Neuronal development in the *Drosophila* compound eye: *rap* gene function is required in photoreceptor cell R8 for ommatidial pattern formation

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In the compound eye of *Drosophila*, cell–cell interactions are thought to play an important role in the determination of neuronal cell fate and pattern morphogenesis. Recent work on the *bride of sevenless* (*boss*) gene has demonstrated an inductive role for photoreceptor R8 in the differentiation of photoreceptor R7. These studies have shown that while R8 differentiates early in the scheme of ommatidial assembly, it continues to play an active role in subsequent patterning events. We describe studies on a new genetic locus *rap* (*retina aberrant in pattern*), whose functions are critical for normal pattern formation in the developing eye. Mutations in the *rap* gene perturb the early stages of pattern formation and lead to a variable number of photoreceptor cells (R cells) in each ommatidium. Experiments with a temperature-sensitive allele have shown that *rap* gene function is required during the period of development when pattern formation occurs. In addition, a somatic mosaic analysis of *rap* has shown that its function is required only in photoreceptor cell R8 for normal ommatidial patterning. These studies suggest an important role for *rap* in the initial events leading to pattern formation and are consistent with R8 playing a central role in directing ommatidial pattern formation.

[Key Words: *Drosophila*; photoreceptor neurons; *rap* genes; pattern formation]

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The nervous systems of multicellular organisms are comprised of complex networks of diverse cell types with unique positions and patterns of connectivity. Understanding how these intricate patterns develop is an important problem in cell and developmental biology. The compound eye of *Drosophila* is well suited for studying the genetic and molecular basis of pattern formation. The adult eye consists of a unit structure, the ommatidium, that is repeated nearly 800 times in a regular symmetrical array (Fig. 1A). In addition to an invariant number of accessory cells, each hexagonally shaped ommatidium contains eight photoreceptor cells (R cells) that can be segregated into three classes (R1–R6, R7, and R8) on the basis of their position, spectral sensitivity, and synaptic connectivity (Trujillo-Cenoz and Melamed 1966; Braitenberg 1967; Harris et al. 1976). The plasma membrane of these cells is multiply folded to form the rhabdomere. These structures are arranged in a stereotypic trapezoidal pattern (Fig. 1C) and contain the light-sensitive photopigments that are unique to each cell type.

The adult compound eye develops from a monolayer

of undifferentiated epithelial cells called the eye imaginal disc. During the third larval instar, pattern formation begins as a wave of morphogenesis moves from posterior to anterior across the disc. This wave is marked by a depression on the disc surface (the morphogenetic furrow) behind which small and precisely distributed cell clusters differentiate into photoreceptor neurons. Anatomical studies employing neuron-specific antibodies have shown that the R cells differentiate in a sequential manner. Photoreceptor cell R8 expresses neural antigens first and is thereafter joined in a pairwise fashion by cells R2 and R5, and R3 and R4. Subsequently, R1 and R6 differentiate, with R7 joining last to form the mature eight-cell cluster (Tomlinson and Ready 1987a). These events involve the precise movement of cell nuclei along the disc epithelium and are thought to require specific cell–cell contacts (Tomlinson 1985).

Genetic studies have provided important insights to the mechanisms employed in the specification of R cell fates. Mosaic analysis has shown that there are no strict lineage relationships between R cells within an ommatidium (Ready et al. 1976; Lawrence and Green 1979). This finding has led to the view that cell–cell interactions and environmental cues play an important role in the determination of R cell fates. Studies of mutations that affect retinal development support this inference by

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Figure 1. Morphology and anatomy of wild-type and *rap* compound eyes. Scanning electron micrographs of (A) wild-type (X185) and (B) *rap3* (X210) compound eyes. The wild-type eye has modular structure with a smooth hexagonal array, whereas the *rap* mutants show unevenly sized ommatidia with aberrant spacing. Tangential sections of (C) wild-type, (D) *rap3* (X300) compound eyes as viewed under transmission electron microscope. Note the regular trapezoidal arrangement of R cells in each ommatidium. In *rap* eyes (D), the ommatidia are irregular and are comprised of variable number of R cells.

revealing the presence of genes that are involved in specific inductive interactions between developing R cells (Reinke and Zipursky 1988; Tomlinson et al. 1988), and have provided clues to the nature of molecular strategies involved (Banerjee et al. 1987a,b; Hafen et al. 1987; Basler and Hafen 1988).

