Cell-fate determination in the developing Drosophila eye: role of the rough gene

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Summary

The homeobox-gene rough is required in photoreceptor cells R2 and R5 for normal ommatidial assembly in the developing Drosophila eye. We have used several cell-type-specific markers and double mutant combinations to analyze cell-fate determination in rough. We show that the cells that would normally become R2 and/or R5 express a marker, a lacZ insertion in the seven-up (svp) gene, which is indicative of the R1/3/4/6 cell fate. In addition, the analysis of mitotically induced svp,ro double mutant clones in the eye indicates that in rough all outer photoreceptors are under the genetic control of the svp gene. These results show that, in the absence of rough function, R2 and R5 fail to be correctly determined and appear to be transformed into cells of the R3/4/1/6 subtype. This transformation and the subsequent developmental defects do not preclude the recruitment of R7 cells. However, the presence of ommatidia containing more than one R7 and/or R8 cell in rough implies a complex network of cellular interactions underlying cell-fate determination in the Drosophila retina.

Key words: Drosophila, eye development, photoreceptor cell fate, homeobox.

Introduction

Genes containing the highly conserved homeobox play a variety of important roles in development. During Drosophila embryogenesis homeobox genes are involved in processes such as the generation of positional information along the anteroposterior axis, segmentation, and the specification of segment identity (for review see Gehring, 1987; Ingham, 1988). More recently, Drosophila homeobox genes such as fushi tarazu (Doe et al. 1988a), even-skipped (Doe et al. 1988b) and cut (Blochlinger et al. 1988), and the C. elegans gene mec-3 (Way and Chalfie, 1988) have been associated with cell-fate determination in the nervous system. In the absence of their gene products, specific sensory neurons or CNS neurons appear to undergo a transformation in neuronal identity. In this report, we analyze the role of the homeobox gene rough in determining the fate of specific photoreceptor neurons in the Drosophila eye.

The Drosophila retina is a repetitive array of a simple structure, the unit eye or ommatidium. Each ommatidium in turn contains a stereotyped arrangement of photoreceptor neurons and their accessory cells. Cells in the retina are not related by lineage (Ready et al. 1976; Lawrence and Green, 1979); rather, they gain their identities through cell interactions (for reviews see Tomlinson, 1988; Ready, 1989; Rubin, 1989; Banerjee and Zipursky, 1990). In the larval eye imaginal disc, differentiation occurs in discrete steps through a process thought to involve sequential recruitment of uncommitted cells into ommatidial clusters. The eight photoreceptor cells (R cells) differentiate first: cell R8 is followed by the pairwise addition of R2/R5, R3/R4, R1/R6, and finally by the addition of R7. Cone and pigment cells join the cluster in subsequent steps (Tomlinson and Ready, 1987a; Cagan and Ready, 1989). This differentiation process starts at the posterior edge of the disc and proceeds anteriorly across the disc epithelium over a two day period. A dorsal–ventral indentation, the morphogenetic furrow, marks the front edge of the differentiation process.

The photoreceptor cells in the adult ommatidium can be subdivided into three groups according to morphological, physiological and genetic criteria (Heisenberg and Wolf, 1984; Harris et al. 1976). The R1–R6 cells elaborate large, peripherally located rhabdomeres, they express the Rh1 opsin (Mismer and Rubin, 1987) and their axons terminate in the first optic neuropil, the lamina. The rhabdomeres of R8 and R7 are small and located in the center of the ommatidium, the R7 rhabdomere occupying the distal portion and the R8 rhabdomere the proximal portion of the retina. The axons of both R7 and R8 terminate in the second optic ganglion, the medulla. R7 cells express, in non-overlapping subsets, opsins encoded by the Rh3 and Rh4 rhodopsin genes (Montell et al. 1987; Pollock and Benzer, 1988), and are specifically affected by mu-
tations in sevenless (sev, Harris et al. 1976), bride of sevenless (boss, Reineke and Zipursky, 1988) and seven in absentia (sina, Carthew and Rubin, 1990).

A further subdivision of R1–R6 into two groups containing R2 and R5, and R1, R3, R4 and R6 has been proposed based on the symmetries of cellular contacts in the adult retina (Ready et al. 1986). Mutations that specifically eliminate the cells of one of these subgroups have not been isolated; however, genes that are specifically required in the cells of each subgroup for normal ommatidial assembly have been described. The homeobox gene rough (ro) is required in R2 and R5 (Tomlinson et al. 1988), while the seven-up (svp) gene, a member of the steroid receptor superfamily, is required in R1, R3, R4 and R6 (Mlodzik et al. 1990) for proper ommatidial development. The absence of svp function leads to the transformation of cells R1, R3, R4 and R6 toward the R7 cell fate (Mlodzik et al. 1990).

