Blue- and Green-Absorbing Visual Pigments of *Drosophila*: Ectopic Expression and Physiological Characterization of the R8 Photoreceptor Cell-Specific Rh5 and Rh6 Rhodopsins

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Color discrimination requires the input of different photoreceptor cells that are sensitive to different wavelengths of light. The *Drosophila* visual system contains multiple classes of photoreceptor cells that differ in anatomical location, synaptic connections, and spectral sensitivity. The Rh5 and Rh6 opsins are expressed in nonoverlapping sets of R8 cells and are the only *Drosophila* visual pigments that remain uncharacterized. In this study, we ectopically expressed Rh5 and Rh6 in the major class of photoreceptor cells (R1–R6) and show them to be biologically active in their new environment. The expression of either Rh5 or Rh6 in "blind" *ninaE*^{17} mutant flies, which lack the gene encoding the visual pigment of the R1–R6 cells, fully rescues the light response. Electrophysiological analysis showed that the maximal spectral sensitivity of the R1–R6 cells is shifted to 437 or 508 nm when Rh5 or Rh6, respectively, is expressed in these cells. These spectral sensitivities are in excellent agreement with intracellular recordings of the R8p and R8y cells measured in *Calliphora* and *Musca*. Spectrophotometric analyses of Rh5 and Rh6 *in vivo* by microspectrophotometry, and of detergent-extracted pigments *in vitro*, showed that Rh5 is reversibly photoconverted to a stable metarhodopsin (λ_{max} = 494 nm), whereas Rh6 appears to be photoconverted to a metarhodopsin (λ_{max} = 468 nm) that is less thermally stable. Phylogenetically, Rh5 belongs to a group of short-wavelength-absorbing invertebrate visual pigments, whereas Rh6 is related to a group of long-wavelength-absorbing pigments and is the first member of this class to be functionally characterized.

Key words: Drosophila melanogaster*; fruit fly; rhodopsin; visual pigment; spectral tuning; green-absorbing rhodopsin; blue-absorbing rhodopsin; protein expression*

Color vision is the ability of an organism to distinguish differences in wavelength independent of differences in light intensity (Jacobs, 1981). With the exception of the use of oil droplets or screening pigments in some photoreceptor cells, color vision is dependent on the expression of spectrally distinct forms of the visual pigment rhodopsin in different photoreceptor cells (Jacobs, 1981; Hardie, 1985; Nathans et al., 1986a,b; Nathans, 1992). This prerequisite for color vision is fulfilled in the *Drosophila* compound eye, which is composed of ~750 ommatidia (for review, see Wolff and Ready, 1993). Each ommatidium contains a bundle of 8 photoreceptor cells and 12 auxiliary cells. The photoreceptor cells differ in their position within the ommatidium, their synaptic connections within the optic lobes of the brain, and the opsin genes they express. Each photoreceptor cell contains a rhabdomere, a microvillar structure that contains the visual pigment and serves as the compartment for visual transduction. The rhabdomeres are arranged concentrically with the six peripheral rhabdomeres of the R1–R6 cells surrounding one central rhabdomere formed by the R7 or R8 cells in the distal or proximal portion of the ommatidium, respectively. The rhabdomeres of the R1–R6 photoreceptor cells span the length of the ommatidium and contain the major visual pigment of the *Drosophila* compound eye, the blue-absorbing Rh1 rhodopsin (*ninaE*) (O'Tousa et al., 1985; Zuker et al., 1985; Feiler et al., 1988). The R7 cells express either Rh3 or Rh4, UV-absorbing visual pigments, in nonoverlapping subsets of cells (Fryxell and Meyerowitz, 1987; Montell et al., 1987; Zuker et al., 1987; Feiler et al., 1992). The R8 cells express either Rh5 or Rh6 in nonoverlapping subsets of cells (Chou et al., 1996, 1999; Huber et al., 1997; Papatsenko et al., 1997). A minor class of ommatidia, located along the dorsal margin, contains R7 and R8 cells that both express Rh3 (Fortini and Rubin, 1990; Feiler et al., 1992). Rh2 encodes a violet-absorbing visual pigment that is expressed in the ocelli, simple eyes located on the vertex of the head (Cowman et al., 1986; Feiler et al., 1988).

The present studies were undertaken to characterize the spectral and photochemical properties of the R8 photoreceptor cell-specific rhodopsins Rh5 and Rh6. Previous work in larger flies, *Calliphora* and *Musca*, suggested that these visual pigments are likely to have unique spectral properties (for review, see Hardie, 1985, 1986). The characterization of these pigments provides insight into the relationship between visual pigment structure and the regulation of spectral tuning. Furthermore, phylogenetic anal-

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yses of Rh5 and Rh6 place both of these pigments into newly defined clades of visual pigment genes that have not been well characterized. Thus the characterization of Rh5 and Rh6 provides a basis for examining other cloned invertebrate pigments and completes the spectral characterization of the visual pigments of the *Drosophila* eye that is essential for a detailed examination of color vision in *Drosophila*.

MATERIALS AND METHODS

Ectopic expression of the Rh5 and Rh6 opsin genes. Flies expressing the Rh5 opsin gene in the R1–R6 photoreceptor cells of *ninaE*^{17} mutants (*y w; ninaE*^{17}*; P[Rh1 + 5, y*$^{+}$*]*) have been described previously (Chou et al., 1996). Flies expressing the Rh6 opsin gene (*y w; ninaE*^{17}*; P[Rh1 + 6, y*$^{+}$*]*) were generated in a similar manner. Briefly, the Rh6 cDNA (a 1.4 kb *Eco*RI/*Kpn*I fragment from clone 4.1A, containing the entire Rh6-coding region and polyA tail) was inserted immediately 3′ of an expression cassette containing the Rh1 promoter (2.4 kb including 33 bp of the 5′-untranslated region). The transcriptional fusion was subcloned into the *y*$^{+}$-marked P-element vector "C4" obtained from Pam Geyer (University of Iowa, Ames, IA). The construct was injected into *y w; sr ninaE*^{17} mutant embryos, and multiple independent P-element-mediated germline transformants were obtained using standard techniques (Karess and Rubin, 1984).

Histology. The immunohistochemistry and confocal imaging were performed as described previously (Chou et al., 1996). Head sections were incubated overnight at 4°C with rabbit anti-Rh1 (1:250 dilution), monoclonal anti-Rh5 (clone 7F1; IgG1 subclass; 1:20 dilution), and monoclonal anti-Rh6 (clone 14C5; IgM subclass; 1:20 dilution). After washes in PBS containing 0.1% saponin, the sections were incubated for 1 hr at room temperature with three secondary antibodies: Texas Red-conjugated goat anti-rabbit IgG (1:100 dilution), Cy5-conjugated goat anti-mouse IgG1 (1:100 dilution), and FITC-conjugated goat anti-mouse IgM (1:100 dilution). The confocal images were collected using a Bio-Rad MRC-1024 (Hercules, CA) with a Nikon Labphot-2 microscope (Melville, NY). Secondary antibodies and other immunological reagents were obtained from Jackson ImmunoResearch (West Grove, PA) and Southern Biotechnology (Birmingham, AL).

Sequence alignment and phylogenetic analysis. Amino acid sequences were aligned using Clustal X (Thompson et al., 1997). Phylogenetic trees were constructed with PAUP* 4.0b2a (Swofford, 1998) using maximum parsimony (unweighted) and neighbor-joining methods. To ensure robustness of the results and to estimate confidence intervals, we bootstrapped all trees 100 times. For each parsimony bootstrap replicate, five random additions and tree-bisection reconnection branch swapping were performed. In all analyses, bovine rhodopsin was designated an outgroup.

Electrophysiology. Electroretinogram (ERG) recordings were obtained from immobilized white-eyed (*w*) flies using glass microelectrodes filled with normal saline (0.9% NaCl, w/v) as described previously (Chou et al., 1996; Townson et al., 1998). Flies were stimulated with light from a xenon arc lamp (450 W; Osram; Oriel, Stratford, CT), using interference and neutral density filters to select specific wavelengths and intensities of light. Light intensity was measured using a calibrated silicon photodiode (model 71883; Oriel) and an optical power meter (OPM model 70310; Oriel).

Spectral sensitivity was measured using a modification of the voltage-clamp method of Franceschini (1979, 1984), which we have described in detail elsewhere (Townson et al., 1998). Briefly, the amplitude of the ERG response to a flickering (10 Hz) monochromatic stimulus was maintained at a criterion level by continuously adjusting the light intensity, as the wavelength of stimulating light was varied during a scan. In previous experiments, we have found that directly measuring and "clamping" the area (amplitude) of the ERG response can be problematic. This is because the scans require several minutes to complete and there is a significant problem in defining the baseline from which the ERG area or amplitude is calculated, especially when the baseline drifts. In the measurements in this study, for each ~0.3 sec window (ERG response to approximately three flickers), we averaged all of the ERG voltages during this period and used the SD as an estimate of the amplitude (width) of the response (Press et al., 1992). The SD is a function of the variance between the individual data points and the mean voltage during the sinusoidal ERG response. Thus, the SD is related to the response amplitude, although we have found that it is much less sensitive to baseline drift and noise.

