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Phylogeny and Physiology of *Drosophila* Opsins

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Abstract. Phylogenetic and physiological methods were used to study the evolution of the opsin gene family in *Drosophila*. A phylogeny based on DNA sequences from 13 opsin genes including representatives from the two major subgenera of *Drosophila* shows six major, well-supported clades: The "blue opsin" clade includes all of the *Rh1* and *Rh2* genes and is separated into two distinct subclades of *Rh1* sequences and *Rh2* sequences; the ultraviolet opsin clade includes all *Rh3* and *Rh4* genes and bifurcates into separate *Rh3* and *Rh4* clades. The duplications that generated this gene family most likely took place before the evolution of the subgenera *Drosophila* and *Sophophora* and their component species groups. Numerous changes have occurred in these genes since the duplications, including the loss and/or gain of introns in the different genes and even within the *Rh1* and *Rh4* clades. Despite these changes, the spectral sensitivity of each of the opsins has remained remarkably fixed in a sample of four species representing two species groups in each of the two subgenera. All of the strains that were investigated had R1–6 (*Rh1*) spectral sensitivity curves that peaked at or near 480 nm, R7 (*Rh3* and *Rh4*) peaks in the ultraviolet range, and ocellar (*Rh2*) peaks near 420 nm. Each of the four gene clades on the phylogeny exhibits very conservative patterns of amino acid replacement in domains of the protein thought to influence spectral sensitivity, reflecting strong constraints on the spectrum of light visible to *Drosophila*.

Key words: Opsin — Visual pigments — Gene family — Evolution — Phylogeny — Spectral sensitivity

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Introduction

Visually mediated behavior in animals begins with the absorption of light by rhodopsins. These sensory molecules are embedded in specialized membranes in photoreceptor cells and consist of two essential components: The protein component, or opsin, and a light-sensitive chromophore, usually 11-*cis* retinal or 3-hydroxy retinal. Like other G-protein coupled receptors, opsins have seven membrane-spanning alpha helices. The chromophore is covalently attached to a lysine in the seventh transmembrane domain of all rhodopsins that have been investigated (reviewed in Applebury and Hargrave 1986; Nathans 1987). Absorption of a photon of light by rhodopsin initiates a cascade of biochemical events that results in a change in the membrane potential of the photoreceptor cell (reviewed in Stryer 1986; Smith et al. 1991).

Many animals have multiple opsin genes encoding independent visual pigments that are expressed in different photoreceptor cell types and respond to distinct wavelengths of light. Models designed to explain differences in spectral sensitivity among opsins suggest

that the distribution of charged and polar amino acids in membrane-spanning domains of the protein determines the amount of energy needed to excite the electrons associated with the chromophore, and therefore the wavelength of light required to elicit a response by the visual pigment (Hubbard and Sperling 1973; Nathans 1987). For example, mammalian rhodopsins normally show a maximal response to light of approximately 480 nm. A conserved glutamic acid residue in the second transmembrane domain of all mammalian rhodopsins is thought to act as a counterion to the chromophore. Replacement of the negatively charged glutamic acid with glutamine, a neutral amino acid, produces a pigment that requires higher-energy light of 380 nm to elicit a response (Sakmar et al. 1991; Zhukovsky and Oprian 1989; Nathans 1990a). The distribution of charged and polar amino acids at specific sites in vertebrate red and green visual pigments has also been shown to affect spectral properties of these molecules (Neitz et al. 1991; Chan et al. 1992; Winderickx et al. 1992). However, systematic mutagenesis of bovine rhodopsin has shown that not all membrane-bound amino acid sites influence spectral sensitivity (Nathans 1990b).

Comparative and experimental data suggest that the role of opsins as integral membrane proteins and as primary photoreceptor molecules places significant constraints on their evolution, and that selection for responses to different wavelengths of light can effect changes in the amino acid sequence. To investigate in detail the molecular and functional evolution of opsins in a defined taxon of organisms, we have undertaken a study of several *Drosophila* species to determine the range of spectral sensitivities in the different photoreceptor cell types, as well as the nature of amino acid substitutions in the corresponding opsins. *Drosophila* is an excellent system for studying molecular evolution of opsins. The phylogeny of several species groups has been thoroughly investigated (Beverly and Wilson 1984; DeSalle 1992; Grimaldi 1990) and provides an excellent framework for comparative studies. In addition, the visual system of *Drosophila melanogaster* has been the subject of intense investigation (reviewed by Smith et al. 1991).

There are four classes of photoreceptor cells in the *Drosophila* visual system, and the opsins expressed in three of these cell types have been cloned and characterized. Each ommatidium in the compound eye has eight photoreceptor cells (R1–8) arranged in a stereotypical pattern: R1–6 are the peripheral photoreceptors in this array, and R7 and R8 reside in the center of the ring formed by R1–6 (reviewed in Pak and Grabowski 1978). The major opsin, encoded by the *ninaE* (*Rh1*) locus, is expressed in R1–6 (O'Tousa et al. 1985; Zuker et al. 1985) and responds maximally to 480-nm light (Harris et al. 1976). Rh3 and Rh4 are ultraviolet (345 nm and 375 nm, respectively) receptors expressed in nonoverlapping sets of R7 cells (Montell et al. 1987; Zuker et al. 1987; Pollock and Benzer 1988; Fortini and Rubin 1990; Feiler et al. 1992). The R8 opsin gene has not been cloned (Fortini and Rubin 1990). The three dorsally located simple eyes, the ocelli, express the Rh2 opsin (Cowman et al. 1986; Fryxell and Meyerowitz 1987), which is sensitive to 420-nm light (Hu et al. 1978). Rh1 and Rh2 are sensitized to ultraviolet light by nonopsin pigments in R1–6 and ocelli (Hu et al. 1978; Kirschfeld et al. 1977; Stark et al. 1977). Figure 1 summarizes much of the available information on the expression, phylogeny, and spectral properties of *Drosophila melanogaster* opsins.

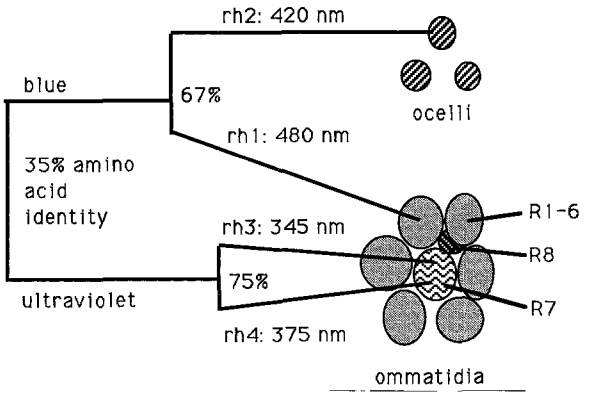


Fig. 1. Phylogeny, gene expression, and spectral properties of *Drosophila melanogaster* rhodopsins. Each of the 700 or so ommatidia in the compound eye of Drosophila has eight photoreceptor cells arranged in this stereotypical pattern. The distinct types of photoreceptor cells indicated by differential shading are classified by the opsin that is expressed and by genetic criteria that group cells together based on mutations that affect individual cells or groups of cells (Fortini and Rubin 1990). The phylogeny is modeled after Zuker et al. (1987) as well as our own results, and the branch lengths do not reflect degree of sequence divergence. *Rh3* and *Rh4* are expressed in nonoverlapping sets of R7 cells, so an individual R7 never as both opsins.

