strongly in 30% of ommatidia throughout the eye, but in the dorsal region also exhibited weaker expression in the remaining 70% (Pichaud and Desplan, 2001). This weaker expression was lost with the −137 construct, suggesting that −160 to −137 carries an element responsible for weak expression in the y ommatidia within the dorsal region of the eye. A deletion analysis of the *rh4* promoter (−1900, −373, −276, −159, and −63) confirmed that 159 bp of upstream sequence was the minimal sequence able to faithfully reproduce *rh4* expression in yR7 cells (Fortini and Rubin, 1990). A −276 bp promoter fragment was the minimal sequence capable of reproducing proper *rh5* expression, while deletion to −236 or −176 led to weaker pR8 expression. Finally, deletion analysis of the *rh6* promoter (−555, −455, −364, −246, and −157) revealed that −246/+121 was the minimal promoter that still mimicked wild-type expression, albeit at considerably reduced levels compared to longer promoters.

Previous analysis of the *rh3* and *rh4* promoters had identified several unique elements that are conserved among various species and are important for their regulation (Fortini and Rubin, 1990). These, referred to as RUS and RCS elements, are indicated in Figure 1. We cloned the *rh5* and *rh6* promoters from *D. virilis* and compared them with the newly released *D. pseudoobscura* sequences. Comparing all inner PR *rhs* between the three *Drosophila* species, we identified a highly conserved K_{50} element (TAATCC; Treisman et al., 1989; Wilson et al., 1996), which was present in several copies in the *rh3*, *rh5*, and *rh6* promoters, but absent in the *rh1*, *rh2*, and *rh4* promoters (Figure 1). TAAT is the core target site for most homeoproteins (Desplan et al., 1988; Wilson et al., 1996). Additional homeodomain binding specificity is provided by the residue at its position 50, which contacts two additional base pairs to extend its DNA binding site to TAATNN. In most cases (e.g., in Hox proteins), this is a Gln (Q_{50}) and the recognized sequence is TAATTG. In a few cases, however, a Lys (K_{50}) in this position alters the DNA binding specificity to TAATCC (Treisman et al., 1989; Wilson et al., 1996). We show below that the K_{50} sites act as horizontal (y versus p ommatidia) elements. Another element detected by our analysis was a motif shared between the R8 genes *rh5* and *rh6* (seq56; Figure 1). Seq56 binds the atypical homeoprotein Prospero and serves as a vertical control element (R7 versus R8 cells) necessary for preventing expression of R8 *rhs* in R7 cells (Cook et al., 2003).

K_{50} Sites Play a Major Role in the Expression of Pale *rh* Genes

The minimal *rh3* promoter contains four K_{50} sites (KI–KIV). KIV is included within the RCSI/P3 element, sharing its TAAT core HD site, while KI and KII fall within the previously identified RUS3A site (Figure 1). Interestingly, mutation of RUS3A did not affect *rh3* reporter activity in the context of a larger promoter (−376; Fortini and Rubin, 1990), nor did mutation of the KIII and KIV sites

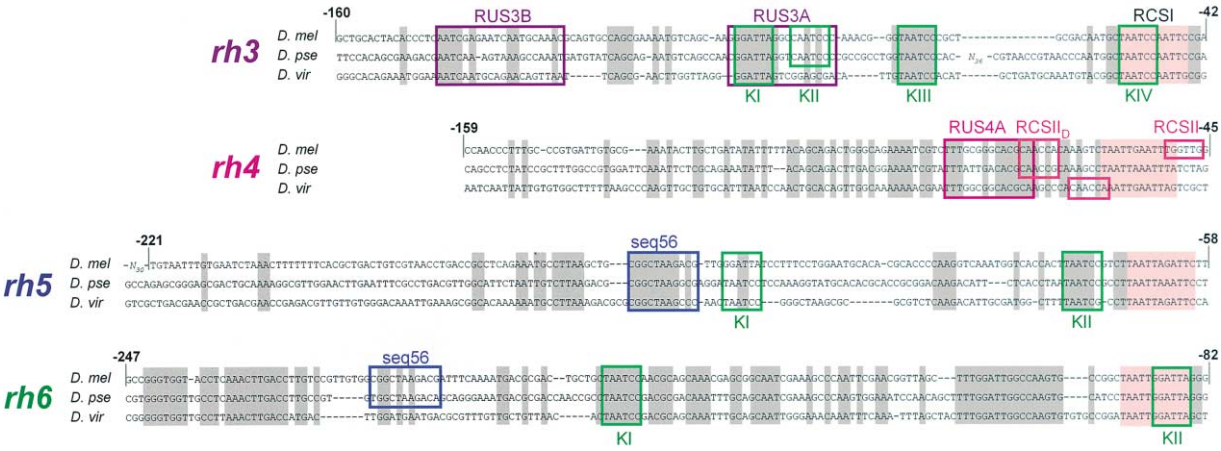

Figure 1. Conserved Elements in the Promoters of *rhodopsin* Genes

rh3, *rh4, rh5, and rh6* promoter sequences of several *Drosophila* species (*D. melanogaster*, *D. virilis, and D. pseudoobscura*) were compared. The sequences that are conserved in all species are boxed. The previously described conserved elements identified by Fortini and Rubin (1990) in *rh3* and *rh4* (RUS3A, RUS3B, RUS4A, RCSI, and RCSII) are boxed. In addition, we identified an additional RCSII element ($RCSII_D$) upstream of the *rh4* RCSI site. Sequence analysis was performed by using DNASTAR software. The *rh3* KIV and *rh6* KII share their TAAT with the RCSI sequence. RUS, *rhodopsin* upstream sequence; sequence homologies between these boxes are noted by common color codes. RCS, *rhodopsin* common sequence; the RCSI sequence, boxed in pink, is recognized by a dimer of the Pax6 homeodomain. Finally, Seq56 represents a repressor site of R8 *rhs* present in *rh5* and *rh6* (Cook et al., 2003).

within the context of the minimal −160 promoter (Figure 2A). We only disrupted KIV by mutating its CC sequence, as mutation of its TAAT core would also affect the RCSI/P3 site and abolish promoter activity (Fortini and Rubin, 1990; Papatsenko et al., 2001). However, disrupting the three upstream sites (KI–KIII) led to a complete loss of *rh3* activity, both in pR7 and in the dorsal rim area (Figure 2B). We also changed all K_{50} sites (I–IV) by replacing the CC nucleotides after the TAAT by TG, thus changing the specificity of these sites from K_{50} to Q_{50} homeoprotein targets. Again, *rh3* activity was abolished in the retina, although this modified promoter was now expressed in a specific pattern in the brain that was observed in all transgenic lines (data not shown). Therefore, the K_{50} sites are redundantly required for *rh3* expression.

The *rh5* promoter also contains two conserved K_{50} sites. KI is distal to the promoter, while KII is adjacent to, but nonoverlapping with RCSI/P3 (Figure 1). Mutation of both KI and KII, or only of the KI site, resulted in a nonfunctional *rh5* promoter (Figure 2D), while mutation of KII alone had no effect on its expression (Figure 2C). These data indicate that, like for *rh3*, a K_{50} site is critical for *rh5* expression. As *rh3* and *rh5* are expressed in the same p subset of ommatidia, the K_{50} sites may serve as elements that distinguish p from y *rhs*, and a common K_{50} homeoprotein regulator should act through these sites to specify the *p* subtype.

