Analysis of cis-acting requirements of the Rh3 and Rh4 genes reveals a bipartite organization to rhodopsin promoters in Drosophila melanogaster

Mark E. Fortini and Gerald M. Rubin

Howard Hughes Medical Institute, Department of Molecular and Cell Biology, University of California, Berkeley, California 94720 USA

The rhodopsin genes of Drosophila melanogaster are expressed in nonoverlapping subsets of photoreceptor cells within the insect visual system. Two of these genes, Rh3 and Rh4, are known to display complementary expression patterns in the UV-sensitive R7 photoreceptor cell population of the compound eye. In addition, we find that Rh3 is expressed in a small group of paired R7 and R8 photoreceptor cells at the dorsal eye margin that are apparently specialized for the detection of polarized light. In this paper we present a detailed characterization of the cis-acting requirements of both Rh3 and Rh4. Promoter deletion series demonstrate that small regulatory regions (<300 bp) of both R7 opsin genes contain DNA sequences sufficient to generate their respective expression patterns. Individual cis-acting elements were further identified by oligonucleotide-directed mutagenesis guided by interspecific sequence comparisons. Our results suggest that the Drosophila rhodopsin genes share a simple bipartite promoter structure, whereby the proximal region constitutes a functionally equivalent promoter “core” and the distal region determines cell-type specificity. The expression patterns of several hybrid rhodopsin promoters, in which all or part of the putative core regions have been replaced with the analogous regions of different rhodopsin promoters, provide additional evidence in support of this model.

[Key Words: Drosophila; compound eye; rhodopsin promoters; cell-type specific gene expression; oligonucleotide-directed mutagenesis; polarized light detection]

Received November 9, 1989; revised version accepted December 29, 1989.

Studies of mammalian transcription factors and their target promoter sequences suggest that eukaryotic gene regulation is achieved via combinatorial control mechanisms, whereby a modest number of regulatory factors interact with a much larger number of downstream genes (for review, see Dynan 1989; Mitchell and Tjian 1989). Exactly how particular combinations of transcription factors are used to generate strikingly different temporal and spatial patterns of gene expression, however, is still poorly understood. We have performed a systematic analysis of the rhodopsin gene promoters of Drosophila melanogaster in the hope of elucidating an underlying logic to combinatorial control in transcription. In contrast to other well-characterized Drosophila genes, which are generally regulated at the level of whole tissues or organs (discussed in Fischer and Maniatis 1988), the four rhodopsin genes isolated to date are expressed with exquisite specificity in different photoreceptor cell subpopulations of the Drosophila larval and adult visual systems. Our investigations have concentrated primarily on patterns of rhodopsin gene expression within the adult visual system, which consists of the compound eyes and ocelli. The ocelli are three small light-sensing organs located on the vertex of the adult head.

The photoreceptor cells of the Drosophila adult comprise a small set of well-defined cell types (Fig. 1). The compound eye, itself, consists of a repetitive hexagonal array of ~700 unit eyes or ommatidia, each containing a regular trapezoidal array of eight photoreceptor cells (Ready et al. 1976). Spectral, morphological, and genetic criteria divide the eight ommatidial photoreceptors into three distinct cell types: cells R1–R6, cell R7, and cell R8 (Harris et al. 1976; Heisenberg and Wolf 1984). These three cell types differ from each other with respect to the positions of their rhabdomeres within the ommatidium, their axonal projection patterns to the optic lobes, their spectral sensitivities, and their specific absence or degeneration in certain mutants. By the same criteria, the ~90 photoreceptor cells associated with each ocellus comprise a fourth class of adult photoreceptor cell type (Schmidt 1975; Hu et al. 1978; Stark et al. 1989).

The different properties of the adult photoreceptor cell types extend to their pattern of rhodopsin gene expression. Rh1 is expressed only in the outer photoreceptors R1–R6, Rh2 is expressed only in the ocellar photoreceptors, and Rh3 and Rh4 are expressed in nonoverlapping subsets of the R7 photoreceptor cell population (Mismer and Rubin 1987; Montell et al. 1987; Mismer et al. 1988; Pollock and Benzer 1988). Three of these rho-
dopsins, namely Rh1, Rh3, and Rh4, are also expressed in the larval photoreceptor organs [Mismer and Rubin 1987; Pollock and Benzer 1988]. Extensive study of the analogous visual systems of Musca domestica and Calliphora erythrocephala [for review, see Hardie 1985, 1986] suggests that the four Drosophila rhodopsin genes isolated so far can account for all visual pigment deposition in three of the four Drosophila adult photoreceptor cell types, namely the R1–R6, R7, and ocellar photoreceptors. Spectral properties of the R8 cells in these larger dипterans indicate that two rhodopsin genes expressed in nonoverlapping R8 subpopulations of the Drosophila retina have yet to be identified.

Previous analysis of the regulatory regions of the Rh1 and Rh2 genes has shown that their expression in particular photoreceptor cell types is transcriptionally mediated and that small promoter regions contain all of the required cis-acting elements for their respective patterns [Mismer and Rubin 1987; Mismer et al. 1988]. This report concerns the cis-acting requirements of the two R7-specific rhodopsin genes Rh3 and Rh4. We show that the expression of these two genes in nonoverlapping photoreceptor R7 subpopulations is transcriptionally mediated by promoter regions <300 bp in length. Promoter fusions of these two genes to the bacterial reporter gene lacZ were used to clarify previously obscure details of their expression patterns, allowing each of the Drosophila R7 opsins to be assigned as the functional homolog of one of the two R7 opsins of Musca and Calliphora.

The promoter regions of all four rhodopsin gene homologs of Drosophila virilis were isolated and sequenced to identify potentially conserved cis-acting elements. In addition, we have found that the D. virilis Rh3 and Rh4 promoters direct R7-specific gene expression when introduced into the D. melanogaster genome. Oligonucleotide-directed mutagenesis of the R7 opsin promoters, guided by cross-species sequence homologies, has identified several functional cis-acting elements for these two promoters. The results of these experiments lead us to propose that the proximal region of each rhodopsin promoter constitutes an interchangeable promoter "core," with cell-type specificity determined by upstream sequences unique to each promoter. Additional support for this model is furnished by the expression patterns of several hybrid promoter constructs that combine the core and the cell-type specificity regions of two different rhodopsin promoters.

**Results**

Reconstitution of Drosophila R7-specific opsin expression patterns using Rh3–lacZ and Rh4–lacZ fusions

RNA blot analysis has previously shown that in the adult, Rh4 is expressed exclusively in R7 photoreceptor cells and Rh3 is expressed predominantly in R7 cells [Fryxell and Meyerowitz 1987; Montell et al. 1987; Zuker et al. 1987]. Rh3 transcripts were also detected at lower abundance in sevenless (sev) heads, which lack all R7 photoreceptors, and at trace levels in adult body tissues. The non-R7 cell expression of Rh3 was not localized further. In situ hybridization of gene-specific Rh3 and Rh4 probes to serial tissue sections of the adult retina demonstrated that these two genes are expressed in nonoverlapping R7 cell subpopulations [Montell et al. 1987]. As a first step toward a systematic analysis of the regulatory regions of Rh3 and Rh4, putative promoter sequences were fused to the bacterial reporter gene lacZ (encoding -β-galactosidase) and introduced into D. melanogaster by P-element-mediated germ line transformation. These constructs were made with two goals in mind: (1) to refine our understanding of the wild-type Rh3 and Rh4 expression patterns, especially by localizing the Rh3 expression detected in sev heads, and (2) to define large promoter fragments that confer every feature of the endogenous Rh3 and Rh4 expression patterns on a bacterial reporter gene.

Two independent transformants of an Rh3–lacZ fusion containing a -2.6-kb to +18-bp promoter fragment were obtained, where +1 denotes the start site of transcription. These transformants, termed P[Rh3.2600lacZ]1 and P[Rh3.2600lacZ]2, were analyzed by histochemical staining with the chromogenic substrate X-gal [see Materials and methods]. Within the retina, both
[\textit{P}[\text{Rh}3.2600\text{lacZ}]] lines exhibit staining of individual R7 photoreceptors, as judged by their characteristic morphology and localization to the distal half of the retina (Fig. 2A). Throughout most of the retinal field, \~30\% of the R7 cells are stained in an apparently stochastic distribution pattern. The presence of unstained R7 cells in the \textit{P}[\text{Rh}3.2600\text{lacZ}] retinas was confirmed by double staining of semithin tissue sections with X-gal and Hoechst dye (Fig. 2B). The Hoechst dye labels all nuclei and reveals that only a subset of nuclei in the R7 nuclear layer, which is located in the apical portion of the retina just beneath the R1–R6 nuclear layer, corresponds to R7 cell bodies expressing the \textit{Rh}3–\text{lacZ} fusion. With long staining times, it is possible to follow the thread-like axonal projections of these cells as they emerge from the R7 cell bodies, traverse the proximal retina and the lamina, cross over each other at the optic chiasma, and enter the medulla to make their synaptic connections with second-order neurons (similar to Fig. 2J). The precisely aligned synaptic terminals of these axons at a single layer of the medulla are especially evident, as is their characteristic swelling prior to entering this optic ganglion (Fig. 2A,F). Within the medulla neuropil, the stained axonal terminals of \textit{Rh}3-expressing R7 cells form a broken line punctuated by gaps that presumably reflect the presence of unstained synaptic terminals of \textit{Rh}4-expressing R7 photoreceptor neurons. The axonal staining in the medulla observed for \textit{P}[\text{Rh}3.2600\text{lacZ}] is in marked contrast to transformants bearing \textit{Rh}1–\text{lacZ} fusions, which display staining only in the lamina, where R1–R6 photoreceptor axons terminate (Misser and Rubin 1987).