Electroretinograms (ERGs) were used to determine the spectral properties of compound eyes and ocelli of white-eyed strains of *Drosophila simulans, D. pseudoobscura, D. mercatorum,* and *D. virilis.* Together with *D. melanogaster* data available from previous studies (Harris et al. 1976), this sample includes representatives of two species groups in each of the two major subgenera of Drosophila: *D. melanogaster* and *D. simulans* representing the *melanogaster* group in the subgenus *Sophophora; D. pseudoobscura* from the *obscura* group in the *Sophophora; D. virilis* from the *virilis* group of the subgenus *Drosophila;* and *D. mercatorum* from the *repleta* group in the *Drosophila* (Grimaldi 1990). We also used the polymerase chain reaction (PCR) to obtain DNA sequences for *Rh1* from each of the strains subjected to the physiological analyses and for *Rh2* from *D.*

simulans and *D. pseudoobscura*. Multiple sequence alignments and phylogenetic analyses of these opsins, as well as additional opsin sequences available from the literature, were used to analyze patterns of amino acid replacements and nucleotide substitutions relative to existing structural and functional models for opsins. Most of the discussion is focused on *Rh1* and *Rh2*, but the ultraviolet opsins *Rh3* and *Rh4* will also be discussed.

Materials and Methods

Drosophila Strains. To analyze the ERG by chromatic adaptation, white-eyed strains of *Drosophila* were used. White-eyed *Drosophila mercatorum* was a spontaneous mutation obtained from Susan Lawler (Washington University); white-eyed *Drosophila pseudoobscura* was from the Bowling Green Stock center; the white-eyed *Drosophila simulans* was a spontaneous mutation obtained in the Hartl laboratory; two white-eyed *D. virilis* strains were used, one from the Bowling Green Stock center, and another provided by Elena Lozovskaya (Harvard University). Flies were reared on standard medium in which the zeaxanthin in yellow cornmeal plus a supplement of β-carotene (1.25 mg/ml) insures an adequate vitamin A supply for complete visual receptor development (Stark et al. 1988).

Electroretinography. The electroretinograph (ERG) measures the response of the entire compound eye or ocellus to specific wavelengths of light. To isolate spectra from different photoreceptor cells in the compound eye (R1–6, R7, and R8), chromatic adaptation was used to sequentially eliminate specific receptor contributions to the mass ERG response (Stark 1975; Stark et al. 1976). The spectral components so isolated have been shown to correspond to specific receptor cells by genetic dissection (Harris et al. 1976; Stark et al. 1976). In *Drosophila melanogaster,* chromatic adaptation has the extreme effect of inactivating specific receptor types with a prolonged depolarizing afterpotential (PDA). For example, maximal conversion of the 480-nm absorbing rhodopsin in R1–6 to its stable photoproduct, the 570-nm absorbing metarhodopsin (Stark and Johnson 1980), induces a PDA that eliminates all electrical responses from R1–6 (Stark and Zitzmann 1976). Since R1–6 generally dominate the ERG response of the compound eye, the PDA allows the ERG responses of R7 and R8 to be examined. Similarly, maximal conversion of UV visual pigments of R7 (Harris et al. 1976) to their photoproducts induces an R7 PDA (Stark 1977) and inactivates R7. This operation thus isolates the R8 contribution to the ERG. In this study ERGs elicited by dim stimuli (in flies that were previously adapted to 625-nm, 470-nm, and 370-nm light) were used to obtain spectral sensitivities dominated by R1–6, R7 + R8, and R8, respectively.

The compound eye of 4-day posteclosion flies was carefully located at the focal plane of the optical stimulator using 625 nm at $\log_{10}I = 16.25$ (quanta/cm^2*s) for insertion of the glass micropipette into the retinula cell layer. The fly was then dark adapted for 40 min before collecting the spectral sensitivity for a 2-mV ERG peak-to-peak criterion. The PDAs were induced by applying the full intensities at 370 and 470 nm of about 14.5 and 16.0 $\log_{10}$quanta/cm^2*s, respectively, for 2 s. These adaptations were applied just half a minute or less before each test stimulus to obtain the adapted spectral sensitivities. Since there were no on- and off-transients in the short-wavelength adapted state, only the remaining negative wave contributed to the criterion ERG potential of 2 mV. The PDA was repolarized by 570-nm light at an average intensity of about 16.3 $\log_{10}$quanta/cm^2*s. The ocellar ERG was obtained generally from the left lateral ocellus, although pilot experiments established that no differences were found among ocelli. A micropipette was inserted locally into the ocellus as had been done previously for wild-type *D. melanogaster* as well as a number of visual mutants (Hu et al. 1978). The ERG criterion was 1.0 mV.

Amplification and Sequencing of Opsin Genes. The polymerase chain reaction (PCR) was used to amplify *Rh1* (all four strains) and *Rh2* (*D. simulans* and *D. pseudoobscura*) sequences from the same *Drosophila* strains that were used for the spectral sensitivity experiments. The PCR primers were based on sequence conservation between *D. melanogaster* and *D. pseudoobscura* opsins (Carulli and Hartl 1992), and their sequences are as follows:

*Rh1*U: 5′ AATGGATCGGTGGTGGATAAGGT 3′
*Rh1*L: 5′ GCCAAAGACGCAGCAAGGACACTT 3′
*Rh2*c028: 5′ CTACTGCCCGACATGGCGC 3′
*Rh2*c041: 5′ TCCGGCTTGGGCTCATCCGT 3′

The PCR products were cloned (*D. virilis Rh1, D. mercatorum Rh1*) prior to sequencing, or were sequenced directly after purification from the rest of the reaction components (*D. simulans Rh1* and *Rh2, D. pseudoobscura Rh1* and *Rh2,* one strand of *D. mercatorum Rh1*). PCR products for cloning were gel purified using NA45 paper (Schleicher and Schuell; Sambrook et al. 1989), blunt-ended using the Klenow fragment of DNA polymerase I (Sambrook et al. 1989), and ligated into the *Sma*I site of pBluescript (Stratagene). Plasmids were transformed into XL1 Blue cells by electroporation (BioRad). Plasmids for sequencing were purified by alkaline lysis minipreps (Sambrook et al. 1989). Two independent transformants from a pool of PCR products were sequenced on both strands for *D. virilis Rh1*. For *D. mercatorum Rh1,* both strands of a single transformant and a single strand of an independently amplified PCR product were sequences. PCR products for sequencing were prepared by phenol extraction of the reaction products followed by precipitation of DNA in the presence of 2.5 M NH_4OAc and 50% ethanol. Both strands of each product were sequenced. Sequencing was carried out on an Applied Biosystems (ABI) 373A sequencer using the ABI Taq Dyedeoxy Terminator cycle sequencing kit according to manufacturer's specifications.

Sequence Analysis. All sequences were transferred directly from the ABI fluorescent sequencer to MacVector (IBI) software, where they were aligned with opsin sequences from *D. melanogaster* or *D. pseudoobscura* using the Pustell DNA matrix alignment program in MacVector and/or the GAP alignment function in GCG (Devereux et al. 1984). Amino acid sequences were inferred using the GAP program in GCG. Additional sequences were obtained from our previous studies (Carulli and Hartl 1992) or from Genbank (release 72).

Multiple alignments of DNA and amino acid sequences were performed with Clustal software (Higgins and Sharp 1988). DNA sequence alignments for phylogenetic analyses were refined by hand to correspond to the amino acid alignments. Phylogenetic analyses were carried out using PAUP, version 3.0r (Swofford 1992). DNA sequences rather than protein sequences were used to maximize the number of characters, and two separate analyses of the sequences were performed. In one analysis, third base positions were excluded to eliminate homoplasies due to multiple substitutions at synonymous nucleotide sites, and transitions and transversions were weighted equally. The branch and bound algorithm, which always produces the shortest tree, was used to search for trees and for bootstrapping the trees. The complete DNA sequences were used in the second set of

analyses, which included one analysis with transitions and transversions weighted equally and a second with transversions assigned five times the weight of transitions to approximate the transition/transversion ratio observed in a subset of the data. The branch and bound algorithm was used to search for the shortest tree, but time constraints (because of the larger data set) required the use of the heuristic search option for bootstrap analysis.