Recent studies have shown that while R8 is the first cell to differentiate during ommatidial assembly, it continues to play an active role in subsequent patterning events. For instance, a mutation in the *bride of sevenless* (*boss*) locus results in the loss of cell R7, the last cell to be added to the maturing R cell cluster. Elegant genetic mosaic studies have shown that the *boss*⁺ gene product is required in cell R8 for normal development of R7 (Reinke and Zipursky 1988). Thus these studies demonstrate the cell nonautonomous nature of *boss* and stress the significance of R8 in patterning events that follow its own differentiation.

While these and other studies have provided insights to the nature of cell–cell interactions and their importance in differentiation, the mechanisms that initiate ommatidial patterning events are poorly understood. Anatomical and immunocytological studies have shown that pattern formation begins with the differentiation and precise positioning of photoreceptor R8. Thus, it is likely that one or more R8 specific functions are required for these processes. By identifying genes that play a role in these early developmental events, it may be possible to determine the mechanism by which pattern formation is initiated.

In this paper we describe the genetic and phenotypic characterization of a new locus, *rap* (retina aberrant in pattern), that is necessary for proper patterning in the compound eye. The *rap* locus exhibits many of the attributes we would expect of a gene involved in ommatidial pattern initiation. Specifically, we present evidence showing that *rap* gene function is required during the stage of development when ommatidial assembly is known to occur. Moreover, immunocytological analysis of eye-imaginal discs show that a mutation in the *rap* gene results in a disruption of patterning events directly behind the morphogenetic furrow. These initial findings prompted a mosaic analysis of the *rap* gene. The results of these studies show that *rap* gene function must be present in photoreceptor cell R8 for proper ommatidial assembly. Together, the data presented here detail the significance of *rap* gene function in eye pattern formation and contribute to the growing body of evidence that shows that photoreceptor cell R8 plays a central role in ommatidial development.

Results

Isolation and mapping of rap

The *rap* mutation is P-element induced The four alleles of *rap* (*rap1*, *rap2*, *rap3*, *rapR22ts*) used in this study, were independently isolated from a P–M hybrid dysgenic cross. Such crosses involve the mating of males of a P strain with M strain females. P-transposable elements are mobilized in the germ line of the subsequent

F₁ progeny and mutations are observed in the F₂ generation (Engels and Preston 1979; Bingham et al. 1982; Rubin et al. 1982). Two methods were used to screen for eye mutations. To search for abnormalities in the internal arrangement of R cells, the optical method of deep pseudopupil (Franceschini and Kirschfeld 1971) was adapted for rapid screening of large numbers of flies (Banerjee et al. 1987a). In addition, a visual screen under a dissecting microscope was used to detect changes in the external eye morphology. Mutant candidates were individually mated to attached-X females of the P cytotype and propagated as stable lines. From a screen of 40,000 male progeny three alleles of *rap* (*rap1*, *rap2*, and *rap3*) were independently isolated. A temperature-sensitive allele (*rapR22ts*) was isolated in a second dysgenic cross (see below).

Mutations that arise from hybrid dysgenesis may result from either the insertion of a transposable element or the deletion of genomic sequences by imprecise P-element excision events. These mechanisms can be distinguished on the basis of the rate at which the mutant phenotype reverts to the wild type. When insertion mutants are placed under dysgenic conditions, P-elements may be remobilized, resulting in a high frequency of partial and complete revertants (Engels and Preston 1979; Tsubota and Schedl 1986). In contrast, when deletion mutants are placed under identical conditions, revertants are rarely observed. To determine whether the rough-eye phenotype found in *rap1* was the result of a P-element insertion, a dysgenic cross between *rap1*(P) males and attached-X y,f(M) females was performed. The male progeny from the F₂ generation were screened and the rate of reversion of the *rap1* allele was estimated. Flies that showed both a normal pseudopupil and wild-type external eye morphology were categorized as complete revertants, whereas those that exhibited an abnormal pseudopupil but wild-type external eye morphology were classified as partial revertants. A screen of 11,712 F₂ males showed a high frequency of reversion (1 : 400) characteristic of insertional mutants of this type. From a collection of 20 revertant lines, 14 were judged to be complete revertants and 6 were partial revertants. These results indicate that the mutation creating the *rap1* allele is the result of an insertion of a P element.