During an experiment, as the monochrometer was stepped through a scan, the SD of the ERG response to three pulses of light (during 0.3 sec) was calculated and compared with a criterion set point. The ERG response was maintained near the set point during the scan by constantly adjusting the light intensity using a proportional-integral-derivative algorithm (Corripio, 1990). Spectral sensitivity (SS) was defined as the inverse of the light flux required to produce the criterion response, taking into account the wavelength and intensity of the stimulating light [i.e., SS $\propto$ 1/(light intensity (μW/cm^2) $\times$ wavelength)]. Raw sensitivity data were normalized to an amplitude of 1.0 at the wavelength of maximum sensitivity, multiple individual measurements were averaged, and values that differed by >10% from the mean value of a 10 nm window were filtered out. The filtered spectra were then smoothed with a window of 12 nm.

Microspectrophotometry. Heads from white-eyed flies were bisected (maintaining the retinas intact) and mounted between quartz coverslips in PBS. A single eye was illuminated antidromically so that light passed through the specimen into the objective lens of the single-beam microspectrophotometer [Leitz (MPV2; Wetzler, Germany) equipped with Zeiss ultrafluar optics and a Products for Research photomultiplier (RCA model C31034A02; Danvers, MA)]. To adapt the specimen, light emitted from a super-pressure mercury arc lamp (HBO 100 W; Osram, Berlin, Germany) and passed through an interference filter was used to photoconvert the visual pigment from its rhodopsin (R) to its metarhodopsin (M) state. The wavelength (λ_1) of the adapting light was selected to shift the photosteady state preferentially between R and M. To measure the transmission through the specimen, light from a tungsten halogen source (12 V/100 W; model 64623; Osram) was passed through a 1/8-m monochromator (model 7420; Oriel) and directed through the specimen. Transmission was measured continuously with a photomultiplier as the monochromator scanned from 350 to 700 nm. The measuring light did not significantly alter the steady state between the two photopigment states. A second adapting light at a different wavelength (λ_2) was then used to shift the photosteady state of the R and M states back toward the R state, and the transmission of the specimen was measured again. A difference spectrum ΔE (λ) was calculated from these measurements: $\Delta E(\lambda) = [\log (I_{\lambda 2}(\lambda)/I_{\lambda 1}(\lambda)]$, where $I_{\lambda 1}(\lambda)$ and $I_{\lambda 2}(\lambda)$ are the light intensities transmitted through the specimen after adaptation at λ_1 and λ_2, respectively. The difference spectra were normalized, averaged, and smoothed with a window of 10 nm.

Electrophysiological and spectroscopic data acquisition and instrument control were performed with a Power Macintosh 7600/120 (Apple Computer, Cupertino, CA) equipped with a National Instruments (Austin, TX) PCI-MIO-16XE-50 multifunction input/output board running LabView 5.0.

Preparation of visual pigment extracts and spectrophotometry. Drosophila ninaE^{17} mutants, white-eyed (*w*^{1118}) control flies, and transgenic strains expressing different *Drosophila* visual pigments in a *ninaE*^{17} mutant background were dark adapted for 48 hr. Between 200 and 1000 eyes were dissected under dim red light (>665 nm) and collected in water. The water was removed, and 200 μl of homogenization buffer (10% sucrose and 0.1 M sodium phosphate buffer, pH 6.0) was added. The samples were homogenized and then centrifuged for 20 sec at low speed (1000 $\times$ *g*) to spin down the chitin debris and unhomogenized tissue. The supernatant was removed, and the procedure was repeated twice. The combined supernatants were centrifuged for 10 min at 100,000 $\times$ *g*, and the membrane pellet was extracted with 40 μl of 4% digitonin in sodium phosphate buffer, pH 6.0, for 40 min at 20°C in the dark. Difference spectra were recorded with a Kontron Uvikon 930 spectrophotometer at 10°C (for measuring absorbance changes of extracts containing Rh1, Rh3, Rh4, or Rh5 rhodopsin) or at 1°C (for extracts containing Rh6 rhodopsin or extracts obtained from *ninaE*^{17} mutants). The extracts were irradiated in the cuvette with monochromatic light (Schott interference filters) using a 50 W xenon lamp until a steady state was achieved (2–4 min) at the wavelengths indicated in the figure legends. To ensure that there was little or no effect on the photosteady state of the pigment caused by measuring the spectrum, before each experiment we measured and then immediately measured again the spectrum of the dark-adapted pigment. The difference spectrum of these two measurements is shown as the "baseline" in relevant figure panels.

Rhodopsin nomogram modeling. Rhodopsin and metarhodopsin absorption spectra were calculated from the difference spectra recorded from the microspectrophotometry (MSP) and detergent-extracted pigments using the exponential function described by Stavenga et al. (1993). Briefly, the spectral shape of the rhodopsin α-band absorption can be described by the following log normal function: $\alpha = A \exp[-a_0x^2(1 + a_1x$

$+ a_2x^2)$], where $x = {}^{10}\log(\lambda/\lambda_{max})$, $A = 1$, $a_0 = 380$, $a_1 = 6.09$, and $a_2 = 3a_1^2/8$. Taking this relationship and assuming that the spectral shape of metarhodopsin is also described by this equation (but with different A and λ_{max}), we fit the measured difference spectra to an equation in which the α-band absorption of rhodopsin was subtracted from that of metarhodopsin. A curve-fitting routine was implemented in KaleidaGraph (version 3.08d; Synergy Software, Reading, PA) using the Levenberg–Marquardt (nonlinear least-squares) algorithm (Press et al., 1992). The computer solved for the λ_{max} and amplitude of both the rhodopsin and metarhodopsin absorption spectra and calculated the SD for each variable and the correlation coefficient (Pearson's r).

Stavenga et al. (1993) have shown that an improved fit can be obtained in the lower wavelength region by incorporating additional terms for the rhodopsin β-band absorption. These terms were not incorporated in the curve fitting because too little is known about the characteristics of M β-band absorption.

RESULTS

Ectopic expression of the Rh5 and Rh6 opsins in transgenic flies

The *Drosophila* visual system has proven to be an extremely useful tool for studying novel invertebrate opsins *in vivo* (Feiler et al., 1988, 1992; Britt et al., 1993; Townson et al., 1998). To characterize the spectral and biophysical properties of the Rh5 and Rh6 opsins, we expressed the genes encoding these visual pigments in the R1–R6 photoreceptor cells of a mutant strain of flies (*ninaE*^{17}). By coupling the structural gene of Rh5 or Rh6 to the promoter region from the Rh1 opsin gene, we targeted the expression of the novel opsin to the R1–R6 photoreceptor cells. These cells comprise the major class of photoreceptor cells in *Drosophila*. The R1–R6 photoreceptor cells dominate the physiological and photochemical properties of the compound eye and are primarily responsible for the optomotor behavior of fruit flies (Heisenberg and Wolf, 1984). The *ninaE*^{17} mutant strain serves as an appropriate host for the expression of the Rh5 and Rh6 opsins because these flies lack the Rh1 visual pigment that is normally expressed in the R1–R6 cells. White-eyed flies *(w)* were used in these experiments because the removal of the red-screening pigments of the eye increases the animal's sensitivity to light, allows for the measurement of rhodopsin difference spectra by MSP and from visual pigment extracts, and also simplifies the immunofluorescence studies of visual pigment expression patterns.

To characterize the expression pattern of the endogenous and ectopically expressed opsins, we used specific polyclonal and monoclonal antibodies directed against the C termini of the opsin proteins (see Materials and Methods). As shown in Figure 1*A*, the Rh1 opsin is expressed throughout the eye of the control flies (*y w*; *red*). Specifically, these opsins are expressed in the rhabdomeres of the R1–R6 cells, which extend the full length of the retina and constitute the majority of the photoreceptor cells found in the compound eye (Fig. 1*A*). In contrast, the endogenous Rh5 (Fig. 1*A*, *blue*) and Rh6 (*green*) opsins localize to the R8 cells, which comprise only a subset of the total photoreceptor cell population and are located proximally within the retina. Not labeled in these figures are the endogenous opsins that localize to the R7 cells, a subset of photoreceptor cells that are located in the distal retina. The host strain (*y w; ninaE*^{17}) lacks Rh1 expression in the R1–R6 photoreceptor cells because of a deletion within the Rh1 gene, but this strain does express the endogenous opsins found in the R7 and R8 cells (Fig. 1*B*). In the transgenic flies, the Rh5 and Rh6 opsins (*y w; ninaE*^{17} *P[Rh1 + 5, y*$^+$*]* and *y w; ninaE*^{17} *P[Rh1 + 6, y*$^+$*],* respectively) are expressed throughout the compound eye, in the R1–R6 photoreceptor cells of *ninaE* mutant flies (Fig. 1*C,D*). Note that the expression of the endogenous opsins in the R8 cells is not disrupted in the transgenic flies and there is no endogenous Rh1 opsin expressed in the retina.

