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Molecular cloning of *Drosophila* Rh6 rhodopsin: the visual pigment of a subset of R8 photoreceptor cells

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Abstract By screening retinal cDNA libraries for photoreceptor-specifically expressed genes we have isolated and sequenced a cDNA clone encoding the rhodopsin (Rh6) of a subset of R8 photoreceptor cells of the *Drosophila* compound eye. Compared to the other visual pigments of *Drosophila*, this rhodopsin is equally homologous to Rh1 and Rh2 (51% amino acid identity) but shows only 32% and 33% amino acid identity with Rh3 and Rh4, respectively. The open reading frame codes for a protein of 369 amino acids (MW = 41 691). The primary structure of Rh6 displays sites typical for rhodopsin molecules in general, for example, a chromophore binding site in transmembrane domain VII, sequence motifs in the intracellular loops 2 and 3 required for the binding of a heterotrimeric G-protein, and a glycosylation site near the N-terminus which seems to be important for protein transport and maturation. Since R8 cells are founder cells in the developing compound eye, the isolation of a *rhodopsin* gene expressed in these cells may aid the understanding of terminal differentiation of photoreceptor cells.

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Key words: *Drosophila*; G-protein; Phototransduction; Rhodopsin; Vision; Visual pigment

1. Introduction

Rhodopsins, the visual pigments of animal photoreceptors, belong to the superfamily of G-protein-coupled receptors which are characterized by a 7-helix transmembrane topology and by the ability to activate heterotrimeric G-proteins. Within the compound eye of *Drosophila* and of other dipteran flies, the rhodopsin-containing compartments, designated rhabdomeres, of eight photoreceptor cells are arranged in a highly regular trapezoidal array. Differentiation into physiologically and functionally distinct photoreceptor subtypes is accompanied by the expression of at least five different rhodopsins. To date three *Drosophila* rhodopsins expressed in the compound eye and one expressed in small ocellar eye spots located on the vertex of the head (ocelli) have been cloned (reviewed in [1,2]). According to their sequence of cloning these rhodopsins are referred to as Rh1–Rh4. Rh1, the major, blue-sensitive visual pigment of the fly eye, is expressed in the photoreceptor cells R1-6 of the compound eye which form the peripheral rhabdomeres of the open rhabdom. *Rh3* and *Rh4* genes encode ultraviolet-sensitive rhodopsins of non-overlapping sets of R7 cells which form the apical part of the central rhabdomere. By reporter gene analysis, *Rh3* expression was also detected in a small population of R8 cells located at the extreme dorsal anterior margin of the compound eye [3]. R8 cells are located basal of R7 cells and form rhabdomeres fused to the basal part of the rhabdomeres of R7 cells. The *Rh2* gene, originally suggested to encode a rhodopsin of R8 cells, was shown by in situ hybridization to be expressed in the ocelli [4]. Thus, despite an intensive search in several laboratories, the genes encoding the rhodopsins of the majority of R8 cells are still elusive. Isolation of these genes is of particular interest not only in order to complement the information about the expression pattern of visual pigments in the best-studied model system for invertebrate phototransduction, but also for a better understanding of eye development. Within the process of cell fate specification in the developing compound eye, R8 cells serve as ommatidial founder cells [for reviews see [5,6]]. R8 cells are the first photoreceptor cells to express neuron-specific antigens and therefore appear to become terminally differentiated prior to R1-7 cells. Part of the signaling pathway leading to the generation of the complex, highly regular pattern in which the photoreceptor cells of the compound eye are arranged has been unfolded by genetic dissection. However, the molecular mechanism underlying the terminal differentiation into photoreceptor cells which express different visual pigments is completely unknown. The identification of *rhodopsin* genes expressed in the R8 founder cells is one prerequisite for gaining better insight into these mechanisms. In the present study we describe the molecular cloning of a novel *Drosophila* rhodopsin which is expressed in a subset of R8 photoreceptor cells of the compound eye.

2. Materials and methods

An oligo(dT)-primed cDNA library in vector Lambda ZAP II (Stratagene) was produced from heads of *Drosophila melanogaster* Oregon R/white. Screening of the library with a *Calliphora* cDNA probe was carried out according to standard protocols [7] using low stringency conditions (hybridization in 5×SSC (1×SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 0.1% laurylsarcosinate, 0.02% sodium dodecyl sulfate (SDS), 1% blocking reagent (Boehringer Mannheim) at 55°C; final washing step in 0.5×SSC, 0.1% SDS at 52°C). cDNA sequencing was performed by the dideoxy chain termination method [8] using templates generated by nested deletions and sequence-specific primers for sequencing of the coding and reverse strand, respectively.