Results

ERG Waveform Analyses

An exhaustive analysis of the compound-eye spectral sensitivities and PDA properties of the four species is presented in Fig. 2. The major findings suggest that each of these species have compound-eye receptor properties much like those of *D. melanogaster* (Harris et al. 1976). These findings are: (1) Dark-adapted spectra, which are dominated by R1–6, have ultraviolet and blue sensitivity maxima indicative of 480-nm rhodopsins (Stark and Johnson 1980) sensitized to ultraviolet by an additional pigment (Kirschfeld et al. 1977; Stark et al. 1977). (2) Blue adaptation induces a PDA and inactivates R1–6. (3) In the blue-adapted condition, a UV-peaking spectral sensitivity is obtained, indicating that the R7 + R8 ERG so isolated is dominated by a UV receptor type, presumably R7. (4) In *D. simulans* (Fig. 2C) and *D. virilis* (Fig. 2E), UV adaptation causes a further depression in UV sensitivity, suggesting isolation of R8's contribution to the compound-eye ERG. For *D. mercatorum,* and *D. pseudoobscura,* UV adaptation increases sensitivity (relative to blue adaptation) at all wavelengths, suggesting partial reactivation of the R1–6 ERG. Therefore, the functions of R7 and R8 could not be dissected with the same resolution as previously done for *D. melanogaster* (Harris et al. 1976).

The ERG spectral sensitivities of ocelli for the four different species are given in Fig. 3. The ocellar spectra in all of these species are similar to those observed for *D. melanogaster* (Hu et al. 1978), with blue and UV maxima. The data are consistent with the UV sensitization of a blue rhodopsin. The ocellar rhodopsin has a shorter-wavelength maximum (420 nm) than the blue peak mediated by the R1–6 rhodopsin. Figure 4 shows the spectral sensitivity of *D. mercatorum* after maximal chromatic adaptation. Intense 370- and 470-nm adaptation decreases overall sensitivity in this selected species but does not change the shape of the spectrum. The same experiment was replicated on the other three species in this study and produced similar results. This finding parallels the *D. melanogaster* result (Hu et al. 1978) and is consistent with the presence of one rhodopsin in ocelli which is encoded by the *Rh2* gene (Feiler et al. 1988; Pollock and Benzer 1988).

Molecular Organization of Drosophila Opsin Genes

The intron/exon organization of all sequenced *Drosophila* opsins is shown in Fig. 5. As expected, *D. simulans* and *D. pseudoobscura* opsins have introns in the same position as the orthologous opsins in *D. melanogaster.* Interestingly, the *D. virilis* and *D. mercatorum* opsins have an additional intron in the *Rh1* gene which interrupts the same amino acids as the first intron in the *Rh2* loci. There are also interspecific differences in the intron/exon structure of *Rh4,* as shown by Neufeld et al. (1991). For the parts of the genes that were sequenced, there are no insertions or deletions of more than one amino acid between any orthologous pairs.

Sequence Alignment and Amino Acid Replacements

The complete alignment of 13 *Drosophila* opsin amino acid sequences (this study; O'Tousa et al. 1985; Zuker et al. 1985; Cowman et al. 1986; Montell et al. 1987; Zuker et al. 1987; Fryxell and Meyerowitz 1987; Neufeld et al. 1991; Carulli and Hartl 1992), one Calliphora opsin sequence (Huber et al. 1990), and two molluscan opsin sequences (Ovchinikov et al. 1988; Hall et al. 1991) (included as outgroups for the phylogenetic analysis) are shown in Fig. 6. While the sequences in this figure are not complete, they include each of the seven transmembrane domains as well as several amino acids from the cytoplasmic and extracellular tails of the protein. For the 322 amino acid sites (including gaps) that are compared here, 61 sites are conserved among all of the opsins examined and an additional 37 sites are conserved among the Dipteran opsins.

Among the five *Drosophila* Rh1 sequences that were analyzed, there are only nine polymorphic amino acids. Rh2 is slightly more variable than Rh1. Even though only three Rh2 sequences from species in a single subgenus were examined, 13 amino acid replacements are observed. Previous analyses (Neufeld et al. 1991; Carulli and Hartl 1992) have shown that the cytoplasmic and extracellular tails of the opsins are more variable than the transmembrane regions, but the location of the PCR primers excludes most of the cytoplasmic and extracellular tails from the current analysis. Replacements involving charged or polar amino acids are enumerated in Table 1 and Table 2. Among the *Drosophila Rh1* sequences there is only one replacement involving a polar amino acid, serine, replacing the nonpolar alanine in the fifth transmembrane domain of *D. mercatorum.* Despite the similarity in spectral properties between *Drosophila Rh1*'s, which absorb maximally at 480 nm (Fig. 2; Harris et al. 1976) and *Calliphora Rh1,* which absorbs maximally at 488 nm (Huber et al. 1990), there are several replacements involving charged or polar

amino acids in *Calliphora* relative to the highly conserved *Drosophila* sequences. The transmembrane domains of *Rh2* are slightly more variable than those of *Rh1,* with three sites that are variable for polar amino acids among the three species that were examined.

Phylogeny of the Drosophila Opsins

Phylogenetic analysis of the opsin DNA sequences yields a tree with two large clades that are supported by all of the bootstrap replications: *Rh1* and *Rh2* sequences cluster together, and *Rh3* and *Rh4* sequences cluster together. Within the *Rh1/Rh2* clade, all *Rh1* sequences fall into a monophyletic group in 100% of the bootstrap replications and all *Rh2* sequences fall into a monophyletic group in all of the bootstraps. Likewise, within the *Rh3/Rh4* clade, the *Rh3* and *Rh4* sequences fall into two separate, fully supported monophyletic groups (Fig. 7). The same topology, with 100% support for each of the major clades, was observed with third positions of codons included (Fig. 7b) and with third positions of codons excluded (Fig. 7a) from the analysis. The *Drosophila Rh1* sequences are clearly delineated from the *Calliphora* sequence, but the extremely low rates of nucleotide substitution in this gene result in a phylogeny within the *Drosophila Rh1* clade that does not reflect the well-substantiated evolutionary relationships (Grimaldi 1990; DeSalle 1992) among the species involved. The sibling species *D. melanogaster* and *D. simulans* form a strongly supported clade, but the rest of the *Rh1* sequences do not follow the expected pattern of grouping *Sophophora* (*melanogaster* and *obscura* group) species in a monophyletic group and *Drosophila* (*virilis* and *repleta* group) species in a separate but closely related monophyletic group. When the data set that includes synonymous sites is analyzed (Fig. 7b), the taxonomically correct tree (not shown) is only one step longer than the shortest tree (2,076 steps vs 2,075 steps). Weighting transversions in the data set with all characters included resulted in a tree with a topology that is identical to that obtained with the unweighted characters.