K_{50} Sites Are Required to Repress *rh6* Expression in Outer PRs

The *rh6* promoter, which is not expressed in the *p* subtype but instead in yR8, also contains K_{50} sites, a distal KI site and KII, which, like *rh3*KIV, shares its TAAT sequence with its RCSI/P3 element (Figure 1). In contrast with the loss of expression observed when we mutated the K_{50} sites in *rh3* and *rh5*, we found that mutation of either the core TAAT of KI or the flanking CC led to a dramatic expansion of the reporter throughout the entire retina (Figure 3E). Mutation of the CC sequence in the proximal KII did not affect the *rh6* promoter (see also Papatsenko et al., 2001). To characterize this expanded expression, we analyzed the differential projection patterns to the optic lobes: the KI-mutated *rh6-lacZ* construct was expressed in PRs whose axonal projections terminated both in the medulla and the lamina (compare Figures 3B and 3E, arrow). As only outer PRs project to the lamina, this indicated that *rh6* expression was expanded to outer PRs. To more accurately define this expression, we fused to Gal4 the wild-type −555 *rh6* promoter, or versions carrying mutations of the K_{50} sites, and crossed the resulting transgenic lines with a UAS-GFP line. This allowed us to use in vivo fluoroscopy to assess the expression of GFP at single-cell resolution (Pichaud and Desplan, 2001). The control *rh6*-Gal4 construct led to GFP expression in ~70% of the R8 PRs (Figure 3C), while the mutated transgene led to expansion to many, but not all outer PRs (Figure 3F; see also below). The mutated promoter constructs were still expressed in a normal ratio of ~70% of inner PRs (Figures 3C and 3F, white and yellow arrows), a pattern consistent with gaps observed in the projection pattern in the medulla (Figure 3E). R7 and R8 project to two distinct layers in the medulla, but we could only detect projections to the R8 axonal projection layer. Therefore, the KI-mutated *rh6* promoter, although expanded to many outer PRs, appears to remain restricted to a subset of R8 cells in inner PRs. These experiments illustrate the versatile role played by the K_{50} motif in regulating *rh* genes, as they either mediate activation (*rh3*/*rh5*) or repression (*rh6*).

K_{50} Sites Mediate Activation of *rh3*/*rh5* and Repression of *rh6* by Orthodenticle

The identification of functional K_{50} sites in *rh3, rh5, and rh6* suggested that a K_{50} homeoprotein recognizes these

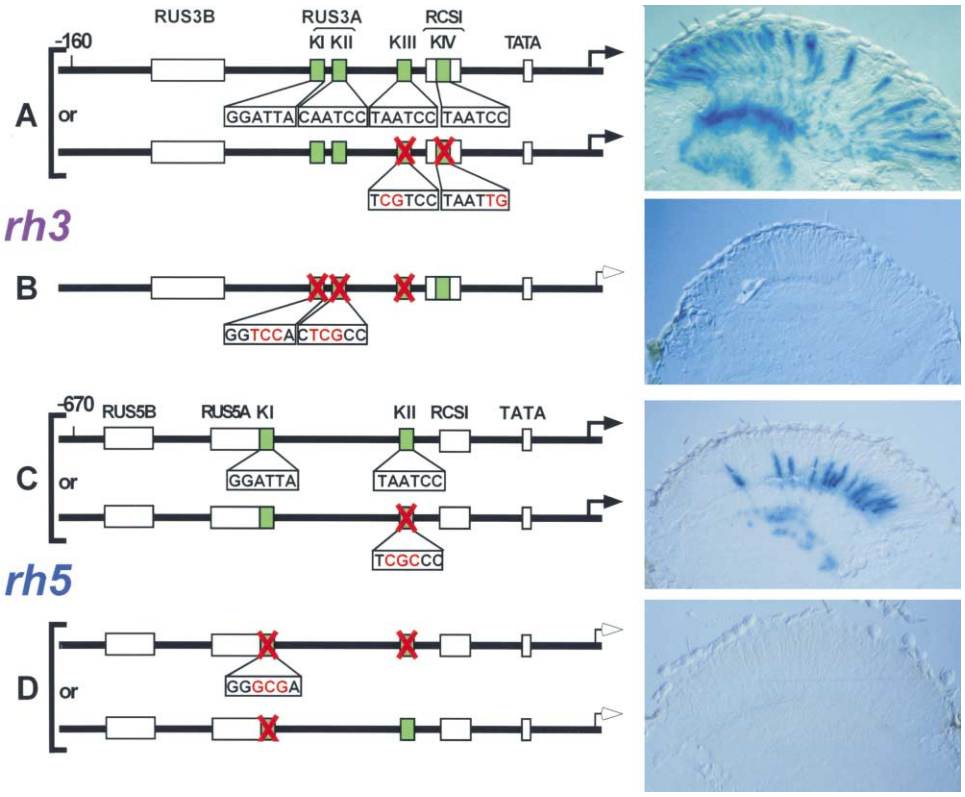

Figure 2. Mutation of K_{50} Sites Abolishes Expression of p-Subtype *rhs* (*rh3* and *rh5*)

(A) Wild-type pattern of *rh3*(−160/+18)-*lacZ* expression in a subset of R7 cells. The cryosection (A) shows expression from a wild-type construct. This expression was not affected by mutations of KIII alone or KIII + KIV (not shown). Expression was abolished when the TAAT core of three K_{50} sites (KI, KII, and KIII) was mutated (B). The TAAT of KIV located within the RCSI was not mutated. When the two nucleotides CC flanking the TAAT core were replaced by TG in all K_{50} sites, the activity of the *rh3* promoter was lost in the retina; however, β-gal staining was now detected in a narrow stripe of cells around the brain (not shown). The *rh5*(−670/+32)-*lacZ* construct displayed a normal expression pattern in the pR8 subtype (cryosection shown in [C]). This expression was not affected by mutation of the KII site (not shown); however, mutation of both K_{50} sites (cryosection shown in [D]) or of KI alone (not shown) completely abolished *rh5* promoter activity.

sites. There are few K_{50} homeoproteins in *Drosophila* that can bind to K_{50} sites: Bicoid (Berleth et al., 1988), Goosecoid (Goriely et al., 1996), Orthodenticle (Finkelstein et al., 1990), and Pitx1 (Vorbruggen et al., 1997). Among these, *otd* is the only one whose expression has been reported in the adult eye (Vandendries et al., 1996). An eye-specific *otd* allele, otd^{uvi}, results from the partial deletion of the eye-specific enhancer and leads to a strong reduction of Otd protein expression in the eye (Vandendries et al., 1996). otd^{uvi} flies exhibit both a loss in UV sensitivity, a function normally served by R7 cells, as well as a dramatic phenotype in PR morphogenesis, resulting in disorganized and duplicated rhabdomeres in all PRs and an upward movement of R8 cells (Vandendries et al., 1996). This suite of phenotypes is strongly reminiscent of the function described for Crx, one of the vertebrate orthologs of Otd, which not only regulates PR cell morphogenesis but also directly regulates many PR-specific gene products in both rod and cone cells (Furukawa et al., 1997; Chen et al., 1997).

