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# TWO OPSINS FROM THE COMPOUND EYE OF THE CRAB *HEMIGRAPSUS SANGUINEUS*

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## Summary

**The primary structures of two opsins from the brachyuran crab *Hemigrapsus sanguineus* were deduced from the cDNA nucleotide sequences. Both deduced proteins were composed of 377 amino acid residues and included residues highly conserved in visual pigments of other species, and the proteins were 75 % identical to each other. The distribution of opsin transcripts in the compound eye, determined by *in situ* hybridization, suggested that the mRNAs of the two opsins were expressed simultaneously in all of the seven retinular cells (R1–R7) forming the main rhabdom in each ommatidium. Two different visual pigments may be present in one photoreceptor cell in this brachyuran crab. The spectral sensitivity of the compound eye was also determined by recording the electroretinogram. The compound eye was maximally sensitive at about 480 nm. These and previous findings suggest that both opsins of this brachyuran crab produce visual pigments with maximal absorption in the blue-green region of the spectrum. Evidence is presented that crustaceans possess multiple pigment systems for vision.**

Key words: opsin, visual pigment, photoreceptor, retina, compound eye, brachyuran crab, *Hemigrapsus sanguineus*, crustacean.

## Introduction

There are several experiments supporting the hypothesis that some crustaceans have multiple visual pigments in their retinas. For example, microspectrophotometric and electrophysiological studies have revealed the presence of multiple spectral classes of photoreceptors in the retinas of crustaceans: some decapods have at least two colour receptors (Cummins and Goldsmith, 1981; Martin and Mote, 1982) and many stomatopod species have as many as 10 spectral classes of photoreceptors (Cronin and Marshall, 1989; Cronin *et al.* 1993). Behavioural evidence for colour discrimination exists for some crabs (von Buddenbrock and Friedrich, 1933; Hyatt, 1975) and can be accounted for by multiple receptor systems.

Most vertebrates possess multiple visual pigments consisting of one rod pigment (rhodopsin) and several cone pigments with different spectral types (e.g. Dartnall *et al.* 1983; Hárosi and Hashimoto, 1983; Okano *et al.* 1992), and vertebrate opsins as the apoproteins of visual pigments can be classified into at least five groups according to amino acid identity (Okano *et al.* 1992; Hisatomi *et al.* 1994). The difference in the spectral sensitivities of photoreceptors is attributed to the spectral properties of the visual pigment and, in most cases, various receptor classes express genes coding for different opsins. Insects, which have compound eyes like those of crustaceans, also possess several colour receptors and different kinds of opsins in their retinas (Hardie, 1985; Arikawa *et al.* 1987*a*, 1995; Pollock and Benzer, 1988). Thus, crustaceans with multiple colour receptors may have multiple visual pigment systems involving different types of opsin proteins. However, little is known about the primary structures of crustacean opsins (Hariyama *et al.* 1993) and, to our knowledge, there are no reports showing the presence of more than one type of opsin protein in the crustacean retina.

In the present study, we used molecular techniques to investigate whether crustaceans possess multiple visual pigments in their retinas. We determined the nucleotide sequences of opsin cDNAs from the brachyuran crab *Hemigrapsus sanguineus* to deduce the primary structures of the proteins. We used both the polymerase chain reaction (PCR) and rapid amplification of cDNA ends (RACE) (Frohman *et al.* 1988; Ohara *et al.* 1989), using retinal cDNA as a template, and obtained cDNAs of two different opsin proteins. We examined the distribution of opsin transcripts in the retina by *in situ* hybridization. The two opsin mRNAs seemed to be expressed simultaneously in all of the seven retinular cells (R1–R7) forming the main rhabdom in each ommatidium. The spectral sensitivity of the compound eye was also measured by recording the electroretinogram (ERG). The compound eye was maximally sensitive at about 480 nm. The findings are discussed in relation to the characteristics of the visual pigments of crustaceans.

## Materials and methods

### *Animals*

Brachyuran crabs, *Hemigrapsus sanguineus* (de Haan), of both sexes (carapace width 20–25 mm) were used throughout these studies. They were captured locally and kept at 20 °C under a cycle of 12 h of light and 12 h of darkness as described by Arikawa *et al.* (1987*b*).