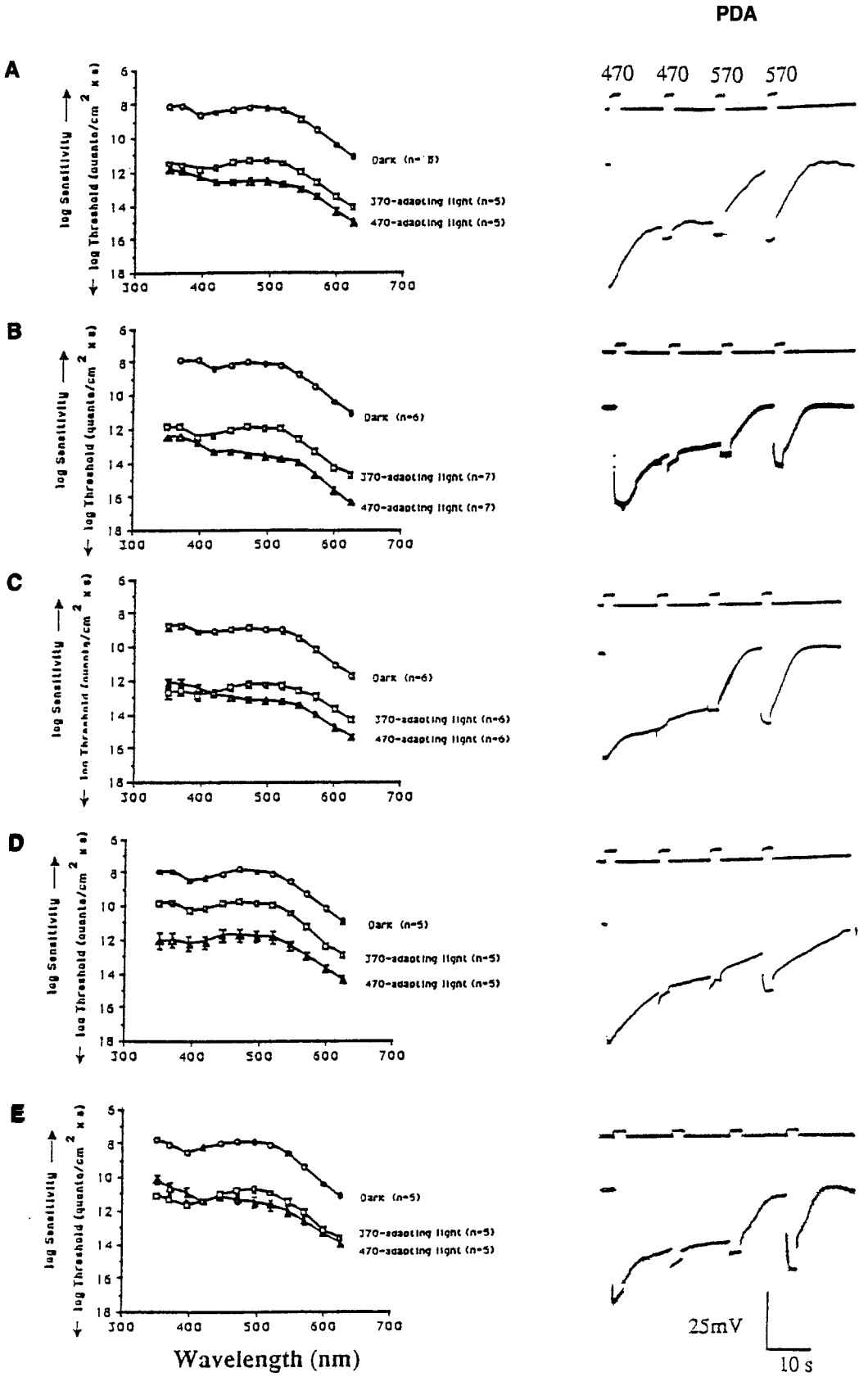
Discussion

Evolution of the Opsin Gene Family in Drosophila

The four opsin genes known from *Drosophila melanogaster* as well as other *Drosophila* species (Zuker et al. 1987; Fortini and Rubin 1990; Neufeld et al. 1991; Carulli and Hartl 1992) are at least as old as the genus *Drosophila.* Phylogenetic analysis of the gene family suggests all of the gene duplications occurred prior to the cladogenetic events that gave rise to the extant species groups. The opsins evolved through a series of duplications, the first of which gave rise to two major lineages: One lineage that includes the blue-absorbing *Rh1* and *Rh2,* and a second lineage that includes the ultraviolet opsins *Rh3* and *Rh4.* Within the blue opsin clade, a subsequent duplication gave rise to *Rh1* and *Rh2,* and these genes have evolved different cell-specific expression patterns and distinct absorption spectra. The relatively closely related *Rh3* and *Rh4* genes, which are expressed in the same photoreceptor cell type (R7), are the product of a separate duplication that also occurred before the origin of the two major subgenera of *Drosophila* approximately 60 million years ago (Beverly and Wilson 1984). The robustness of the tree that separates orthologous opsins into independent clades suggests that mechanisms of concerted evolution have not had significant effects on these genes during *Drosophila* radiation (Sanderson and Doyle 1992).

The molecular organization of the opsin genes has changed in several interesting ways during the divergence of the genes and the species that carry them. The number and position of introns vary not only among the different members of the gene family but also among *Rh1* genes in different lineages and *Rh4* genes in different lineages. *Rh1* has four introns in *D. melanogaster, D. simulans,* and *D. pseudoobscura* but has an additional intron in *D. virilis* and *D. mercatorum* (Fig. 5). Interestingly, the additional intron in the *D. mercatorum* and *D. virilis Rh1* genes interrupts the gene at precisely the same position as the first intron of *Rh2.* It appears likely that this intron was present in both *Rh1* and *Rh2*

Fig. 2. Spectral sensitivity (*left*) and PDA (*right*) analyses of the compound-eye ERGs of five strains of flies: **A** *Drosophila mercatorum;* **B** *Drosophila pseudoobscura;* **C** *Drosophila simulans;* **D** the *Drosophila virilis* strain which lacks R7; and **E** the *Drosophila virilis* strain which does have R7. The ordinate on the spectra shows sensitivity plotted upward as the inverse of the threshold (downward) for the criterion ERG voltage (in units of $\log_{10}$quanta/cm^2*s). Sensitivities as a function of wavelength (nm) for dark adapted, blue (470 nm)-adapted, and UV (370 nm)-adapted conditions are clearly labeled for each strain along with the number of animals averaged for each curve (with SE's shown). The PDAs (*right*) are photographs of the oscilloscope face annotated for the test wavelengths (shown as upward deflections on the *upper trace* in each case). The *lower trace* shows the large ERG potentials recorded on a slow time scale (note calibration axes in *lower right corner*) elicited by maximal intensities of the stimulus wavelengths labeled; negative is plotted downward. The sustained negativity after 470-nm stimulation relative to the prestimulus baseline or to after 570-nm stimulation is the PDA (prolonged depolarizing afterpotential) generated by R1–6 photoreceptors in the compound eye.


PDA
470 470 570 570
A
B
C
D
E
log Sensitivity
log Threshold (quanta/cm² x s)
Dark (n=5)
370-adapting light (n=5)
470-adapting light (n=5)
Dark (n=6)
370-adapting light (n=7)
470-adapting light (n=7)
Dark (n=6)
370-adapting light (n=6)
470-adapting light (n=6)
Dark (n=5)
370-adapting light (n=5)
470-adapting light (n=5)
Dark (n=5)
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470-adapting light (n=5)
300 400 500 600 700
Wavelength (nm)
25mV
10 s