In eye discs from ro larvae, R8, R2 and R5 appear to differentiate normally in the majority of ommatidia (Tomlinson et al. 1988); R3 and R4, however, are not properly recruited. This observation, together with the genetic requirement for ro in the R2 and R5 cell, led to the hypothesis that ro was required for the generation of developmental signals in R2 and R5 necessary for the development of R3 and R4 (Tomlinson et al. 1988). Ectopic ro expression in the R7 cell precursor causes this cell to develop as a photoreceptor of the R1–6 class, suggesting that ro can specify the outer photoreceptor cell fate (Kimmel et al. 1990; Basler et al. 1990). However, available cell markers did not allow the distinction of R2 and R5 from the other outer photoreceptors such as R1, R3, R4 and R6. Moreover, these experiments did not directly address the question of the cell fate of the presumptive R2 and R5 cells in ro mutants, and what effect these early developmental defects have on the recruitment and fate determination of other photoreceptors in mutant clusters.

In this paper, we use cell-type-specific markers and various double mutant combinations to study the rough phenotype in more detail. Our results suggest that in rough ommatidia the cells that would normally develop as R2 and/or R5 do not adopt their correct identity and in the majority of cases appear to be transformed toward the R3/R4/R1/R6 fate. In addition, we show that this transformation does not preclude the recruitment of the R7 photoreceptor. However, clusters containing abnormal numbers of both R8 and R7 cells are observed in rough, suggesting a complex network of positive and negative interactions underlying cell-fate determination in the Drosophila retina.

Materials and methods

Fly stocks
Marker mutations and balancer chromosomes are described in Lindsley and Grell, 1968. ro163 is an X-ray-induced protein-null allele containing a 22 bp deletion that results in a frameshift after amino acid 30 in the ro protein; this allele was used unless otherwise stated. The developmental defects in ro163 were studied at the light microscope level after staining mutant eye discs with neuron-specific antibodies (Kimmel, 1990; Heberlein and Rubin, 1991). This analysis revealed no major differences with the description available for ro1 (Tomlinson et al. 1988). Moreover, the analysis of somatically induced ro163 mosaic eyes (Kimmel, 1990) was completely consistent with the results obtained with ro1 (Tomlinson et al. 1988). Alleles sev22 (Gerrersheim, 1984) and svp22 (formerly cK102, Gausz et al. 1981) were used. Construction of double mutant combinations were done using standard genetic crosses. Strains that express R7-specific markers are: P[Rh3.247 CAT]7, P[Rh3.945 CAT]1, P[Rh4.373 CAT]13, P[Rh4.1900 CAT]6, P[Rh4.1900 lacZ]6 (Fortini and Rubin, 1990). Line AE-127 is a P-lacZ enhancer trap line inserted in the svp gene (M.M., J. S. Heilig and G.M.R., unpublished). β-galactosidase in the AE-127 line is localized to the nucleus, which allows easier identification of expressing cells. Line rK519, also a P-lacZ enhancer trap line (L. S. Higgins and G.M.R., unpublished), has not been fully characterized.

Histochemistry and immunocytochemistry
Horizontal frozen sections: 12 µm sections of adult heads and β-galactosidase histochemistry were performed using previously described techniques (Misher and Rubin, 1987). Tangential thin sections: Adult heads were bisected and fixed in 3.7 % formaldehyde, 0.1 % glutaraldehyde in PBS for 2–3 h on ice. Heads were then infiltrated with 2.3 m sucrose in PBS for 3–5 h on ice, after which 1 µm thick sections were cut on a ultracryostat. Sections were collected on gelatin-coated slides and air-dried for several hours. Sections were then blocked for 15 min with 10 % normal goat serum (Jackson Immuno Research) and incubated with the anti-β-galactosidase monoclonal antibody (1:250 dilution, Promega) at 4°C for 1 h. After two 5 min washes in PBS, the sections were incubated for 1 h at room temperature with a goat anti-mouse secondary antibody coupled to horse radish peroxidase (HRP) (1:250 dilution, New England Biolabs). Sections were washed twice in PBS and HRP was visualized with a solution containing 0.5 % diaminobenzidine, 0.02 % CoCl2·6H2O and 0.003 % H2O2 in PBS. The reaction was stopped after 10 min by washing in PBS, after which sections were dehydrated by immersion in 30, 50, 70 and 100 % ethanol and mounted with DPX.