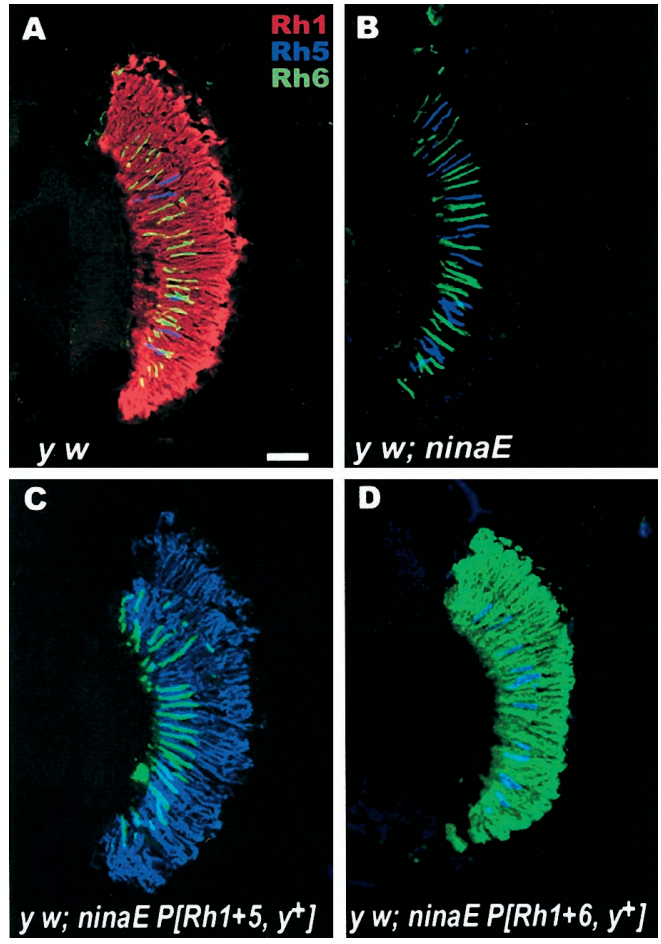

Figure 1. Expression of Rh5 and Rh6 in the compound eye of *Drosophila*. Shown are confocal immunofluorescence images of longitudinal sections of retinas from white-eyed flies. *A,* In control flies (*y w)*, Rh1 (*red*) is expressed in the rhabdomeres of the R1–R6 photoreceptor cells. Endogenous Rh5 (*blue*) and Rh6 (*green*) opsins are expressed in the rhabdomeres of the R8 cells, which occupy the proximal half of the retina. Rh3 and Rh4 are expressed in the rhabdomeres of R7 photoreceptor cells, which occupy the distal half of the retina (not labeled). *B,* In the mutant host strain (*y w; ninaE*^{17}), a portion of the Rh1 gene (*ninaE)* has been deleted, and Rh1 is not expressed in the R1–R6 cells. However, the endogenous minor opsins, localized to the R7 (not labeled) and R8 (Rh5 and Rh6 shown as *blue* and *green*, respectively) cells, are still expressed. *C,* In transgenic flies that ectopically express Rh5 under the control of the Rh1 promoter (*y w; ninaE*^{17} *P[Rh1 + 5, y*$^+$*]*), Rh5 (*blue*) is found throughout the retina in the R1–R6 photoreceptor cells. *D,* Similarly, in transgenic flies that ectopically express Rh6 (*y w; ninaE*^{17} *P[Rh1 + 6, y*$^+$*]*), Rh6 (*green*) is expressed in the R1–R6 cells. Because the host strain for the expression experiment is mutant for *ninaE*^{17} (as in *B*), there is no detectable Rh1 opsin expressed in either of the transgenic strains (*C, D*). Scale bar in *A*, 50 μm.

To test whether Rh5 and Rh6 are biologically active in their new cellular environment, ERGs were recorded from several different lines of control and transgenic flies. Figure 2 shows wild-type ERGs recorded from white-eyed (*y w)* flies, which express the Rh1 opsin in the R1–R6 photoreceptor cells (Fig. 2*A, top row* of *traces*). In these ERGs, a hyperpolarizing "on-" and a depolarizing "off-"transient appears at the onset and termination of the light stimulus, respectively. In addition there is a robust depolarization that is maintained for the duration of the stimulus.

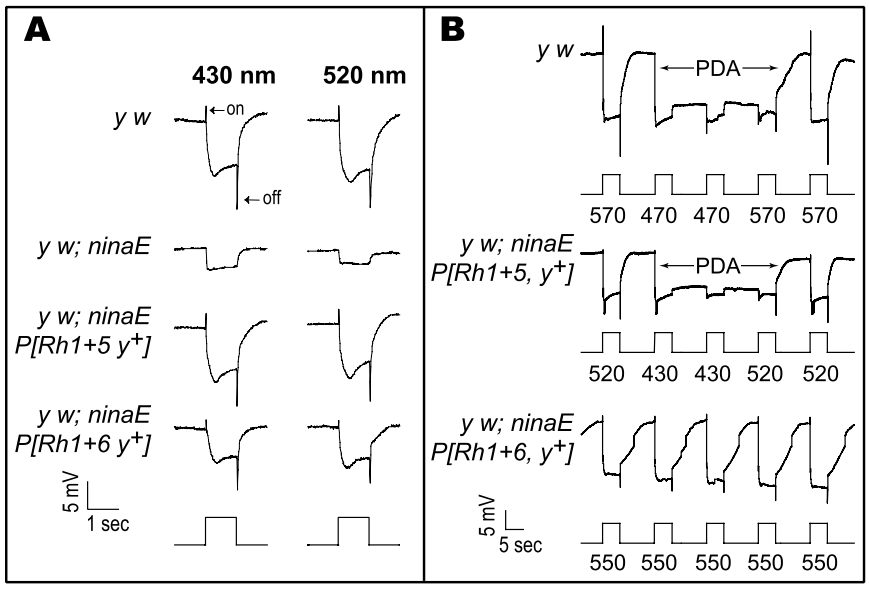

Figure 2. ERGs recorded from transgenic flies expressing Rh5 and Rh6. *A*, Responses to different wavelengths are arranged in *columns*, and responses recorded from specific genetic backgrounds are arranged in *rows*. Control *y w* flies (*top row* of *traces*), which express the Rh1 opsin in the R1–R6 photoreceptor cells, have a robust response to light at both 430 and 520 nm. The depolarization is preceded and followed by on- and off-transients, respectively, which originate in the lamina and reflect the activation of the R1–R6 cells (Heisenberg, 1971; Heisenberg and Wolf, 1984; Laughlin, 1989). The mutant host strain that lacks the Rh1 opsin, *y w; ninaE*^{17} (*second row* of *traces* from the *top*), has a dramatically reduced receptor potential and lacks both on- and off-transients. The residual response is derived from the R7 and R8 photoreceptor cells that are unaffected by the *ninaE*^{17} mutation. The transgenic flies that express Rh5 or Rh6, *y w; ninaE*^{17} *P[Rh1 + 5, y*$^{+}$*]* (*third row* of *traces* from the *top*) and *y w; ninaE*^{17} *P[Rh1 + 6, y*$^{+}$*]* (*fourth row* of *traces* from the *top*), display robust, wild-type photoresponses at either wavelength of light, demonstrating that both Rh5 and Rh6 are functional when expressed in the R1–R6 photoreceptor cells. Response amplitudes are not directly comparable between strains because of differences in the transgene expression levels. All of the strains were stimulated with the same intensity of light, which was ~1.09 μW/cm^2 at 430 nm and 0.9 μW/cm^2 at 520 nm. *B,* When a control fly (*y w*; *top trace*) is stimulated with intense light at 570 nm, there are a robust depolarization and immediate repolarization at the end of the stimulus. A PDA is induced when the fly is stimulated with light at 470 nm (thus producing a large amount of activated M-form). The depolarization is maintained after the cessation of the stimulus, and the photoreceptor cells are relatively inactivated to further stimuli. When the fly is again stimulated with 570 nm light, the PDA is terminated by the photoconversion of the M-form back to the R-form. Transgenic flies expressing Rh5 (*y w; ninaE*^{17} *P[Rh1 + 5, y*$^{+}$*]*; *second trace* from the *top*) can undergo a PDA when stimulated with light at 430 nm, and this can be terminated by stimulation with light at 520 nm. Transgenic flies expressing Rh6 (*y w; ninaE*^{17} *P[Rh1 + 6, y*$^{+}$*]*; *third trace* from the *top*) do not show a PDA when stimulated with 550 nm light. This wavelength would be expected to produce maximal conversion of Rh6 from the R- to the M-form with minimal photoconversion back to the R-form (see Fig. 4, for Rh6 extract difference spectra). Stimulation of Rh6 transgenic flies at 350, 430, 470, 520, and 570 nm was also insufficient to induce a PDA (data not shown). Light intensity was unattenuated in these experiments and was ~0.5 mW/cm^2 at each of the wavelengths tested. The *bottom row* in *A* and the *row below* each *trace* in *B* show the stimulus, with the time and voltage scales indicated on the *bottom left* of each *panel*.