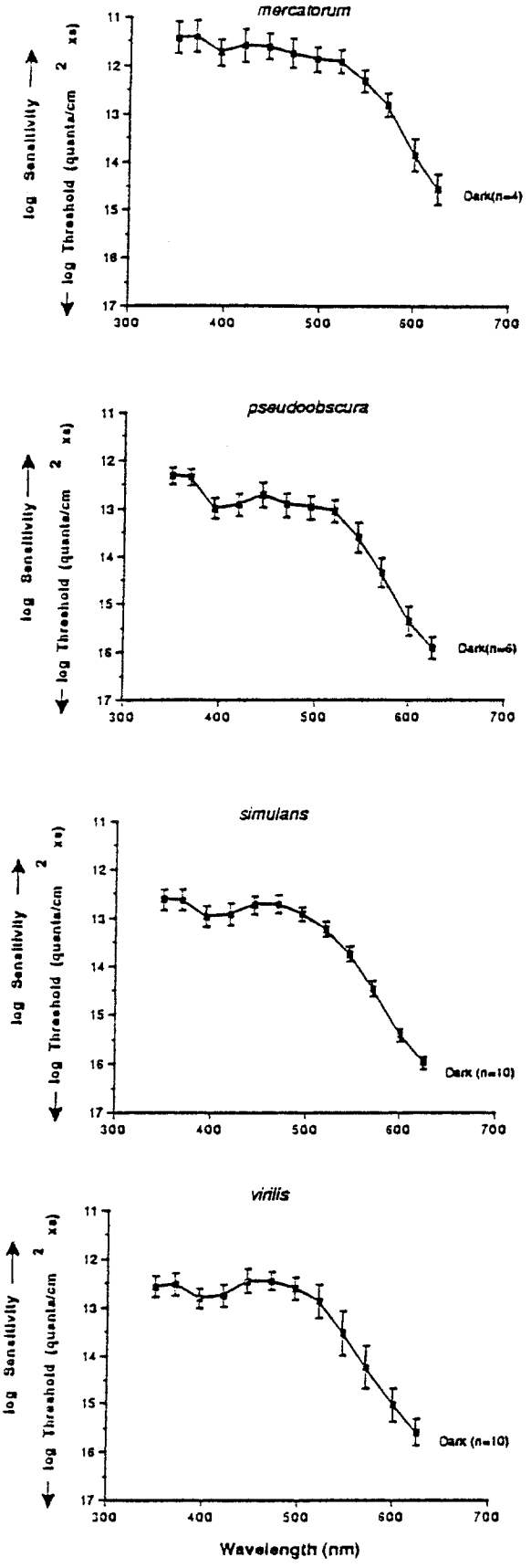

Fig. 3. The ERG ocellar spectral sensitivities of the four species of *Drosophila* as labeled. All analyses are as in Fig. 3.

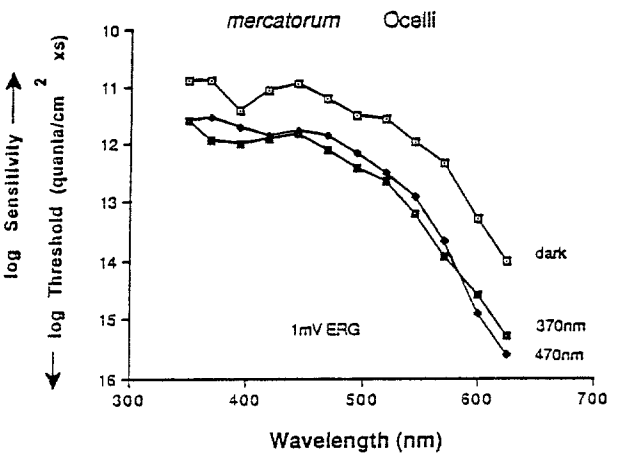

Fig. 4. A typical experiment showing adaptation in the ocellar ERG's spectral sensitivity for *Drosophila mercatorum.* The sensitivity for dark-adapted animals is highest. Maximal blue (470 nm) and UV (370 nm) adaptations depress overall sensitivity but do not change the shape of the spectral sensitivity curve.

at the time of the duplication but was subsequently lost from *Rh1* in the lineage leading to the subgenus *Sophophora.* The alternative to this scenario, that introns independently invaded the exact same site in two different genes in two separate events, is unlikely. The *Rh4* genes also exhibit a loss or gain of an intron during the 60 million years of Drosophila evolution. The large intron in *D. melanogaster Rh4* has another gene, *seven in absentia* (*sina*), nested within it (Carthew and Rubin 1990), and this arrangement appears to be conserved in *D. pseudoobscura* (Carulli and Hartl 1992). The *D. virilis Rh4* gene has no introns (Neufeld et al. 1991).

Spectral Sensitivities are Highly Similar Among Divergent Drosophila Species

The spectral mechanisms of all photoreceptor types are remarkably similar among the *Drosophila* species that were examined. R1–6 spectra (Fig. 2) suggest that all the species analyzed have the blue (470–480 nm)-absorbing rhodopsins and UV-sensitizing pigments that have been previously observed in *D. melanogaster* (Hardie 1985; Kirschfeld et al. 1977; Stark et al. 1977; Stark and Johnson 1980). The PDAs of the species analyzed are also quite similar to that of *D. melanogaster* (Stark and Zitzmann 1976), and the repolarization of the PDA by 570-nm adaptation (Fig. 2) suggests that all species have similar long-wavelength metarhodopsins. In all *Drosophila* species that have been studied the PDA is very long-lived, which differentiates *Drosophila* PDAs from those of white-eyed mutants of other dipterans (Stark et al. 1977).

R7 is an ultraviolet receptor in all of the presently

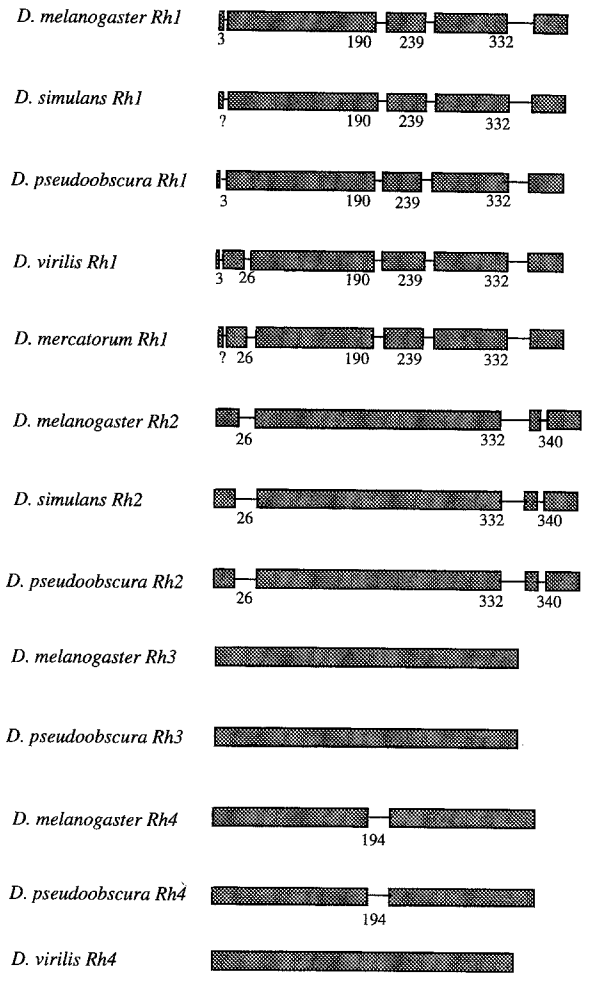

Fig. 5. Intron-exon structure of *Drosophila* opsin genes. Exons are *shaded* and introns are denoted by *thin lines*. The size of each of the exons is roughly proportional to their size in nucleotide bases, but the size of the introns is not proportional to their actual sizes because the length of the lines was modified to align introns that are in homologous locations in the different genes. The *numbers* beneath each gene indicate the amino acid position (numbered according to *D. melanogaster Rh1*) that is interrupted by the intron. *Question marks* indicate that a portion of the gene was not sequenced, and the presence or absence of that intron is not known. The positions of the first two introns in *D. virilis Rh1* were independently observed by M. Fortini and G. Rubin (personal communication).

studied species, as demonstrated by the blue-adapted ERG spectra (Fig. 2), which are nearly identical to those of *Drosophila melanogaster* (Harris et al. 1976). In *D. melanogaster Rh3* and *Rh4* have distinct absorption spectra with *Rh3* absorbing maximally at 345 nm and *Rh4* absorbing maximally at 375 nm (Feiler et al. 1992). Our data identify the pooled response of all R7 cells in the compound eye and cannot distinguish between R7 pigments that may absorb at different wavelengths. For *D. simulans* and *D. virilis,* the R8 spectral mechanism is similar to that of *D. melanogaster* (Harris et al. 1976). This can be inferred from the preferential depression in UV sensitivity after intense UV adaptation. The R8 spectral mechanism of *D. mercatorum* and *D. pseudoobscura* cannot be uncovered by chromatic adaptation in the ERG. However, given the conservative mode of evolution for the other photoreceptor cell types, it would not be surprising if these latter two species also have similar R8 spectra.