To test whether Otd was the factor that recognizes the K_{50} sites in *rh3*, *rh5*, and *rh6*, we analyzed *rh* expression in flies using antibodies to the various Rh proteins or by visualization of different *rh-lacZ* or -GFP transgenes. As previously reported, the shape of all eight PR rhabdomeres was abnormal in these mutants. However, *rh1* expression in outer PRs (Figures 4A, 4B, and 5A) and *rh2* expression in the ocelli (data not shown) appeared normal. *rh4* expression also remained restricted to yR7 cells, as an *rh4-lacZ* transgene exhibited relatively abundant staining in the distal portion of the retina and projected to a subset of R7 terminations in the medulla (Figures 4E, 4F, and 5A). By contrast, the expression of *rh3*, *rh5*, and *rh6* was dramatically affected in otd^{uvi} flies, and their expression was fully consistent with the expression of reporter genes carrying mutations in the K_{50} sites: the expression of both *rh3* and *rh5* was completely lost (Figures 4C, 4D, 4G, 4H, and 5A), whereas *rh6* expression was broadly expanded (Figures 4I, 4J, and 5B–5D). Using an *rh6-lacZ* reporter gene, we also observed projections to both the medulla (Figure 4J, arrow) and the lamina (Figure 4J, white arrowhead; Figure 5F, green arrowhead), again indicating that *rh6* expression was expanded to outer PRs. However, Rh6 expression did not expand to all inner PRs (Figures 5B and 5D). Consistent with this observation, the medulla projections of *rh6-lacZ* only reached a subset of the R8 layer, with ~70% of the projections visible (Figure 5F, white arrow shows gaps in the R8 projection layer). Interestingly, we also observed a few instances where *rh6-lacZ* was

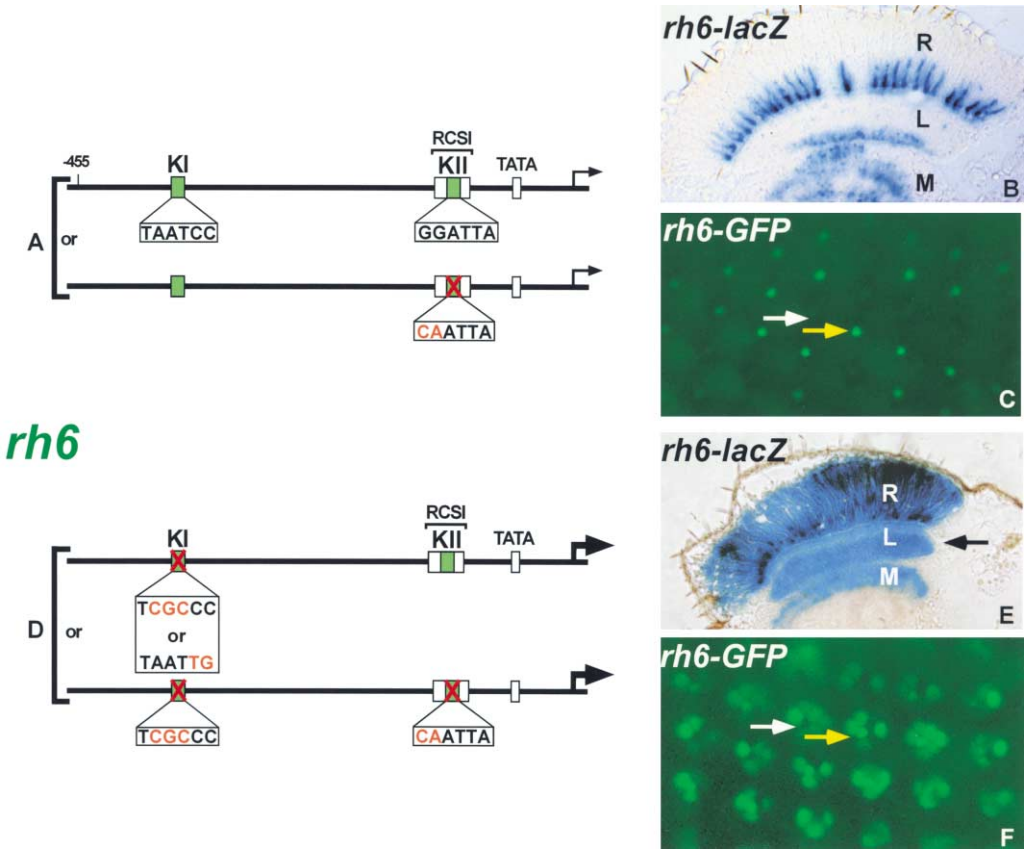

Figure 3. A K_{50} Site Is Required for the Repression of *rh6* in Outer Photoreceptors

Rh6(−455/+121)-*lacZ* contains two K_{50} sites (A). It is expressed in ~70% of R8 cells (B) that project exclusively to the medulla (in [B], the blue band at the base of the lamina below the L represents inner PR axons en route to the medulla). This expression is not affected by mutation of the CC base pairs in the proximal KII site that overlaps the RCSI sequence (not shown). An *rh6*-GFP reporter analyzed by in vivo fluoroscopy shows the wild-type proportion of R8 cells expressing the reporter ([C], yellow versus white arrow). Mutation of the TAAT core of KI (shown in [D]) or change of specificity of both K_{50} sites to Q_{50} sites by replacing CC to TG (not shown) both lead to expansion of *rh6* expression to the outer PRs, as demonstrated by strong lamina staining for the KI mutant ([E], black arrow). The medulla staining (M) shows that *rh6* is still expressed in a subset of inner PRs. In vivo fluoroscopy with *rh6*-GFP clearly shows expansion of the reporter to the outer PRs (F) but not to all inner PRs, as many R8 express *rh6* (yellow arrow) while others do not (white arrow). This strongly suggests that *rh6* is still restricted to yR8 cells. R, retina; L, lamina; M, medulla.

detected in R7 terminations (Figure 5F, yellow arrow). Rh proteins in *otd*^{uvi} mutants reflected the *lacZ* reporter results: expression of Rh4 was not affected (Figures 5A and 5G), Rh3 and Rh5 expression was lost (Figures 5G and 5H), and Rh6 was present in outer PRs and in a subset of R8 cells (Figures 5B–5D and 5H). We also generated whole mutant eyes for the null *otd*^{2} allele present on an FRT chromosome using the eye-specific source of FLP recombinase, ey-Flp (Newsome et al., 2000); Rh4 expression was maintained in a subset of R7 cells, Rh3 and Rh5 expression was lost, and Rh6 was expanded to outer PRs (Figures 5G and 5H).

Thus, the expression patterns of *rh3*, *rh5*, and *rh6* in *otd* mutants (Figures 4 and 5) precisely recapitulated those observed when mutating the K_{50} sites in the individual *rh* promoters (Figures 2 and 3). To confirm direct binding of Otd to the *rh* promoters, we performed gel shift experiments (Figure 4K) using individual K_{50} sites found in the *rh3, rh5*, or *rh6* promoters, and found that a GST-Otd homeodomain fusion protein bound specifically in vitro to these sites with high affinity, and that binding was similar for the activator sites present in *rh3* and *rh5* and for the repressor site in *rh6* (compare lanes 2 and 3 with lane 4 in Figure 4K). Furthermore, interaction was lost when we mutated the *rh5* K_{50} site (Figure 4K, lane 6). These data strongly suggest that Otd directly interacts with K_{50} sites within the inner PR *rhs*, and participates in two different mechanisms of *rh* regulation that determines the complex pattern of *rh3*, *rh5*, and *rh6* expression: activation of the p-type *rhs* (*rh3* and *rh5*) and repression that restricts *rh6* to inner PRs.