Although most of the retina of these \textit{Rh}3–\text{lacZ} transformants exhibits staining of an apparently random sub-

**Figure 2.** Histochemical analysis of \textit{Rh}3–\text{lacZ} and \textit{Rh}4–\text{lacZ} transformant lines. Cryostat sections (10–14 \text{"m}) of head tissues were prepared and stained as described in Materials and methods. (A] Horizontal section through the compound eye and optic lobes of the transformant line \textit{P}[\text{Rh}3.2600\text{lacZ}], showing staining of \~30\% of the R7 cell bodies in the distal retina [re] and their synaptic terminals in the medulla [me]. (la) Lamina; (oc) optic chiasma. (B] Higher magnification view of a \textit{P}[\text{Rh}3.2600\text{lacZ}] retina counterstained with Hoechst, demonstrating R7 nuclei corresponding to unstained R7 cells. Note the two unstained R7 cell nuclei indicated by the solid arrowheads flanking the nucleus of a stained R7 cell. The right-most portion shows the most apical region of the retina, which contains nuclei of R1–R6 cells, pigment cells, and cone cells. The R7 nuclei are located just beneath this dense nuclear layer, and the R8 nuclei are located in the basal region of the retina [left-most portion]. (C) Sagittal section through a \textit{P}[\text{Rh}3.2600\text{lacZ}]/2 retina demonstrating \textit{Rh}3–\text{lacZ} expression in paired R7 and R8 photoreceptors at the dorsal margin (between solid arrows; dorsal at top, anterior to the left). (D) Frontal section through the dorsal eye margin of \textit{P}[\text{Rh}3.2600\text{lacZ}] [dorsal at top], showing stained dorsal marginal photoreceptors [between solid arrows]. (E) Frontal section through the dorsal eye margin of \textit{P}[\text{Rh}3.2600\text{lacZ}] at a deeper level than in D, counterstained with Hoechst (orientation as in D). Because of the curvature of the eye, the line of stained dorsal marginal R8 cells [between open arrows] is interrupted by a region of R8 nuclei corresponding to unstained, nonmarginal R8 cells. Synaptic terminals of dorsal marginal central photoreceptors display pronounced staining in the dorsal medulla in such sections [small solid arrow]. Stained nonmarginal R7 photoreceptors are present in the region between the large solid arrows. (F) Horizontal section through a \textit{P}[\text{Rh}3.2600\text{lacZ}] dorsal medulla showing dorsal marginal R7 and R8 synaptic terminals at the periphery of this optic lobe [circled terminals], as well as staining of a random subset [\~30\%] of nonperipheral R7 synaptic terminals. Additional staining on the right is attributable to central photoreceptor axons swelling prior to entering the medulla. [Inset] An expanded view of the posterior (bottom of F) set of circled dorsal marginal synaptic terminals. The two dorsal marginal R8 terminals [open arrows] make synaptic connections at a slightly more shallow level of the medulla than that of the dorsal marginal R7 terminals [solid arrow]. (G) Sagittal section through the dorsal marginal retina of \textit{seva}2; \textit{P}[\text{Rh}3.2600\text{lacZ}]/2, showing stained R8 cells [cf. C] same orientation]. (H) Frontal section through a \textit{P}[\text{Rh}3.2600\text{lacZ}] retina at same level and orientation as in D. Staining is only observed in dorsal marginal R8 cells. (I) Sagittal section through dorsal head tissues of \textit{seva}2; \textit{P}[\text{Rh}3.2600\text{lacZ}]. Axonal projections of stained dorsal marginal R8 cells in the retina [re] extend through the lamina [la], and terminate in the two dorsal-most rows of the medulla neuropil [me], as indicated by the solid arrowheads. The regularly spaced dots of staining at the lamina–medulla interface are additional dorsal marginal R8 axons passing through the plane of the section near the optic chiasma [dorsal at top, anterior to the right]. (J) Horizontal section through \textit{P}[\text{Rh}4.1900\text{lacZ}] head tissues, counterstained with Hoechst. With long staining times, the thin axonal fibers of individual R7 cells are visible as they traverse the proximal retina and the lamina on their way to the medulla. (K) Higher magnification view of a \textit{P}[\text{Rh}4.1900\text{lacZ}] retina counterstained with Hoechst, confirming the presence of unstained R7 cells and their nuclei [solid arrowheads; orientation as in B]. (L) Sagittal section through \textit{P}[\text{Rh}4.1900\text{lacZ}] head tissues. Note that although the majority of R7 cells and their synaptic terminals are stained, the dorsal marginal retina and its corresponding medulla neuropil are devoid of staining regions between solid arrowheads; cf. C, E, F, G, and I. (M) Tangential section through the dorsal retina of \textit{P}[\text{Rh}4.1900\text{lacZ}]5, showing that a stochastically distributed majority of the ommatidia contain stained R7 cells. Because of the curvature of the eye, the central area of this section passes through the proximal retina, which contains R8 instead of R7 cell bodies and hence shows little staining [dorsal at top, anterior to the right]. (N) Horizontal section through a \textit{P}[\text{Rh}4.1900\text{lacZ}]2 medulla, displaying an abundance [\~70\%] of stained synaptic terminals complementary to those observed for the typical \textit{P}[\text{Rh}3.2600\text{lacZ}] medulla shown in F. (O) Horizontal section through a \textit{P}[\text{Rh}3.2600\text{lacZ}]/1/2 \textit{P}[\text{Rh}4.1900\text{lacZ}]2 compound eye and optic lobe structures, displaying staining of all R7 cells and their synaptic terminals. \textit{P} scanning EM photograph of a wild-type \textit{D. melanogaster} compound eye [courtesy of B. Kimmel]. Specialized dorsal marginal R7 and R8 photoreceptors are present in a single row of ommatidia adjacent to and extending along the dorsal head cuticle, as indicated by open arrows.
Figure 2. [See facing page for legend.]
population of ∼30% of the R7 photoreceptors, the dorsal marginal region of the retina shows a strikingly nonrandom pattern of staining. A single row of ∼25 ommatidia along the extreme dorsal anterior eye margin, adjoining the head cuticle of the vertex and frons, exhibits staining of all R7 and R8 photoreceptor cell pairs (Fig. 2C,D,E,F). The presence of reporter gene activity in dorsal marginal R8 cells was confirmed by histochemical analysis of $P[Rh3.2600lacZ]$ in a sev$^{de}$ genetic background. To ensure that the sev$^{de}$ phenotype is fully penetrant in the dorsal marginal ommatidia, eyes of these individuals were examined by antidromic illumination after optical neutralization of the cornea (Franceschini and Kirschenfeld 1971a,b) prior to sectioning and staining. As expected, flies of genotype sev$^{de}$, $P[Rh3.2600lacZ]$ display staining only in the dorsal marginal R8 cells and in no other region of the retina (Fig. 2G–I). This finding, together with the number of Rh3-expressing R7 cells throughout the entire retina, indicates that these specialized R8 cells could account for the Rh3 transcription previously detected in sev heads. Examination of serial sections of $P[Rh3.2600lacZ]$ in both wild-type and sev$^{de}$ genetic backgrounds reveals that after passing through the optic chiasma, the axonal projections of these dorsal marginal cells terminate in the two most peripheral synaptic rows extending along the dorsal posterior neuropil of the medulla (Fig. 2E,F,I). These synaptic terminals generally exhibit more pronounced staining than those of nonmarginal Rh3-expressing R7 cells present in the same preparation (Fig. 2E). We were unable to localize the body expression of Rh3 detected previously (Montell et al. 1987) using $P[Rh3.2600lacZ]$ because of high levels of endogenous β-galactosidase activity in many body tissues.

An analogous histochemical study was performed for Rh4, utilizing a −1.9-kb to +7-bp promoter fragment fused to lacZ. Eleven independent transformant lines were analyzed in a wild-type genetic background. As with the Rh3–lacZ fusion, this Rh4–lacZ construct labels a subpopulation of R7 photoreceptor cell bodies, their axonal projections, and their synaptic terminals in the medulla (Fig. 2J,L–N). The $P[Rh4.1900lacZ]$ lines, however, stain ∼70% of the total photoreceptor R7 cell population in an apparently random distribution across the retina, as confirmed by X-gal/Hoechst double staining (Fig. 2K). Several of the Rh4–lacZ transformant lines were also analyzed in a sev$^{de}$ genetic background, and no staining was detected (data not shown).

A previous in situ hybridization study using Rh3 and Rh4 gene-specific probes has demonstrated that these two genes are expressed in complementary subsets of R7 cells within a small sampled region of ∼40 ommatidia (Montell et al. 1987). Our histological analysis of Rh3–lacZ and Rh4–lacZ expression over the entire retinal field is consistent with this pattern. As expected, double transformants of genotype $P[Rh3.2600lacZ]1/P[Rh4.1900lacZ]2$ display staining of all R7 cell bodies and synaptic terminals (Fig. 2O). Furthermore, examination of serial sections of $P[Rh4.1900lacZ]$ reveals that although the majority of R7 cells across the surface of the retina are stained, the Rh3-specific dorsal marginal region of the eye and the corresponding region of the dorsal medulla neuropil are devoid of staining (Fig. 2L).

**Determination of minimal R7 opsin regulatory regions by promoter deletion analysis**

The Rh3–lacZ and Rh4–lacZ analysis described above has defined large (>2000 bp) promoter fragments able to confer apparently wild-type opsin gene expression patterns on a bacterial reporter gene. Extensive 5′-deletion analysis of each of these fragments was next undertaken to identify the smallest functional promoter region for both Rh3 and Rh4. The bacterial reporter gene chloramphenicol acetyltransferase (CAT) was chosen instead of lacZ for the deletion analysis to take advantage of the higher transformation efficiencies and sensitive quantitative assays available for this reporter gene. Although good histochemical assays are not available for the CAT enzyme, localization of promoter activity to R7 cells is easily monitored in transformant lines by simply measuring CAT activity in wild-type heads versus sev heads (for autosomal inserts) or bride of sevenless (boss) heads (for X-linked inserts).

Eight independent transformant lines of a 2.6-kb Rh3 promoter–CAT fusion yield a CAT activity profile in wild-type and sev$^{de}$ or boss$^{sev}$ adult heads consistent with the Rh3–lacZ staining pattern as well as the endogenous Rh3 expression pattern ($P[Rh3.2600CAT]$–1–8; Fig. 3A). Approximately 90% of the CAT activity in these lines is localized to the R7 photoreceptor cells, the remaining 10% presumably reflects Rh3 expression in dorsal marginal R8 cells. Some lines also show low but detectable CAT activity in body tissues, again consistent with the native Rh3 transcript distribution. CAT activity was observed in dissected thoracic and abdominal tissues of these lines, indicating that Rh3 body expression is not highly localized to a specific tissue or group of cells (data not shown). One line, $P[Rh3.2600CAT]4$, exhibits unusually high CAT activity in both the body and the head, although the proper cell-type specificity is preserved within the photoreceptors. The behavior of this line typifies the occasional position effect associated with P-element-mediated germ line transformation (Spradling and Rubin 1983; Hazelrigg et al. 1984; Levis et al. 1985).

Deletions of this full-length Rh3 promoter fragment to −945, −583, −343, and −247 bp upstream of the transcriptional start site do not significantly affect any aspect of promoter function in this assay (Fig. 3A). Further deletion to −137 bp leads to a slight (two- to three-fold) decrease in wild-type expression and the complete loss of dorsal marginal R8 expression in 12 of 14 lines examined (<0.1% of reference value in sev$^{de}$ or boss$^{sev}$ head samples; see $P[Rh3.137CAT]$1–14, Fig. 3A). Deletion of the Rh3 promoter to −99 bp completely abolishes expression in four of seven transformant lines. The remaining three lines of this construct exhibit low to moderate CAT activity levels localized to the R7 cells ($P[Rh3.99CAT]$2, 4, and 5; Fig. 3A). Further deletion of
Figure 3. Histograms summarizing the deletion analysis of the *D. melanogaster Rh3* promoter (A), the *D. melanogaster Rh4* promoter (B), and the *D. virilis Rh3* and *Rh4* promoters (C). CAT assays were performed as described in Materials and methods. Each position along the axis of the abscissa represents a single transformant line, with head CAT activity levels plotted above the axis and body CAT activity levels plotted below the axis. CAT activity levels measured in wild-type tissues are shown as shaded bars, solid bars show the amount present in *sev*<sup>a2</sup> tissues (for autosomal insertions) or *boss*<sup>3991</sup> tissues (for X-linked insertions). Each bar represents the average of at least two CAT assays on five heads or bodies of a given transformant line; error is estimated to be not greater than 15%. If no bar is present, CAT activity was <0.1% for that sample. All independent transformant lines of a single construct, followed by their average CAT activity profile (A), are enclosed within the underlying bracket. Each bracket is labeled with information corresponding to the name of the transformant lines, where *X* is the number of nucleotides upstream of the transcriptional start site present in each promoter fragment (5'-deletion end point) and *Y* is the numerical designation of each transformant line (in the same order as in the histograms). CAT activity levels of all transformant lines here and in Fig. 5 are normalized to the wild-type head value of the standard reference line P[Rh3.2600CAT]1 (100%; the first hatched bar of A).
the Rh3 promoter to −66 bp eliminates retinal expression \([P\{Rh3.66CAT\}_{1-5}; \text{Fig. 3A}]\).