### *Amplification of opsin cDNA fragments by PCR*

Opsin cDNA fragments were amplified using the polymerase chain reaction (PCR). Retinas were dissected from 120 animals, quickly frozen and kept in liquid nitrogen until used. About 24 $\mu$g of total RNA was isolated by guanidinium thiocyanate extraction followed by centrifugation through a cushion of CsCl (Sambrook *et al.* 1989). cDNA was made from this total RNA template using an oligonucleotide primer [T-primer; 5′-GCCGAATTCGTCGACAAGCTTTTTTTTTTTTTTTT-3′]. The reaction mixture was composed of 10 $\mu$g of denatured total RNA template, 20 pmol of T-primer, 1000 units of reverse transcriptase (SuperScript RT, 8053SA, Gibco/BRL), 50 mmol $l^{-1}$ Tris–HCl (pH 8.3), 75 mmol $l^{-1}$ KCl, 3 mmol $l^{-1}$ $MgCl_2$, 10 mmol $l^{-1}$ dithiothreitol and 0.5 mmol $l^{-1}$ each of the four dNTPs in a total volume of 100 $\mu$l, and was incubated at 42 °C for 2 h. This cDNA was used as a template for PCRs.

For each PCR, the mixture, containing 50 pmol of each primer, 0.2 mmol $l^{-1}$ of each of the four dNTPs, 1 unit of Taq DNA polymerase (Takara, Japan), 50 mmol $l^{-1}$ KCl, 10 mmol $l^{-1}$ Tris–HCl (pH 8.8), 1.5 mmol $l^{-1}$ $MgCl_2$, 0.1 % Triton X-100 and sterilized water, was added to the template cDNA solution to give a final volume of 50 $\mu$l. For template DNA, we used one-twentieth of the cDNA which had been synthesized from 10 $\mu$g of the total RNA template. To amplify DNA, thermal cycling was performed for 45–50 cycles of denaturation (94 °C, 1 min), annealing (55–37 °C, 1.5 min) and extension (72 °C, 3 min). PCR products were cloned into a pUC 18 plasmid vector for sequencing.

To obtain cDNAs encoding the full length of the opsins, four portions of the cDNA which partially overlapped each other were amplified using the PCR and RACE methods (Fig. 1). The strategy was as follows.

*PCR using degenerate oligonucleotide primers.* First, we performed PCRs using degenerate oligonucleotide primers corresponding to amino acid sequences highly conserved in invertebrate visual pigments. We designed three degenerate oligonucleotide primers: [IVRH-F1 (forward); 5′-TGTGA-ATTCGANC(A/T)(A/G)GCNAA(A/G)A(A/G)NATNAA-3′], [IVRH-R1 (reverse); 5′-TGTAAGCTTA(C/T)N(G/C)C-(A/G)TANA(C/T)NA(C/T)NGG(A/G)T-3′] and [IVRH-F2 (forward); 5′-GCGAATTCAAT(C/T/A)TN(C/T)TNGTN-(C/G)TNAA-3′] (where N is A/C/G/T), corresponding to the amino acid sequences (E/D/A)(Q/M)AK(K/R)(M/L)N, (N/D)P(I/F/M)(V/I)Y(G/A/S)(I/V) and N(L/I/M)(L/F)(V/I)-(I/L/V)N, respectively.

PCR was first carried out between IVRH-F1 and IVRH-R1 primers on annealing at 37 °C (Fig. 1A). Two types of cDNA fragments were amplified. We then synthesized one forward and one reverse primer specific to both cDNAs amplified between IVRH-F1 and IVRH-R1: [Crab-F1 (for amplification of the 3′ end cDNA); 5′-CGGGATCCGCATCGCTAAGA-CTGC-3′] and [Crab-R2; 5′-TCCAAAGCTTGAACCACA-GAGAAAC-3′], respectively. The second PCR was performed between the primers IVRH-F2 and Crab-R2 (Fig. 1C) on annealing at 50 °C. We also obtained two types of cDNA fragments, and synthesized a reverse primer specific for both cDNAs amplified between IVRH-F2 and Crab-R2: [Crab-R3 (for amplification of the 5′ end cDNA); 5′-CACAAGCTTC-GCTGAAGCAGTTGTA-3′].