Northern blot analysis with digoxigenin-labeled cRNA probes was performed as has been described previously [9]. In situ hybridization of polytene salivary gland chromosomes was performed according to Bloomquist et al. [10]. For in situ hybridization of tissue sections, *Drosophila* heads were cryofixed and cut in a cryomicrotome according to Wolfrum [11]. The sections were fixed onto polylysine-coated coverslips with 3% paraformaldehyde in PBS (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.2) for 5 min at 22°C

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The cDNA sequence of the Drosophila Rh6 gene has been submitted to the EMBL data library under accession number Z86118.

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and were then rinsed 3× in PBS. The sections were prehybridized for 1 h at 42°C in 50% formamide, 2×SSC, 0.1% polyvinylpyrrolidone, 0.1% Ficoll 400, 0.2% bovine serum albumin, 5% dextran sulfate, 0.5 mg/ml sheared herring testis DNA, and were then hybridized in the same solution supplemented with 5 ng/μl digoxigenin-labeled antisense cRNA for 20 h at 52°C. Following hybridization the sections were washed in 2×SSC at 22°C, in 1×SSC at 22°C and in 0.1×SSC, 0.1% Tween-20 at 55°C (1 h for each wash). Hybridized probe was detected with an alkaline phosphatase-coupled anti-digoxigenin antibody (Boehringer Mannheim) using nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as chromogen. Cell nuclei were labeled by adding the fluorescent dye 4,6-diamidino-2-phenylindol (DAPI) prior to mounting the sections in Mowiol 4.88. The sections were analyzed in a Zeiss Axiovert fluorescence microscope.