As with Rh3, the large Rh4 promoter fragment extending from −1.9 kb to +7 bp drives CAT expression in a manner consistent with both the endogenous Rh4 transcriptional profile and the Rh4–lacZ staining pattern. Discounting minor position effects, fifteen independent transformant lines of \([P\{Rh4.1900CAT\}_{1-15}; \text{Fig. 3B}]\). The CAT activity levels detected among the different lines are fairly uniform and are quantitatively comparable to those measured in genetically wild-type Rh3–CAT transformant lines. Deletions of the Rh4 promoter to −472, −373, and −276 bp do not appreciably impair either the strength or cell-type specificity of this promoter \(\text{[Fig. 3B]}\). Further deletion to −159 bp has two effects, suggesting that Rh4 promoter function has now been perturbed slightly \([P\{Rh4.159CAT\}_{1-11}; \text{Fig. 3B}]\). First, the 11 independent transformant lines of this deletion construct display relatively high variation in wild-type head CAT activity. Second, CAT activity is detected in sev\(_{22}\) and boss\(_{999}\) heads of these transformants, demonstrating a partial disruption of the stringent R7 cell specificity obtained with larger Rh4 promoter constructs. Both of these effects may reflect the increased sensitivity of a crippled promoter to the regulatory properties of neighboring genomic sequences at its site of insertion. Further deletions of the Rh4 promoter to −123, −87, and −63 bp completely eliminate retinal expression of the Rh4–CAT fusion genes \(\text{[Fig. 3B]}\).

This deletion analysis shows that for both Rh3 and Rh4, proper cell-type-specific gene expression may be generated by sequences residing in a very small regulatory region. An Rh3 promoter fragment from −247 to +18 bp and an Rh4 fragment from −276 to +7 bp appear to contain all the cis-acting information needed to reproduce fully the wild-type R7 opsin gene expression patterns. Moreover, deletion of either of these small promoters by an additional 100 bp still fails to undermine seriously their basic specificity for the R7 photoreceptor cell type.

Isolation, sequencing, and functional characterization of D. virilis rhodopsin promoters

Having defined very small functional promoters for both Rh3 and Rh4, we sought to identify the discrete cis-acting elements responsible for the regulatory properties of these small promoter regions. Systematic mutagenesis of all nucleotides from −119 to −2 bp of the Rh1 promoter has proved to be an informative but laborious method for locating important cis-acting elements \(\text{[Mismer and Rubin 1989]}\). Instead, we have searched for putative cis-acting elements of the D. melanogaster rhodopsin genes by comparing the DNA sequences of their promoters to the corresponding promoter regions of the distantly related species D. virilis. This approach has proved quite effective in the analysis of regulatory domains of the Drosophila dopa decarboxylase gene \(\text{[Ddc; Scholnick et al. 1986]}\).

The Rh1–4 homologs of D. virilis were isolated from a D. virilis genomic library using the corresponding D. melanogaster probes, as described in Materials and methods. DNA sequence was obtained for ∼250 bp of the D. virilis Rh1 promoter, ∼260 bp of the D. virilis Rh2 promoter, ∼500 bp of the D. virilis Rh3 promoter, and ∼300 bp of the D. virilis Rh4 promoter. Complete DNA sequence was also obtained for a D. melanogaster Rh3 promoter fragment extending to −1039 bp and a D. melanogaster Rh4 promoter fragment extending to −472 bp. Pairwise homology searches among the eight rhodopsin gene promoters were performed in all possible combinations. As shown in Figure 4, most short stretches of high sequence similarity are clustered within the 150 bp immediately upstream of the transcriptional start site of each promoter.

To test whether the conserved DNA sequences reflect an actual conservation of promoter function, small putative promoter regions of the D. virilis Rh3 and Rh4 genes were fused to the CAT reporter module, introduced into D. melanogaster, and assayed for promoter function in wild-type and sev\(_{22}\) or boss\(_{999}\) genetic backgrounds. For the D. virilis Rh3 gene, promoter fragments of 500 and 180 bp confer a largely R7-specific expression pattern on the reporter gene \(\text{[Fig. 3C]}\). These 3 \([P\{Rh3v.500CAT\}_{1-11}]\) and 1 \([P\{Rh3v.180CAT\}_{1-11}]\) transformant lines display considerable quantitative variation in their wild-type levels of expression, which generally range from 5% to 20% of the wild-type levels measured for D. melanogaster nondeleted Rh3–CAT constructs. Expression of the D. virilis constructs is reduced approximately 10-fold in sev\(_{22}\) or boss\(_{999}\) heads, reminiscent of the 10% contribution of the dorsal marginal R8 cells to the overall level of D. melanogaster Rh3 retinal expression. The possibility that the small D. virilis Rh3 promoters are active in the specialized dorsal marginal R7 and R8 photoreceptors has not been examined further. D. virilis Rh4 promoter fragments of 300 and 190 bp confer a completely R7-specific expression pattern on the CAT reporter module at levels comparable to those of D. melanogaster Rh4–CAT constructs \(\text{[Fig. 3C]}\). The analysis of these D. virilis Rh3–CAT and Rh4–CAT transformant lines demonstrates that small D. virilis promoters interact functionally with the D. melanogaster transcriptional machinery to generate R7-specific patterns of gene expression.

Oligonucleotide-directed mutagenesis of the R7-specific opsin gene promoters

The finding that the Rh3 and Rh4 promoters of D. virilis are active in the D. melanogaster compound eye in an R7-specific manner argues that the evolutionarily conserved sequences of the R7 opsin promoters are strong candidates for cis-acting regulatory elements. This assertion is supported by the fact that exhaustive mutagenesis of the D. melanogaster Rh1 proximal promoter region \(\text{[Mismer and Rubin 1989]}\) has failed to discover any functional cis-acting elements outside of a small region consisting of two sequences conserved in the D. virilis Rh1 promoter and the four nucleotides separating...
Figure 4. Sequence homologies detected among the rhodopsin promoters of *D. melanogaster* and *D. virilis*. All possible pairwise homology searches were performed among the eight available rhodopsin promoters, as described in the text. Evolutionarily conserved sequences clustered in the more proximal promoter regions are presented here. Additional homologies detected in the distal enhancers of the *D. melanogaster* and *D. virilis* Rh1 promoters [M. Fuchs and G.M. Rubin, unpubl.] and in nonessential upstream regions of the Rh3 and Rh4 promoters of both species are not shown. Promoter sequences are aligned at the TATA box and the transcriptional start sites of the promoter-CAT fusions analyzed in Fig. 3 are indicated above the sequence (solid arrowheads). The sources of these promoter sequences are as follows: Rh1 mel [Misser and Rubin 1987], Rh1 vir [M. Fuchs, D. Schneider, and G.M. Rubin, unpubl.], Rh2 mel [Misser et al. 1988], Rh2 vir [this study], Rh3 mel [this study; Fryxell and Meyerowitz 1987; Zuker et al. 1987], Rh3 vir, Rh4 mel, and Rh4 vir [this study].
Fortini and Rubin

them [RUS1A and RCS I, Fig. 4]. For these reasons, cross-species sequence homologies were used as a guide in further investigations of the role of individual cis-acting elements in the Rh3 and Rh4 promoters of D. melanogaster.

Oligonucleotide-directed mutagenesis was used to alter each individual conserved sequence of the D. melanogaster Rh3 gene. Several nonconserved regions were also altered to assess directly the validity of this targeted mutagenesis approach. In general, the mutagenesis was designed to maximize the number of mismatched nucleotides between wild-type and mutant sequences of a given region without affecting the nucleotide composition of the region. For Rh3, mutagenesis was performed on the promoter fragment extending from −343 to +18 bp. To ensure that this small promoter is truly wild-type for all features of the Rh3 expression pattern, it was fused to lacZ and analyzed histochemically in transformed flies. Two independent transformants of [P[Rh3.343lacZ]] display staining of a stochastic subpopulation of R7 photoreceptor cells, as well as all dorsal marginal R7 and R8 cells, exactly as described for transformants of the 2.6-kb Rh3 promoter–lacZ fusion gene (data not shown).

Mutagenesis of each of the three conserved regions of the Rh3 promoter reveals that two are required for proper promoter function. Mutation of the Rh3 rhodopsin core sequence I (RCS I; Fig. 4), an element common to each of the four rhodopsin promoters of both D. melanogaster and D. virilis, virtually destroys Rh3 promoter function [P[Rh3.343m8CAT]]−6; Fig. 5A]. The more distal Rh3-specific conserved sequence, termed RUS3B [rhodopsin upstream sequence B of Rh3], exhibits extraordinarily high promoter activity in body tissues when mutated [P[Rh3.343m1CAT]]−6; Fig. 5A]. Mutation of the more proximal Rh3-specific conserved element RUS3A has little or no effect on the expression pattern of the Rh3 promoter in this assay [P[Rh3.343m5CAT]]−15; Fig. 5A]. The failure of this mutation to alter Rh3 promoter function is surprising, considering the highly palindromic character of RUS3A and the perfect conservation of half of the palindromic sequence at an equivalent position of the D. virilis Rh3 promoter. To test the possibility that a mutant phenotype of RUS3A is masked by functional redundancy of the upstream RUS3B element, the double mutant of RUS3A and RUS3B was constructed and analyzed. Aside from a very modest reduction in head expression levels, four independent transformants of this construct reveal no additional phenotype other than the high body expression found for mutagenesis of RUS3B alone [P[Rh3.343m1&5CAT]]−4; Fig. 5A].

Five nonconserved regions of the D. melanogaster Rh3 promoter in the −124- to −57-bp segment were also individually mutagenized and found to have no role in Rh3 promoter function. Transformants bearing these mutant constructs display CAT activity profiles indistinguishable from those of nonmutagenized Rh3–CAT fusions [P[Rh3.343m2, m3, m4, m6, and m7CAT]]−1; Fig. 5A]. A puzzling result of this analysis is that 5′ deletion of sequences between −137 and −66 bp abolishes Rh3 promoter activity, yet virtually complete mutagenesis of this region yields no loss-of-expression phenotypes. One possible explanation is that wild-type sequences between −343 and −137 bp in these mutant Rh3 promoters can substitute for mutagenized cis-acting elements in the −137- to −66-bp region.