Derepression of *rh1* in Inner Photoreceptors in *otd*^{uvi} Mutants

Several studies have shown that rhabdomere formation and maintenance require the presence of rhodopsin proteins (Chang and Ready, 2000; Kumar and Ready, 1995; Leonard et al., 1992). Because no inner *rh* expression was found in many p-type inner PRs, one might have expected that the rhabdomeric defects in the *otd*^{uvi} mutant resulted from the lack of rhodopsin expression.

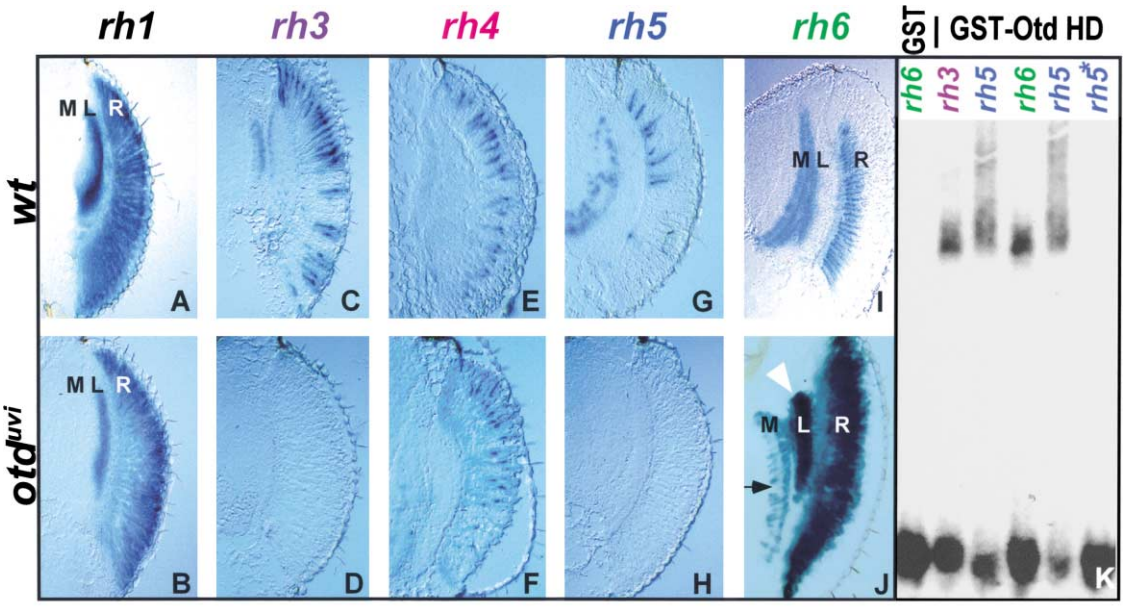

Figure 4. *otd* Is Required for the Activation of *rh3* and *rh5* and for the Repression of *rh6* in Outer Photoreceptors

The expression of *rh1* (A and B), *rh3* (C and D), *rh4* (E and F), *rh5* (G and H), and *rh6* (I and J) (using the same promoters as in Figures 2 and 3) was assessed in females containing one copy of otd^{uvi} (A, C, E, G, and I) and in mutant males hemizygous for otd^{uvi} (B, D, F, H, and J). *rh1* (B) and *rh4* (F) expression is not affected, whereas *rh3* (D) and *rh5* (H) expression is completely abolished. *rh6* expression is expanded to the outer PRs as demonstrated by *lacZ* staining in the lamina ([J], white arrowhead). Expression is confined to a subset of inner PRs, as shown by the gaps in medulla staining ([J], black arrow). *rh2* expression, normally restricted to ocelli, is not affected (data not shown). R, retina; L, lamina; M, medulla.

(K) Gel shift analysis of a GST::OtdHD fusion protein revealed binding to wild-type portions of the *rh3*, *rh5*, and *rh6* promoters carrying the distal K_{50} sites, whereas binding was abolished using a mutated form of the *rh5* K_{50} element (*rh5**). No binding was observed using GST as a control (lane 1 and data not shown).

However, electron microscopy studies have demonstrated that all PR rhabdomeres form in otd^{uvi} mutants, despite their misshapen and duplicated appearance, do not degenerate (Vandendries et al., 1996). This suggested that another *rh* was expressed in p-type inner PRs to allow rhabdomere formation in the absence of *rh3* and *rh5*. As we found that neither *rh4* nor *rh6* was expanded to the p subset (Figure 5), and because we did not detect *rh2* in the retina (data not shown), *rh1* was the only possible alternative. We used an *rh1-lacZ* reporter gene in otd^{uvi} to analyze its ectopic expression in inner PR axon terminations in the medulla, as the misshapen rhabdomeres would complicate analysis in the retina. A subset of axons that projected to the medulla was clearly, though weakly, stained with *rh1-lacZ* in otd^{uvi} flies, suggesting that *rh1* was expanded to a subpopulation of inner PRs (Figure 5E). Using both *rh1-lacZ* and *rh4*-GFP transgenes, we could detect *rh1-lacZ* expression in the p subset, but also in some yR7 where it was coexpressed with *rh4*. Thus, in the absence of *otd*, the pR7 only express low levels of *rh1* while the yR7 coexpress *rh4* and low levels of *rh1* (not detectable in the retina using the anti-Rh1 antibody; Figure 5A). We conclude that Otd normally participates in repression of *rh1* in inner PRs, and that its removal perturbs but does not abrogate this repression, which might involve other factors.

Dual Role of *otd* in Late Photoreceptor Differentiation

otd is involved in several PR developmental processes that take place during pupation, including a general role in PR morphogenesis, defects in R8 cell positioning (Vandendries et al., 1996), and *rh* expression defects. Thus, although *otd* is expressed and required in all PRs (Vandendries et al., 1996), some of its actions are highly specific to subsets of PRs. To test whether *rh* misregulation was independent from rhabdomere misformation, and to establish the role of *otd* in PR differentiation, we examined the temporal requirement for *otd* throughout PR differentiation. Rhabdomere formation starts around 35% of pupation (Chang and Ready, 2000), while high levels of Rh expression are established only after 70% pupation (Kumar and Ready, 1995; Sheng et al., 1997a). Although there are no temperature-sensitive alleles of *otd*, it is possible to fully rescue the otd^{uvi} phenotype with a *heat shock-otd (hs-otd)* construct (Vandendries et al., 1996). We devised a rescue regimen by heat pulsing otd^{uvi} flies containing an *hs-otd* transgene at different times during pupation and examined the expression of various *rh* reporter constructs. Batches of developmentally synchronized flies were heat shocked twice for 1 hr at 12 hr intervals, from 0 hr to 84 hr after puparium formation (APF; Figure 6). For maximum rescue, we also treated otd^{uvi} flies by heat shocking five times at 12 hr intervals from 24 hr to 84 hr APF, as described by Vandendries et al. (1996). The flies were analyzed 24 hr after eclosion, and 3 days later to assay for the continued requirement for *otd*.