3. Results and discussion

In the course of isolating and sequencing cDNA clones

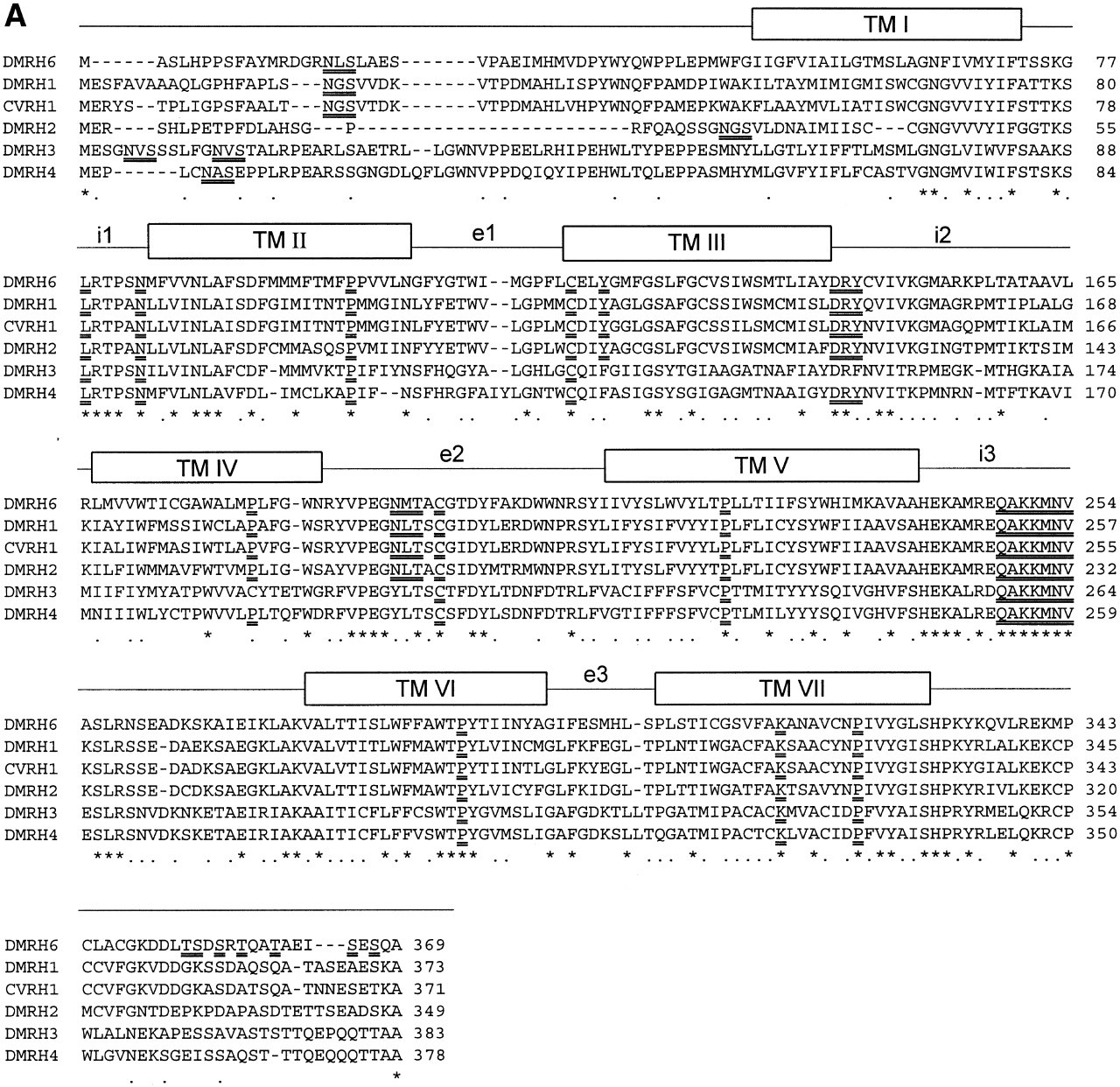

Fig. 1. A: Amino acid sequence alignment of fly rhodopsins. DMRH1-DMRH6, *Drosophila melanogaster* rhodopsins Rh1, Rh2, Rh4, Rh6; CVRH1, *Calliphora vicina* Rh1 rhodopsin. Amino acids are given in single letter code. Asterics (*) and dots (·) indicate amino acids conserved in all or at least 4 sequences, respectively. The transmembrane domains (TM I–TM VII), cytoplastmic (i1–i3) and extracellular (e1–e3) loops are depicted above the sequences. Functionally important residues which are discussed in the text are double underlined. The alignment was performed with the programm 'Clustal' of the PC-GENE software package (IntelliGenetics Inc.). B: Evolutionary relationship of representative invertebrate rhodopsins. The phylogenetic relationship of the fly rhodopsins and of rhodopsins from *Apis mellifera* (BEE), *Camponotus abdominalis* (ANT 1), *Cataglyphis bombycina* (ANT 2) *Sphodromantis sp.* (MANTIS), *Schistocerca gregaria* (LOCUST 1, 2), *Limulus polyphemus* lateral (LIMULUS 1) and ocellar (LIMULUS 2) eye, and *Hemigrapsus sanguineus* (CRAB RH1, RH2) was calculated by aligning the deduced amino acid sequences as shown for the fly rhodopsins in (A). The SwissProt or EMBL accession numbers for the respective sequences are: DMRH1, P06002; DMRH2, P08099; DMRH3, P04950; DMRH4, P29404; DMRH6, Z86118, CVRH1, P22269; BEE, U26026; MANTIS, P35362; LOCUST 1, X80071; LOCUST 2, X80072; ANT 1, U32502; ANT 2, U32501; CRABRH1, D50583; CRABRH2, D50584; LIMULUS 1, P35360; LIMULUS 2, P35361.

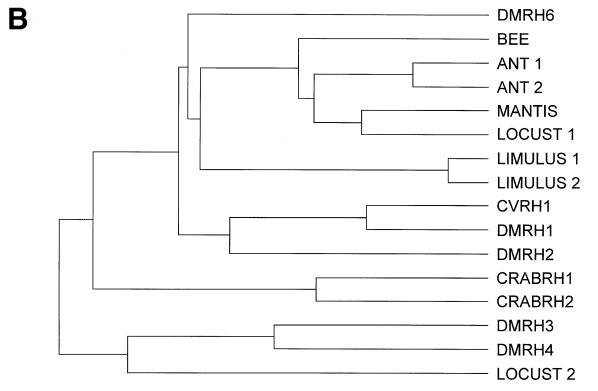

Fig. 1 (*continued*).