Oligonucleotide-directed mutagenesis of the Rh4 promoter fragment extending from −472 to +7 bp reveals that all three highly conserved sequences are necessary for proper R7-specific gene expression. Mutation of the Rh4-specific upstream element RUS4A completely eliminates promoter activity in 12 independent transformant lines [P[Rh4.472m1CAT]]−12; Fig. 5B]. Expression of an Rh4 promoter bearing a mutagenized RCS I is reduced 5- to 50-fold relative to nonmutagenized Rh4 promoters and is no longer localized to R7 photoreceptor cells [P[Rh4.472m2CAT]]−11; Fig. 5B]. In spite of the large quantitative variations among the 11 different lines, individual lines display CAT activity levels in sev or boss heads virtually identical to those in wild-type heads. Mutation of the Rh4 RCS II produces a phenotype similar to but less severe than mutation of RCS I [P[Rh4.472m3CAT]]−19; Fig. 5B]. Expression is reduced 3- to 20-fold relative to nonmutagenized Rh4–CAT constructs and R7 cell-type specificity is only par-

Figure 5. Histograms summarizing the mutagenesis data for the D. melanogaster Rh3 promoter (A), the D. melanogaster Rh4 promoter (B), and the RCS I–RCS II region exchanges between the Rh3 and Rh4 promoters (C). CAT assay data were obtained and plotted as described in the legend to Fig. 3 and in Materials and methods. Mutagenesis was performed on a wild-type Rh3 promoter fragment extending from −343 to +18 bp [see P[Rh3.343CAT]] and on a wild-type Rh4 promoter fragment extending from −472 to +7 bp [see P[Rh4.472CAT]] and P[Rh4.472m2CAT]]. The wild-type sequence of the mutagenized region [wt] and the sequences of the individual mutagenizing oligonucleotides (mut) are aligned beneath (A and B) or beside (C) the histogram. The asterisks [*] in C denote the individual nucleotides mutated in each construct. In A and B, transformant lines corresponding to each mutagenized promoter construct are encompassed by the underlying bracket and are labeled with the construct designation used in the text. Solid lines indicate the mutagenized sequence corresponding to each bracketed set of transformant lines. In A, max & min refers to an Rh3 promoter doubly mutagenized with both m1 and m3. CAT activity levels in body tissues of P[Rh3.343m1CAT] and P[Rh3.343m1&5CAT] are 1.5- to 2.5-fold less in sev than in wild type; we have not yet determined whether this effect is due to the sev mutation or to genetic modifiers present in the sev[sev] parental strain. In B, the head CAT activity levels of the P[Rh4.472m2CAT]] lines in wild-type and sev or boss genetic backgrounds have been offset to reveal their similarity. As in Fig. 3, shaded bars show CAT activity levels in wild-type tissues, and solid bars show CAT activity levels in sev or boss tissues, absence of bars means <0.1% CAT activity, and A denotes the average values for all transformant lines of a given construct. All CAT activity levels are normalized to the wild-type head value of the standard reference line P[Rh3.2600CAT]] (Fig. 3A). Evolutionarily conserved sequences are boxed and labeled as in Fig. 4. Arrows denote the direction of transcription.
Bipartite structure of rhodopsin promoters

Figure 5. (See preceding page for legend.)
tially impaired. As with the Rh3 promoter mutagenesis, alteration of two different nonconserved sequences within the proximal Rh4 promoter region does not affect promoter function significantly [P[Rh4.472m4 and m5CAT], Fig. 5B].

The mutant phenotypes of RCS I and RCS II of the Rh4 promoter suggest that RCS II may serve as an accessory element for RCS I. Consistent with this proposal, RCS II is located immediately adjacent to one end of RCS I [Fig. 4]. Further evidence for an accessory function of RCS II is provided by the analysis of transformants bearing Rh4-CAT fusions with mutated RCS I and RCS II sequences in a glass^3 [g^3] genetic background, which eliminates all photoreceptor cells [Moses et al. 1989]. A total of 39 selected transformant lines bearing rhodopsin promoter-CAT fusions have been analyzed in g^3 flies, representing a diverse collection of D. melanogaster Rh3 and Rh4 nonmutagenized promoters of different sizes, D. virilis Rh3 and Rh4 promoters, Rh3 and Rh4 promoters mutant for each of the conserved sequences, and a single Rh1 promoter bearing a mutagenized RCS I sequence [from Mismer and Rubin 1989]. With the exception of the Rh4 RCS I and RCS II mutants, CAT activity levels of all these transformants were much lower in g^3 heads, as compared to sev^2 or boss^991 heads [data not shown], consistent with most ectopic non-R7 expression in these lines being localized to other photoreceptor cells. In contrast, all five transformants mutant for Rh4 RCS I [P[Rh4.472m2CAT]1, 7, 8, 10, and 11] and both mutant for Rh4 RCS II [P[Rh4.472m3CAT]5 and 14] displayed 1.5- to 2-fold more expression in g^3 heads than in wild-type, sev^2 or boss^991 heads [data not shown]. Although we do not understand the basis of this effect, it suggests a functional relationship between RCS I and RCS II. No CAT activity was detected in sine oculis heads of a single tested transformant line of the Rh4 RCS I mutant construct [P[Rh4.472m2CAT]5], indicating that the ectopic non-R7 expression of this fusion gene detected in sev^2, boss^991, and g^3 heads is still localized to visual system structures [data not shown]. The contiguous arrangement of RCS I and RCS II in the Rh4 promoter, as well as their similar phenotypic behavior when mutated, suggest that these two cis-acting elements comprise a single functional unit. The finding that D. virilis Rh4 promoter, in which this unit is inverted, is functionally equivalent to its D. melanogaster counterpart demonstrates that the RCS I–RCS II unit is capable of working in an orientation-independent manner.

RCS I and RCS II exchanges and hybrid promoter experiments

Evolutionary considerations and promoter mutagenesis data immediately suggest an attractive model for combinatorial control of transcription in the Drosophila rhodopsin genes. All four paired rhodopsin promoters of D. melanogaster and D. virilis contain an RCS I element, in some cases coupled with RCS II, immediately upstream of the TATA box [Fig. 4]. More distal RUS elements, on the other hand, are specific to each pair of corresponding rhodopsin gene promoters from the two species. The mutagenesis results presented here and elsewhere [Mismer and Rubin 1989] have demonstrated the functional importance of all RCS and RUS elements tested, with the exception of RUS3B, in the D. melanogaster Rh1, Rh3, and Rh4 promoters. For each rhodopsin gene, the proximal regulatory region containing RCS I [or RCS I–RCS II] and the TATA box may therefore constitute a functionally equivalent promoter core, with cell-type specificity determined by unique upstream sequences. Support for this model is furnished by two additional lines of investigation.

In the first set of experiments, oligonucleotide-directed mutagenesis was used to exchange the RCS I and RCS II sequences of Rh3 and Rh4 to test directly their functional equivalency. The D. melanogaster and D. virilis Rh3 RCS I sequence CTAATCCAAATT differs from the RCS I sequences of the remaining six rhodopsin promoters, which define the consensus RCS I sequence CTAATTGAATT [Fig. 4]. The D. melanogaster Rh4 RCS I sequence matches this consensus. Seven transformant lines of a 472-bp Rh4-CAT construct in which the Rh4 RCS I has been altered to match the Rh3 RCS I exhibit normal R7-specific gene expression [P[Rh4.472ex CAT]1–7, Fig. 5C]. CAT activity levels in heads of most of these transformants are elevated 1.5- to 2.5-fold relative to levels detected in heads of transformants bearing the nonmutagenized 472-bp Rh4-CAT construct. Because conversion of the Rh4 RCS I to the Rh3 RCS I entails only a 2 bp substitution [TG to CC], this quantitative effect most likely reflects a slightly higher binding affinity of the Rh3 RCS I sequence for the putative trans-acting factor[s] interacting with this element.

The converse RCS exchange was performed by mutagenizing the RCS I sequence of the 343-bp Rh3 promoter to match that of Rh4 (and the consensus) and to replace simultaneously the adjacent 3’ hexanucleotide with an RCS II element. This Rh3 promoter, now bearing an Rh4-like RCS I–RCS II region, was fused to CAT and assayed in 16 independent transformant lines [P[Rh3.343exCAT]1–16, Fig. 5C]. All lines display an expression profile similar to nonmutagenized Rh3–CAT transformants. Approximately 90% of head CAT activity is localized to R7 photoreceptor cells, with the remaining 10% presumably attributable to dorsal marginal R8 cell expression. However, the quantitative levels of head CAT activity in these transformants are generally 1.5- to 2.5-fold higher than in transformants bearing the nonmutagenized 343-bp Rh3–CAT construct. This increase is most easily interpreted as the net effect of two alterations made to the Rh3 promoter. Conversion of the Rh3 RCS I to an Rh4 RCS I is expected to reduce promoter strength slightly [by analogy to P[Rh4.472exCAT], Fig. 5C], whereas inclusion of RCS II is expected to increase promoter activity moderately [by analogy to P[Rh4.472m3CAT], Fig. 5B]. Disregarding minor quantitative effects, these experiments clearly demonstrate that the Rh3 RCS I and the Rh4 RCS
I–RCS II unit are functionally interchangeable despite their differing DNA sequences.

As a second test of the bipartite functional organization of the rhodopsin promoters, hybrid promoters consisting of distal (putative cell-type specificity) regions and proximal (putative core) regions of different rhodopsin promoters were constructed, fused to CAT, and assayed in transformed flies. The D. melanogaster Rh2, Rh3, and Rh4 promoters were chosen for these experiments, as cell-type specificity of the resulting hybrid promoters is easily determined by measuring CAT activity in wild-type transformants, transformants lacking R7 photoreceptors (sev or boss), and transformants lacking ocellar photoreceptors [oc for autosomal inserts or noc for X-linked inserts]. Proximal and distal promoter segments were derived from an Rh2 promoter extending from −183 to +32 bp, an Rh3 promoter extending from −137 to +18 bp, and an Rh4 promoter extending from −159 to +7 bp. Previous analysis has shown that this Rh2 promoter fragment is active at wild-type levels in ocellar photoreceptors (Mismer et al. 1988). The Rh3 and Rh4 promoter fragments selected for these experiments are the smallest ones still capable of generating nearly wild-type levels of R7-specific gene expression. A BgIII restriction site was inserted immediately 5′ to the RCS I element in each of these promoters, such that the last 2 bp of the BgIII site (AGATCT) coincides with the initial CT nucleotides of RCS I, and was used to construct all six possible pairwise hybrid promoter–CAT fusions.