The on- and off-transients have been shown to originate in the first optic neuropile (the lamina) and are induced only by activation of the R1–R6 photoreceptor cells (Heisenberg, 1971; Heisenberg and Wolf, 1984; Laughlin, 1989). The maintained depolarization is generated directly at the level of the retina by the action of all three classes of photoreceptors contained within the compound eye. In the ERGs recorded from the *y w*; *ninaE*^{17} host strain, no on- or off-transients can be detected, and the magnitude of the depolarization is dramatically reduced (Fig. 2*A, second row* of *traces*). The remnant depolarization recorded from these flies arises from the R7 and R8 photoreceptor cells, which still express functional opsins (Fig. 1*B*). When either Rh5 or Rh6 is introduced into the *ninaE*^{17} mutant host strain, the light response in the ERG is completely restored (Fig. 2*A*, *third, fourth rows* of *traces*). This indicates that these genes encode functional opsins capable of forming rhodopsins that are activated by light and that couple to the downstream components of the phototransduction cascade within the R1–R6 photoreceptor cells.

Spectral and photochemical analysis of Rh5 and Rh6

To characterize the spectral properties of the Rh5 and Rh6 opsins, we used both spectrophotometric and electrophysiological techniques. To determine the spectral sensitivity of transgenic flies expressing either the Rh5 or Rh6 opsin, we used a modified version of Franceschini's voltage-clamp technique (Franceschini, 1979, 1984). As shown in Figure 3, *top left* (Rh1), white-eyed control flies (*y w*), which express Rh1 in the R1–R6 photoreceptor cells, have a dual sensitivity in the UV and visible region of the spectrum. The sensitivity peak in the visible region has a maximum near 475 nm, which is well fit by a rhodopsin nomogram having an absorption maximum at 478 nm. The sensitivity in the UV region is thought to arise as the result of a sensitizing pigment that transfers the energy of an absorbed photon to the Rh1 chromophore, thereby inducing isomerization and visual pigment activation (Kirschfeld et al., 1977; Minke and Kirschfeld, 1979). In contrast, the spectral sensitivity of flies expressing the Rh5 or Rh6 minor opsins in the R1–R6 photoreceptor cells has been markedly altered (Fig. 3, *bottom left*). In the case of flies expressing Rh5, there is a peak of sensitivity near 440 nm, which is well fit by a rhodopsin nomogram having an absorption maximum at 437 nm. The spectral sensitivity of flies expressing Rh6 shows a peak in the green region near 510 nm, which is well fit by a rhodopsin nomogram having an absorption maximum at 508 nm.

To test whether either the Rh5 or Rh6 rhodopsins are capable of coupling to the sensitizing pigment and thus conferring UV sensitivity to the R1–R6 cells, we examined the spectral sensitivity of Rh5 and Rh6 when expressed in a special host strain. As described above and shown in Figure 2*A*, *ninaE*^{17} flies have functional R7 and R8 cells that are unaffected by the *ninaE*^{17} mutation and produce a small depolarization in the ERG. The R7 photoreceptor cells have a pronounced UV sensitivity that could potentially interfere with the analysis of Rh5 and Rh6 in the UV region of the spectrum. Therefore, in transgenic flies expressing either Rh5 or Rh6, the *norpA*-encoded phospholipase C that is required for visual transduction in all photoreceptor cells of the compound eye was removed genetically (by the *norpA* mutation) (Bloomquist et al., 1988), and then this activity was restored only in the R1–R6 cells by expressing the *norpA* cDNA under the control of the *ninaE* (Rh1) promoter (Pearn et al., 1996). Because these flies also lack the Rh1 visual pigment gene in the R1–R6 cells (*ninaE*^{17} mutant), all of the light-induced response was

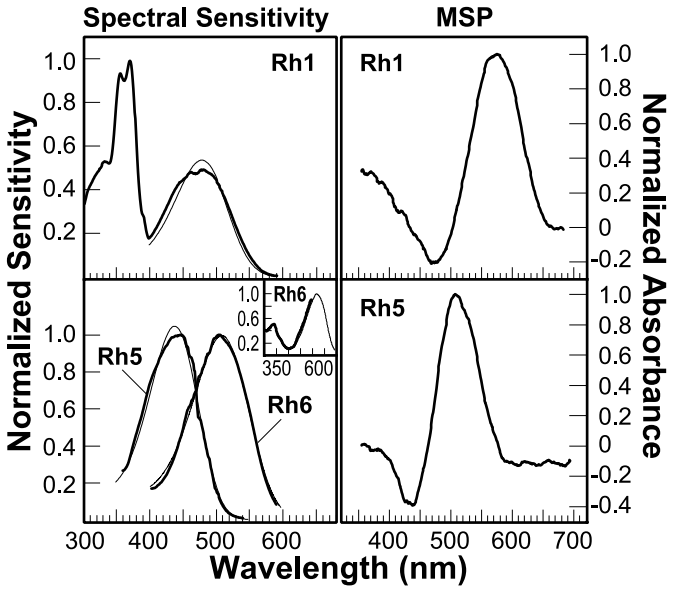

Figure 3. Spectral sensitivity and *in vivo* spectroscopy of Rh5 and Rh6. Spectral sensitivity was measured using a modification of the voltage-clamp method of Franceschini (1979, 1984) (see Materials and Methods for details). *Top Left* (Rh1), Recordings averaged from *y w* control flies ($n = 2$). These animals express the Rh1 (*ninaE*) opsin in the R1–R6 photoreceptor cells and have prominent sensitivity peaks in the blue and UV regions of the spectrum (*bold trace*). The single peak at ~475 nm (blue) is attributable to direct absorption by, and activation of, the Rh1 pigment. The doublet at ~355 and 370 nm (UV) is attributable to a UV-sensitizing pigment. The sensitizing pigment is thought to transfer the energy of absorbed UV quanta to the Rh1 chromophore, thereby inducing isomerization and visual pigment activation (Kirschfeld et al., 1977). The peak of Rh1 sensitivity in the blue region is fit well by a calculated rhodopsin α-band absorption (*fine trace*) having a maximal absorption at 478 nm ($r = 0.983$) (Stavenga et al., 1993). The measured spectral sensitivity is somewhat broader than the calculated absorption, and this could be caused by waveguide or self-screening effects (Smakman and Stavenga, 1986). *Bottom Left,* The averaged spectral sensitivities of transgenic flies expressing either Rh5 ($n = 4$) or Rh6 ($n = 5$) in the R1–R6 cells (same genotypes indicated in Fig. 2). Flies expressing Rh5 have a prominent peak of sensitivity at ~440 nm, whereas flies expressing Rh6 have a prominent peak at ~510 nm (*bold traces*). The spectral sensitivities of Rh5 and Rh6 were well fit by calculated rhodopsin α-band absorptions (*fine traces*) having absorption maxima at 437 nm ($r = 0.995$) and 508 nm ($r = 0.998$) for Rh5 and Rh6, respectively. When Rh5 or Rh6 is expressed in a host strain that lacks the response of the R7 and R8 cells (*w norpA*^{P24}; *ninaE*^{17} *P[Rh1+norpA cDNA, w*$^{+}$]; see Results for description), we find that Rh5 transgenic flies display no UV sensitivity (data not shown). *Inset*, In contrast, Rh6 transgenic flies showing UV sensitivity. The *fine trace* shows the same curve shown in the *main panel,* and the *bold curve* is a high-resolution scan through the lower wavelength region. The amplitude of the high-resolution scan was normalized to that of the short-wavelength limb of the visible region scan (*fine trace*) for comparison. The *y w; ninaE*^{17} mutant host strain did not have a detectable spectral sensitivity in this assay, because the amplitude of the ERG response was not high enough to meet the criterion of the recording paradigm (see Materials and Methods). *Right*, Rhodopsin and metarhodopsin difference spectra measured *in vivo* by MSP. *Top Right,* Control flies (*y w*; Rh1; $n = 3$). *Bottom Right,* Rh5 transgenic flies (*y w; ninaE*^{17} *P[Rh1 + 5, y*$^{+}$*]*; $n = 2$). Rh1-expressing flies were illuminated with adapting lights (λ_1 and λ_2) of 475 and then 580 nm, to shift the photosteady state from the Rh1 R-form to the M-form, and then vice versa. Rh5 transgenic flies were illuminated with adapting lights (λ_1 and λ_2) of 418 and 524 nm. No difference spectrum could be generated from flies expressing the Rh6 opsin construct after adaptation at multiple wavelengths (see Results).

derived from Rh5 or Rh6 expression in the R1–R6 cells. Flies expressing Rh5 have no detectable sensitivity in the UV region (data not shown). We would expect that even a small peak of UV sensitivity would be detectable because there would be little overlap between the short-wavelength limb of the sensitivity peak of Rh5 transgenics and that attributable to the sensitizing pigment. By contrast, flies expressing Rh6 do show a small sensitivity peak in the UV (Fig. 3, *bottom left, inset*). This most likely reflects the coupling of Rh6 to the sensitizing pigment in the R1–R6 cells, although the efficiency of coupling appears reduced in Rh6 transgenics in the UV region compared with Rh1, as demonstrated by relative sensitivity ratios of ~2:1 (UV/visible) for Rh1 versus ~0.5:1 for Rh6. Furthermore, we did not observe the expected fine structure in the UV region for Rh6-expressing flies that is normally observed for other rhodopsins that couple to the sensitizing pigment and has also been observed in the R8y photoreceptor cells of larger flies (Hardie and Kirschfeld, 1983; Feiler et al., 1988). Our inability to detect any fine structure may be caused by the relatively low UV sensitivity of the Rh6-expressing flies.