To characterize further the revertant lines, flies were grown at 17°C and 29°C to determine whether any of the lines represented a temperature-sensitive allele of *rap*. One revertant, *rapR22ts*, exhibited a temperature-sensitive eye phenotype. At 17°C, *rapR22ts* falls in the category of a partial revertant, showing a normal exterior morphology (Fig. 2A) and an abnormal pseudopupil. There is considerable variation in the degree of this latter phenotype; roughly 64% of the *rapR22ts* flies (reared at 17°C) show a wild-type pseudopupil pattern, whereas the remaining 36% exhibit a pattern that is indistinguishable from the original mutant, *rap1*. However, when reared at 29°C, *rapR22ts* flies show a mild rough-eye phenotype (Fig. 2B) and severely disrupted pseudopupil pattern.

Figure 2. Phenotype of the temperature-sensitive allele *rapR22ts*. Scanning electron micrograph of a compound eye of *rapR22ts* reared at (A) 17°C (X209) and (B) at 29°C (X185).

Further studies on *rapR22ts* show it to be an allele of our original mutant *rap1*. *rapR22ts/rap3* trans-heterozygotes raised at 29°C fail to complement and exhibit a mild rough-eye phenotype. Furthermore, deletion mapping localizes *rapR22ts* to the same region of the X chromosome as the original mutant *rap1*, from which it was derived (see below). Thus, both mapping and complementation data support the notion that the temperature-sensitive revertant is an allele of *rap* and not the result of a mutation at a second locus (i.e., a second site suppressor).

Mapping and complementation

The *rap* locus maps between 4C7 and 4C15 on the X chromosome The *rap* locus was mapped by recombination using the markers *yellow* (*y*), *white* (*w*), *vermillion* (*v*) and *forked* (*f*). By scoring 838 F₂ male recombinants (48.3% of total male progeny) the position of the *rap* gene was determined to be 5.3–11.7 cM from the distal tip of the X chromosome (Fig. 3A). Finer mapping of *rap* was accomplished using a series of overlapping X chromosome deficiencies. As shown in Figure 3B, when *rap* was made heterozygous with Df(1)JC70 or Df(1)rb1, females were indistinguishable from wild-type (Canton S) flies. In contrast, when *rap1* was heterozygous with either Df(1)Hf366, Df(1)A113, Df(1)RC40, Df(1)biD2, or Df(1)rb13, females exhibited the rough-eye phenotype and an abnormal pseudopupil. From this pattern of complementation, the cytological position of the *rap* locus

has been narrowed down to the region between 4C7 and 4C15 on the X chromosome. In situ hybridization of *rap* mutants support our mapping data. When polytene chromosomes taken from the salivary glands of third-instar *rap1* mutant larvae were probed with radiolabeled P-element DNA, a hybridization site was observed in the region where *rap* has been genetically localized (data not shown).

The position of *rap* on the X chromosome places it in close proximity with two previously characterized rough-eye mutants, *rugose* (*rg*, 11.0) and *pebbled* (*peb*, 7.3). To determine whether *rap* was allelic with either of these loci, *rap1/peb* and *rap1/rg* heterozygotes were constructed to test for complementation. Results indicate that *rap* is distinct from these two loci as both heterozygotes were phenotypically wild type.

rap mutants exhibit a variable number of R cells in each ommatidium

The relatively smooth texture of the wild-type compound eye results from the precise and ordered distribution of 800 hexagonally shaped ommatidia. These ommatidia are arranged in rows and show a high degree of homology in both their size and shape (Fig. 1A). In contrast, the configuration of ommatidial units found in the *rap* compound eye is extremely disordered. The *rap* mutant ommatidia are aberrant in their size, shape, and alignment with respect to neighboring units. These

Figure 3. (A) Recombination map position of *rap*. Eight-hundred and thirty-eight recombinants (43% of total progeny) were obtained from a cross between *rap1* males and female flies carrying X chromosome linked markers, *y*, *v*, and *f*. Of these 117, 369, and 794 recombinants represented crossovers between *rap* and the three markers, respectively. From this information, *rap* was mapped to a region between 6.7 and 11.7 map units from the distal tip of the X chromosome. Subsequent crosses between *rap* and *w* (*w*, 1.5) gave only 13 recombinants in 341 progeny, placing *rap* 3.8 units to the right of *w* at position 5.3. (B) Deficiency map of *rap*. The *rap1* allele was made heterozygous with X chromosomes carrying deficiencies listed above. Deficiencies that uncover *rap* are shown (shaded areas). These results locate *rap* cytologically to the region between 4C7 and 4C15.

gross changes in structure and pattern are accompanied by abnormalities in the distribution of mechanosensory bristles and a slight reduction in the total surface area of the eye. As a result of these events, the exterior eye morphology of the *rap* mutant is extremely rough (Fig. 1B).