The expression patterns of four of these hybrid promoter constructs in different genetic backgrounds are consistent with the model presented above. Hybrid promoters in which the distal promoter segment of Rh2 is fused to the proximal promoter regions of either Rh3 or Rh4 are active exclusively in ocellar photoreceptor cells [P[Rh2/3CAT]1–9 and P[Rh2/4CAT]1–6; Fig. 6]. In contrast, hybrid promoters combining the Rh3 distal promoter region with proximal promoter segments of either Rh2 or Rh4 display R7-specific expression patterns [P[Rh3/2CAT]1–6 and P[Rh3/4CAT]1–2; Fig. 6]. The activities of these hybrid promoter–CAT constructs are difficult to quantitate accurately, as they are expressed at very low levels relative to their parental Rh2, Rh3, and Rh4 promoters (precluding a lacZ analysis). Nevertheless, the transformant lines exhibiting higher levels of expression demonstrate convincingly that cell-type specificity is dictated by the distal promoter component for each of these four hybrid promoters. The remaining two hybrid promoters, in which the distal promoter segment of Rh4 is matched with the proximal promoter re-

---

### Table 1: Expression Patterns of Hybrid Rhodopsin Promoter–CAT Constructs

| Hybrid Promoter | % CAT Activity |
|-----------------|----------------|
|                  | Bodies          |
|                  | Wild-type | sev or boss | or noc |
| P[Rh2/4CAT]1     | <0.1         | 10.3       | 20.5    | <0.1 |
| 2                | <0.1         | 8.3        | 11.7    | <0.1 |
| 3                | 0.1          | 130.0      | 117.0   | <0.1 |
| 4                | 0.3          | 4.6        | 13.5    | 0.4  |
| 5                | 0.3          | 138.7      | 150.8   | <0.1 |
| 6                | 0.1          | 44.6       | 56.6    | <0.1 |
| Average          | <0.1         | 56.1       | 61.7    | <0.1 |
| P[Rh2/3CAT]1     | 0.2          | 1.1        | 1.1     | 0.2  |
| 2                | <0.1         | <0.1       | <0.1    | <0.1 |
| 3                | <0.1         | 6.7        | 5.9     | 0.2  |
| 4                | 0.3          | 8.7        | 4.2     | <0.1 |
| 5                | 0.1          | 9.2        | 7.5     | <0.1 |
| 6                | 0.4          | 0.5        | 0.8     | <0.1 |
| 7                | <0.1         | 1.5        | 1.8     | <0.1 |
| 8                | <0.1         | 0.6        | 0.9     | <0.1 |
| 9                | <0.1         | 1.5        | 1.4     | 0.2  |
| Average          | 0.1          | 3.3        | 2.6     | <0.1 |
| P[Rh3/4CAT]1     | <0.1         | 53.7       | 0.6     | 30.0 |
| 2                | 0.8          | 25.2       | <0.1    | 13.5 |
| Average          | 0.4          | 39.5       | 0.3     | 21.8 |
| P[Rh3/2CAT]1     | <0.1         | 0.5        | <0.1    | <0.1 |
| 2                | 0.2          | 1.5        | <0.1    | 0.7  |
| 3                | <0.1         | 6.2        | <0.1    | 1.7  |
| 4                | <0.1         | 6.1        | 0.4     | 1.9  |
| 5                | 0.3          | 0.8        | <0.1    | 1.4  |
| 6                | <0.1         | 3.8        | 0.5     | 4.5  |
| Average          | <0.1         | 3.2        | 0.2     | 1.7  |
| P[Rh4/3CAT]1-7   | <0.1         | <0.1       | <0.1    | <0.1 |
| P[Rh4/2CAT]1-13  | <0.1         | <0.1       | <0.1    | <0.1 |

### Figure 6: Expression Patterns of Hybrid Rhodopsin Promoter–CAT Fusion Genes

The left-most column lists all independent transformant lines obtained for the six hybrid promoter–CAT fusion constructs. Each construct is named according to the sources of the distal and proximal promoter segments joined at RCS I. For example, P[Rh2/4CAT]1 contains a distal Rh2 promoter fragment joined to a proximal Rh4 promoter fragment. The exact composition of each hybrid promoter may be found in the text and in Materials and methods. The four numerical columns show transformant line CAT activity levels in wild-type bodies, wild-type heads, heads lacking R7 cells [sev or boss], and heads lacking ocelli [oc or noc]. Shaded columns reveal the genotypes in which CAT activity is eliminated, allowing the expression of four hybrid promoter constructs to be localized to either R7 cells or the ocelli. Because of the quantitatively low levels of expression of these hybrid promoter–CAT fusions, duplicate CAT assays were performed for 5 hr instead of 2 hr and CAT activity is expressed relative to wild-type head levels of a 4.3-kb Rh2 promoter–CAT fusion [P[ry; Rh2(−4300/+32)]CAT2; Mismer et al. 1988]. For comparison, the wild-type head CAT activity of P[Rh3.2600CAT]1, the standard reference line of Figs. 3 and 5, was extrapolated from head extract dilutions included in these assays and is also shown. Error is estimated to be not greater than 30%; <0.1 means that CAT activity was not detected above background level.
regions of either Rh2 or Rh3, are completely nonfunctional in this assay (P[Rh4/2CAT]1–13 and P[Rh4/3CAT]1–7, Fig. 6). Proper function of the Rh4 distal promoter region may thus depend on sequences specific to the Rh4 proximal promoter segment, such as RCS II, or on particular spatial requirements among different cis-acting elements not preserved in these two hybrid promoter constructs.

Discussion

Combinatorial control of Drosophila rhodopsin gene regulation

The major result of this study is that the four rhodopsin promoters all share a simple bipartite structure. The 60- to 70-bp region immediately upstream of the transcriptional start site apparently constitutes a functionally equivalent proximal promoter core for each rhodopsin gene. Determinants of cell-type specificity generally reside within a 100- to 200-bp segment of each promoter immediately distal to the core region. The best evidence for this model is provided by the expression patterns of hybrid promoter constructs that mismatch proximal and distal promoter regions of the Rh2, Rh3, and Rh4 genes. Cell-type-specific expression of four such hybrid promoter constructs in either R7 or ocellar photoreceptors is dictated by the distal promoter segment in each case.

A striking feature of all four D. melanogaster rhodopsin promoters is that cis-acting DNA sequences sufficient for highly cell-type-specific gene expression reside within very small (<300 bp) upstream regulatory regions (Rh1, Misher and Rubin 1987; Rh2, Misher et al. 1988; Rh3 and Rh4, this report). D. virilis Rh3 and Rh4 promoter fragments of comparable sizes also function in the appropriate R7-specific manner when introduced into D. melanogaster. The small sizes of the rhodopsin gene promoters are in marked contrast to those of many developmentally regulated Drosophila genes (see, e.g., Hiromi et al. 1985) and may reflect their relatively simple expression patterns in single differentiated cell types. In practical terms, the small sizes of these promoters have greatly facilitated further characterization of their cis-acting requirements by oligonucleotide-directed mutagenesis of evolutionarily conserved sequences.

Between this report and a previous study (Misher and Rubin 1989), a total of 31 sequences of the Rh1, Rh3, and Rh4 promoters have been separately mutagenized and functionally assayed in vivo. Mutagenesis seriously impairs promoter function for 7 of the 8 sequences conserved between D. melanogaster and D. virilis, whereas mutagenesis does not alter normal expression patterns for the 23 nonconserved regions. Moreover, the one conserved sequence that fails to reveal a mutant phenotype in our assay (RUS3A) is specifically protected from DNase I digestion by a factor present in Drosophila adult head nuclear extracts [E. O’Neill, G.M. Rubin, and R. Tjian, unpubl.]. However, a limitation of this approach is revealed by the D. melanogaster Rh4 promoter deletion analysis. This analysis has localized essential regulatory sequences between −159 and −123 bp, a region containing no obvious homologies to the D. virilis Rh4 promoter. Nevertheless, our data indicate that evolutionary considerations are a useful guide for designing deletion and mutagenesis studies of gene regulatory regions.

The dominant feature of each rhodopsin proximal promoter region, aside from the TATA box, is the presence of a motif [RCS I] corresponding to the consensus sequence CTAATTGAATT. For Rh1, mutagenesis of RCS I leads to a pronounced reduction in promoter strength with no loss of cell-type specificity (Misher and Rubin 1989). Mutation of the Rh3 RCS I essentially eliminates promoter function, and mutation of the Rh4 RCS I results in low levels of ectopic expression in adult visual system structures. The mutant phenotype of the Rh2 RCS I has not yet been examined.

Whereas RCS I and other proximal core sequences do not determine photoreceptor subclass specificity, they may nevertheless serve as less restrictive tissue-specificity determinants. Sequence motifs closely matching the RCS I consensus have been noted [Misher and Rubin 1989; M. Fortini and C.S. Zuker, unpubl.] in the proximal 5′ regions of several Drosophila genes expressed specifically in all or part of the photoreceptor cell population, namely trp [Montell and Rubin 1989], ninaA (Schneuwly et al. 1989; Shieh et al. 1989), ninaC [Montell and Rubin 1988], chp [Reinke et al. 1988], and arrestin [Smith et al. 1990]. RCS I may thus confer on a promoter the ability to be potentially activated in all photoreceptor cells, with more restricted cell-type specificities determined by upstream cis-acting elements.

The exact roles played by these distal promoter RUS elements in restricting rhodopsin gene expression to individual photoreceptor cell types are not well understood. Three different mutations affecting RUS1A have been found previously to cause ectopic expression of Rh1–CAT constructs in body tissues without perturbing the specificity of Rh1–lacZ expression in R1–R6 photoreceptor cells [Misher and Rubin 1989]. The fact that the ninaA gene—which, within the retina, is also expressed only in R1–R6 photoreceptors—contains two RUS1A-like sequences and one RCS I-like motif within the region immediately upstream of the TATA box [M.E. Fortini and C.S. Zuker, unpubl.] suggests that RUS1A may nevertheless contribute to the restricted expression of Rh1 in outer photoreceptors only. The Rh3-specific element RUS3B, like RUS1A, displays a mutant phenotype of elevated body expression. On the other hand, partial deletion of RUS3B in P[Rh3.137CAT] reveals a preferential loss of dorsal marginal R8 expression with no increase in body expression. Whether RUS1A and RUS3B serve to repress the Rh1 and Rh3 genes in body tissues and whether they participate in the regulation of these genes in photoreceptor cells of the eye are still unresolved issues. Mutagenesis of RUS4A yields a complete loss-of-expression phenotype, demonstrating only that this element is somehow required for Rh4 promoter function.
The hybrid promoter experiments avoid these ambiguities by demonstrating in a positive assay those cell-type-specific regulatory features of distal promoter sequences that can be conferred on a heterologous proximal promoter core. The results of these experiments provide a glimpse into the underlying logic of combinatorial control in the transcriptional regulation of a small set of eukaryotic genes. In every photoreceptor cell, common transcription factors might be expected to interact with rhodopsin gene proximal promoter sequences such as RCS I and RCS II. All of the rhodopsin genes in the cell would then be poised for activation, but only one would be activated via specific interactions of its distal promoter sequences (such as RUS elements) with a particular constellation of trans-acting factors characteristic of that photoreceptor cell type. Interactions of factors bound upstream with components of the transcriptional apparatus, such as TATA factors and RNA polymerase II, may be mediated through the factor(s) associated with RCS I or RCS I–RCS II. Distal Rh3 and Rh4 promoter sequences are unable to direct a coherent expression pattern of any sort when RCS I has been removed by mutagenesis. Furthermore, upstream enhancer regions of Rh1 are nonfunctional when fused to a heterologous hsp70 promoter TATA box region (Misser and Rubin 1989). Precedents for a similar mediation of the regulatory effects of factors bound distally via those bound more proximally have been noted in the mammalian SV40 promoter (Courey et al. 1989), the present in a variety of different tissues and cell types.

The essential constraint is that a full complement of trans-acting factors capable of activating one of the rhodopsin promoters must be found only in the appropriate photoreceptor cell type. Within the photoreceptor cell population, the factor(s) interacting with RCS I and perhaps RCS II are expected to be present in all cell types. At least some of the factors interacting with the distal RUS regions of the rhodopsin promoters should exhibit more restricted distributions within these cells, although not necessarily to single photoreceptor cell types.