Ocellar spectra of the species we examined (Fig. 3) are also highly similar to those of *Drosophila melanogaster* (Hu et al. 1978). Thus the ocelli have a rhodopsin with a broad absorption spectrum that peaks at approximately 420 nm, and which is sensitized to ultraviolet light by an accessory pigment. *D. melanogaster* has a single ocellar rhodopsin (Feiler et al. 1988; Hu et al. 1978; Pollock and Benzer 1988) which photointerconverts with a long-wavelength metarhodopsin (Hu et al. 1978). Chromatic adaptation experiments with *D. simulans, D. pseudoobscura, D. mercatorum,* and *D. virilis* (Fig. 4) are consistent with the presence of a single opsin in the ocelli of each of these species.

Patterns of Amino Acid Replacement in the Opsins Reflect Strong Constraints on Spectral Sensitivity

Patterns of amino acid replacement in opsins are consistent with functional evidence for strong constraints on the spectrum of visible wavelengths in *Drosophila.* The extremely conservative mode of evolution of the opsins is apparent when the amino acid identity between the *D. melanogaster* and *D. virilis Rh1* and *Rh4* proteins, 97.5% and 94.0%, respectively, is considered relative to other genes that have been sequenced in the same species. Amino acid identities for comparisons between these two species are as low as 36% for the *transformer* locus (O'Neil and Belote 1992), and 55% for the *period* locus (Colot et al. 1988). Among the more than 10 genes that have been sequenced in both of these species, only *Hsp* 82 (Blackman and Meselson 1986) and *seven in absentia* (Neufeld et al. 1991) have amino acid identities of greater than 90% (reviewed by O'Neil and Belote 1992).

Despite the extremely conservative mode of evolution of the *Drosophila* opsins there are some amino acid replacements in orthologous proteins that involve polar residues. However, none of these replacements appears to have any effect on the spectral properties of the opsins. In *Rh1, D. mercatorum* has an alanine at position 233 and all of the other species have a serine. In *Rh2,* replacement of the nonpolar alanine with a serine at position 128 (Table 2) might be expected to produce a slight red shift, but no shift in spectral sensitivity is observed in *D. simulans Rh2* relative to other *Drosophila* species. The replacements of polar residues at sites 128 (third transmembrane domain) and 240 (fifth transmembrane domain) might be expected to produce a

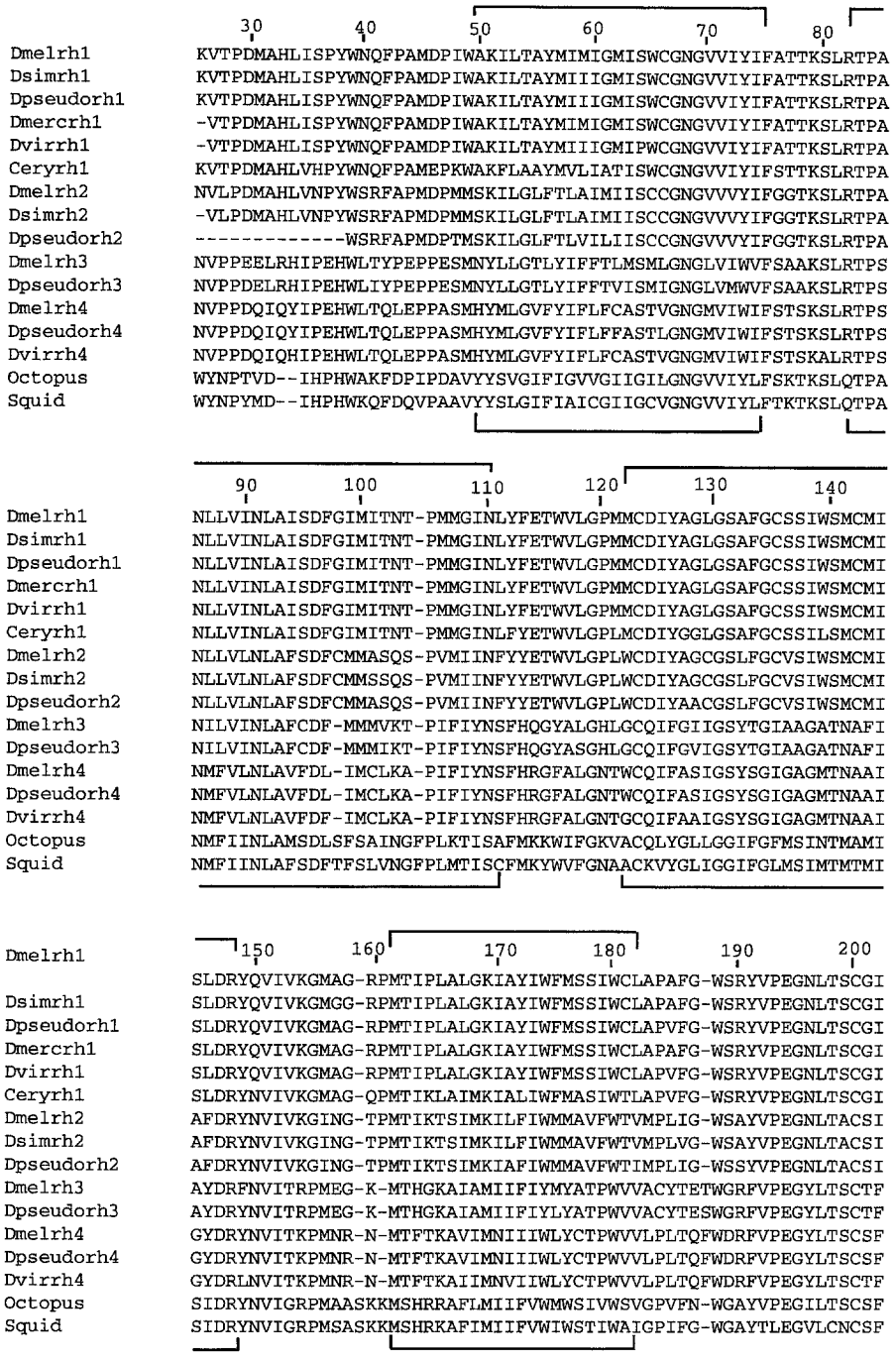

Fig. 6. Alignment of 13 *Drosophila* opsin sequences, one *Calliphora* opsin sequence, and two molluscan opsin amino sequences, created using Clustal software (Higgins and Sharp 1988). The numbers above the alignment mark the *D. melanogaster Rh1* amino acid positions used as a standard throughout this paper. *Brackets* above and below the alignment indicate transmembrane domains as determined in the original *D. melanogaster* references, modified slightly to more closely approximate transmembrane domains in well-studied vertebrate opsins (B. Chang, personal communication).

blue shift in *D. pseudoobscura Rh2* relative to the other *Rh2* opsins, but no spectral sensitivity difference is observed among these opsins either.