We observed almost complete rescue of all otd^{uvi} phenotypes after five successive heat shocks. The rhabdomeres appeared normal in shape and were not split. In vivo visualization with *rh3*-GFP indicated that *rh3* expression was restored in 30% of R7 cells (Figure 6D).

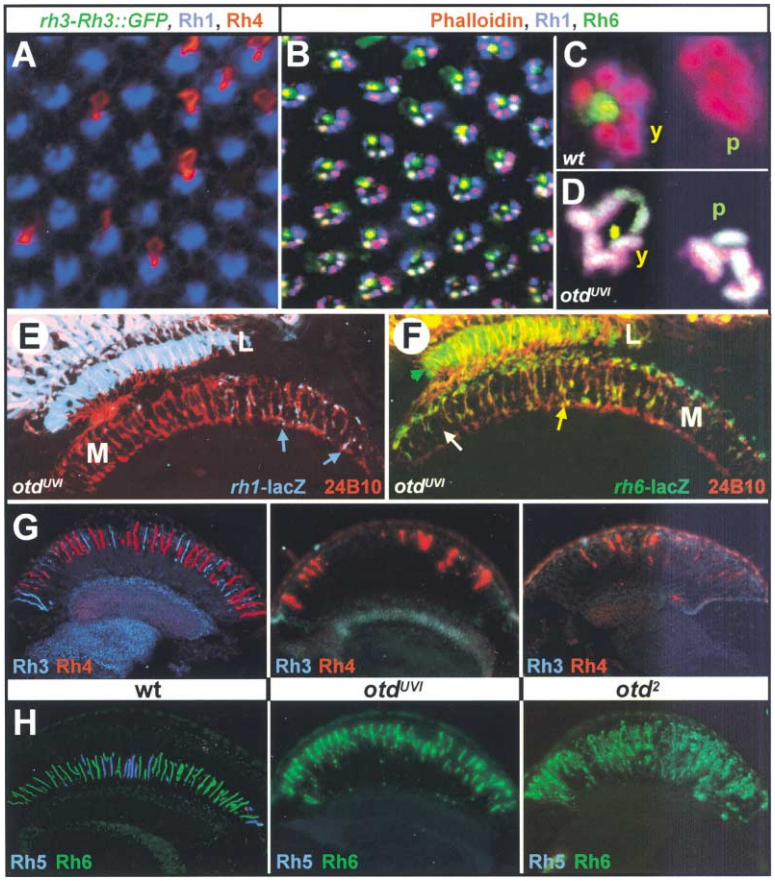

Figure 5. Altered *rhodopsin* Expression in *otd* Mutants

Confocal microscopy performed after staining with antibodies against Rh1, Rh4, and Rh6 on whole-mounted retina preparations from otd^{uvi} hemizygous flies.

(A) Rh1 expression (in blue) is maintained in outer PRs and Rh4 (red) is expressed in a subset of R7 (yR7). An *rh3*-Rh3::GFP transgene is not expressed in the absence of *otd* function.

(B) Phalloidin (red) reveals all PR rhabdomeres. Rh6 (green) remains restricted to yR8 in inner PRs but is dramatically expanded to outer PRs (which also express Rh1 in blue).

(C) Wild-type expression of *rh6*.

(D) *rh6* in otd^{uvi}. Note that, in both cases, we can detect ommatidia without (p) or with (y) Rh6-expressing R8, suggesting that *rh6* remains limited to its yR8 subset.

(E) Staining of projections of an *rh1-lacZ* construct in an otd^{uvi} mutant clearly show that *rh1* is not only expressed in outer PRs that project to the lamina (L), but also in some inner PRs that project to both R7 and R8 layers of the medulla (M; blue arrows indicate staining in the R7 layer).

(F) Staining of projections of an *rh6-lacZ* construct in an otd^{uvi} mutant clearly shows that *rh6* is expressed in outer PRs projecting to the lamina (green arrowhead) and in a subset of R8 cells (white arrow points to blank R8 PR projections). In a few instances, projections to the R7 layers can also be observed (yellow arrow).

(G and H) Staining of endogenous Rh3 and Rh4 (G) or Rh5 and Rh6 (H) in wild-type, otd^{uvi}, or whole mutant eyes generated with ey-Flp and the null otd^2 allele recombined on an FRT chromosome.

rh6 expression was significantly reduced (although not silenced) in outer PRs as observed by the loss of *lacZ* staining in the lamina from an *rh6-lacZ* transgene (compare Figures 6H and 6I). This staining became stronger again in older flies (data not shown), indicating a continued requirement for the repression by Otd of *rh6* in outer PRs.

We next examined the rescue achieved with short heat shock regimens. We were able to discriminate two short time windows when *otd* was needed. *otd* was necessary before 48 hr (50% pupation) to achieve proper rhabdomere formation. Using water immersion (Pichaud and Desplan, 2001), we could see that many PRs had rescued rhabdomeres with early heat shocks at +12 hr, +24 hr, and +36 hr APF (Figures 6A and 6E, white arrow), but *rh* expression was not rescued; expression of *rh3*-GFP was strongly diminished and the *rh6*-GFP reporter was expanded to outer PRs (Figures 6A, 6B, and 6E). Instead, *rh* expression was rescued with heat shocks performed between +48 hr and +72 hr (Figures 6A and 6C). Repression of *rh6* in outer PRs was also partially rescued with heat shocks between 48 hr and 72 hr APF when the flies were observed immediately after eclosion (Figure 6F), although *rh6*-GFP expression increased when the flies were analyzed 3 days later (Figure 6G). It must be noted that no dominant phenotype was observed when we expressed *otd* with the full regimen of *hs-otd*, even in the presence of a wild-type copy of the gene, confirming that *otd* is expressed and required in all PRs, but that it is not sufficient to induce *rh3* and *rh5* expression. Thus, other factors are required, along with Otd, to activate p-type *rhs*. Interestingly, later heat shocks (e.g., >48 hr APF) failed to rescue the rhabdomeric defects (see Figure 6F; arrowhead shows an ommatidium with strongly disrupted rhabdomeres), and thus suggest that *otd* is involved in several PR developmental processes at different times, namely rhabdomere formation prior to 48 hr APF, and *rh* gene expression after 48 hr. As no rescue of *rh* gene expression was observed with late heat shocks (>84 hr APF), *otd* appears to be required at the time of initiation of *rh* expression for their appropriate expression and maintenance in the different PR subtypes.