Regulation of R7 opsin expression patterns

The expression patterns of Rh3–lacZ and Rh4–lacZ fusion genes presented here provide further insight into the organization of the Drosophila visual system. The relative abundance of Rh3-expressing (30%) and Rh4-expressing (70%) R7 photoreceptors across most of the retina, as well as expression of Rh3 in dorsal marginal R7 and R8 cells, allows the Rh3 and Rh4 opsins to be assigned as the respective Drosophila homologs of the Musca and Calliphora 7p and 7y opsins (Kirschfeld et al. 1978). In vivo epifluorescence studies have shown that R7 photoreceptors of the 7p and 7y classes are distributed in an apparently random fashion across most of the compound eye in M. domestica (Franceschini et al. 1981; Hardie et al. 1981; Hardie 1985). The distribution pattern displays no dorsoventral or bilateral symmetries within a single eye, and no reproducible patterns are noticed among different eyes, even between the two eyes of the same individual. Spectral sensitivity measurements in the larger dipterans unequivocally demonstrate the presence of two distinct R8 visual pigments, termed the 8p and 8y opsins, each expressed specifically in R8 cells corresponding to a particular subclass of overlying R7 cells (Smola and Meffert 1979; see also Hardie 1985, 1986). The central photoreceptor cell populations of Musca and Calliphora thus comprise a random distribution of matched 7p/8p and 7y/8y pairs. The cis-acting regulatory regions of these four opsin genes must be capable of directing highly specific expression of the R7 and R8 opsin genes in strictly nonoverlapping subpopulations of their respective photoreceptor cell types while ensuring that the two resulting stochastic patterns coincide completely with each other. Isolation of the corresponding R8 opsin genes of D. melanogaster and comparison of their regulatory regions with the Rh3 and Rh4 promoters may provide some insight into this remarkable expression pattern.

Models may be devised envisioning the stochastic pattern of R7 opsin gene expression as the consequence of rather direct regulatory interactions between their promoters in terminally differentiated R7 photoreceptor cells. For example, competition between the Rh3 and Rh4 promoters for a limited supply of common trans-acting factors or antagonistic effects among different factors that activate one promoter while repressing the other could, in principle, generate the observed mutually exclusive distributions of Rh3- and Rh4-expressing R7 subclasses. However, the coordinate expression of R7 and R8 opsin genes assumed for Drosophila by analogy to other dipterans makes such scenarios unlikely. The compound eye photoreceptors are recruited from a homogeneous population of pluripotent precursor cells of the late third-instar larval eye disc and are not related by lineage (see Tomlinson 1988; Ready 1989; Rubin 1989; Zipursky 1989). For this reason, we favor a model in which early regulatory events of a stochastic nature establish the subclass identity of one of the undifferentiated central photoreceptor cells, which, in turn, communicates this decision to the other member of the pair in the developing ommatidial cluster. For example, local fluctuations in inductive signals across the eye disc may partition the R8 cells, the first ommatidial cells to undergo cell fate specification, into two differentiation.
subclasses having a ratio of ~70:30. Next, the sev- and boss-mediated signaling event by which the R8 cell induces R7 cell formation might be modulated differently for each R8 subclass, causing R7 precursors to correctly adopt either the Rh3- or the Rh4-expressing differentiation pathway. Thus, within the regulatory hierarchies controlling development of these cell types, the processes that ultimately generate the stochastic expression patterns of the Drosophila R7 (and presumably R8) opsin genes may operate considerably upstream of the rhodopsin promoters. Consistent with this idea, the Drosophila R7 opsin gene promoters seem as different from each other in terms of functional organization as they are from the promoters of the R1–R6 and ocellar rhodopsin genes (Fig. 4). Moreover, detailed characterizations of Musca and Calliphora central photoreceptor cells suggest that the two subclasses of both R7 and R8 cells display subtle differences in numerous anatomical and physiological features, including rhabdomere length and twist, the fine structure of the transition point between R7 and R8 rhabdomeres, nuclear location, and the presence of photosensitizing and screening accessory pigments (Kirschfeld et al. 1977, 1978; Wunderer and Smola 1982a; Hardie 1985). In light of these considerations, the assignment of R7 (and R8) subclasses as members of single photoreceptor cell types may be somewhat artificial.

Functional properties of Drosophila R7 opsins

The rich variety of spectral types that distinguish the dipteran central photoreceptors from the spectrally uniform outer photoreceptors has led numerous investigators to speculate that they may be involved in color vision, similar to the cones of the vertebrate retina (Menne and Spatz 1977; Franceschini 1984; Hardie 1985, 1986). Defective color-specific phototactic responses are, in fact, observed for the Drosophila sev mutant (Schäumperl 1973; Harris et al. 1976; Heisenberg and Buchner 1977; Fischbach 1979). The idea that the two R7 cell subclasses may comprise part of a dipteran color vision system would gain credence if it could be demonstrated that their inputs are processed independently by the brain. Interestingly, Golgi impregnations of Drosophila photoreceptor neurons have revealed long and short synaptic terminal variants in both the R7 and R8 target layers of the medulla (Fischbach and Dittrich 1989). The long variants were found to occur more rarely; however, this technique did not allow the two variants to be correlated with specific Rh3- or Rh4-expressing R7 cells. The use of rhodopsin promoter-driven histological marker genes capable of differentially labeling the axonal projections of each R7 subclass holds much promise for this line of investigation.

Expression of Rh3–lacZ constructs in a small group of ~25 dorsal marginal R7 and R8 photoreceptor pairs constitutes a striking deviation from the stochastic R7-specific expression of Rh3 across the remainder of the Drosophila compound eye. An analogous region of R7 and R8 cells (termed 7marg/8marg), both containing the 7p opsin, have been described in Musca and Calliphora (Hardie 1985, 1986). The labeling of these cells with Lucifer yellow (Hardie 1984) and cobalt (Strasfeld and Wunderer 1985) has revealed that they project long axons with enlarged terminals to the dorsal posterior medulla, consistent with the β-galactosidase staining patterns of the Rh3–lacZ transformant lines described in this paper. A more detailed description of the Rh3–lacZ staining pattern in the medulla neuropil subserving these specialized central photoreceptor cells of D. melanogaster will be presented elsewhere (M.E. Fortini and G.M. Rubin, in prep.).

Specialized dorsal marginal ommatidia with greatly enlarged central rhabdomeres have been identified in a number of dipteran species, including Drosophila (Wada 1971, 1974). Detailed characterization of these photoreceptors in Musca and Calliphora has shown that the paired R7 and R8 cells are extremely sensitive to different e-vector directions of polarized light (Wunderer and Smola 1982b; Hardie 1984). Similar polarized light-sensitive photoreceptors have also been found in the dorsal marginal eye regions of bees and desert ants. Selective masking experiments have shown that in these insects, the dorsal marginal ommatidia are used to navigate by the ultraviolet polarized light patterns of the sky (Labhart 1980, Wehner 1982, 1989). D. melanogaster is also apparently capable of orienting with respect to the e-vector direction of polarized light (Stephens et al. 1953, Wolf et al. 1980). Navigation not only requires that the polarized light composition of the sky be measured with respect to two different planes but also that absorbance differences due to changes in e-vector direction be distinguished from those due to changes in intensity or wavelength of the light (Autrum and Stumpf 1950; Wehner 1982, 1989; Hardie 1984). If both central photoreceptors contain the same rhodopsin, the latter absorbance differences are automatically normalized such that any net absorbance difference between the two paired cells can only be due to polarization of light in the dorsal contralateral visual field. These constraints provide a clear rationale for expression of Rh3 in paired R7 and R8 photoreceptors at the dorsal eye margin in Drosophila.

Concluding remarks

Recent genetic and molecular studies have implicated sev, boss, and other loci in the early events of ommatidial assembly in D. melanogaster (for review, see Tomlinson 1988, Ready 1989, Rubin 1989, Zipursky 1989). The analysis of the regulatory regions of rhodopsin genes expressed in specific subsets of adult photoreceptor cells offers a complementary approach toward investigating ommatidial development. The expression patterns of these genes must reflect, to a large extent, unique gene regulatory properties of the ommatidial photoreceptor cells established during their earlier determination and differentiation. Identification of transcription factors that interact with the rhodopsin promoters may thus help narrow the gap in our understanding of how genes
such as sev and boss exert their regulatory effects via sets of downstream genes. Through this approach we hope ultimately to reconstruct the hierarchy of molecular events, whereby positional cues and cell–cell communication in the developing eye disc eventually give rise to the precise array of terminally differentiated cell types of the adult compound eye.

Materials and methods

DNA manipulations

DNA manipulations, including restriction endonuclease digestions, ligations, nick-translations, bacterial transformations, and plasmid DNA isolations, were performed according to standard procedures [Maniatis et al. 1982].

Isolation of D. virilis rhodopsin genes

The D. virilis rhodopsin genes were isolated from a D. virilis genomic DNA library prepared in λ EMBL-3 [gift of Dr. M. Scott, University of Colorado, Boulder] by screening with nick-translated D. melanogaster probes under the following conditions: 2 × SSC, 20% formamide, 0.2% SDS, 5 × Denhardt’s solution [0.1% Ficoll, 0.1% polyvinylpyrrolidone [PVP], 0.1% bovine serum albumin [BSA]], 100 μg/ml herring sperm DNA at 45°C, followed by three 1-hr washes in 2 × SSC, and 0.2% SDS at 45°C. A precise description of the D. melanogaster rhodopsin gene probes used will be available elsewhere [Fortini 1990]. A limited amount of protein-coding region sequence was obtained for the D. virilis opsins genes to allow each one to be correctly identified as the homolog of a particular D. melanogaster opsin gene.

DNA sequence analysis

DNA sequencing was performed according to the dideoxy chain-termination method [Sanger et al. 1977]. Promoter sequences obtained during this study (see legend to Fig. 4) were determined on both DNA strands. Pairwise homology searches were performed in all combinations among the eight available D. melanogaster and D. virilis rhodopsin promoters on a SUN computer, using IntelliGenetics sequence analysis software.

Construction of rhodopsin promoter fusion genes

All 45 rhodopsin promoter fusions described in this paper were made by a simple three-step cloning procedure. First, appropriate DNA manipulations were performed to generate the desired promoter fragments. Second, the promoter fragments were fused to CAT or lacZ in the vector pHSS7 [Seifer et al. 1986]. For all promoter fusions to CAT, we used the pDM47 vector [Mismar et al. 1988], a pHSS7 derivative bearing a CAT transcriptional fusion module. The polylinker of pHSS7 is bordered by NotI sites, which are not found in any of the rhodopsin promoter or reporter gene sequences used here. Third, the resultant promoter fusions were removed from pHSS7 as intact NotI fragments and subcloned into the unique NotI site of the modified Carnegie 20 vector pDM30 [Misser and Rubin 1987].