Conservation and Divergence Between Rh1 and Rh2

Much can be learned about *Drosophila* opsins by comparing the 14 sequences now available to the more intensively studied vertebrate opsins. The Schiff's base counterion has been identified as glutamate-113 in bovine rhodopsin, and it has been suggested that the tyrosine at the putatively homologous position in *D. melanogaster Rh1* and *Rh2* acts as the counterion in these proteins (Zhukovsky and Oprian 1989). The tyrosine at this position (126) is conserved in all of the *Rh1* and *Rh2* opsins that have been sequenced, which lends support to this argument. Three sites have been

```
                  210       220       230       240       250       260
Dmelrh1           DYLERDWNPRSYLIFYSIFVYYIPLFLICYSYWFIIAAVSAHEKAMREQAKKMNVKSLRS
Dsimrh1           DYLERDWNPRSYLIFYSIFVYYIPLFLICYSYWFIIAAVSAHEKAMREQAKKMNVKSLRS
Dpseudorh1        DYLERDWNPRSYLIFYSIFVYYIPLFLICYSYWFIIAAVSAHEKAMREQAKKMNVKSLRS
Dmercrh1          DYLERDWNPRSYLIFYSIFVYYIPLFLICYAYWYIIAVVSAHEKAMREQAKKMNVKSLRS
Dvirrh1           DYLERDWIPRSYLIFYSIFVYYIPLFLICYSYWFIIAVVSAHEKAMREQAKKMNVKSLRS
Ceryrh1           DYLERDWNPRSYLIFYSIFVYYLPLFLICYSYWFIIAAVSAHEKAMREQAKKMNVKSLRS
Dmelrh2           DYMTRMWNPRSYLITYSLFVYYTPLFLICYSYWFIIAAVAAHEKAMREQAKKMNVKSLRS
Dsimrh2           DYMTRLWNPRSYLITYSLFVYYTPLFLICYSYWFIIAAVAAHEKAMREQAKKMNVKSLRS
Dpseudorh2        DYMTRQWNPRSYLITYSLFVYYTPLFMICYSYWFIIATVAAHEKAMRDQAKKMNVKSLRS
Dmelrh3           DYLTDNFDTRLFVACIFFFSFVCPTTMITYYYSQIVGHVFSHEKALRDQAKKMNVESLRS
Dpseudorh3        DYLTDNFDTRLFVACIFFFSFVCPTTMITYYYSQIVGHVFSHEKALRDQAKKMNVDSLRS
Dmelrh4           DYLSDNFDTRLFVGTIFFFSFVCPTLMILYYYSQIVGHVFSHEKALREQAKKMNVESLRS
Dpseudorh4        DYLSDNFDTRLFVGTIFLFSFVVPTLMILYYYSQIVGHVFNHEKALREQAKKMNVESLRS
Dvirrh4           DYLTDNFDTRLFVGTIFFFSFVCPTLMIIYYYSQIVGHVFSHEKALREQAKKMNVESLRS
Octopus           DYLSTDPSTRSFILCMYFCGFMLPIIIIAFCYFNIVMSVSNHEKEMAAMAKRLNAKELRK
Squid             DYITRDTTTRSNILCMYIFAFMCPIVVIFFCYFNIVMSVSNHEKEMAAMAKRLNAKELRK

                  270       280       290       300       310       320
Dmelrh1           SEDAEK-SAEGKLAKVALVTITLWFMAWTPYLVINCMGLFKFEGL-TPLNTIWGACFAKS
Dsimrh1           SEDAEK-SAEGKLAKVALVTITLWFMAWTPYLVINCMGLFKFEGL-TPLNTIWGACFAKS
Dpseudorh1        SEDADK-SAEGKLAKVALVTISLWFMAWTPYLVINCMGLFKFEGL-TPLNTIWGACFAKS
Dmercrh1          SEDAEK-SAEGKLAKVALVTISLWFMAWTPYLIINCMGLFKFEGL-TPLNTIWGACFAKS
Dvirrh1           SEDAEK-SAEGKLAKVALVTISLWFMAWTPYLVINCMGLFKFEGL-TPLNTIWGACFAKS
Ceryrh1           SEDADK-SAEGKLAKVALVTISLWFMAWTPYTIINTLGLFKYEGL-TPLNTIWGACFAKS
Dmelrh2           SEDCDK-SAEGKLAKVALTTISLWFMAWTPYLVICYFGLFKIDGL-TPLTTIWGATFAKT
Dsimrh2           SEDCDK-SAEGKLAKVALTTISLWFMAWTPYLVICYFGLFKIDGL-TPLTTIWGATFAKT
Dpseudorh2        SEDCDK-SAENKLAKVALTTISLWFMAWTPYLIICYFGLFKIDGL-TPLTTIWGATFAKT
Dmelrh3           NVDKNKETAEIRIAKAAITICFLFFCSWTPYGVMSLIGAFGDKTLLTPGATMIPACACKM
Dpseudorh3        NVDKSKEAAEIRIAKAAITICFLFFASWTPYGVMSLIGAFGDKTLLTPGATMIPACTCKM
Dmelrh4           NVDKSKETAEIRIAKAAITICFLFFVSWTPYGVMSLIGAFGDKSLLTQGATMIPACTCKL
Dpseudorh4        NVDKSKETAEIRIAKAAITICFLFFVSWTPYGVMSLIGAFGDKSLLTPGATMIPACTCKL
Dvirrh4           NVDKSKDTAEIRIAKAAITICFLFFVSWTPYGVMSLIGAFGDKSLLTPGATMIPACTCKL
Octopus           AQAGA--SAEMKLAKISMVIITQFMLSWSPYAIIALLAQFGPAEWVTPYAAELPVLFAKA
Squid             AQAGA--NAEMKLAKISIVIVTQFLLSWSPYAVVALLAQFGPIEWVTPYAAQLPVMFAKA

                  330       340
Dmelrh1           AACYNPIVYGISHPKYRLALKEKCPC
Dsimrh1           AACYNPIVYGISHPKYRLALKEKCPC
Dpseudorh1        AACYNPIVYGISHPKYRLALK-----
Dmercrh1          AACYNPIVYGIRHPKSRLALKEKC--
Dvirrh1           AACCNPIVYGISHPKYRLALKEKSPC
Ceryrh1           AACYNPIVYGISHPKYGIALKEKCPC
Dmelrh2           SAVYNPIVYGISHPKYRIVLKEKCPM
Dsimrh2           SAVYNPIVYGISHPKYRIVLKEKCPM
Dpseudorh2        SAVYNPIVYGISHPKYRLVLKEKCPM
Dmelrh3           VACIDPFVYAISHPRYRMELQKRCPW
Dpseudorh3        VACIDPFVYAISHPRYRMELQKRCPW
Dmelrh4           VACIDPFVYAISHPRYRLELQKRCPW
Dpseudorh4        VACIEPFVYAISHPRYRMELQKRCPW
Dvirrh4           VACIDPFVYAISHPRYRMELQKRCPW
Octopus           SAIHNPIVYSVSHPKFREAIQTTFPW
Squid             SAIHNPMIYSVSHPKFRERIASNFPW
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Fig. 6. Continued.

identified in vertebrate green and red opsins where replacement of nonpolar amino acids with polar amino acids results in a shift of the visual pigment sensitivity toward longer wavelengths (Neitz et al. 1991; Chan et al. 1992). Interestingly, alignment of the *Drosophila* sequences with vertebrate sequences (not shown) shows parallel differences between the *Rh1* and *Rh2* opsins at putatively homologous sites. Specifically, green visual pigments have alanine at position 184 while red pigments have serine at this site. In *Drosophila,* the putatively homologous position (177) and the adjacent site (178) are occupied by polar amino acids in *Rh1* and nonpolar amino acids in the shorter-wavelength-absorbing *Rh2.* Wavelength shifts associated with these amino acids would be expected to be quite small compared with the 60-nm difference between these pigments, but these residues may contribute to the overall wavelength shift. The *Calliphora Rh1* protein has a nonpolar alanine at 177, but it does have the serine at 178 as well as a polar amino acid at another position (293) that is homol-