Immagical disc: Staining of immaginal eye-antennae complexes with antibodies was carried out as described by Tomlinson and Ready (1987a) using the same anti-β-galactosidase antibody and incubation conditions described for the staining of thin frozen sections.

Histology
Fixation and sectioning of adult heads were performed as described (Tomlinson and Ready, 1987b). For scanning electron microscopy, flies were dried for several weeks in vials containing Drierite, mounted with TV tube coat (Ted Pella, Inc) onto SEM stubs, and sputter-coated with a 25 nm-thick platinum coat. Samples were viewed and photographed on an International Scientific Instruments DS-130 SEM.

CAT assays
Virgin females of genotype sev22/sev22; TM3,ry506/ro163,ry506 were crossed to males of genotype +/Y: P[Rh.CAT,ry1]/+, ro163,ry506/ro163,ry506. Two duplicate sets of 5 progeny flies of the following genotypes were analyzed for CAT activity: females sev22/++; P[Rh.CAT,ry1]+/+, TM3,ry506/ro163,ry506 (sev+/ro163), females sev22/++; P[Rh.CAT,ry1]+/+, ro163,ry506/ro163 (sev+/ro163), males sev22/+; P[Rh.CAT,ry1]+/+, TM3,ry506/ro163,ry506 (sev+/ro163), and males sev22/Y; P[Rh.CAT,ry1]+/+, ro163,ry506/ro163,ry506 (sev+/ro163). Flies
not carrying the Rh.CAT transposons, and thus rosy− were used as negative controls. Extracts and CAT assays were carried out according to published procedures (Gorman et al. 1982; Misler and Rubin, 1987).

Somatic recombination and mosaic analysis

The ro, svp double mutant analysis was facilitated by the fact that both genes are located on chromosome arm 3R at positions 97D5−7 and 87B4−6, respectively. A 3rd chromosome carrying alleles of both genes (svp62 and ro63) was generated by standard meiotic recombination. Males heterozygous for svp and ro (svp62, ro63/TM3, Sb) were crossed to virgin females homozygous for both the w118 mutation on the X chromosome and a P[w+] transposon, P[w(y)D]3 at position 90E (Levis et al. 1985). In an otherwise white background, half of the male progeny were trans-heterozygous for the svp, ro chromosome and the P[w+] transposon. X-ray irradiation (1000 rad) of late first instar larvae was used to induce somatic recombination, generating mutant cells lacking both svp and ro function and also the P[w+] transposon. Clones that develop from these cells can be identified in the adult eye as patches of tissue that lack pigment surrounded by pigmented, wild-type tissue. Mutant clones were observed at a frequency of about 1 in 50 eyes. Fixation and sectioning were performed as described by Tomlinson and Ready (1987b), except that 1 or 2 μm sections were cut. The sections were examined with phase contrast microscopy.

A very similar strategy was used to generate svp− clones in a ro− eye. A chromosome carrying the ro63 allele and the P[w+] transposon at 90E was generated by standard meiotic recombination. Females homozygous for this 3rd chromosome and white− on the X chromosome were crossed to svp, ro heterozygous males (svp62, ro63/TM3, Sb) and clones were induced as described above.

Results

The rough phenotype

The compound eyes of flies carrying a null allele of rough (ro63) are slightly smaller than wild-type and the arrangement of facets is clearly disorganized (Fig. 1; Heberlein and Rubin, 1991). A tangential section through one such eye (Fig. 1D) shows that the structure of individual ommatidia is aberrant; the defects, however, are not uniform among different clusters. On average a rough ommatidium contains 3.7±1.2 (n=311) photoreceptor cells as judged by the number of rhabdomeres. This number is in good agreement with the number of cells per ommatidium that differentiate neurally in rough eye discs, 6.0±0.9 (n=113) (data not shown), as determined after staining with a monoclonal antibody, MAbBP104, that specifically labels neurons (Hortsch et al. 1990). This correlation suggests that the cells that initiate neuronal differentiation in the eye disc develop into mature photoreceptor cells. The photoreceptors in adult rough ommatidia can be further subdivided by morphological criteria into cells that elaborate large rhabdomeres, of the R1−R6 class, and small rhabdomeres, of the R7/R8 class. The average rough ommatidium contains 3.4±0.7 (n=311) large rhabdomeres and 2.3±0.9 (n=311) small rhabdomeres. In wild-type ommatidia only one small rhabdomere can be observed in each plane of section: the rhabdomere of R7 in the distal portion, and the rhabdomere of R8 in the proximal portion of the retina. In serial reconstructions of a few rough ommatidia, we observed that the number of small rhabdomeres was the same in distal and proximal sections (data not shown). This suggests that the rhabdomeres of these putative R7 and R8 cells extend throughout most of the retina, probably due to the general disorder in rough ommatidia.