We examined the transgenic flies expressing Rh5 and Rh6 using microspectrophotometry (MSP) to determine the absorption profiles of rhodopsin and metarhodopsin for each of the novel pigments. Photoconversion of R produces the activated form of the visual pigment M. For many invertebrate pigments, the M-form of the visual pigment is thermally stable, and its absorbance λ_{max} is dramatically shifted from that of the R-form of the pigment (Stavenga, 1989, 1992). In addition the R- and M-forms are photointerconvertible, so that when the λ_{max} of R and M differ significantly the ratio of the rhodopsin molecules in the R- and M-forms can be manipulated using different illumination conditions. In the photosteady state, the ratio of the R- and M-forms is dependent on the spectral composition of the illuminating light and the absorption profiles of the two states of the pigment (Hamdorf et al., 1973; Hamdorf, 1979). For example, when the Rh1 rhodopsin (expressed in *y w* control flies) is illuminated with blue light near 480 nm, ~70% of the Rh1 R-form is converted to the M-form at steady state (Huber et al., 1990). Subsequent illumination with orange light near 580 nm photoconverts 100% of the M-form back to the R-form of rhodopsin. When absorption spectra are recorded before and after an illumination that shifts a substantial amount of the R-form to the M-form and these spectra are subtracted from each other, the resulting difference spectrum reflects the decrease in R-form absorption and the increase in M-form absorption (Fig. 3, *top right*). We used MSP to measure the difference spectrum of flies expressing the Rh5 opsin and found that Rh5 is photoconverted to a thermally stable metarhodopsin that absorbs maximally near 500 nm (Fig. 3, *bottom right*). No difference spectrum could be generated for Rh6-expressing flies by MSP, suggesting either (1) that there is not enough Rh6 expressed to be detectable by MSP, (2) that the M-form of Rh6 is not stable, or (3) that there is sufficient overlap in the absorption spectra of the R- and M-forms that illumination at many different wavelengths (402, 418, 430, 442, 456, 475, 499, 524, 563, and 584 nm; data not shown) did not lead to a detectable shift in the ratio of R and M in the photosteady state.

To examine the photoconversion between the R- and M-forms of Rh5 and Rh6 further, we attempted to generate prolonged depolarizing afterpotentials (PDAs) in transgenic flies expressing these pigments. A PDA is only generated in white-eyed flies when a substantial amount of rhodopsin has been photoconverted to metarhodopsin. During a PDA, the depolarization is maintained after the cessation of the light stimulus, and the response is

inactivated to further stimuli (Pak, 1979). The PDA is thought to result from the inadequate inactivation of the activated metarhodopsin state. Metarhodopsin is inactivated in part by a stoichiometric interaction with an abundant, cytosolic protein, arrestin. Because arrestin is expressed at a fraction of the concentration of rhodopsin, excessive conversion of the visual pigment to its activated M-form is thought to overcome the ability of arrestin to inactivate it, and this produces a PDA (Dolph et al., 1993). This requires that (1) the visual pigment must be expressed and activated at sufficiently high levels that the concentration barrier to PDA generation is overcome, (2) the absorptions of the R- and M-forms are sufficiently different that illumination creates a photosteady state in which a high percentage of the visual pigment is present in the M-form, and (3) the M-form of the pigment must be stable for the period of time in which the PDA is observed. Figure 2*B, top trace*, shows that when *y w* control flies are illuminated with intense orange light at 570 nm, which does not shift a significant amount of the R-form to the M-form, there is a robust depolarization that ends abruptly at the end of the stimulus. When the animals are illuminated with blue light at 470 nm, converting 70% of R to M, there is a robust depolarization that is maintained after the stimulus ends, and the fly eye is relatively inactivated to further stimuli. When orange light is used to photoconvert all of M to R, the PDA is immediately terminated, and the response returns to baseline. When flies expressing Rh5 are stimulated with appropriate wavelengths of light, a similar "retuned" PDA can be generated. The PDA can be initiated with intense light at 430 nm, converting ~50% of the Rh5 R-form to M, and this response can be terminated by illumination at 520 nm (photoconverting the M-form back to the R-form) (Fig. 2*B, second trace* from the *top*). This retuning of the PDA to wavelengths of light that can shift the steady state ratio of the R- and M-forms of ectopically expressed pigment has been demonstrated in previous studies with transgenic flies expressing Rh2, Rh3, and Rh4 (Feiler et al., 1988, 1992). Interestingly, stimulation of Rh6 transgenic flies at many wavelengths (Fig. 2*B, third trace* from the *top*; 550 nm is shown) was insufficient to induce a PDA. As was the case with the MSP studies, this suggests either that the pigment is not being expressed or activated at high levels, that there is a significant overlap between R- and M-form absorption, or that the M-form is unstable. To resolve these possibilities we examined the absorption profiles of the *Drosophila* Rh5 and Rh6 opsins in detergent extracts prepared from the retinas of dark-adapted flies (Fig. 4). We found that the Rh5 R-form can be reversibly photoconverted to a stable M-form (Fig. 4, *top left*), whereas photoactivation of Rh6 at a reduced temperature (1°C) produces an M-form that was not photoconverted back to the R-form in detectable amounts, and no stable M-form could be detected in extracts at higher temperatures (10°C) (Fig. 4, *top right*). This finding suggests that the M-form of Rh6 is not as stable as that of Rh5, at least when the pigments are expressed ectopically in the R1–R6 cells and extracted in digitonin. This finding provides one possible explanation for our inability to detect the Rh6 M-form by MSP and to induce a PDA in Rh6-expressing flies.

An alternative explanation of these findings may be that there is too little Rh6 expressed in the transgenic flies to induce a PDA or be detectable by MSP. The evidence that this may be the case is based on the absorbance change, corresponding to the formation of metarhodopsin, observed in visual pigment extracts. We calculated the amount of rhodopsin per eye, assuming that the molar extinction coefficients of the M-forms of the *Drosophila* rhodopsins are approximately the same as has been determined for the *Calliphora* Rh1 rhodopsin, i.e., 72,000 M^{-1} cm^{-1} (Stavenga and Schwemer, 1984). Although wild-type flies contain ~220 fmol of Rh1 per eye, the amount of photoconvertible Rh5 and Rh6 rhodopsin expressed in the R1–R6 and R8 cells of the transgenic strains is only 45 and 9 fmol/eye, respectively. So although the relatively low amount of Rh5 present may be at the lower limit of the amount of photoconvertible rhodopsin required to induce a PDA (~10–20% of wild-type Rh1 levels), the even smaller amount of functional Rh6 present may be the major reason for our inability to generate a PDA in these flies.

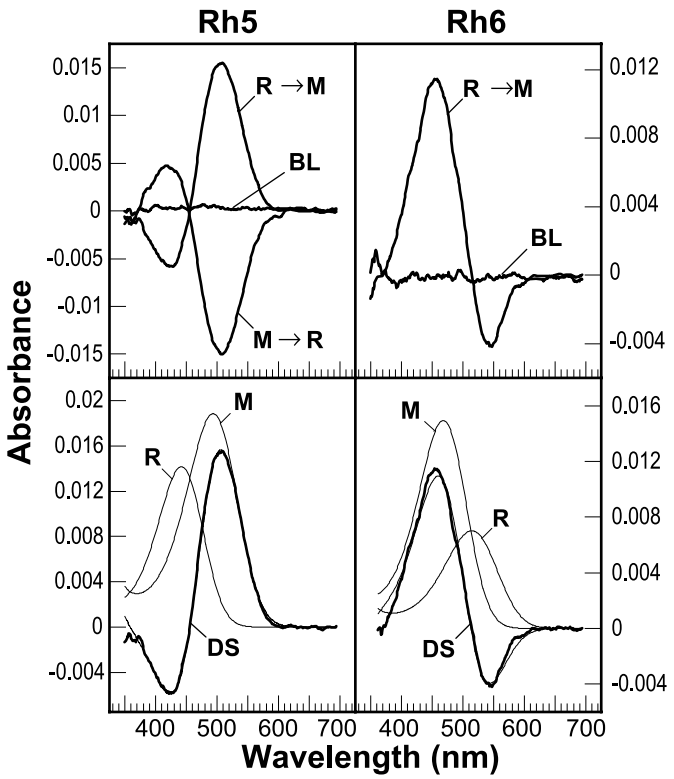

Figure 4. *In vitro* spectroscopy of Rh5 and Rh6 visual pigment extracts. *Top*, Difference spectra obtained from digitonin extracts of Rh5 (*left*) and Rh6 (*right*) transgenic flies are shown. Extracts were prepared in dim red light from 260 or 700 hand-dissected eyes of flies expressing Rh5 or Rh6, respectively, in the R1–R6 cells. Measurement of the absorption spectrum had no measurable effect on the samples, as shown by the baseline (*BL*) difference spectra calculated from sequential scans of the sample without other illumination. For both Rh5 and Rh6, when the extracts were illuminated with light selected to convert preferentially the R-form to the M-form ($R \rightarrow M$) (4 min at 421 nm and 2 min at 560 nm for Rh5 and Rh6, respectively), a difference spectrum is generated. For Rh5, but not Rh6, the difference spectrum could be generated at 10° C. The Rh6 difference spectrum could only be generated at a reduced temperature (1° C). When the extracts were illuminated with light selected to convert preferentially the M-form back to the R-form ($M \rightarrow R$) (4 min at 560 nm for Rh5; not shown for Rh6), a difference spectrum could be generated for Rh5 but not for Rh6. *Bottom*, The Rh5 and Rh6 difference spectra (*DS*) were fit with calculated rhodopsin and metarhodopsin absorption profiles as described in Materials and Methods (curve fits for Rh5 and Rh6 are shown as *fine traces* overlying the *DS* in the *bottom right* and *left panels*, respectively). The Rh5 difference spectrum was best fit with R- and M-form absorptions having maxima at 442 and 494 nm, respectively ($r = 0.998$). The calculated ratio of R- and M-form extinctions (R/M) at the λ_{max} for each form was 1:1.3. The Rh6 difference spectrum was best fit with R- and M-form absorptions having maxima at 515 and 468 nm, respectively ($r = 0.991$). The calculated ratio of R/M extinctions at the λ_{max} for each form was 1:2.1.