which encode proteins present in blowfly (*Calliphora*) photoreceptor membranes [12], we isolated a clone which encodes a polypeptide with significant homology to previously reported sequences of fly rhodopsins (data not shown). Analyzing the homology between the partial protein sequence deduced from this clone with *Calliphora* Rh1 rhodopsin and with the *Drosophila* rhodopsins revealed substantially less amino acid identities (between 31% and 65%) than would have been expected if this clone was the homologue of one of the previously identified *Drosophila* rhodopsins Rh1 to Rh4. Usually, the inter-species conservation of rhodopsin homologues is higher (e.g. 86% amino acid identity between *Drosophila* and *Calliphora* Rh1) than the conservation of rhodopsins which serve different functions in the same species (e.g. 67% amino acid identity between *Drosophila* Rh1 and Rh2). This finding prompted us to investigate whether the newly isolated *Calliphora* clone encodes the homologue of a novel *Drosophila* rhodopsin.

The isolated *Calliphora* cDNA clone was used to screen a *Drosophila* head cDNA library and the longest of the obtained cDNA clones (1409 bp) was sequenced. It contained 121 bp of the 5′-untranslated region with an in-frame stop codon 44 nucleotides before the first AUG start codon, an open reading frame coding for 369 amino acids (MW=41 691), and a 181 bp 3′-untranslated region. Comparison of this sequence with DNA sequences listed in the EMBL data library indicated that we had isolated a novel *Drosophila* gene that displayed high homology to invertebrate *rhodopsin* genes. For deducing the amino acid sequence, the translation initiation site was assigned to the first AUG codon (GAAAUG) of the open reading frame which fits well with the consensus sequence for translation initiation sites in *Drosophila*, (C/A)AA(A/C)AUG [13]. Alignment of the amino acid sequence deduced from the novel cDNA clone with the sequences of other fly rhodopsins (Fig. 1A) revealed sequence identities of 51% with *Drosophila* Rh1, 53% with *Calliphora* Rh1, 51% with *Drosophila* Rh2, 32% with *Drosophila* Rh3, and 33% with *Drosophila* Rh4. As is evident from the evolutionary relationship of representative invertebrate rhodopsins (Fig. 1B), the protein analyzed here clearly belongs to a group comprising the known arthropod rhodopsins. It is most closely related to the long-wavelength absorbing (λmax at 520–530 nm) rhodopsins of the honeybee, ants, mantis, locust and *Limulus* which apparently constitute a group separated from blue–green and ultraviolet-absorbing rhodopsins [2,14].

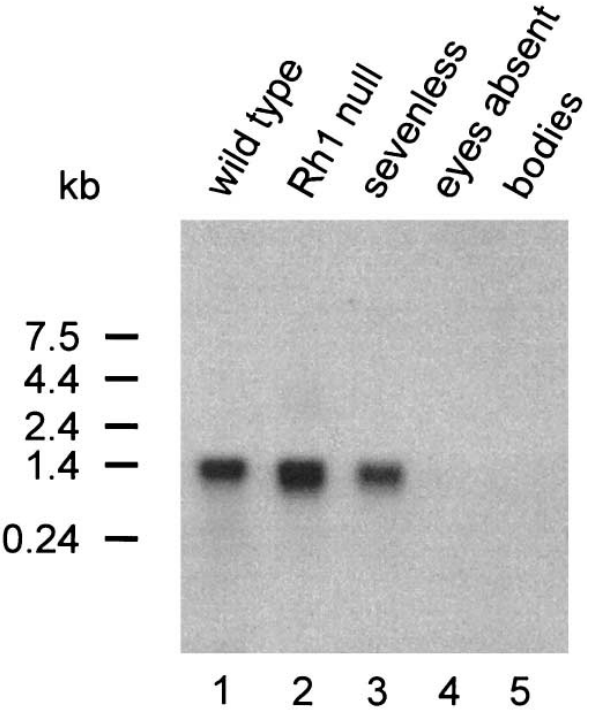

Fig. 2. Northern blot hybridization of *Drosophila* Rh6 mRNA. RNA was isolated from heads of wild-type flies (lane 1), *ninaE*^{oi17} (lane 2), *sev*^{d2} (lane 3), *eya*^{1} (lane 4) mutants and from *Drosophila* bodies (lane 5). RNA (3 μg) was size fractionated on a denaturing agarose gel, blotted onto nylon membrane and hybridized with digoxigenin-labeled antisense cRNA transcribed from Rh6 cDNA. Figures on the left side indicate the migration of RNA size markers in kilobases (kb).