The underlying basis for the change in ommatidial size and shape was revealed when mutant eyes were sectioned and examined by transmission electron microscopy. Whereas tangential sections of wild-type compound eyes show each ommatidium to contain the stereotypic number and position of photoreceptor cells (Fig. 1C), the number of R cells per ommatidia in *rap* mutants varies considerably (Fig. 1D). Furthermore, the R cell position and rhabdomere arrangement in *rap* mutants is altered. This aspect of the *rap* phenotype makes it difficult to assign R cell identities to the photoreceptor cells without the aid of cell-specific markers.

The four alleles of *rap* vary in the severity of their rough-eye phenotype and thus, may represent various levels of gene function. The temperature-sensitive allele (*rapR22ts*) shows a milder phenotype than *rap1* or *rap2*. These latter two alleles are in turn, less severe than *rap3* (i.e., *rap3* > *rap2*, *rap1* > *rapR22ts*). By comparing the effects of gene dosage on the severity of the rough-eye phenotype in various alleles, we believe *rap3* may represent a null condition. Homozygous *rap3* flies are phenotypically indistinguishable from hemizygous *rap3* males and *rap3* females that carry the mutation over a deficiency chromosome. In contrast, *rap1* homozygotes (and heteroallelic *rap1/rap2* flies) exhibit a moderately rough-eye phenotype which is easily distinguished from the severe rough-eye of the hemizygous males and *rap1/Df* females. These genetic results suggest that both *rap1* and *rap2* are hypomorphs and have retained some portion of their function.

Mutation in the *rap* gene affects early cell recruitment events

The various stages of R cell differentiation and cluster formation can be visualized in a single disc by staining with monoclonal antibodies (mAb) that recognize specific antigens in the eye disc (Zipursky et al. 1984; Venkatesh et al. 1985). We used neuron-specific monoclonal antibody mAb22C10 to investigate pattern formation in eye-antennal discs taken from third-instar *rap* mutant larvae. The staining patterns observed with mAb22C10 show that the process by which cells are sequestered into clusters behind the furrow is abnormal. Typically in wild-type discs, clusters differentiate in a spatially symmetrical fashion along both the posterior-anterior and lateral axes. Five-cell preclusters are observed a few rows behind the furrow and more mature clusters, containing seven and eight cells, are tightly packed together in more posterior regions of the disc (Fig. 4C). As shown in Figure 4D, many of the normal attributes of disc patterning are lost in the *rap* mutant. We have observed that the arrangement of photoreceptor preclusters in *rap* discs is extremely disorganized. Clusters are no longer distributed in an ordered array, and the progressive increase in cluster size that is observed in wild-type tissues is absent in the *rap* mutant. In addition, the level at which photoreceptor neurons differentiate appears to have been altered. Typically, the cell bodies of the developing neurons observed in wild-type tissue are apically positioned within the developing disc (Fig. 4A,C). This is not the case in the *rap* mutant. Using camera lucida reconstructions of the mAb22C10 stained disc, we observed that the R cells of *rap* eye imaginal discs differentiate at various levels within the tissue (Fig. 4B,D).

Examination of regions close to the furrow suggests

Figure 4. Developing R-cell clusters stained with mAb22C10. (A) Wild-type disc showing an ordered array of R-cell clusters with mature eight-cell clusters at the posterior region of the disc. (B) *rap* mutant disc showing single cells near the furrow and variably sized clusters distributed in the region posterior to the furrow. (C and D) Camera lucida reconstructions of mAb22C10 staining cells from A and B. Squared-off regions represent the actual areas within the disc from which cells were traced. The different colors represent the various levels dorso-ventrally within the disc epithelium. (Red) basal; (blue) apical; (black) intermediate. Note in *rap* discs, R cells differentiate at different levels. Bar, 10 μ m.

that the pattern abnormalities observed in *rap* mutants occur early during ommatidial assembly and may result from a disruption in the events involved in the differentiation of R8. Single cells, can be observed behind the morphogenetic furrow, yet their distribution and orientation is unlike that found in the wild-type disc. In wild-type tissue, the staining pattern of cells directly behind the furrow is concentrated at single points, thus making it difficult to observe the neuronal soma (Fig. 4A). In contrast, mAb22C10 staining of *rap* eye imaginal discs clearly outlines the cell body of single cells, suggesting that the orientation of these cells or the distribution of antigen recognized by mAb22C10 is altered (Fig. 4B).