Fate of the R2/R5 precursor cells in rough

The early steps of ommatidial differentiation in eye discs from ro differentiated as described above. In the eye disc, L-galactosidase expression was evident in 3 or 4 cells. The R2 and R5 cells are closely associated with each other and form a unit that fails to differentiate is often excluded from the cluster. The differentiation of ro63 eye discs has been examined by light microscopy and found to be similar to that of ro2 (Kimmel, 1990). Consistent with a requirement for rough function during the early steps of ommatidial assembly is the finding that rough protein is expressed broadly in the morphogenetic furrow, and, slightly later, in the R2, R5, R3 and R4 precursor cells (Kimmel et al. 1990).

As a marker to visualize the differentiation of R3 and R4 in rough, we used a lacZ-enhancer-trap insertion in the svp gene (line AE127, see Methods). Flies carrying this insertion express β-galactosidase in the nuclei of the same cells in the eye disc that normally express svp transcripts (Mlodzik et al. 1990). In eye discs from wild-type larvae, expression is first detectable in two cells, the R3 and R4 precursors, just posterior to the morphogenetic furrow (Fig. 2A). In older clusters, located 3−4 columns behind the morphogenetic furrow, β-galactosidase is expressed in the developing R1 and R6 cells in addition to R3 and R4 (Fig. 2A). Expression of β-galactosidase in the AE127 line in a rough genetic background was qualitatively different than in wild-type (Fig. 2B). On first inspection, a general increase in lacZ-expressing cells could be observed in and near the morphogenetic furrow; however, it is difficult to establish the identity of the cells in the morphogenetic furrow. In clusters located just posterior to the furrow, expression could often be observed in 3 or 4 cells. The position of these cells is consistent with their identity as precursors of R2, R3, R4 and R5 or a subset thereof (Fig. 2B). We presume that the vast majority of clusters in which only three cells are stained correspond to those in which only one of the R3/R4 pair becomes incorporated into the ommatidium (Tomlinson et al. 1988) or to those in which a second cell differentiates as an R5-like cell (see below). In the posterior region of the eye disc, β-galactosidase expression was evident in 3−5 cells per cluster. The aberrant development of rough ommatidia, however, precludes the establish-
ment of cellular identities in these older clusters. In summary, svp expression, as inferred from the lacZ expression in the AE127 line, was derepressed in eye discs from rough larvae. More specifically, expression was observed in cells that occupy the positions of cells that would develop as R2 and/or R5 in wild type. Indistinguishable results (data not shown) were obtained with the original V-tacZ insertion in the svp gene (line H162, Mlodzik et al. 1990).

The altered svp expression pattern in ro discs suggested that in ro some R2 and R5 precursors may adopt a R3/4/1/6 fate. In wild type, the development of R3-R4, R1 and R6 depends on svp+ function. In a svp− genetic background, these four outer photoreceptors are at least partially transformed towards the R7 cell fate resulting in ommatidia that contain up to five R7-like cells. Such svp− ommatidia contain, in addition to several R7-like cells and R8, two to four outer photoreceptors: R2, R5 and one or two additional cells (Mlodzik et al. 1990). It has been proposed (Mlodzik et al. 1990) that these extra cells correspond to the so-called 'mystery cells' (Tomlinson et al. 1987), which fail to leave the cluster when R3 and R4 are svp−. The mystery cells are normally located between R3 and R4, they do not express neural antigens and are lost during the steps that lead to the assembly of the five-cell precluster in wild type. Due to the embryonic lethality of svp, its function and phenotype in the eye can only be observed in somatically induced svp− clones. To test if the remaining outer photoreceptors in ro (3–4 per cluster) depend on svp+ activity as would be expected if they are indeed transformed toward the R3/4/1/6 identity, we generated clones that were doubly mutant for both ro and svp in an otherwise wild-type eye (for experimental details see Materials and methods).