These results suggest, that the isolated cDNA clone encodes a long-wavelength absorbing rhodopsin. It will hereafter be referred to as *Drosophila* Rh6 rhodopsin[1].

In line with the proposal that the *Rh6* gene encodes a visual pigment, hallmarks of rhodopsin molecules are found to be conserved in Rh6: a chromophore binding site in transmembrane domain (TM) VII is found at Lys^{317} which is conserved throughout all visual pigments. A tyrosine residue (Tyr^{123}) in TM III may serve as a counter ion for the protonated Schiff base, according to the model proposed by Hall et al. [15]. Conserved proline residues in the membrane-embedded portion of Rh6 are present in TM II (Pro^{102}), TM IV (Pro^{181}), TM V (Pro^{223}), TM VI (Pro^{289}) and TM VII (Pro^{324}). Due to their ability of *cis-trans* isomerization, it has been suggested that these prolines serve a function in signal transduction within the protein when conformational changes of the molecule are induced by photon absorption [2]. Two extracellular cysteine residues, the homologues of which were shown to form a disulfide bridge necessary for protein stability in bo-

[1]During the reviewing process of this paper the isolation of another *Drosophila rhodopsin* gene was published (Chou et al. (1996) *Neuron* 17, 1101–1115). This rhodopsin is expressed in a subset of R8 photoreceptor cells which are paired with Rh3-expressing R7 cells (R7p). It is not identical to the rhodopsin analyzed here which is most likely expressed in the other subset of R8 cells paired with Rh4-expressing R7 cells (R7y). Since the rhodopsin isolated by Chou et al. was termed Rh5, the rhodopsin analyzed here is referred to as Rh6, in order to avoid confusion in the nomenclature of *Drosophila* rhodopsins.