The disruption in early patterning events is propagated to other regions within the disc. Just posterior to

the furrow, two- and three-cell clusters are frequently found adjacent to groups containing larger numbers of differentiated R cells. Conversely, in regions where one typically finds eight-cell clusters in the wild-type disc, small clusters containing fewer cells are visible. These groups are often intermixed with clusters containing more than eight photoreceptor cells, suggesting that *rap* mutants lack the ability to restrict cell number to the normal wild-type complement.

rap gene function is required during the third-instar stage of larval development

Previous studies have shown that ommatidial pattern formation takes place during the third-instar stage of larval development (Ready et al. 1976). If *rap* function were specific to eye development, we would expect its function to be required during that period. To test this, the *rapR22ts* allele was used in temperature-shift experiments to identify the time during development when *rap* gene function was necessary for normal eye morphology. As mentioned previously, the eye phenotype of *rapR22ts* allele is temperature sensitive. When grown at permissive temperature (17°C), *rapR22ts* flies show normal external eye morphology (Fig. 2A). In contrast, when *rapR22ts* flies are grown at 29°C, their eyes show a rough phenotype (Fig. 2B). Two kinds of temperature shift experiments were performed with *rapR22ts* flies. In temperature upshift experiments, *rapR22ts* flies were grown at 17°C and shifted to 29°C at the specific times indicated (Fig. 5). In the complementary downshift experiments, *rapR22ts* flies were grown at 29°C and shifted to 17°C (Fig. 5). The results show that when *rapR22ts* flies are shifted to the higher temperature at any time prior to pupation, normal eye development is forfeited and the adult is rough-eyed. In contrast, *rapR22ts* flies shifted from 17°C to 29°C after larvae have entered pupation exhibit a normal (wild-type) exterior. In the downshift experiment (from 29°C to 17°C), it was observed that flies transferred to 17°C prior to the third-instar larval stage exhibited a wild-type exterior morphology typical of *rapR22ts* revertants maintained at low temperature over the full course of their development. This was not the case when flies were shifted to the permissive temperature during later stages. Flies downshifted during pupation always showed a rough-eye phenotype over the entire surface of the eye. These two experiments show that the third-instar stage of larval development is the period in which *rap* function is necessary for normal eye morphology. When third-instar larvae of *rapR22ts* flies were downshifted, we commonly found a disruption in eye pattern that was confined to the posterior region of the eye while a wild-type morphology was exhibited more anteriorly (Fig. 6A,B). This distribution of roughness is consistent with the observation that pattern formation progresses anteriorly across the eye imaginal disc and emphasizes the point that normal *rap* function in one quadrant of the eye fails to rescue the mutant phenotype in regions where *rap* function is absent.

Figure 5. Temperature-shift experiments with *rapR22ts*, showing that *rap* gene function is specifically required only during third larval instar for normal ommatidial patterning. (Upshift) 17°C to 29°C; (downshift) 29°C to 17°C. Eggs were collected for 12 hr and then shifted at appropriate times. Points on the curve represent stages in development when shift occurred. Time during development when *rap* gene function is required for normal ommatidial development is shown (shaded area).

rap gene function is required in photoreceptor cell R8 for proper ommatidial patterning

The immunocytological studies described above suggested that *rap* function is involved in the early patterning events of the eye imaginal disc. To determine whether *rap* expression is required in all cells or is instead necessary in a discrete subclass of photoreceptors, mosaic flies were constructed using the *rap1* allele.

Mosaic eyes that contain patches of genotypically mutant cells bordered by wild-type tissue frequently contain ommatidia of mixed genotype. Mutant and wild-type cells can be distinguished by placing a recessive, closely linked, cell autonomous marker on the *rap*⁻ chromosome. In our studies, *white* (*w*), the gene that is required for the formation of pigment granules, was used to identify cells carrying the *rap* mutation. Using the scheme described in Figure 7a (see below), *rap1*, *w* mosaics were generated at a frequency of 1 per 15 flies. Mosaic eyes were embedded in plastic and serially sectioned (4 μm) for light microscopy. Our analysis then focused on scoring phenotypically normal ommatidia that contained both *rap*⁺ and *rap*⁻ cells (Fig. 7B). Phenotypically abnormal ommatidia were excluded from this study because of the difficulty in determining R cell identity in such clusters.