The theoretical absorption spectra of the R- and M-forms of

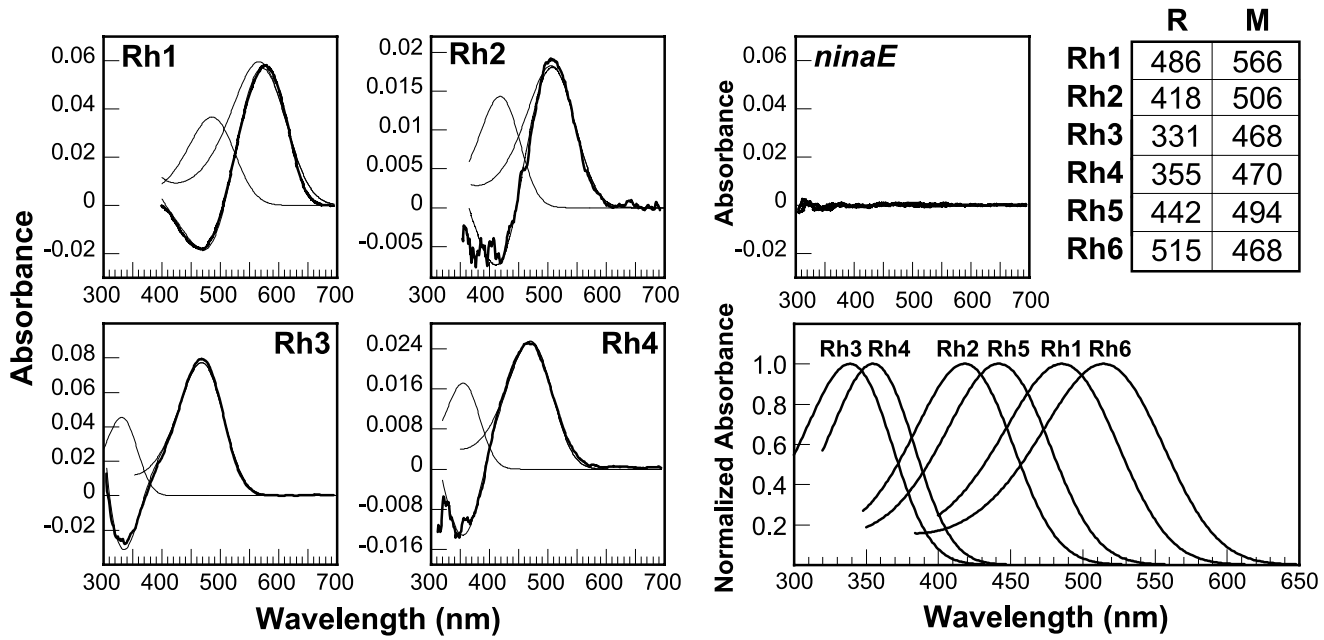

Figure 5. Spectroscopy of the *Drosophila* Rh1–Rh4 visual pigments. *Left*, *Middle*, Difference spectra that were measured from digitonin extracts of flies expressing Rh1, Rh3, and Rh4 and *ninaE*^{17} mutant controls and MSP of flies expressing Rh2 are shown. In each case the difference spectra were calculated from spectra measured after illumination with adapting lights (using a single-adapting light and subtracting from the dark-adapted state or with λ_1 and λ_2 as described above), which were 461 nm (for Rh1), 418 and 524 nm (for Rh2), 344 nm (for Rh3), 384 nm (for Rh4), and 560 and 442 nm (for *ninaE*). Difference spectra reflecting the R → M conversion were generated from flies expressing Rh1–Rh4; however no difference spectrum was generated from the *ninaE*^{17} mutant host strain. Each difference spectrum (*bold trace* in each *panel*) was fit with calculated rhodopsin and metarhodopsin absorption profiles as described in Materials and Methods (curve fits of the difference spectrum and the R- and M-forms are shown as *fine traces* in each *panel*). *Table*, *Top Right*, The calculated absorption maxima of the R- and M-forms for each pigment are indicated. The *r* values for the fits and the ratio of the R- and M-form extinctions λ_{max} were as follows: Rh1, $r = 0.996$ and R/M = 1:1.6; Rh2, $r = 0.0.987$ and R/M = 1:1.5; Rh3, $r = 0.998$ and R/M = 1:1.7; and Rh4, $r = 0.997$ and R/M = 1:1.5. *Bottom Right,* The calculated rhodopsin α-band absorptions based on the absorption maxima listed in the *table* are shown. As discussed in the text, the calculated R- and M-form absorption maxima differ somewhat between the different measurement methods. Difference spectroscopy would be expected to yield a fairly accurate estimate of M-form absorption, because its extinction coefficient is greater than that of the R-form. In the absence of waveguide or self-screening effects, the spectral sensitivity measured physiologically would be expected to yield a fairly accurate estimate of the R-form absorption. For reference, the maximal sensitivities of flies expressing Rh1–Rh6 are 478 nm (Rh1), 420 nm (Rh2), 345 nm (Rh3), 375 nm (Rh4), 437 nm (Rh5), and 508 nm (Rh6) (Feiler et al., 1988, 1992; this paper).

each pigment were derived from the difference spectra by fitting the data to a model in which the absorption of the R-form was subtracted from the absorption of the M-form. The absorptions of the R- and M-forms were calculated using an exponential function that describes the shape of the rhodopsin absorption curve as a function of wavelength (described in Materials and Methods) (Stavenga et al., 1993). We found that the difference spectrum of the detergent-extracted Rh5 was fit well by R- and M-forms having absorption maxima at 442 and 494 nm, respectively (Fig. 4, *bottom left*). This is in fairly good agreement with a similar analysis of the MSP data [R (λ_{max}) = 450 nm; M (λ_{max}) = 496 nm] and the spectral sensitivity measurements determined electrophysiologically (λ_{max} = 437 nm). Differences in the calculated absorption spectra of the R-form could be caused by detergent effects, or waveguide effects within the photoreceptor rhabdomere, although they more likely are the result of the derived nature of the difference spectrum and the fact that the extinction coefficient of the R-form is approximately one-half that of the M-form. Similar analysis of the detergent-extracted Rh6 difference spectrum showed that it was fit well by R- and M-forms having absorption maxima at 515 and 468 nm, respectively (Fig. 4, *bottom right*). Again, the maximal absorption of the R-form differs slightly from the spectral sensitivity determined electrophysiologically (λ_{max} = 508 nm).

Photoactivation of rhodopsin to metarhodopsin is associated with the isomerization of the chromophore from the 11-*cis* to the all-*trans* form as well as with conformational changes within the rhodopsin protein that allow it to couple to and activate the G-protein transducin. These structural changes are associated with changes in the absorption of the visual pigment, which are likely to reflect changes in the interaction of the chromophore with amino acid residues within the chromophore-binding pocket. Thus, the study of R- and M-form absorption changes in different visual pigments may potentially provide important insight into the activation process. Analyses of the difference spectra obtained from extracts or MSP of transgenic flies expressing Rh1–Rh4 and the absorption spectra of the calculated R- and M-forms are shown in Figure 5. The absorption maxima for both states of each pigment are also indicated in tabular form (Fig. 5, *top right*). It is interesting to note that all of the *Drosophila* rhodopsins undergo a bathochromic (red) shift after photoactivation, except for Rh6 that undergoes a hypsochromic (blue) shift. This hypsochromic shift in the metarhodopsin absorption of Rh6 is typical for pigments having a rhodopsin absorption maxima at wavelengths longer than 500 nm (Stavenga, 1989, 1992).

Figure 5, *bottom right,* shows the calculated rhodopsin absorption curves for Rh1-Rh6 based on the R-form curve fits to the MSP and extract data. The *Drosophila* visual pigments have absorption maxima that differ by almost 200 nm, ranging from the UV to the green region of the spectrum. This remarkable range of spectral sensitivity is shared by many invertebrate organisms; however fruit flies are the first invertebrate organism from which

the complete complement of all known visual pigment genes has been expressed and characterized in detail.