In these svp−,ro− ommatidia, we detected only photoreceptors of the central type (R7 or R8) as judged by their morphology (Fig. 2C, for comparison to svp−,ro+ ommatidia see inset). Strikingly, in mosaic ommatidia, whenever we observed one or more
Determination of photoreceptor cell fate in rough

Fig. 2. Interaction between rough and seven-up. Eye discs from wild-type (panel A) and ro<sup>63</sup> (panel B) third instar larvae carrying a P-lacZ enhancer-trap insertion in the svp gene (line AE127) were stained with a monoclonal antibody directed against β-galactosidase. (A) In a wild-type disc, lacZ expression is observed in the nuclei of the R3 and R4 cells just posterior to the morphogenetic furrow (MF, arrow). Several rows behind the MF lacZ expression is evident in four nuclei, corresponding to the R3, R4, R1 and R6 cells. Two typical clusters are circled. (B) In a ro<sup>63</sup> eye disc, an increased number of lacZ-expressing cells can be observed just posterior to the MF (arrow). Several single clusters located just posterior to the MF are shown at higher magnification below panels A and B. The left-most cluster is wild type, all others are ro<sup>63</sup>. In the mutant clusters lacZ expression is observed in the nuclei of 3 or 4 cells, which according to their position correspond to the presumptive R3, R4, R2 and R5 cells or a subset thereof. We used line AE127 in this experiment because the nuclear localization of β-galactosidase allows easier establishment of cellular position in the cluster. However, identical results were obtained with line H162 (Mlodzik et al. 1990), which displays stronger cytoplasmic β-galactosidase expression. Anterior is to the left in panels A, B and the panels showing single ommatidia. (C) Eye phenotype of a svp,ro double mutant clone. Phase contrast image of a tangential section through a mosaic eye is shown. The pigment indicative of a white<sup>+</sup>, and thus svp<sup>+</sup> and ro<sup>+</sup> genotype is evident as black granules in the cytoplasm adjacent to the rhabdomeres of the photoreceptor cells and as highly refractile granules in the pigment cells that surround each ommatidium. Mosaic clusters containing one or two w<sup>+</sup> photoreceptors are indicated by arrows. For reference, two svp<sup>−</sup>,ro<sup>−</sup> ommatidia are shown in the inset (Mlodzik et al. 1990). Bar is 20 μm in A and B, and 12 μm in C).
photoreceptors with large rhodobemes they contained pigment and thus were genotypically $syp^+, ro^+$ (examples are indicated by arrows in Fig. 2C). Very similar results were obtained in $syp^-$ clones induced in the eyes of $ro^-$ flies (data not shown). The overall number of photoreceptors in each ommatidium was the same in $syp^- ro^-$ and $syp^+, ro^-$. We detected on average $5.6 \pm 0.87 \ (n=138)$ photoreceptors in double mutant ommatidia, as compared to $5.7 \pm 1.2 \ in \ ro^-$. In summary, these observations indicate that no R2 and R5 cells are present in $ro^-$ since all the existing outer photoreceptors require $syp^+$ function for their development and thus, by this criterion, are of the R3/4/1/6 type.

**Differentiation of R7 cells in rough**

The R7 cell is the last photoreceptor to join differentiating ommatidia. To test if the disruption in the photoreceptor assembly process in $ro$ affected the differentiation of the R7 cell, we analyzed the expression of R7-specific markers in $ro$. Fusions of the Rh3 and Rh4 opsin promoters to reporter genes encoding bacterial chloramphenicol acetyl transferase (CAT) and $\beta$-galactosidase ($lacZ$) have been shown to reproduce the wild-type Rh3 and Rh4 expression patterns in transgenic flies carrying these fusion genes (Fortini and Rubin, 1990).

The levels of Rh3 and Rh4-CAT expression in wild-type and $ro$ flies are very similar and, as in wild-type, this expression is dependent on $sev^+$ (Table 1) and $sina^+$ (data not shown) function. The residual expression of Rh3-CAT in the $sev; ro$ double mutant flies is presumably due to expression in the specialized R8 cells of the dorsal marginal region of the eye as has been reported for $sev$ flies (Fortini and Rubin, 1990). Thus, $ro$ eyes appear to contain approximately wild-type numbers of R7 cells, and the development of these cells is under the same genetic control as in wild-type flies.