Fifty-four phenotypically normal ommatidia containing both *rap*⁺ and *rap*⁻ R cells were scored from 8 mosaic eyes (Fig. 7C). The data, summarized in Table 1, show that no correlation exists between the wild-type ommatidia pattern and the presence of the *rap*⁻ allele in any of the photoreceptor cells R1–R7. In contrast, in all of the 54 ommatidia examined, R8 was found to be *rap*⁺. The likelihood that a distribution thus skewed would arise by chance is less than 0.1% ($P < 0.001$).

Thus these results indicate that *rap* expression is required only in R8 for normal ommatidial pattern formation.

In addition to demonstrating the R8-specific requirement for *rap* expression, mosaic studies have led to a second interesting observation. As expected, our mosaic flies contained four major classes of ommatidia: (1) phenotypically wild-type ommatidia containing only wild-type (*rap*⁺) R cells; (2) wild-type ommatidia containing a mixture of *rap*⁺ and *rap*⁻ R cells; (3) mutant ommatidia containing *rap*⁺ and *rap*⁻ R cell types; and (4) mutant ommatidia containing only mutant (*rap*⁻) cell types. Unexpectedly, a fifth class of ommatidia was observed. In five of the eight mosaic eyes examined, we found cases where phenotypically mutant ommatidia contained only wild-type (*rap*⁺) R cells (Fig. 7B). The number of *rap*⁺ R cells in these clusters varied greatly with examples of three-, four-, six-, and eight-cell clusters being observed. Furthermore, although all of these clusters were localized to regions that were in close proximity to the border separating mutant and wild-type tissue, their position was not limited to either side.

Figure 6. (A) Scanning electron micrograph of a mosaic compound eye (X155) induced by shifting *rapR22ts* flies from non-permissive temperature to permissive temperature (29°C to 17°C) during third instar. (B) A high magnification (X760) view of the mosaic eye showing mutant–wild-type border region. The eye is oriented with the posterior on the left.

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Table 1. Summary of results from mosaic analysis

R cell	<i>rap</i> ⁻	Frequency (%)
R1	18	33
R2	24	44
R3	26	48
R4	25	46
R5	26	48
R6	19	35
R7	16	30
R8	0	0

The first column gives the name of the particular R cell studied; the second column gives the number of instances that the R cells were found to be *rap*⁻ in phenotypically normal ommatidia; and the third column provides the frequency that the number of instances represents as a percentage of the total number of R cells studied.

Discussion

In this paper we have described the genetic and phenotypic characterization of *rap*, a locus whose functions are required for normal *Drosophila* eye development. Mutations in the *rap* locus cause a disruption in the early patterning events of the eye imaginal disc. The aberrant pattern observed behind the morphogenetic furrow invariably leads to ommatidia containing abnormal numbers of R cells and a rough-eye phenotype in the adult. In addition, the timing and cell-specific requirements for *rap* function have been addressed. *rap* gene function has been shown to be required during the third-instar stage of larval development. Moreover, a correlation has been observed between normal ommatidial development and a *rap*⁺ genotype in photoreceptor R8. These latter findings support our premise that *rap* gene function is specific to eye development and is necessary in photoreceptor R8 for normal pattern development.

rap is essential for ommatidial assembly

Our data indicate that the *rap* gene product plays an essential role in eye pattern formation. The timing of *rap* function overlaps the interval in which ommatidial assembly is initiated in the eye imaginal disc and thus presents a correlation between *rap* function and the emergence of ommatidial pattern. With temperature-downshift experiments we have generated *rapR33ts* flies with half-mutant/half-wild-type eyes. In these eyes the rough phenotype is confined to the posterior regions and the border that separates mutant and wild-type tissue is aligned in a dorsoventral manner. These features argue that *rap* function is closely associated with the patterning events taking place behind the morphogenetic furrow and thus make it unlikely that the *rap* gene product is a diffusible substance that is distributed throughout the disc prior to morphogenesis.