Table 1. Polymorphisms involving charged or polar amino acids in transmembrane domains of dipteran Rh1 and Rh2 opsins

Opsin	Position[a]	Common amino acid	Replacement amino acid
Rh1	54	Thr in all *Drosophila*[b]	Ala in *Calliphora*
	61	Gly in all *Drosophila*	Ala in *Calliphora*
	62	Met in all *Drosophila*	Thr in *Calliphora*
	164	Pro in all *Drosophila*	Lys in *Calliphora*
	168	Gly in all *Drosophila*	Met in *Calliphora*
	172	Tyr in all *Drosophila*	Leu in *Calliphora*
	177	Ser in all *Drosophila*	Ala in *Calliphora*
	233	Ser in most *Drosophila* and in *Calliphora*	Ala in *D. mercatorum*
	293	Leu in all *Drosophila*	Thr in *Calliphora*
Rh2	101	Ala in *D. melanogaster* and *D. pseudoobscura*	Ser in *D. simulans*[c]
	128	Gly in *D. melanogaster* and *D. simulans*	Ala in *D. pseudoobscura*
	240	Ala in *D. melanogaster* and *D. simulans*	Thr in *D. pseudoobscura*

[a] Amino acid position according to the numbering scheme for *D. melanogaster* Rh1 (O'Tousa *et al.* 1985)
[b] "All Drosophila" refers to those Drosophila sequences reported in this paper
[c] This position is also occupied by a serine in an additional strain of *D. simulans* (Carulli and Hartl unpublished)

Table 2. Charged or polar amino acids that differ between Dipteran Rh1 and Rh2 opsins

Position[a]	Rh1	Rh2
50	Ala	Ser
56	Tyr	Phe
57	Met	Thr
61	Gly	Leu or Met
65	Trp	Cys
108	Gly	Ile
129	Leu	Cys
136	Ser	Val
145	Ser	Ala
164	Pro[b]	Lys
165	Leu	Thr
166	Ala	Ser
168	Gly[b]	Met
172	Tyr[b]	Phe
177	Ser[b]	Ala
178	Thr or Ser	Val
225	Ile or Leu	Thr
240	Ala or Val	Thr or Ala
242	Ser	Ala
281	Val	Thr
321	Ala	Ser
323	Cys	Val

[a] Amino acid position according to the number scheme for *D. melanogaster* Rh1 (O'Tousa *et al.* 1985)
[b] Sites that are polymorphic for charged or polar amino acids among Dipteran Rh1 opsins (see Table 1)

ogous with a site that has been shown to be important for the wavelength shift in human red and green pigments. *Rh3* and *Rh4*, which also differ in spectral sensitivity (Feiler et al. 1992), show a similar replacement at amino acid 177: The 345-nm-absorbing *Rh3* has a nonpolar alanine at this position, while the 375-nm-absorbing *Rh4* has a polar cysteine.

Patterns of amino acid replacement in *Drosophila* opsins reflect severe constraints on the spectral properties of the photoreceptors. These loci are actively evolving in many ways: The intron/exon structure of *Rh1* and *Rh4* vary within the genus, and relatively large numbers of amino acid replacements have accumulated in the cytoplasmic and extracellular domains of each of the opsins (Neufeld et al. 1991; Carulli and Hartl 1992). Numerous changes have taken place between the paralogous opsin genes in the time since their origin by gene duplication before the evolution of the genus, resulting in differences in spectral sensitivity maxima ranging from 345 nm for Rh3 to 480 nm for Rh1. However, when the transmembrane domains of orthologous *Drosophila* opsins are examined, there are few amino acid replacements and most of those observed are of a highly conservative nature. The visible spectrum in *Drosophila* appears to be limited to the ultraviolet and blue range, and this is associated with exceptionally low rates of amino acid replacement in functionally important domains of the opsin proteins.

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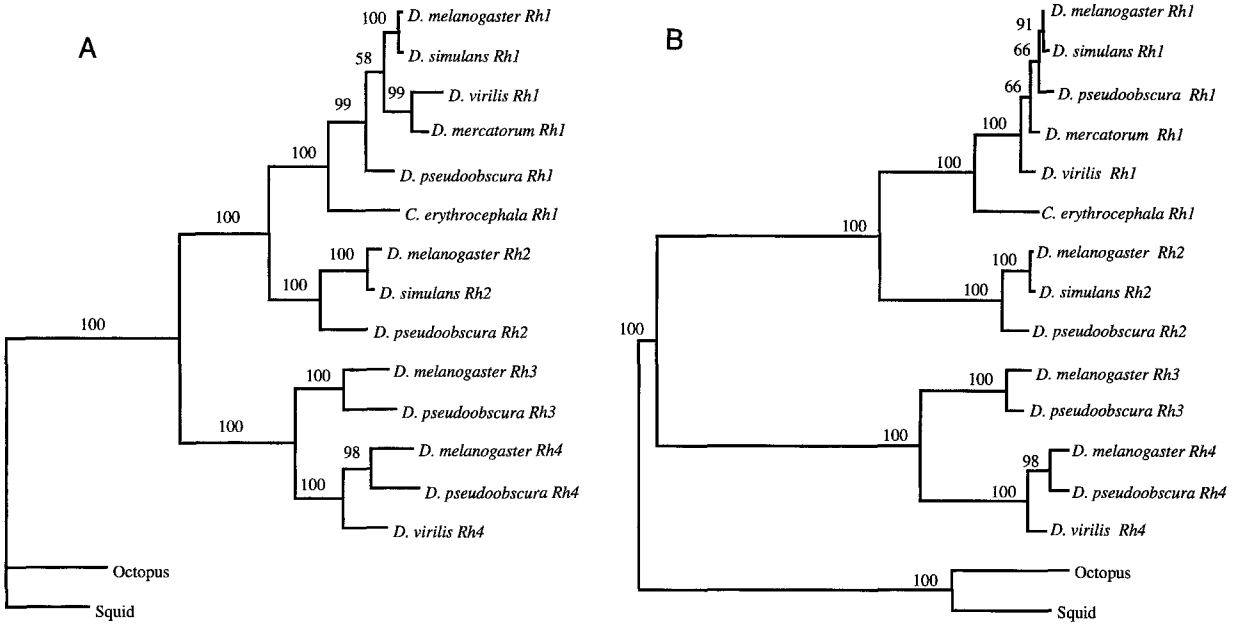

Fig. 7. Phylogeny of *Drosophila* opsins determined from DNA sequences. Sequences were aligned as described in the text, and phylogenies were inferred using the branch and bound procedure in PAUP 3.0r software, which guarantees the shortest tree (Swofford 1990). The sequences used for phylogeny reconstruction include only coding DNA (no introns were included in the phylogenetic analysis) and correspond to amino acids 37–345. The mollusk opsins were used as an outgroup for this analysis because parsimony analysis of a large number of opsins, including vertebrate, molluscan, and non-insect arthropod sequences, showed that other arthropod opsin sequences fall within the *Drosophila* opsin clade. The single rhodopsin in squid and octopus represents the closest outgroup to the Drosophila gene family (Chang and Crandall unpublished). The *numbers* at the nodes indicate the percentage of 2,000 bootstrap replications that reproduced that node. **A** Phylogeny inferred from nonsynonymous nucleotide sites only. The tree is 785 steps long, and the consistency index of 0.823 and retention index of 0.914 indicate low levels of homoplasy. Examination of 100 trees between 785 and 795 steps in length failed to produce a tree that did not retain each of the six major clades. **B** Phylogeny inferred from all nucleotide sites, without any character weighting. The tree is 2,075 steps long with a consistency index of 0.640 and a retention index of 0.741, exhibiting more homoplasy than the tree which only has nonsynonymous sites. Examination of all trees up to 2,095 steps failed to produce a tree that did not have each of the six major clades.

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