Cloning steps were designed to minimize nonessential differences among the various DNA constructs. All promoter fusions described here were inserted into pDM30 in the same orientation [head to head with respect to the rosy* [ry+] selectable marker gene of pDM30]. All promoter–CAT fusions also utilize the same KpnI site of pDM47 as the sequence junction. The three rhodopsin promoter–lacZ fusions are also equivalent to one another in terms of sequences present at the junction. Moreover, all constructs using a given promoter [e.g., D. melanogaster Rh3, D. virilis Rh4] are identical with respect to 5′ untranslated leader sequences and polylinker sequences present at the promoter–reporter gene junction [see below for details].

Rh3–lacZ and Rh4–lacZ fusions

For Rh3, a genomic HindIII–MspI promoter fragment extending from −2.6 kb to + 18 bp of the 22-bp Rh3 untranslated leader sequence was subcloned into HindIII-AccI-cleaved pIC20R [Marsh et al. 1984]. The entire insert was then removed as a BgIII–EcoRI fragment and transferred into BamHI/EcoRI-cleaved pHSS7. A lacZ transcriptional fusion module was removed as a 4.0-kb EcoRI fragment from pDM79 [Misser and Rubin 1987] and inserted into the EcoRI site of this pHSS7 construct. The resulting Rh3 (−2.6 kb to + 18 bp)–lacZ fusion was transferred into pDM30 as an intact NotI fragment to create p[Rh3.2600lacZ].

A smaller Rh3 promoter HindIII–KpnI fragment extending from −343 to + 18 bp was used to replace the Rh1 promoter HindIII–KpnI fragment of an Rh1–lacZ fusion in pHSS7 [Misser and Rubin 1989]. The lacZ module in this construct is identical to that in p[Rh3.2600lacZ]. The resulting Rh3–lacZ fusion was subcloned into pDM30 as a NotI insert to make p[Rh3.343lacZ].

For Rh4, a 3.5-kb genomic fragment containing 1.9 kb of upstream Rh4 sequences in pUC19 was digested with XmlI, which produced a blunt-ended cleavage precisely at the transcriptional start site. A synthetic linker composed of the sequence AGTTCAGGTACCTGCAG was inserted into this cleavage site to reconstitute the first 7 bp of the 87-bp translated leader sequence, followed by unique KpnI and PstI restriction sites. The resultant Rh4 promoter BgIII–KpnI fragment extending from −1.9 kb to + 7 bp was used to replace the Rh1 promoter BamHI–KpnI fragment of the Rh1–lacZ fusion in pHSS7 [see above, Misser and Rubin 1989]. The ensuing Rh4–lacZ fusion was transferred into pDM30 as a NotI fragment to make p[Rh4.1900lacZ].

D. melanogaster Rh3 and Rh4 promoter deletion series

p[Rh3.2600CAT] was constructed by subcloning an Rh3 promoter XhoI–KpnI fragment extending from −2.6 kb to + 18 bp into the CAT fusion vector pDM47 and then transferring the resultant fusion into pDM30 as a NotI fragment. p[Rh3.945CAT], p[Rh3.583CAT], p[Rh3.343CAT], p[Rh3.247CAT], p[Rh3.137CAT], and p[Rh3.66CAT] were constructed in an analogous fashion by utilizing restriction endonuclease sites that occur naturally within the Rh3 upstream DNA sequences. p[Rh3.99CAT] was similarly derived from a precursor of p[Rh3.343m4CAT] in which oligonucleotide-directed mutagenesis was used to introduce a ClnI site at position −104 bp of the Rh3 promoter (described below, Fig. 5A).

p[Rh4.1900CAT] was made by inserting an Rh4 promoter ClaI–KpnI fragment extending from −1.9 kb to + 7 bp into pDM47 and subcloning the resultant Rh4–CAT fusion into pDM30 as a NotI fragment. p[Rh4.472CAT] was constructed in an analogous manner by utilizing an EcoRI site at −472 bp of the Rh4 promoter. Exonuclease III digestion [Rogers and Weiss 1980] of the Rh4 promoter from this EcoRI site yielded 5′ deletion end points used to make p[Rh4.373CAT], p[Rh4.276CAT], and p[Rh4.159CAT] by the same cloning protocol. Oligonucleotide-directed mutagenesis of a 472-bp Rh4 promoter fragment in Bluescript(+) [Stratagene] (described in detail below) was used to generate three further deletions as

GENES & DEVELOPMENT 459
follows. \(p[\text{Rh}4.123\text{CAT}]\) and \(p[\text{Rh}4.87\text{CAT}]\) were derived from mutagenesis products in which synthetic \(BglII\) sites were introduced at \(-125\) and \(-92\) bp of the \(Rh4\) promoter. \(p[\text{Rh}4.63\text{CAT}]\) was made from a mutagenized 472-bp \(Rh4\) promoter bearing a \(BglII\) site at \(-67\) bp, originally designed to facilitate the construction of hybrid rhodopsin promoters (see below).

For all \(Rh3\) and \(Rh4\) promoter fragments \(<500\) bp in size, the precise 5'-deletion end points were determined by DNA sequencing. In some cases, vector polylinker sequences at the deletion end point fortuitously replace several nucleotides deleted from the \(Rh3\) or \(Rh4\) promoter. For all constructs, 5'-deletion end point numbers reported here reflect the number of base pairs identical to those of the native rhodopsin promoter sequences. The 3' ends of all \(D.\) melanogaster \(Rh3\) promoter fragments are identical, consisting of 18 bp of untranslated leader sequence followed by 23 bp of \(pIC\)-20R polylinker sequences (from the \(AccI\) site to the \(KpnI\) site). The 3' ends of all \(D.\) melanogaster \(Rh4\) promoter fragments are also identical, consisting of 7 bp of untranslated leader sequence terminating in a \(KpnI\) site.

\(D.\) virilis \(Rh3\)-CAT and \(Rh4\)-CAT fusions

A 517-bp \(XbaI\)-\(Sau\)3AI fragment of the \(D.\) virilis \(Rh3\) promoter was subcloned into \(XbaI\)/\(BamHI\)-cleaved \(Bluescript\,+J\), and the amount of 3' polylinker sequences was reduced by \(Small\)/\(Hin\)clI digestion followed by religation. This \(Sau\)3AI site of \(D.\) virilis \(Rh3\) occurs 42 bp downstream of the \(TATA\) box and 26 bp upstream of the putative translation initiation ATG codon. The \(-500\)-bp \(D.\) virilis \(Rh3\) promoter module was inserted into \(pDM47\) as an \(XbaI\)-\(KpnI\) fragment, and the resulting fusion was subcloned into \(pDM30\) as a \(NotI\) fragment to create \(p[Rh3v.500\text{CAT}]\). \(p[Rh3v.180\text{CAT}]\) was similarly derived from the same \(pDM47\) subclone after first removing the \(D.\) virilis \(Rh3\) sequences between the \(XbaI\) site and a \(Clai\) site located 142 bp upstream of the \(TATA\) box.

Oligonucleotide-directed mutagenesis was used to insert a unique \(KpnI\) site into the \(D.\) virilis \(Rh4\) promoter region at a position \(-47\) bp downstream of the \(TATA\) box and 10 bp upstream of the putative translation initiation ATG codon. A 310-bp \(EcoRI\)-\(KpnI\) fragment and a 202-bp \(XbaI\)-\(KpnI\) fragment of this construct were each fused to \(CAT\) in \(pDM47\), and the resulting fusions were inserted into \(pDM30\) to produce \(p[Rh4v.300\text{CAT}]\) and \(p[Rh4v.190\text{CAT}]\).

\(D.\) melanogaster \(Rh3\) and \(Rh4\) promoter oligonucleotide-directed mutagenesis

Oligonucleotide-directed mutagenesis was performed essentially as described in Laski et al. (1986). For the \(Rh3\) and \(Rh4\) promoter mutagenesis summarized in Figure 5, the two \(NotI\) fragments containing the promoter-\(CAT\) fusions of \(p[Rh3.343\text{CAT}]\) and \(p[Rh4.472\text{CAT}]\) (described above) were transferred into \(Bluescript\,+J\) in the desired orientation. The \(Rh3\)-\(CAT\) single-stranded template was mutagenized separately with nine different oligonucleotides, each consisting of the mutagenizing sequence flanked on either side by 10 bp of the appropriate wild-type sequences. The \(Rh4\)-\(CAT\) template was also mutagenized separately with six different oligonucleotides.

Mutagenized promoter fragments were identified by differential hybridization to the corresponding oligonucleotide probe and verified by DNA sequencing. Because the \(CAT\) module of the single-stranded templates was found to undergo rearrangements occasionally during the mutagenesis procedure, properly mutagenized \(Rh3\) and \(Rh4\) \(Clai\)-\(KpnI\) promoter fragments were re-fused to \(CAT\) in \(pDM47\). These mutagenized promoter-\(CAT\) fusions were transferred as \(NotI\) fragments into \(pDM30\) to generate the \(p[Rh3.343\text{CAT}]\), \(p[Rh4.472\text{CAT}]\), \(p[Rh3.343\text{CAT}]\), and \(p[Rh4.472\text{CAT}]\). The \(p[Rh3.343\text{CAT}]\), \(p[Rh4.472\text{CAT}]\), and \(p[Rh4.472\text{CAT}]\) was constructed by identical procedure after single-stranded DNA of the oligonucleotide m5-mutagenized \(Rh3\)-\(CAT\) fusion was subjected to a second round of mutagenesis with oligonucleotide m1 (Fig. 5A).

Hybrid promoter-\(CAT\) fusion series

Small \(Clal\)-\(KpnI\) fragments consisting of the \(Rh2\) promoter, extending from \(-183\) to \(+32\) bp [Mismer et al. 1988], the \(Rh3\) promoter, extending from \(-137\) to \(+18\) bp, and the \(Rh4\) promoter, extending from \(-159\) to \(+7\) bp, were subcloned into \(Bluescript\,+J\) and used to prepare single-stranded DNA templates. Oligonucleotide-directed mutagenesis was used to introduce a unique \(BglII\) site immediately upstream of RCS I in each promoter, as described above, except that the mutagenizing oligonucleotide for \(Rh3\) contained 20 bp of 5'-flanking wild-type sequences. Correctly mutagenized promoter sequences were identified by testing for the presence of the \(BglII\) site and verified by DNA sequencing.

All six hybrid promoters were constructed by three-way ligations of the \(Clal\)-\(BglII\) distal segment of one promoter and the \(BglII\)-\(KpnI\) proximal segment of another promoter into \(Clal\)-\(KpnI\)-cleaved \(pDM47\). The resultant hybrid promoter-\(CAT\) fusions were transferred as \(NotI\) inserts into \(pDM30\) to create \(p[Rh2\text{CAT}]\), \(p[Rh3\text{CAT}]\), \(p[Rh2\text{CAT}]\), \(p[Rh4\text{CAT}]\), \(p[Rh3\text{CAT}]\), and \(p[Rh4\text{CAT}]\).

\(P\)-element-mediated germ line transformation

Transformation of \(D.\) melanogaster was performed as described in Spradling and Rubin (1982) and Rubin and Spradling (1982). Prior to injection, the purified DNAs of all constructs derived from oligonucleotide-directed mutagenesis were confirmed by resequencing the appropriate promoter fragment.

Histochemical stainings

Staining for \(\beta\)-galactosidase activity was performed as described by Mismer and Rubin (1987). For \(\beta\)-galactosidase/\(Hoechst\) double staining, sections were not dehydrated after \(X\)-gal staining but were immersed in PBS containing 1.0 \(\mu\)g/ml Hoechst 33258 for 2–3 min, washed twice in PBS, and mounted in 70% glycerol in PBS.