Genetic mosaic studies further support the notion that the *rap* gene plays an important role in pattern formation. Results presented in this paper demonstrate

that *rap*⁺ function is required only in photoreceptor cell R8 for normal ommatidial pattern formation. Although *rap*⁺ function is not required in cells R1–R7, our studies do not rule out the possibility that a low level of functional gene product is made in these cells. This concern can be addressed by using an allele for which the lack of functional gene product has been established. Furthermore, although our analysis does not take into consideration the role of accessory cells, we feel such contributions are unlikely. The pigment and mechanosensory cells that make up the majority of the accessory cells in the compound eye do not differentiate until pupation. Because *rap*⁺ function has been shown to be required during the third-instar stage of larval development, contributions made by accessory cells seem improbable.

During our mosaic analysis we frequently observed mutant ommatidia (i.e., abnormal number and position of R cells) that contained strictly wild-type (*rap*⁺) cells. In these abnormal clusters the specific R cell identities could not be ascertained because of the altered position and number of cells. However, in light of the findings from our mosaic analysis, which show that a *rap*⁺ genotype in R8 leads to normal ommatidial pattern, we would argue that these abnormal clusters do not contain a *rap*⁺ R8.

In addition to the mosaic and developmental data, immunocytological studies of *rap* eye discs support a role for *rap* gene function in eye pattern formation. Antibody staining of discs from third-instar larvae show that a disruption of the *rap* gene function leads to abnormal ommatidial distribution and photoreceptor cell recruitment. These events suggest the *rap* gene function has a central role in pattern formation and provide clues as to its time of action during ommatidial assembly. Specifically, detailed analysis of eye imaginal discs stained with mAb22C10 indicate that pattern irregularities are present in the early stages of ommatidial assembly (i.e., the positioning and spacing of cells just posterior to the morphogenetic furrow).

Role of *rap* function in ommatidial assembly

Our current understanding of pattern formation is based on the model that R cells differentiate in a sequential manner. Specific cell–cell contacts and positions are postulated to play a critical role in these events. An important attribute of this process is the determination of precise positioning of the newly differentiated R8 cells. Under normal conditions, R8 cells localized directly behind the furrow are symmetrically arranged with respect to adjacent R8 cells and larger, more posterior, clusters. Three well-characterized mutants that disrupt retinal pattern formation do so by interrupting recruitment at various stages in ommatidial assembly. For instance *rough* (*ro*) mutants appear to form normal three-cell clusters but fail to complete subsequent recruitment events (Tomlinson et al. 1988). Similarly, *boss* and *sevenless* (*sev*) mutants nearly succeed in forming mature eight-cell clusters yet fall short in their recruitment of photoreceptor cell R7 (Harris et al. 1977; Reinke and Zi-

pursky 1988]. Although these three genes disrupt cell recruitment, the overall pattern of ommatidial spacing in the eye imaginal disc is normal. This is not the case in *rap*⁻ flies. *rap*⁻ mutants exhibit an abnormal distribution of cells directly behind the morphogenetic furrow. This change in the overall spacing of cells behind the furrow may result in the disruption of the subsequent recruitment events that follow. Thus, *rap* gene function appears to be required at a stage prior to that of *ro*, *sev*, and *boss* (Fig. 8).

In view of these observations, we propose two possible models for the role of *rap* function in the developing eye imaginal disc. In the first model, *rap* gene function is important for the proper differentiation of photoreceptor cell R8. *rap* may be responsible for receiving or interpreting developmental cues that direct and determine the position of R8. Mutations in a gene product with these attributes would lead to the improper spacing/positioning of R8 cells behind the morphogenetic furrow and subsequent recruitment abnormalities. In the second model, the *rap* gene product serves as a signal for cellular interactions that lead to the recruitment and proper patterning of the cells R1–R7. Such signals might also include inhibitory signals that prevent cells within a particular radius from differentiating into an R8. As is the case in the first model, mutations in such functions would lead to both abnormal distributions of R8 cells behind the morphogenetic furrow and to aberrant cluster sizes in the adult eye.

Materials and methods

Stocks

The P strain 4A/(2)A/*y,f* from which *rap* mutants were derived was obtained from the Benzer laboratory at Caltech. Deficiency strains Df(1)Hf366 and Df(1)A113 were obtained from Dr. John Postlethwait (University of Oregon). *peb* and *rg* files used in complementation studies were obtained from the Indiana stock center as were deficiency strains Df(1)RC40 and Df(1)JC70. Deficiencies Df(1)biD2, Df(1)rb13 and Df(1)rb1 were donated by the Mahowald lab (Case Western Reserve).

Mutagenesis

A dysgenic cross was performed between 4A/(2)A/*y* (P) males and attached-X *y,f* (M) females. F₁ progeny were mated inter se and F₂ males were screened under a dissecting microscope for gross abnormalities in eye morphology.