Discussion

The Otd/Otx Gene Family

otd is a highly conserved gene whose ancestral function resides in the determination of "anterior" structures. Even in cnidarians (e.g., hydra), which demonstrate a primitive, diploblastic grade of organization, *otx* is expressed in the oral region (Fei et al., 1999). While only one *otd* gene exists in flies, four paralogs are expressed in mice: Otx1, Otx2, and the newly characterized Otx3 (Simeone et al., 1993; Zhang et al., 2002), as well as Crx (see below). From flies to mammals, *otd* and *otx* genes

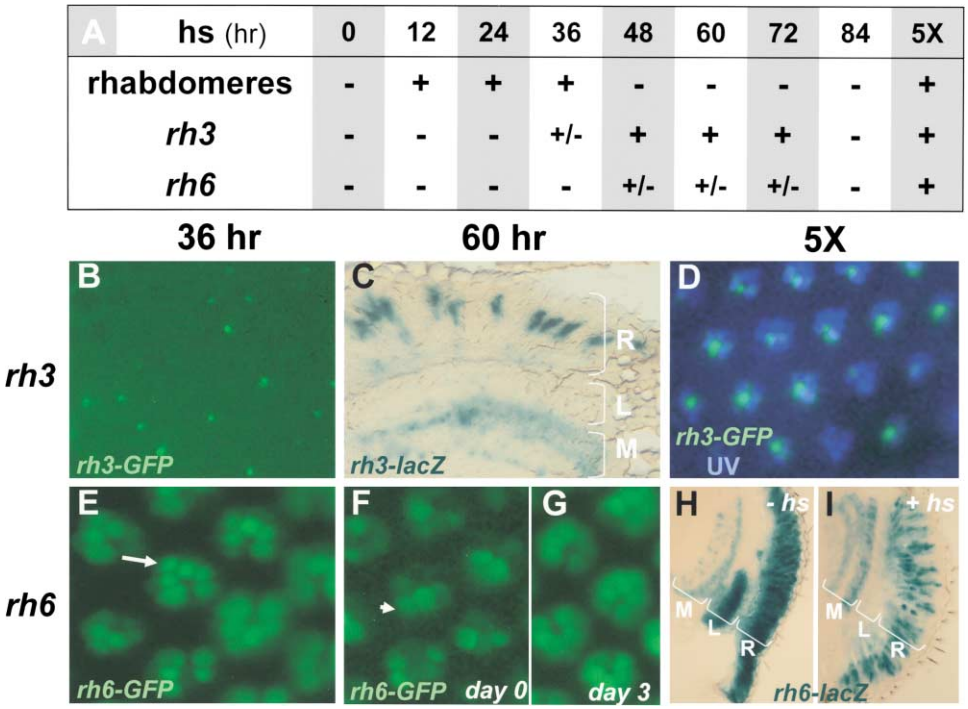

Figure 6. Rescue of *otd*^{uvi} at Different Times during Pupation

(A) A series of rescue experiments was performed by inducing *otd* expression in an *otd*^{uvi} mutant using a *heat shock-otd* transgene. Induction was performed by two 1 hr heat shocks (hs) during a 12 hr period. Each batch of *otd*^{uvi} flies was subjected to a single induction, the first batch at the time of pupation (0 hr), another one 12 hr after puparium (APF), and so on. Flies eclose 96 hr APF. Rescue of *otd*^{uvi} function was assessed using the *rh3* and *rh6* promoter driving either GFP or *lacZ* expression. Rhabdomere rescue is observed with early hs (12–36 hr), while *rh* expression is only rescued with later hs (48–72 hr).

The loss of *rh3* expression in pR7 cells is rescued from 36 to 72 hr (A–C) or with the full regimen of 5× hs (D). At 36 hr, weak *rh3*-GFP expression is detected in some flies (B). By 60 hr, *rh3-lacZ* exhibits full rescue (C). At this time, *rh3*-GFP is also found in a subtype of inner PRs, but the GFP staining is diffuse, due to the disrupted rhabdomere morphology and the mispositioning of R7 cells (data not shown).

The expansion of *rh6* expression to outer PRs is not rescued at 36 hr (E), and is only partially rescued from 48 to 72 hr, still exhibiting some outer PR expression (F and G). Full rescue of *otd*^{uvi} flies is only achieved with 5× hs, with the disappearance of the lamina staining (I) normally observed in *otd*^{uvi} flies (H). It should be noted that while good but partial rescue is observed at 60 hr, immediately after eclosion (F), flies from the same batch sacrificed 3 days later exhibit reexpression of ectopic *rh6*-GFP in outer PRs (G). It should also be noted that *rh6*-GFP could never be detected in inner PRs when using in vivo fluoroscopy, although it is clearly visible by whole-mount immunostaining (Figure 5).

The phenotype of disrupted rhabdomeres is partially rescued by inductions from 12 to 36 hr (the ectopic expression of *rh6*-GFP in outer PRs allows the visualization of nicely shaped rhabdomeres in [E]; arrow), but not by earlier or later inductions ([F] and [G] exhibit fat rhabdomeres at 60 hr; arrowhead). Full rescue is observed with 5× hs: the shape of the rhabdomeres can be seen with the UV autofluorescence of Rh1 in outer PRs ([D], blue).

are involved in anterior brain development, and later in nervous system patterning. In contrast, the role of *otd* in PR morphogenesis appears to have been adopted by another related vertebrate factor, the *Crx* gene. For instance, Otx1/2 pattern the brain, while Crx affects cone and rod PR development as well as PR-specific gene expression (Chen et al., 1997; Freund et al., 1997; Furukawa et al., 1997).

As for Otd, the ability of Crx to regulate PR-specific gene expression requires its binding to conserved K_{50} sites present within their promoters (Chen et al., 1994, 1997; Chiu et al., 1994; Wang et al., 1992). In addition, mutations in the *Crx* gene are responsible for an autosomal dominant form of cone-rod dystrophy (Furukawa et al., 1997; Freund et al., 1997). Some of Crx functions might also be partially redundant with Otx2, which is also expressed in the retina. In *Drosophila*, the various roles of Otx/Crx might be represented by distinct regulatory elements that control *otd* anterior embryonic or eye expression (e.g., *otd*^{uvi} affects an eye-specific enhancer while a distinct enhancer responds to the morphogenetic gradient of Bicoid; Gao and Finkelstein, 1998). Similarly, our data indicate that the roles of Otd in PR morphogenesis and PR-specific gene expression can be temporally separated. We are currently testing whether vertebrate Crx and Otx's are able to rescue *rh3*, *rh5*, and *rh6* expression in an *otd*^{uvi} background. The high homology between pathways involved in *Drosophila* and vertebrate eye development might reveal general principles that are applicable to the vertebrate retina.

Transcriptional Control of *rhodopsin* Gene Expression

The expression of the different *rh* genes is tightly restricted to different subsets of PRs, and this regulation is essentially transcriptional (Montell et al., 1987; Zuker et al., 1987; Fortini and Rubin, 1990). Although the minimal promoters that we identified faithfully reproduced

endogenous expression for *rh4, rh5*, and *rh6*, the *rh3* transgenes exhibited weak pan-R7 expression in the dorsal compartment of the eye, consequently overlapping with Rh4. Although this could reflect the lack of a regulatory element in the reporter construct, we observed the same weak expression with several types of constructs, which included 2.4 kb of upstream sequence, the 3′ UTR, and 1.2 kb of downstream genomic sequences. Furthermore, we could detect low levels of Rh3 in all R7 in the dorsal region using anti-Rh3 antibodies (data not shown). It is interesting to note that, in other species, several *rhs* have been shown to be coexpressed in the same PR. For instance, in the butterfly *papillio* and in bee and mouse, coexpression between Rhs is observed with dorsoventral differences (Applebury et al., 2000; Kitamoto et al., 2000; Briscoe, 2000; Townson et al., 1998). The dorsal portion of the eye is more likely to be exposed to UV-rich wavelengths and thus might have a specialized function.