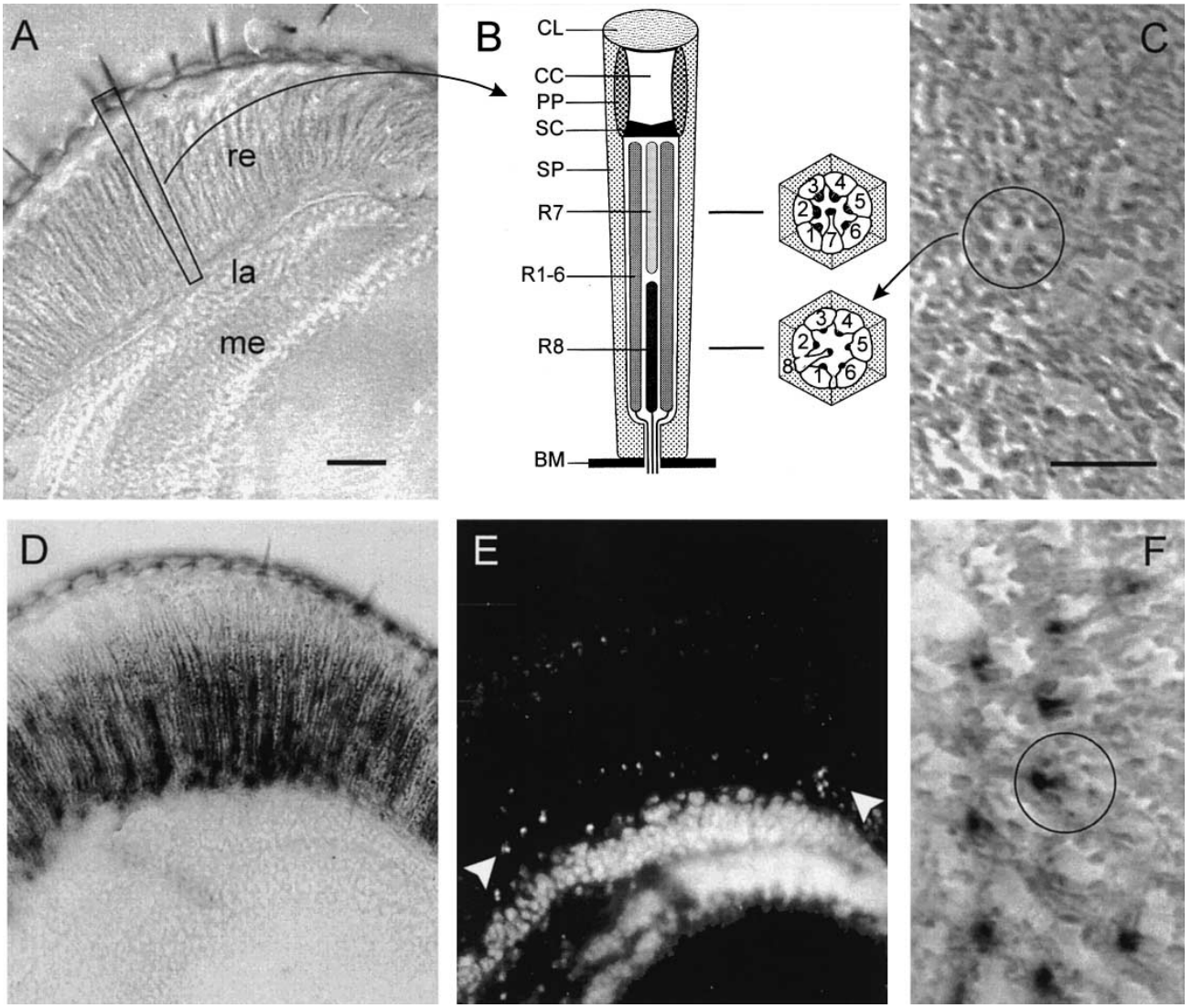

Fig. 3. Spatial distribution of Rh6 mRNA in the compound eye of *Drosophila* revealed by in situ hybridization. (A,C,D,F) Differential interference contrast images of longitudinal sections (A,D) and of cross-sections (C,F) that were incubated with (D,F) or without (A,C) digoxigenin-labeled Rh6 cRNA. In the cross-sections (C,F) one ommatidium is indicated by a circle. Hybridized cRNA was detected with alkaline phosphatase-coupled anti-digoxigenin antibodies using NBT/BCIP as chromogen (dark precipitates in (D) and (F)). *re*, retina; *la*, lamina; *me*, medulla. (E) Fluorescent image of DAPI-stained nuclei corresponding to (D). The row of nuclei of R8 cells is indicated by arrowheads. (B) Schematic drawing of a longitudinal section through an ommatidium (left) and of cross-sections at the indicated height (right). *CL*, cornea lens; *CC*, cristalline cone; *PP*, primary pigment cell; *SC*, semper cell; *R7*, photoreceptor cell R7; *R1-6*, photoreceptor cells R1-6; *R8*, photoreceptor cell R8; *BM*, basement membrane; *1-8* on the right, photoreceptor cells R1-R8. Scale bar (A): 50 μm; scale bar (C): 20 μm.

vine rhodopsin [16], are present at positions 120 and 197. Cytoplasmic domains which are thought to be involved in G-protein binding [17], in particular the D–R–Y motif at the beginning of cytoplasmic loop 2 and the N-terminal half of cytoplasmic loop 3 are well conserved. In this loop the peptide Q–A–K–K–M–N–V is perfectly conserved in all insect rhodopsins as well as in *Limulus* rhodopsins. Cytoplasmic loop 1 also contains a stretch of highly conserved amino acids, two of which, Leu^{78} and Asn^{83}, have been shown to be crucial for proper protein maturation during the biogenesis of *Drosophila* Rh1 [18]. A pair of cysteines located in the C-terminal region of vertebrate rhodopsins which may function as a palmitylation site is absent in Rh6. For vertebrate rhodopsins it has been proposed that palmitylation of these cysteine residues anchors the C-terminal domain to the membrane and may be required for G-protein binding [19]. However, experimental proof for the functional relevance of rhodopsin palmitylation has not yet been provided [20]. In several invertebrate rhodopsins (e.g. *Drosophila* Rh3 and Rh4) corresponding cysteine residues are missing. In addition, mutation of the relevant cysteine residues of *Drosophila* Rh1 does not reveal functional alterations as compared to wild-type Rh1 (Bentrop, unpublished). Seven serine and threonine residues near the C-terminus may be employed for arrestin-mediated rhodopsin phosphorylation as has been shown for *Calliphora* and *Musca* Rh1 rhodopsins [21,22]. Finally, extracellular consensus sequences for N-glycosylation (N–X–S/T) are present in Rh6 near the N-terminus (Asn^{17}) and in loop e2 (Asn^{193}). It has been shown that *Calliphora* Rh1 and *Drosophila* Rh1 are transiently glycosylated at a single N-glycosylation site [23–25]. Rh1 also contains one putative glycosylation site near the N-terminus and one in loop e2. Mutation of the N-terminal glycosylation site of *Drosophila* Rh1 interferes with proper protein maturation [26]. In this mutant, no gly-

cosylated intermediates of rhodopsin biosynthesis are detected [27]. Thus, Rh1 seems to be only glycosylated at the N-terminal glycosylation site. In analogy we assume that the N-terminal site of Rh6 is glycosylated as well.