To determine the distribution of R7 cells in $ro$ eyes, we analyzed the expression patterns of $\beta$-galactosidase in flies carrying Rh4-$lacZ$ (Fig. 3) and Rh3-$lacZ$ (data not shown) fusion constructs. Horizontal sections of flies carrying Rh4-$lacZ$ construct were histochemically stained for $\beta$-galactosidase activity (Fig. 3A,B,C). In wild-type heads, $\beta$-galactosidase was restricted to the distal half of the retina where the R7 cell bodies are located. The normal pathway of R7 axons through the lamina and their termination in the medulla can be observed. Strong $\beta$-galactosidase expression was observed in similar sections of $ro^{63}$ flies carrying the Rh4-$lacZ$ construct, consistent with the high levels of CAT expression observed in flies carrying Rh4-CAT. $\beta$-galactosidase activity, however, was not restricted to the distal portion of the retina, confirming our previous results that suggested that R7 cells extend throughout most of the retina in $ro$. The optic lobes of $ro$ flies are grossly misformed as a consequence of abnormal retinal development (Meyerowitz and Kankel, 1978); the lamina is greatly reduced in size, and the medulla, lobula and lobula plate are rotated about $90^\circ$ relative to wild type. In spite of this defect, the R7 cells in $ro$ still send their axons to the medulla (Fig. 3B and data not shown), resulting in aberrant axonal pathways. The expression of Rh4-$lacZ$ was completely abolished in $sev; ro$ double mutant flies (Fig. 3C).

To precisely localize the R7 cells present in $ro$ to individual ommatidia, we stained thin tangential eye sections of flies carrying Rh4-$lacZ$ with a monoclonal antibody against $\beta$-galactosidase. Fig. 3D and 3E show the distribution of R7 cells in wild-type eyes. A single stained cell with a centrally located rhabdomere is observed in most ommatidia. Clusters that do not stain presumably contain R7 cells as judged by Rh4 expression. The distribution of Rh4-expressing R7 cells in $ro$ is shown in Fig. 3F,G,H. In contrast to wild-type, $ro$ ommatidia often contain more than one R7 cell as judged by Rh4 expression. In the majority of these ommatidia $\beta$-galactosidase was detected in what appear to be adjacent cells, but in several clusters two separate R7 cells were observed (Fig. 3F,G,H). In summary, we show that, in spite of the early developmental aberrations occurring in $ro$ ommatidia, R7 cells are recruited. In many instances, however, more than one cell per ommatidium adopts the R7 cell fate.

**Differentiation of R8 cells in rough**

We have shown that several of the photoreceptor cells that elaborate small rhodobemes in $ro$ ommatidia appear to be R7 cells. Surprisingly, the analysis of eye sections of double mutant $sev; ro$ flies, in which R7 cells should be absent, revealed that a significant fraction of ommatidia still contained more than one cell with a small rhodobeme of the R7/R8 type (Fig. 4D). To test the possibility that these cells correspond to R8 cells, we analyzed the expression pattern of an R8-specific marker in eye discs from $ro$ larvae. Line rK519 is a $P-lacZ$ enhancer-trap line that specifically expresses $\beta$-galactosidase in one cell per cluster, the developing R8 cell (L. S. Higgins, personal communication; Fig. 4A), starting about 4 rows behind the morphogenetic furrow. In a $ro$ mutant background, $lacZ$ expression in the rK519 line was observed in more than one cell in a $30\%$ of the developing ommatidia (Fig. 4B). Similar results were obtained with several other R8-specific markers (U. Gaul, personal communication). These results suggest that in $ro$ more than one cell per ommatidium can adopt the R8 cell fate. We cannot establish the exact time at which this aberrant

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**Table 1. Expression of rhodopsin promoter-CAT fusion genes**

<table>
<thead>
<tr>
<th>genotype:</th>
<th>CAT activity (%)*</th>
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<tbody>
<tr>
<td>$sev^+; ro^+$</td>
<td>100</td>
</tr>
<tr>
<td>$sev^-; ro^-$</td>
<td>95.1</td>
</tr>
<tr>
<td>$sev^-; ro^+$</td>
<td>0.4</td>
</tr>
<tr>
<td>$sev^-; ro^-$</td>
<td>0.4</td>
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</table>

*$P[Rh4.373CAT]$ | 100 | 90.2 | 10.3 | 18.9 |
*$P[Rh4.1900CAT]$ | 100 | 141.4 | 6.5 | 14.8 |
*$P[Rh3.343CAT]$ | 100 | 129.9 | 10.3 | 18.9 |
*$P[Rh3.945CAT]$ | 100 | 90.2 | 10.3 | 18.9 |