Microscopy

Adult eyes were fixed according to the methods of Tomlinson and Ready (1987a). Specimens were thin sectioned (60–70 nm) on a Reichert Ultracut-E, and stained with saturated uranyl acetate for 2 hr at 45°C. Samples were photographed on a Phillips CM-12 electron microscope.

Antibody staining of eye antennal discs

Antibody stainings of eye antennal discs followed the proce-

Figure 8. A schematic diagram showing the sequence of R-cell differentiation and the steps at which various genes are required.

dures of Zipursky et al. (1984). Briefly, eye antennal discs were dissected from third-instar larvae and fixed for 15 min in 2% formalin in phosphate-buffered saline (PBS). Then discs were washed in PBS for 1 hr and permeabilized with 0.5% Nonidet P-40 (NP-40) in PBS for 30 min. A 3-hr incubation with mAb22C10 diluted 1 : 1 with 0.5% NP-40 followed. Subsequent reagents and procedures used to counterstain tissues (e.g., biotinylated rabbit anti-mouse antibody, avidin-HRP) were obtained from Vector Laboratories.

Mapping and complementation

To determine the approximate position of *rap* on the X chromosome, *rap* males were mated with females carrying the markers *y* (*y*, 0.0), *v* (*v*, 33.0), and *f* (*f*, 56.7). F₁ progeny were mated inter se and the eye phenotype in F₂ recombinants were scored. To more rigorously define the position of the *rap* locus, *rap* males were mated with deficiency/balancer strains. Non-bar-eyed female progeny in the F₁ generation were examined under a dissecting microscope for the mutant phenotype. To test whether *rap* males were allelic with *peb* (*peb*, 7.3) or *rg* (*rg*, 11.0), *rap* males were crossed with *peb*, *v*, or *rg* females. Heterozygous F₁ females (*rap/peb* or *rap/rg*) then were examined for exterior and pseudopupil abnormalities.

P-element remobilization

To determine the frequency at which the mutant reverted to the wild type, a dysgenic cross was performed with one of the alleles of *rap*. *rap1* (P) males and attached-X *y,f* (M) females were crossed to remobilize P elements in the *rap1* line. F₁ progeny were mated inter se and F₂ males were screened for the rough-eye phenotype.

Mosaics

w, *rap1* males generated by recombination were mated with Canton S females. First- and second-instar heterozygous female progeny were gamma-irradiated (1000 rad) on a Gammacell 1000. Upon eclosion, female progeny were screened for mosaic patches under a dissecting microscope. To prepare tissue for sectioning, mosaics were placed under bright light for 2 hr to enhance the migration of pigment granules to the apical tips of R cells. Then tissue was fixed in ice-cold 2% glutaraldehyde for 1 hr followed by a 10-min wash in iced PBS. Samples then were dehydrated (successive 5-min incubations in ice-cold 50, 70, 85, 95, and 100% EtOH) and treated with iced propylene oxide for 5 min. Following a 15-min incubation in a propylene oxide–Epon solution (1 : 1), samples were left overnight at 4°C in 100% Epon aroldite. Subsequently, samples were desiccated for 30 min at room temperature and embedded in Epon at 60°C for 17

hr. Four- to five-micrometer sections were then prepared for light microscopy.

Isolation of temperature-sensitive allele of rap and temperature-shift experiments

Revertant lines derived from the remobilization of P elements in *rap1* were reared at 17°C and 29°C to determine whether any of these lines represented a temperature-sensitive allele of *rap*. One revertant (*rapR22ts*) that exhibited a temperature-dependent eye morphology was used in temperature-shift experiments. In upshift studies, *rapR22ts* males were mated to attached-X y,f (P) females for 12 hr at 17°C. Then parents were removed and progeny were transferred to 29°C during successive stages of development (egg, 24 hr; first instar, 72 hr; second instar, 162 hr; third instar, 216 hr; pupae, 272 hr). F₁ progeny remained at 29°C until eclosion, whereupon male progeny were examined for the rough-eye phenotype. In downshift experiments, parents were mated again for 12 hr and progeny were transferred from 29°C to 17°C incubators at 0 hr (egg), 24 hr (first instar), 48 hr (second instar), 72 hr (third instar), or 144 hr (pupae) after the removal of parents. After eclosion, male progeny were examined under a dissecting microscope for abnormal eye morphology.

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