Relationship between opsins from *Drosophila* and other invertebrate species

To examine the relationship between the visual pigments of *Drosophila* and other invertebrate species, we aligned the amino acid sequences for many of the known genes and generated a phylogenetic tree to evaluate their relatedness. As shown in Figure 6, visual pigments that are thought to have similar spectral properties share a high degree of structural relatedness. The Rh5 visual pigment is most similar to other opsins that are thought to be short-wavelength absorbing, including the locust 2, bee blue, and moth Manop3 opsins, to which Rh5 is ~46, 49, and 50% identical, respectively (Fig. 6, *SW clade*). In contrast, Rh5 is only 30–35% identical to the *Drosophila* Rh1, Rh2, and Rh6 opsins and 40–44% identical to the Rh3 and Rh4 opsins. Interestingly, a subset of the SW-absorbing pigments appears to share a common ancestor with the UV-absorbing pigments. The Rh6 visual pigment belongs to a group of opsins that are proposed to form long-wavelength-absorbing rhodopsins (Fig. 6, *LW clade*). As with Rh5, Rh6 shares a greater similarity (>55%) with opsins of the LW group than with other *Drosophila* opsins. Rh6 shares a 51% identity with Rh1 and Rh2 and only a 30–33% identity with Rh3–Rh5 (Chou et al., 1996; Huber et al., 1997).

It is noteworthy that the only cloned invertebrate pigments that have been functionally expressed and characterized are the *Drosophila* rhodopsins Rh1–Rh6 (Feiler et al., 1988, 1992) (this paper) and the honeybee blue- and UV-absorbing pigments (Townson et al., 1998) (Fig. 6, indicated with ←). This group includes a large number of the UV- and SW-absorbing pigments shown in the tree; however Rh6 is the first and only member of the LW group to be formally characterized. As such, Rh6 both confirms and defines this group of pigments as likely having absorption maxima at longer wavelengths of light. In other experiments, we have found that the more distantly related *Limulus* lateral eye opsin (horseshoe crab 1) also confers LW sensitivity when ectopically expressed in *Drosophila* (B. E. Knox, E. Salcedo, W. C. Smith, W.-H. Chou, L. V. Chadwell, S. G. Britt, and R. Barlow, unpublished results).

The characterization of Rh6 as an LW-absorbing invertebrate opsin is particularly important because there is a large group of visual pigments that are within this clade. Although none of these pigments have been expressed and characterized, some of them are thought to encode red-absorbing visual pigments on the basis of their expression pattern (e.g., Pxu Rh3) (Kitamoto et al., 1998), whereas other long-wavelength-absorbing pigments have been characterized spectrophotometrically but have not yet been cloned (Schwemer and Paulsen, 1973; Langer et al., 1986). In addition, it is interesting to note that many of the SW visual pigments are included within clades that share common ancestors either with the UV visual pigments (in the case of Rh5 and its related pigments) or with the LW visual pigments (in the case of Rh1 and its related pigments). This suggests that there may be specific amino acid differences between the SW pigment group (like Rh5) and the UV pigments that are responsible for their different spectral properties. Thus our analysis of the *Drosophila* Rh5 and Rh6 opsins provides a framework in which the other invertebrate pigments can be grouped and studied, which is based on both structural and functional information about the pigments.

DISCUSSION

In this paper, we have described the spectral characterization of two *Drosophila* visual pigments, Rh5 and Rh6. Each protein falls into a structurally related group of rhodopsins that are distinguished from other groups by having absorption maxima at different wavelengths of light. Both Rh5 and Rh6 were ectopically expressed in the R1–R6 photoreceptor cells of blind *ninaE*^{17} flies, and we found that they are capable of restoring the light response of this mutant. Rh5 encodes a visual pigment that confers a maximal spectral sensitivity at 437 nm to the R1–R6 cells, whereas Rh6-expressing cells are maximally sensitive to light at 508 nm. We examined the absorption properties of the Rh5 opsin *in situ* using MSP and in visual pigment extracts and found that it can form a thermally stable metarhodopsin (λ_{max} = 494 nm) that can be reversibly photoconverted. After activation, Rh6 is converted to a metarhodopsin (λ_{max} = 468 nm), which appears to be less stable than the M-forms of other *Drosophila* rhodopsins.

The spectral and physiological properties of the *Drosophila* R8 photoreceptor cells have been difficult to examine because of their proximal location in the ommatidium and their small size. An early analysis of *Drosophila* photoreceptor cell function was undertaken using adapting lights and mutations to eliminate specific classes of photoreceptor cells (Harris et al., 1976). These studies showed that the R8 photoreceptor cells of *Drosophila* are sensitive to blue-green light with a maximal sensitivity near 500 nm. Physiological studies performed in larger flies, *Calliphora* or *Musca,* have identified four different classes of R8 photoreceptor cells. Two minor classes include the R8marg cells, whose rhabdomeres are located beneath those of the R7marg cells along the dorsal margin of the eye. The R8marg cells express a UV-absorbing pigment identical to that found in the R7marg cells (Hardie, 1984). In *Drosophila*, the R7marg and R8marg cells express Rh3 (Fortini and Rubin, 1990; Feiler et al., 1992). The R8r cells have only been found in *Musca* males and express a pigment that is identical to that found in both the R1–R6 cells and the sex-specific R7r cells whose rhabdomeres lie above the R8r rhabdomeres (Hardie, 1983). The two major classes of R8 cells (R8p and R8y) are distinguished on the basis of their paired occurrence within ommatidia that contain the R7p or R7y photoreceptor cells, respectively. Interestingly, we have found that the pairing of different classes of R7 and R8 photoreceptor cells in individual ommatidia also occurs in *Drosophila*. Specifically, the R7p and R8p photoreceptors correspond to the R7 and R8 cells of *Drosophila* that express Rh3 and Rh5 in a pairwise manner, and R7y and R8y correspond to the *Drosophila* R7 and R8 cells that express Rh4 and Rh6 (Chou et al., 1996, 1999). These classes of ommatidia were originally identified on the basis of their appearance under blue illumination and fluorescence (y for yellow; p for pale) (Kirschfeld et al., 1978; Franceschini et al., 1981). The R8p and R8y cells of *Calliphora* and *Musca* have maximum spectral sensitivities at ~440 and 540 nm, respectively (Hardie et al., 1979; Smola and Meffert, 1979). The sensitivity of the R8y cell is thought to be shifted from the predicted rhodopsin λ_{max} (~520 nm) to longer wavelengths (540 nm) because of the screening effects of the overlying R7y cell rhabdomere (Hardie et al., 1979). The R8y cells also displayed a small triplet of peaks in the UV region, indicating the presence of a sensitizing pigment similar to that seen in the R1–R6 cells; however no UV sensitivity was found in the R8p cells (Hardie and Kirschfeld, 1983).

The spectral sensitivities measured in the present study from transgenic flies expressing Rh5 and Rh6 are in excellent agree-