*CAT activity levels are normalized to the value observed for each transformant line in a wild-type background.
Fig. 3. Expression of an R7-specific marker in rough. All sections correspond to adult heads from flies carrying an Rh4–lacZ fusion gene (Fortini and Rubin, 1990). Panels A–C show light micrographs of 12 μm horizontal sections histochemically stained for β-galactosidase activity. The following genotypes are shown: (A) wild-type, (B) re^{63}, and (C) sev^{62}; ro^{63}. Panels D–H show light micrographs of 1 μm tangential sections stained with a monoclonal antibody directed against β-galactosidase. Panels D and E correspond to wild-type flies, panels F, G and H to ro^{63} flies. Bar is 125 μm in A, B and C, 20 μm in D and F, and 8 μm in E, G and H.

differentiation begins due to the late onset of expression of the R8-specific markers available.

Discussion

Inductive interactions between specific cells have been proposed to determine cell fate in the developing Drosophila retina (Tomlinson and Ready, 1987a) and several genes have been characterized recently that provide support for this model (for recent review see Banerjee and Zipursky, 1990). The rough gene is required during an early step in the ommatidial assembly process (Tomlinson et al. 1988). The cells that according to their position correspond to R8, R2 and R5 initiate neural differentiation normally in rough. However, the presumptive R3 and R4 cells, although
Fig. 4. R8 photoreceptor differentiation in rough. Panels A and B are photomicrographs of eye discs from wild-type (panel A) and ro^{rd} (panel B) third instar larvae carrying an R8-specific marker (P-lacZ rK519, see Methods) stained with a monoclonal antibody directed against β-galactosidase. Panels C and D are photomicrographs of tangential 1 μm sections. (C) In this basal section through a wild-type eye, the central small rhabdomere of the R8 cell is observed. (D) A section through an eye from a sev^{rd} ro^{rd} fly displays several clusters (arrows) that contain more than one cell with a small R8-like rhabdomere. At the plane of section shown, several ommatidia appear not to contain an R8 cell. A more basal section shows at least one R8-like cell in each ommatidium; however, the overall morphology in such basal regions of the retina is severely disorganized. Bar is 15 μm in A and B, and 20 μm in C and D.

usually positioned correctly, fail to develop appropriately. From this point onward the assembly process is not consistent from one ommatidium to another leading to the variety of phenotypes observed in ro ommatidia. On average, ro ommatidia contain ~3 to 4 outer photoreceptors with large rhabdomeres, and ~2 to 3 cells of the R7/R8 type, and are thus missing ~2 photoreceptor cells. In this report, we have attempted to establish the identity of the photoreceptors in ro. We were particularly interested in determining the fate of the cells that would normally become R2 and R5, and the consequence of the early developmental defects on the recruitment of the R7 cell.

Two genes are currently known to control proper differentiation of the six outer photoreceptor cells: the ro gene is required in R2 and R5 (Tomlinson et al. 1988), and thesvp gene is required in the remaining four cells. In the absence of svp function, the R3, R4, R1 and R6 cells develop as photoreceptors with small rhabdomeres (Mlodzik et al. 1990). In this paper, we show that the expression of the svp gene, as inferred from the expression of P-lacZ insertions in the svp gene, is altered in ro. Ectopic svp expression in ro is most obvious during the early stages of ommatidial assembly. At the five-cell precluster stage, when svp is normally only expressed in the R3 and R4 precursors, ro ommatidia express svp in 3–4 cells, which according to their positions correspond to the cells that would become R3, R4, R2, R5 or a subset thereof in wild-type discs. If the fate of these cells in ro is in fact regulated by the svp gene, loss of svp function should lead to their transformation into photoreceptors with small rhabdomeres. We found that svp<sup>−</sup>, ro<sup>−</sup> double mutant clones in adult mosaic animals contain only photoreceptors
with small rhabdomeres of the R7/R8 class. Thus, all outer photoreceptors that differentiate in ro− ommatidia require svp+ function, which suggests that they are photoreceptors of the R3/4/1/6 type.

The derepression of svp expression in the absence of ro function suggests that ro normally directly or indirectly represses svp transcription during the initial stages of ommatidial development. Curiously, this repression is not observed in the developing R3 and R4 cells, where ro and svp are coexpressed throughout photoreceptor cell assembly. Since ro has no known function in R3 and R4, this raises the interesting possibility that the activity of ro is differentially modulated in R2/5 and R3/4 resulting in different patterns of gene expression in these two cell types. Such cell-type-specific modulation of activity has recently been observed for another transcription factor required for photoreceptor cell differentiation, the Zn-finger containing glass gene (Moses and Rubin, 1991).