\(\text{CAT enzyme activity assays}\)

To obtain accurate quantitation, \(\text{CAT}\) activity assays were performed simultaneously on a series of \(-30\) transformant lines each. For each transformant line, duplicate \(\text{CAT}\) assays were performed on five heads and five bodies of the appropriate genetic background according to published protocols [Gorman et al. 1982; Mismer and Rubin 1987]. Each series included 12–16 wild-type head samples of the standard reference line \(p[Rh3.2600\text{CAT}]\) as well as \(-8\) nontransformed \(ry^{556}\) negative control samples. For all series, excluding the hybrid promoter construct series, \(\text{CAT}\) assay reactions were performed for 2 hr at 37°C, during which time the reactions were in the linear range. Hybrid promoter \(\text{CAT}\) assays were allowed to proceed for 5 hr at 37°C due to their low levels of expression.

For autosomal inserts, male transformants were assayed, whereas for \(X\)-linked inserts, female transformants were assayed.
sayed to avoid dosage compensation effects [Spradling and Rubin 1983; Hazelrigg et al. 1984]. Standard genetic crosses were employed to produce transfectant flies of the appropriate mutant genotypes using the following stocks: sev[d2], ry506 (sev-r); Banerjee et al. 1987), boss3991 ry506 [boss3991: Reinecke and Zipursky 1988], g38 ry506 [g38; Moses et al. 1989], so; TM3ry506/+ [so: Lindsley and Grell 1968], cm c-toc pti-CIB, ry506 [oc: Lindsley and Grell 1968] and In(ZLR)noce+ b cn sp/Cyo, ry506 [noce; Ashburner et al. 1983].

CAT assay reaction products were separated by thin-layer chromatography [Gorman et al. 1982] and quantitated by liquid scintillation counting. Counts per minute (CPM) data were used to calculate the CAT activity present in each transformant mutant genotypes using the following stocks:

- For the hybrid promoter transformants, CAT activity is expressed relative to the level present in P[ry, Rh2(-4300/+32)]-CAT2 reference heads (normalized to 100%). The CAT activity histograms of Figures 3 and 5 were plotted directly from the normalized scintillation counting data using Cricket Graph software.

Acknowledgments

We thank D. Mismer for providing plasmids and fly strains, C. Zinker for providing Rh3 genomic subclones, and C. Montell for providing Rh4 genomic subclones. We gratefully acknowledge M. Scott for generously providing us with his D. virilis genomic library, S.L. Zipursky for making boss3991 flies available prior to publication, M. Fuchs and D. Schneider for obtaining the D. virilis Rh1 promoter sequence, and D. Lin and M. Rexach for assisting with the D. virilis Rh2 cloning. A special debt of gratitude is owed to J. Hirsh for initially suggesting the isolation of the D. virilis rhodopsin genes. We thank J. Fischer, R. Hardie, I. Harinharan, Y. Hiromi, E. O’Neill, R. Tjian, and C. Zinker for improving the manuscript with their comments. This work was supported by a National Science Foundation predoctoral fellowship to M.E.F. and by National Institutes of Health grant GM-33135 to G.M.R.

Note added in proof

Rhodopsin promoter sequence data described in this paper have been submitted to the EMBL/GenBank Data Libraries under accession numbers X31348 and X51353.

References

Ashburner, M., C. Detwiler, S. Tsubota, and R.C. Woodruff. 1983. The genetics of a small autosomal region of Drosophila melanogaster containing the structural gene for alcohol dehydrogenase. VI. Induced revertants of Scutoid. Genetics 104: 405–431.

Autrum, H. and H. Stumpf. 1950. Das Bienenauge als Analyse eines polarisierten Licht. Z. Naturforsch. 5b: 116–122.

Banerjee, U., P.J. Renfranz, J.A. Pollock, and S. Benzer. 1987. Molecular characterization and expression of sevenless, a gene involved in neuronal pattern formation in the Drosophila eye. Cell 49: 281–291.

Biggin, M.D., S. Bickel, M. Benson, V. Pirotta, and R. Tjian. 1988. Zeste encodes a sequence-specific transcription factor that activates the Ultrabithorax promoter in vitro. Cell 53: 713–722.

Bray, S.J., W.A. Johnson, J. Hirsh, U. Heberlein, and R. Tjian. 1988. A cis-acting element and associated binding factor required for CNS expression of the Drosophila melanogaster dopa decarboxylase gene. EMBO J. 7: 177–188.

Courey, A.J., D.A. Holtzman, S.P. Jackson, and R. Tjian. 1989. Synergistic activation by the glutamine-rich domains of human transcription factor Sp1. Cell 59: 827–836.

Dietrich, W. 1909. Die Facettenaugen der Dipteren. Z. Wiss. Zool. 92: 465–539.

Dyman, W.S. 1989. Modularity in promoters and enhancers. Cell 58: 1–4.

Fischbach, K.-F. 1979. Simultaneous and successive colour contrast expressed in ‘slow’ phototactic behaviour of walking Drosophila melanogaster. I. Comp. Physiol. 130: 161–171.

Fischbach, K.-F. and A.P.M. Dittrich. 1989. The optic lobe of Drosophila melanogaster. I. A Golgi analysis of wild-type structure. Cell Tissue Res. 258: 441–475.

Fischer, J.A. and T. Maniatis. 1988. Drosophila Adh: A promoter element expands the tissue specificity of an enhancer. Cell 53: 451–461.

Fortini, M.E. 1990. Transcriptional regulation of R7 opsin genes in Drosophila melanogaster. Ph.D. thesis. University of California, Berkeley.

Franceschini, N. 1984. Chromatic organization and sexual dimorphism of the fly retinal mosaic. In Photoceptors (ed. A. Borsellino and L. Cervetto), pp. 319–350. Plenum, New York.

Franceschini, N. and K. Kirschfeld. 1971a. Étude optique in vivo des éléments photorécepteurs dans l’œil composé de Drosophila. Kybernetik 8: 1–13.

———. 1971b. Les phénomènes de pseudopupille dans l’œil composé de Drosophila. Kybernetik 9: 159–182.

Franceschini, N., R.C. Hardie, W. Ribi, and K. Kirschfeld. 1981. Sexual dimorphism in a photoreceptor. Nature 291: 241–244.

Fryxell, K.J. and E.M. Meyerowitz. 1987. An opsin gene that is expressed only in the R7 photoreceptor cell of Drosophila. EMBO J. 6: 443–451.

Gorman, C.M., L.F. Moffat, and B.H. Howard. 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. 2: 1044–1051.

Hardie, R.C. 1984. Properties of photoreceptors R7 and R8 in dorsal marginal ommatidia in the compound eyes of Musca and Calliphora. Comp. Physiol. 154: 157–165.

———. 1985. Functional organization of the fly retina. In Progress in sensory physiology (ed. D. Ottoson), vol 5, pp. 1–79. Springer-Verlag, Berlin.

———. 1986. The photoreceptor array of the dipteran retina. Trends Neurosci. 9: 419–423.

Hardie, R.C., N. Franceschini, W. Ribi, and K. Kirschfeld. 1981. Distribution and properties of sex-specific photoreceptors in the fly Musca domestica. J. Comp. Physiol. 145: 139–152.

Harris, W.A., W.S. Stark, and J.A. Walker. 1976. Genetic dissection of the photoreceptor system in the compound eye of Drosophila melanogaster. J. Physiol. (London) 256: 415–439.

Hazelrigg, T., R. Levis, and G.M. Rubin. 1984. Transformation of white locus DNA in Drosophila: Dosage compensation, zeste interaction, and position effects. Cell 36: 469–481.

Heisenberg, M. and E. Buchner. 1977. The role of retinula cell types in visual behavior of Drosophila melanogaster. J. Comp. Physiol. 117: 127–162.

Heisenberg, M. and R. Wolf. 1984. Vision in Drosophila: Genetics of microbehavior. In Studies of brain function (ed. V. Braithenberg), vol. 12, pp. 1–32. Springer-Verlag, Berlin.

Hiromi, Y., A. Kuroiwa, and W.J. Gehring. 1985. Control elements of the Drosophila segmentation gene fushi tarazu.
Tomlinson, A. 1988. Cellular interactions in the developing
*Drosophila* eye. *Development* **104**: 183–193.
Treisman, R. and T. Maniatis. 1985. Simian virus 40 enhancer
increases number of RNA polymerase II molecules on
linked DNA. *Nature* **315**: 72–75.
Wada, S. 1971. Ein spezieller Rhabdomerentyp im Fliegenauge.
*Experientia* **27**: 1237–1238.
—–. 1974. Spezielle randzonale Ommatidien der Fliegen
(Diptera: Brachycera): Architektur und Verteilung in den
Komplexaugen. *Z. Morph. Tiere* **77**: 87–125.
Wehner, R. 1982. Himmelsnavigation bei Insekten. Neurophy-
siologie und Verhalten. In *Neuahrsblatt der naturfors-
chenden Gesellschaft in Zürich* (ed. H.H. Bossard). Natur-
forschende Gesellschaft, Zürich. 184.
—–. 1989. Neurobiology of polarization vision. *Trends
Neurosci.* **12**: 353–359.
Wolf, R., B. Gebhardt, R. Gademann, and M. Heisenberg. 1980.
Polarization sensitivity of course control in *Drosophila me-
lanogaster*. *J. Comp. Physiol.* **139**: 177–191.
Wong, F., E.L. Schaefer, B.C. Roop, J.N. LaMendola, D.
Johnson-Scatone, and D. Shao. 1989. Proper function of the
*Drosophila trp* gene product during pupal development is
important for normal visual transduction in the adult.
*Neuron* **3**: 81–94.
Wunderer, H. and U. Smola. 1982a. Morphological differentia-
tion of the central visual cells R7/8 in various regions of the
blowfly eye. *Tissue Cell* **14**: 341–358.
—–. 1982b. Fine structure of ommatidia at the dorsal eye
margin of *Calliphora erythrocephala* Meigen (Diptera: Cal-
liphoridae). An eye region specialized for the detection of
polarized light. *Int. J. Insect Morphol. Embryol.* **11**: 25–38.
Zipursky, S.L. 1989. Molecular and genetic analysis of *Drosop-
phila* eye development: sevenless, bride of sevenless and
rough. *Trends Neurosci.* **12**: 183–189.
Zuker, C.S., C. Montell, K. Jones, T. Laverty, and G.M. Rubin.
1987. A rhodopsin gene expressed in photoreceptor cell R7
of the *Drosophila* eye: Homologies with other signal-trans-
ducing molecules. *J. Neurosci.* **7**: 1550–1557.
Analysis of cis-acting requirements of the Rh3 and Rh4 genes reveals a bipartite organization to rhodopsin promoters in Drosophila melanogaster.

M E Fortini and G M Rubin

Genes Dev. 1990, 4:  
Access the most recent version at doi:10.1101/gad.4.3.444

References
This article cites 71 articles, 15 of which can be accessed free at:
http://genesdev.cshlp.org/content/4/3/444.full.html#ref-list-1

License

Email Alerting Service
Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or click here.