Role of Otd in *rh3*/*rh5* and *rh6* Expression

Otd is a K_{50} homeoprotein required in the eye at the time of PR differentiation (Vandendries et al., 1996). We showed here that *otd* is absolutely required, but is not sufficient, for turning on the expression of p-type *rhs*, *rh3*, and *rh5*. Thus, Otd is unlikely to act alone to confer spatial regulation. First, it is expressed in all PRs (Vandendries et al., 1996); second, generalized expression of Otd under heat shock control in wild-type flies does not dramatically affect the expression of *rh3* and *rh4*, nor does it affect *rh1* or *rh6*; third, our ability to fully rescue the expression of *rh3* in otd^{uvi} mutants by pulses of *otd* demonstrates that *otd* does not need to be restricted to p-type ommatidia; fourth, *otd* is required in the outer PRs to repress *rh6* and in all PRs for proper rhabdomere morphology; and finally, *otd* is required for preventing *rh1* expression in most, if not all inner PRs, probably through an indirect mechanism. Indeed, we found no K_{50} sites within the *rh1* promoter construct that shows derepression in otd^{uvi} mutants, while, *rh3, rh5*, and *rh6* all exhibit very clear binding sites for Otd.

Although the sequence of the K_{50} sites in *rh3*/*rh5* and *rh6* is identical, they function to activate the p-type *rhs* and to repress *rh6* in outer PRs (and in a subset of R7 cells). While we could not detect conserved flanking sequences associated with the activator K_{50} sites in *rh3* and *rh5*, we found that the *rh6* KI site is associated with a 21 bp sequence that is highly conserved in *D. virilis* and *D. pseudoobscura* (Figure 1). This sequence might represent the site of action of a corepressor binding together with Otd to transform it from an activator into a repressor. While mutation of this element in the context of the −555/+121 or −246/+121 *rh6* promoter did not lead to expansion of reporter activity to outer PRs (data not shown), it remains possible that this site and/or other not yet unidentified elements function redundantly in preventing *rh6* expression in outer PRs.

As *otd* does not provide the spatial specificity to *rhs*, other factors must do so. For instance, a coactivator might be expressed specifically in p-type ommatidia to activate *rh3* and *rh5*, while a corepressor might be needed in outer PRs to repress *rh6*. These cofactors do not have to bind DNA and thus might not have a cognate site in the promoters. Alternatively, Otd could only be permissive for *rh3* and *rh5* expression, while spatial specificity is provided by other proteins that bind to distinct elements within their promoters. RUS3B could represent such an element for *rh3*, as we have shown that this site is essential for expression in pR7 (A.T., T.C., and C.D., unpublished data).

Although Otx family genes mostly act as activators (Mailhos et al., 1998), *Otx2* has been found to repress the expression of *XWnt-5a* (Morgan et al., 1999) through a conserved K_{50} site in its promoter, suggesting that the repression activity of Otd is also conserved in vertebrates, and might depend on similar cofactors. It will be interesting to investigate whether Otx2, together with Crx, can modulate late retinal development and particularly the distribution of cone opsin genes, whose promoters contain conserved K_{50} sites (Chen et al., 1997; Chiu et al., 1994; Wang et al., 1992). Finally, the observation that, in *Drosophila*, Otd is likely to require cofactors for its various functions in the eye, is consistent with the fact that Crx has been shown to function synergistically with a number of factors, including NRL, to activate opsin gene expression (Chau et al., 2000; Mitton et al., 2000). It will be important to identify the ancestral function of Otd/Crx from which the role of these genes in regulating eye development has evolved.

A "Default" Rh1 Allows Inner PR Rhabdomere Development in Otd Mutants

The loss of *rh3* and *rh5* expression in p inner PRs is not compensated by expansion of the y inner PR *rhs*; *rh4* and *rh6* remain largely restricted to the y subset of R7 and R8. This suggests that the p ommatidia remain committed as such but fail to express their *rhs*. This is consistent with the direct binding of Otd to the *rh3* and *rh5* promoters, which are terminal differentiation markers, and also shows that *otd* is not the spatial determinant of p versus y fates. While the loss of p *rhs* should lead to a lack of proper rhabdomere formation or to their degeneration (Kumar and Ready, 1995; Leonard et al., 1992; O'Tousa et al., 1989), this is not observed in otd^{uvi} flies (Vandendries et al., 1996). We suggest that this may be due to the low levels of Rh1 that are induced by the absence of *rh* gene expression in inner PRs lacking *otd*, thus avoiding degeneration. Thus, Rh1 may serve as a "default" rhodopsin whose expression is normally repressed in inner PRs by Otd or through an Rh-mediated exclusion process.

A general rule in many sensory systems is that expression of a sensory receptor molecule in a given cell excludes the expression of all other sensory receptors. For instance, the vertebrate or *Drosophila* olfactory receptors or the *Drosophila rh* genes are generally not coexpressed. Although the vertebrate olfactory receptor molecules themselves do not appear to play a role in the exclusion pathway, it has been argued that they might be directly involved in some step in the specification of olfactory receptor cells, in particular their projection pattern (Mombaerts et al., 1996). The Rhs are, like the olfactory receptors, seven-transmembrane G-coupled receptors, and they might too play an instructive role in the exclusion pathway that is distinct from their role in phototransduction (Chang and Ready, 2000). In

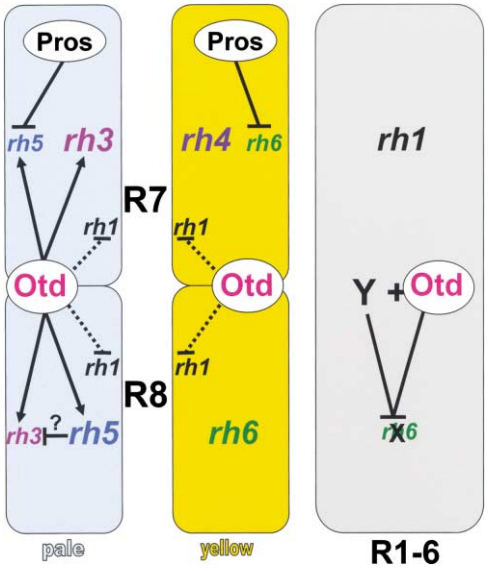

Figure 7. Model for the Role of Otd in the Regulation of Rhodopsins

See text for explanation. A solid line indicates a direct interaction while a dashed line is speculative.

otd mutants, the general coexpression rule is broken as Rh6 and Rh1 coexist in outer PRs, and Rh1 and Rh4 are present together in yR7. This suggests that *otd*-mediated processes are key to the exclusion process.

The Role of Otd in Photoreceptor Subtype Specification

The two different inner PR subtypes (p and y) remain defined in *otd* mutants, as *rh4* and *rh6* remain restricted to 70% of ommatidia, while p ommatidia acquire *rh1* or *rh6*. Thus, *otd* is likely to act downstream of other factors that determine the p subtype. Otd is present and required in all PRs and it is likely that its activation and repression roles are determined by interaction with other proteins that place Otd at the heart of the pathway that specifies the exclusion and coordination of *rhs*. In Figure 7, we propose a model to explain the multiple late roles of Otd; in pR7 and R8, Otd acts along with p-specific cofactors to direct expression of both *rh3* and *rh5*. In pR7, Prospero represses *rh5* and *rh6*, leaving *rh3* as the only *rh* expressed (Cook et al., 2003). In pR8, both *rh3* and *rh5* genes are also turned on, but the exclusion mechanism might only allow *rh5* to be maintained in R8. The mechanism for such regulation remains to be identified and could involve the Rh molecules themselves. In yR7, the Otd p cofactor is not present and *rh3* and *rh5* are not activated. *rh4* does not depend on Otd and must therefore be turned on specifically in yR7 by another system. We have recently identified a gene that is necessary and sufficient to turn on *rh4* in these cells (M.F.W. and C.D., unpublished data). In yR8, *rh6* is expressed by default (Chou et al., 1996; Papatsenko et al., 1997). In all inner PRs, Otd also indirectly represses *rh1*. Finally, in outer PRs, Otd interacts with a corepressor to turn *rh6* off (Figure 7), while strong activators turn on *rh1* at high levels. This model suggests that *otd* functions downstream of the p/y decision pathway, and that specific cofactors are required to allow the spatial determination of the two classes of ommatidia.