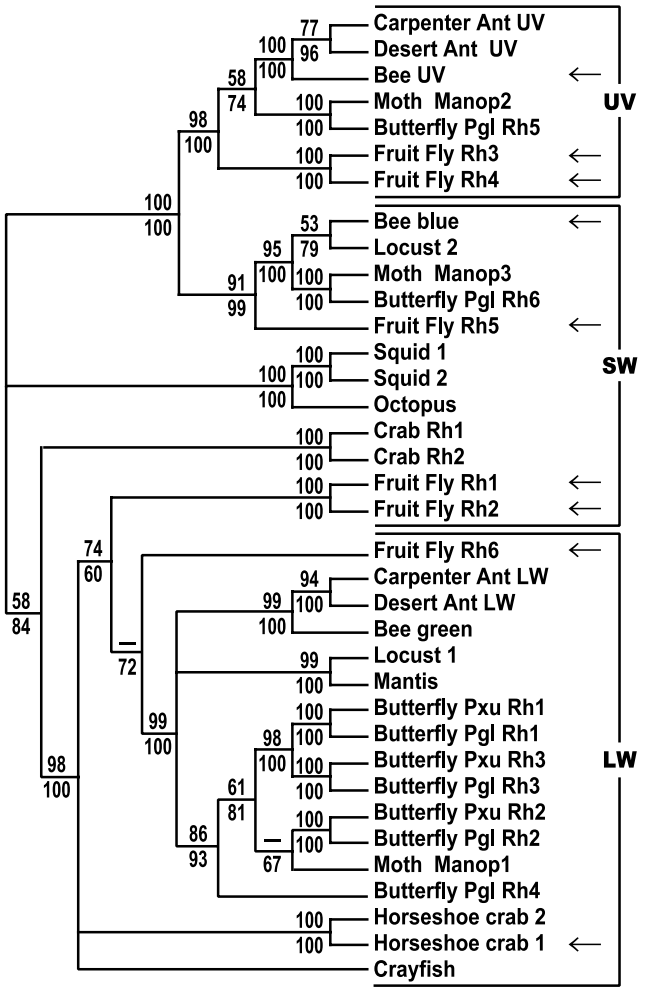

Figure 6. Phylogenetic relationships between selected invertebrate opsins. The deduced amino acid sequences of cloned invertebrate opsin genes were aligned and used to generate a phylogenetic tree, as described in Materials and Methods. Both maximum parsimony (unweighted) and neighbor-joining methods were used, and the *values* at each node represent the percentage of 100 bootstrap replicates that yielded the indicated structure (parsimony/neighbor-joining). Nodes with bootstrap values <60% are indicated as − and if <60% for both analyses were collapsed. Visual pigments were grouped into three families based on their spectral properties. The ultraviolet (*UV*) pigment family includes those with maximal absorptions below 400 nm. The short-wavelength (*SW*) family includes those pigments having maximal absorptions between 400 and 500 nm. The long-wavelength (*LW*) family includes those pigments with maximal absorptions above 500 nm. Inclusion within a family was based on one or more of three criteria: (1) direct characterization of the rhodopsin after ectopic expression in *Drosophila*, (2) well characterized spectral sensitivity or absorption measured from the native organism that is consistent with the placement within the tree, and (3) position within the phylogenetic tree with respect to other better-characterized visual pigments. The first criterion was met by *Drosophila* (fruit fly) Rh1–Rh6, bee blue and UV, and the horseshoe crab 1 pigments (indicated with ←) (Feiler et al., 1988, 1992; Townson et al., 1998) (Knox, Salcedo, Smith, Chou, Chadwell, Britt, and Barlow, unpublished results; this paper). The second criterion was met by the following pigments. Squid 1 and 2 are thought to have a λ_{max} of 494 and 480 nm, respectively (Suzuki et al., 1976; Morris et al., 1993). The desert and carpenter ant UV pigments are thought to have a λ_{max} of 360 nm, whereas the desert and carpenter ant LW pigments are thought to have a λ_{max} of 510 nm (Popp et al., 1996; Smith et al., 1997). The bee green pigment is thought to have a λ_{max} of 526 nm (Chang et al., 1996). The octopus pigment is thought to have a λ_{max} of 475 nm (Koutalos et al., 1989). One or both of the crab pigments are thought to have a λ_{max} of ~480 nm (Sakamoto et al., 1996). The crayfish pigment appears to have a λ_{max} of 533 nm (Hariyama et al., 1993; Zeiger and Goldsmith, 1994). Locust 1 and 2 are thought to have a λ_{max} of 520 and 430 nm, respectively (Towner et al., 1997). The moth Manop1, Manop2, and Manop3 pigments are thought to have a λ_{max} of 520, 357, and 450 nm, respectively (Chase et al., 1997). Butterfly Pxu Rh1 and Rh2 are thought to have a λ_{max} of 515 nm, whereas Pxu Rh3 is thought to have a λ_{max} of 575 nm (Kitamoto et al., 1998; Arikawa et al., 1999). The horseshoe crab 2 pigment is thought to have a λ_{max} of 530 nm (Smith et al., 1993). The remaining pigments were organized on the basis of the third criterion. This group includes the visual pigments cloned from *Papilio glaucus* (Pgl Rh1–Rh6) that are closely related to the Pxu Rh1–Rh3 or the moth Manop1 and Manop2 opsins (Briscoe, 1998, 1999) (A. D. Briscoe, personal communication) and the Mantis opsin, which is closely related to Locust 1. The phylogenetic tree shows that opsins are typically more closely related to pigments that share similar spectral properties than to pigments from the same species. Rh5 belongs to a group of related visual pigments that appear to be *SW* absorbing, whereas Rh6 belongs to a class of *LW*-absorbing pigments. Rh6 is the first opsin of this class to be functionally characterized. GenBank accession numbers: *Apis mellifera* (Bee UV, AF004169; blue, AF004168; green, U26026), *Camponotus abdominalis* (Carpenter Ant UV, AF042788; LW, U32502), *Cataglyphis bombycina* (Desert Ant UV, AF042787; LW, U32501), *Drosophila melanogaster* (Fruit Fly Rh1, P06002; Rh2, P08099; Rh3, P04950; Rh4, P29404; Rh5, U67905; Rh6, Z86118), *Hemigrapsus sanguineus* (Crab Rh1, D50583; Rh2, D50584), *Limulus polyphemus* [Horseshoe crab 1 (lateral eye), L03781; 2 (ventral eye), L03782], *Loligo forbesi* (Squid 1, X56788), *Octopus dofleini* (Octopus, X07797), *Papilio xuthus* (Butterfly Pxu Rh1, AB007423; Pxu Rh2, AB007424; Pxu Rh3 AB007425), *Papilio glaucus* (Butterfly Pgl Rh1, AF077189; Pgl Rh2, AF077190; Pgl Rh3, AF067080; Pgl Rh4, AF077193; Pgl Rh5, AF077191; Pgl Rh6, AF077192), *Procambrus clarkii* (Crayfish, S53494), *Schistocerca gregaria* (Locust 1, X80071; Locust 2, X80072), *Sphodromantis sps* (Mantis, X71665), and *Todarodes pacificus* (Squid 2, X70498). Genome Sequence Database (GSDB) accession numbers (www.ncgr.org/cgi-bin/ff): *Manduca Sexta* (Moth Manop1, 76082; Manop2, 109852; Manop3, 1249561).

ment with the measurements from the R8p and R8y cells of larger flies, respectively, both in terms of their spectral sensitivities and in that Rh6 but not Rh5 appears to couple to the sensitizing pigment. Furthermore, our current results are also in good agreement with previous studies in *Drosophila*. Spectral sensitivity measurements made from *sev; ora* flies, which lack the R7 photoreceptor cells and the Rh1 opsin, demonstrated that the remaining R8 cells had blue-green sensitivity with a maximum near 500 nm (Harris et al., 1976; Washburn and O'Tousa, 1989). Although these experiments did not resolve the diversity of R8 cells present in wild-type flies, the spectral sensitivity is consistent with that of the class of R8 cells that express Rh6 (λ_{max} = 508 nm). Interestingly, in other experiments examining the basis for the paired expression of opsin genes in the R7 and R8 cells of individual ommatidia, we have found that the expression of Rh5 versus Rh6 is dependent on the presence of an R7 cell. Indeed, in *sev* mutant flies (as would be the case in *sev; ora*), we have found that there is a dramatic decrease in the number of R8 cells that express Rh5, and virtually all of the R8 cells express Rh6 (Chou et al., 1996, 1999). And just as we were unable to measure a difference spectrum by MSP *in vivo* or induce a PDA in transgenic flies expressing Rh6, no MSP difference spectrum could be elicited from *sev; ora* flies, and the spectral sensitivity of *sev; ora* flies was unchanged by light adaptation (Harris et al., 1976). These results are consistent with the idea that the Rh6 metarhodopsin may be short-lived and that the primary class of R8 photoreceptor cells that is present in *sev; ora* flies is that expressing Rh6. However, on the basis of the available data we cannot eliminate the possibility that our inability to obtain an MSP spectrum and to induce a PDA in flies expressing Rh6 in R1–R6 cells simply reflects the low level of photoconvertible rhodopsin present in the

eyes of these flies. Whether or not a reduced thermal stability is characteristic of all M-forms derived from the LW class of invertebrate rhodopsins remains to be determined. Complete photoreversibility of the extracted pigments, i.e., photoconversion of R to M and vice versa without decay of substantial amounts of rhodopsin, has only been shown at −15°C for the long-wavelength-absorbing (520 nm) visual pigment of the moth *Deilephila* that forms an M-form with maximal absorbance at 475 nm (Schwemer and Paulsen, 1973).

This work completes the characterization of all of the known, cloned, *Drosophila* opsin genes. Although more opsin genes may potentially be identified as the *Drosophila* genome is completed, there are no known classes of photoreceptor cells that would necessarily require additional genes. The characterization of Rh5 and Rh6 is an important addition to the characterization of invertebrate visual pigments, because they have unique spectral properties and functionally define groups of related pigments that are as yet uncharacterized. As described in Results, Rh6 is the first cloned LW-absorbing invertebrate visual pigment to be functionally characterized. Its position within a well supported clade of visual pigments that are thought to be LW absorbing provides the only available evidence that these genes do in fact encode LW pigments.

One of the primary interests behind the study of the invertebrate visual pigments is the study of color vision. Several invertebrate species have been show to use color vision in specific behaviors. Honeybees are well known to use color vision in foraging and in returning to the hive (for review, see Menzel and Muller, 1996). Swallowtail butterflies, blowflies, and the mantis shrimp have also been shown to use color vision (Fukushi, 1985, 1989; Troje, 1993; Marshall et al., 1996; Kinoshita et al., 1999). Limited evidence suggests that *Drosophila* may also be able to discriminate between different colors (Quinn et al., 1974; Spatz et al., 1974; Menne and Spatz, 1977; Bicker and Reichert, 1978). The visual system of *Drosophila* is ideally suited to an analysis of color vision because of the wide range of genetic and molecular approaches that are available to identify new genes, manipulate them, and study the effects *in vivo*, in the intact fly. These approaches have provided important insights into the basis of photoreceptor cell recruitment and phototransduction (Dickson and Hafen, 1993; Zuker, 1996; Treisman and Heberlein, 1998) and have the potential to reveal important aspects of color vision in *Drosophila*. The characterization of Rh1–Rh6 provides a comprehensive view of the visual pigments and photoreceptor cell sensitivities in the *Drosophila* compound eye and opens up the possibility of conducting molecular and genetic studies of color vision in a well defined, genetically tractable experimental system.

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