In order to evaluate whether or not a *Drosophila* mutant exists that may have a defect in Rh6, we determined the cytogenetical map position of the *Rh6* gene by in situ hybridization to salivary gland chromosomes. The *Rh6* gene is located on chromosome 3 at position 88F. No mutants showing malfunctions in photoreception or eye development have been isolated at or near this cytogenetic location. It is, however, worth noting that all *Drosophila rhodopsin* genes sequenced to date are located on chromosome 3 (positions 73D3-5 for Rh4, 88F for Rh6, 91D1-2 for Rh2, 92 B8-11 for Rh1, and 92D1 for Rh3). This finding may support the hypothesis that the distinct visual pigments of *Drosophila* were generated by gene duplications. The *Drosophila* rhodopsins most closely related to Rh6 are Rh1, which is expressed in R1-6 cells of the compound eye, and Rh2, the visual pigment of the ocelli. In order to determine whether Rh6 is expressed in another subset of photoreceptor cells of the ocelli or in photoreceptor cells of the fly's compound eye, we performed Northern blot analyses and in situ hybridizations with *Drosophila* head sections. Using antisense cRNA transcribed from the Rh6 cDNA clone as a probe, a 1.4 kb mRNA was detected on Northern blots in lanes containing RNA isolated from heads of wild-type flies (Fig. 2, lane 1), Rh1-null mutants (Fig. 2, lane 2), and *sev* mutants (Fig. 2, lane 3). No hybridization signals were observed in lanes which were loaded with RNA obtained from heads of *Drosophila eya* mutants (Fig. 2, lane 4) or from *Drosophila* bodies (Fig. 2, lane 5). *eya* (eyes absent) mutants lack the compound eyes but do have ocelli [28], and *sev* (sevenless) mutants do not develop rhabdomeres of R7 cells [29]. Thus, the Northern blot results reveal that Rh6 is expressed in R8 cells of the compound eye. Expression of Rh6 in the compound eye is also suggested by the fact that the corresponding *Calliphora* clone which is highly homologous to *Drosophila* Rh6 was isolated form a cDNA library prepared from mRNA of the compound eye.

In order to further confirm that the *Rh6* gene encodes an opsin of R8 cells we determined the spatial distribution of Rh6 mRNA expression in the fly eye. Cryosections through *Drosophila* wild-type heads were hybridized with the Rh6-specific antisense cRNA probe (Fig. 3). In longitudinal sections through *Drosophila* heads, hybridization signals were detected in the basal part of the retinula cell layer. This finding is entirely consistent with Rh6 being expressed in R8 cells since these cells are located in the basal half of the retina. The most intense staining was detected in close proximity to the nuclei of the R8 cells. No hybridization signals were observed in the optic ganglia. The examination of cross-sections through the retina showed that hybridization signals were not detected in each ommatidium, indicating that Rh6 is expressed only in a subset of R8 cells. Accordingly, one has to assume that the remaining R8 cells express yet another rhodopsin. Fluorescence studies have revealed that R7 and R8 photoreceptor cells are organized in matched pairs which show either green fluorescence or no fluorescence and are termed R7y/R8y or R7p/R8p, respectively [30]. While both R7 cells have their peak spectral sensitivity in the ultraviolet, the spectral sensitivities of R8y and R8p show a λmax of 530 nm and of 460 nm, respectively [31]. As has readily been demonstrated for R7 cells [3], it is likely that the subclasses R8y and R8p are distinguished by the expression of different *rhodopsin* genes. As discussed above, Rh6 is most homologous to long-wavelength absorbing arthropod rhodopsins. Rh6 may thus represent the 530 nm pigment of R8y cells. A more definite assignment of Rh6 to either R8y or R8p cells will be obtained by ectopic expression of Rh6 in R1-6 cells which should allow to measure the absorption spectrum of Rh6.

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