Experimental Procedures

Cloning of *rh6* Promoter

The *Drosophila melanogaster rh6* promoter was cloned by inverse PCR. One microgram of genomic DNA was digested for 4 hr by different four-cutter enzymes (NdeII, HpaII, and AvaII). The digestion reactions were diluted 1/60 and ligated using T4 DNA ligase (3,600 U). An initial PCR reaction (94°C for 2 min, [94°C for 30 s, 55°C for 1 min, 72°C for 1 min] × 40, 72°C for 5 min) was performed using the primers: +561*rh6*(3′): CAGTAACGATCGTAGGCAATCAG and +732*rh6*(5′): CGGCTGGAATCGATATGTGCCCG. A nested PCR was then performed using primers +561*rh6*(3′) and +774*rh6*(5′): GGT CTATCTCACTCCGCTGCTGAC. The amplified bands were cloned in the TA vector (pCR2.1; Invitrogen).

Cloning of the *Drosophila virilis rh6* Gene

A portion of the *rh6* coding sequence was amplified from *D. virilis* genomic DNA using the degenerate primers DV6+149 (5′) (GTNATH GTNAARGGNATGGCN) and DV6+323 (3′) (CCRTANACDATNGG RTT). Flanking sequences were amplified by inverse PCR as described above to provide the almost complete cDNA sequence (359 amino acids). Sequence alignment with *D. melanogaster rh6* revealed 91% similarity at the amino acid level, demonstrating that this cDNA is the *D. virilis* ortholog of *rh6*. Another round of inverse nested PCR led to the cloning of a 531 nucleotide sequence 5′ to the translation initiation ATG site.

Plasmid Constructs

All *Rh3* deletions were generated by PCR using pDM30*Rh3*(−2500/+18) as a template (kindly provided by Charles Zuker). *Rh4*(−373/+85) was PCR amplified from *D. melanogaster* genomic DNA, and deletions described in the text were generated from this fragment. The *rh5*(−670/+30) construct described by Papatsenko et al. (1997) was used as a template for the *rh5* deletions, and the *rh6* deletions were constructed using the *rh6*(−555/+121) construct described earlier. Site-directed and deletion mutants were generated using PCR. Primer sequences are available upon request. All of the amplified fragments were cloned into pCaspeRΔSalI (Wimmer et al., 1997). The *rh6*(−555/+121) promoter was also cloned upstream of GAL4, which was subsequently subcloned into pCasperΔXbaI, lacking the *lacZ* coding sequence. GAL4 transgenic lines were crossed to UAS-GFP lines and observed using water immersion (Pichaud et al., 2001).

Transgenic Lines and Staining

The transgenic lines were produced by standard procedures (Rubin and Spradling, 1982; Spradling and Rubin, 1982). At least five lines were generated for each construct. Homozygous or heterozygous fly heads were embedded directly in OCT medium and frozen. Sections of 10 μm were transferred to slides and fixed in 0.5% glutaraldehyde for 15 min at room temperature. X-gal staining was performed in 1× PBS, 2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 1 mg/ml X-gal for 30 min to overnight at 37°C. Alternatively, *lacZ* reporters were detected using a rabbit anti-β-galactosidase antibody 1/1000 (Cappel) and goat anti-rabbit Cy3 1/600 (Jackson Laboratories). All lines with the same construct gave similar expression patterns. Cryosection and whole-mounted retina stainings of adult heads from wild-type or *otd*^{uvi} were performed as previously described (Cook at al., 2003). Rh3 expression was assayed with an *rh3*-Rh3::GFP transgene (Pichaud and Desplan, 2001). The following primary antibodies were used: rabbit anti-Rh4 1/40 (a gift from C. Zuker), and rabbit anti-Rh6 (1/5000) and mouse anti-Rh1 (Hybridoma bank). The rabbit anti-Rh6 antibody was generated in our laboratory against a KLH-conjugated peptide LACGKDDLTSDSRTQ and was affinity purified. Anti-rabbit Cy3 (1/600), anti-mouse Cy5, anti-rabbit FITC, and anti-mouse FITC secondary antibodies (Jackson Immunochemicals) were used 1/200, and F-actin was stained with 1 μM phalloidin-TRITC (Sigma). In vivo fluoroscopy of GFP transgenes was performed as previously described (Pichaud et al., 2001).

Gel Shift Experiments

A fragment (amino acids 50–160) containing the homeodomain of Otd was amplified by PCR, subcloned into pGEX-5X-1 (Pharmacia), and transformed into BL21 bacteria (Stratagene). GST::OtdHD protein expression was induced at OD = 0.6 for 90 min at 37°C with 0.2 mM IPTG, and soluble protein was purified using a GST-Sepharose slurry (Pharmacia) following the commercial protocol. Primers of the following sequences were annealed and labeled with [γ-^{32}P]ATP as previously described (Cook et al., 2003): *rh3 K50*, gatcctgtcagcaag GGTATTAggccaatcccaaacgggtag; *rh5 K50*, gatccggctaagacgttgG GATTAtccttcctggaatg; *rh5 K50**, gatccggctaagacgttgGGGCGAtcc-ttcctggaatg; and *rh6 K50*, gatccgacgcgactgctgcTAATCCaacgcag caaacgag.

Half a microgram of GST or GST::OtdHD fusion protein and 0.35 pmol probe were incubated in a 20 μl reaction containing 20 mM HEPES (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 10 mM $ZnCl_2$, 6% glycerol, 0.2 g/l BSA, 50 μg/ml poly(dI-dC) for 20 min at 25°C. Samples were run on a 4% nondenaturing acrylamide gel, dried, and exposed to film for 2 hr at −80°C.

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We are grateful to Charles Zuker for providing us with *Rh3*(−2300/+18)-*lacZ* and *Rh4*(−1900/+7)-*lacZ* transgenic flies and with pDM30{*Rh3*(−2300/+18)} and pDM30{*Rh4*(−1900/+7)} plasmids, and with antibodies against Rh3 and Rh4. Steve Britt also generously provided anti-Rh5 antibodies. We thank Rosemary Reinke for *otd*^{uvi} flies. We are indebted to Terry Blackman and Sheng Wang for their assistance in generating transgenic flies. We thank all the present and former members of the Desplan lab for many discussions, suggestions, and comments on the paper, especially Ernst Wimmer for numerous discussions, ideas, and technical tools. P.B. and F.P. were supported by fellowships from the HFSPO, T.C. by NIH fellowship F32-EY07054, and M.F.W. by a Boehringer fellowship. This work was supported by grant RO1 EY13010 from the National Eye Institute/NIH to C.D.

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