In addition to the abnormal differentiation of the R3/4/1/6 cells, svp− ommatidia often contain one or two extranumerary outer photoreceptors located in the vicinity of R3 and R4. It has been proposed that these correspond to the mystery cells, which fail to be excluded from the mutant cluster (Mlodzik et al. 1990). Double mutant svp−,ro− ommatidia contain the same number of photoreceptor cells as ro− ommatidia, 5–6 per cluster, all of which elaborate small rhabdomeres. This suggests that the mystery cell(s) are properly displaced from double mutant svp−,ro− clusters as they are from most ro− clusters. Although it is currently difficult to explain this observation, the simplest explanation is that in the absence of svp function ro could function in R3 and R4 and enable them to recruit the mystery cells to become photoreceptors. The analysis of svp− and of double mutant svp−,ro− clones in the eye disc will help answer these questions. However, our observations suggest a complicated network of interactions between svp and ro that lead to the specification of the six outer photoreceptor cells.

We used R8 and R7 specific markers to establish the identity of the ~2–3 small rhabdomere cells present in ro ommatidia. Unexpectedly, we found that some ro ommatidia contain more than one R8 cell as judged by the expression of an R8-specific marker in the eye disc, and the presence of multiple central R8-like cells in some sev;ro double mutant ommatidia. The R8 cell is the first cell to express neural antigens (Tomlinson and Ready, 1987a) and it has been proposed that a mechanism involving competition and lateral inhibition results in the specification and the regular spacing of R8 cells in the eye disc (Baker et al. 1990). In ro, the initial spacing of ommatidial preclusters is normal (Tomlinson et al. 1988). We therefore believe that the differentiation of the second R8 cell observed in some ro clusters occurs after the establishment of the R8 ‘foundation photoreceptor’ and therefore does not interfere with the initial spacing mechanisms. Our currently available markers do not allow us to exactly establish the time at which the extranumerary R8 cells adopt their fate. It is possible that one of the cells, which in wild-type would become R2 or R5, sometimes differentiates as an R8 cell rather than a cell of the R3/4/1/6 type. Clusters in which two R8 cells differentiate may correspond to those that fail to form a morphologically normal five-cell preclusters (Tomlinson et al. 1988).

The recruitment of the R7 cell is the last step in the assembly of the eight photoreceptors. The only signal known to date involved in this process originates in the R8 cell and is likely to be mediated by the boss gene (Hart et al. 1990). Besides R8, the two other cells that are in a position to instruct the development of R7 are the R1 and R6 cells. Our data show that R7 cells develop normally in ro. The aberrant and non-reproducible photoreceptor differentiation patterns observed in ro ommatidia make it difficult to postulate an essential role for R1 and R6 in the normal recruitment of R7, and therefore suggest that R8 is the only cell required to instruct R7 cell differentiation.

Thus, it appears that two critical criteria for a cell to differentiate as an R7 cell are: expression of the sev receptor and contact with the R8 cell. However, not every cell in the eye disc that fulfills these criteria, such as R3, R4, R1, R6 and R7 (Tomlinson et al. 1987), becomes an R7 cell. The analysis of the svp− phenotype suggested that wild-type svp function induces proper differentiation of outer photoreceptors R3, R4, R1 and R6 and thus prevents them from differentiating as R7 cells (Mlodzik et al. 1990). Expression of sev protein appears relatively normal in ro (Tomlinson et al. 1988); thus, several cells have the potential to develop as R7 cells. It is therefore possible that in ro discs the cells that fail to develop as outer photoreceptors under the instruction of svp could fulfill the criteria necessary to differentiate as R7 cells, that is, express the sev receptor and be in contact with R8.

Our data, together with previous observations, have started to unravel a hierarchy of genetic interactions that operate to establish the stereotyped developmental process of the ommatidium. Genes such as ro and svp display complex genetic interactions that are essential for appropriate differentiation of the six outer photoreceptor cells. In addition, svp function prevents a subset of outer photoreceptors from developing as R7 cells (Mlodzik et al. 1990). It thus appears that presumptive photoreceptor cells have a relatively broad developmental potential that becomes gradually restricted as cells are directed to adopt particular fates.

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