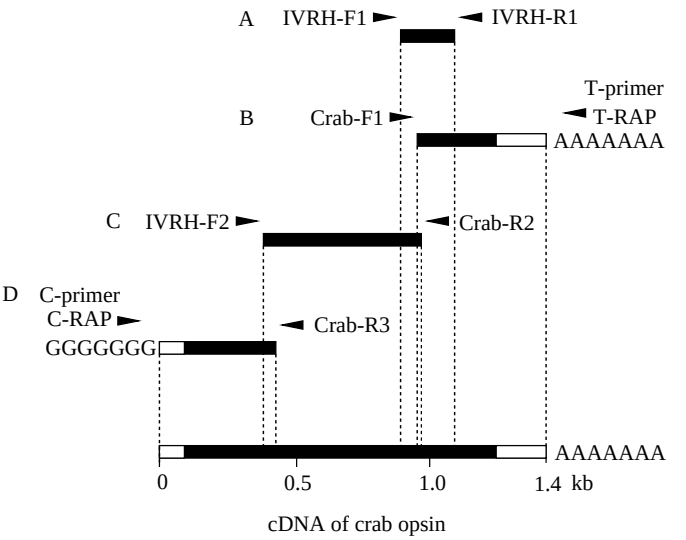


Fig. 1. Cloning strategy for the brachyuran crab opsin cDNAs (for details see Materials and methods). Filled bars represent coding regions.

*Amplification of the 3′ end.* To amplify the 3′ end cDNA, PCR was performed by using the primers Crab-F1 and T-RAP (3′RACE amplification primer) (Fig. 1B), on annealing at 50 °C. T-RAP (5′-GCCGAATTCGTCGACAAGC-3′) is identical to part of the T-primer from which the template cDNA had been made. We obtained two types of cDNA fragments longer than 500 bp containing a poly(A) site.

*Amplification of the 5′ end.* To amplify the 5′ end cDNA, the 5′RACE method was used after slight modification. First, a poly(G) tail was added to the cDNA (Fig. 1D). Half of the cDNA synthesized from 10 $\mu$g of the total RNA template was heated (to hydrolyze the RNA) at 80 °C for 20 min in the presence of 0.3 mol $l^{-1}$ NaOH and 10 mmol $l^{-1}$ EDTA. The solution was neutralized with HCl, and excess T-primer and dNTPs were removed using a Sephadex G-50 spin column. A poly(G) tail was added to the cDNA using terminal deoxynucleotidyl transferase. After heating to 65 °C for 15 min, the buffer was exchanged for 50 $\mu$l of 0.1× TE (1 mmol $l^{-1}$ Tris–HCl, pH 7.5, 0.1 mmol $l^{-1}$ EDTA) using a spin column. This cDNA solution (10 $\mu$l) was used as a template DNA in PCR.

PCR was performed in two steps (Fig. 1D). First, to synthesize the second strand of the cDNA, three thermal cycles (96 °C, 5 min; approximately 25 °C, 5 min; 55 °C,

5 min; 72 °C, 5 min) were carried out, using 5 pmol of C-primer (5′-GCGAATTCGCGGCCGCGGATCCCCCCCCCCCC-3′). Next, 50 pmol of C-RAP (5′RACE amplification primer) and Crab-R3 primers were added to the PCR reaction solution, and 45 cycles of amplification were carried out (94 °C, 1 min; 55 °C, 2 min; 72 °C, 3 min). C-RAP (5′-GCGAATTCGCGGCCGC-GGAT-3′) is identical to part of the C-primer. The other conditions for PCR were as described above. We obtained two types of cDNA fragments longer than 400 bp.

As two different types of cDNA fragments were amplified for all four portions of the opsin cDNA, we obtained eight cDNA fragments in all. For each cDNA fragment, at least six clones were isolated from a plural independent PCR reaction and were sequenced on the forward and reverse strands to confirm the nucleotide sequences of the cDNAs.

### In situ *hybridization*

Compound eyes were fixed in 4 % paraformaldehyde in 0.1 mol $l^{-1}$ phosphate buffer (pH 7.4) with 5 % sucrose for 1 h at room temperature. Fixed eyes were washed with phosphate-buffered sucrose solution and embedded in Tissue-Tek OCT compound (Miles). 10 $\mu$m cryosections were cut.

Antisense and sense cRNA probes (digoxigenin-labelled) were generated using a DIG RNA labelling kit (Boehringer Mannheim, catalogue no. 1175 025). The sense probes (identical to the mRNA target) were used as negative controls. The templates for transcription were a 246 bp PCR-generated cDNA fragment of opsin BcRh1 (BcP-1; between positions 1146 and 1391: see Fig. 2A) and a 242 bp cDNA fragment of opsin BcRh2 (BcP-2; between positions 1147 and 1388: see Fig. 2B). Each cDNA fragment shares at most 40 % identity with the other opsin cDNA. The cDNA fragments were subcloned into the pGEM-3zf(+) vector.

Hybridization was performed according to the methods described by Raymond *et al.* (1993). Slides were treated with 0.01 mg $ml^{-1}$ proteinase K at 37 °C for 2 min, then incubated in 0.1 mol $l^{-1}$ triethanolamine (pH 8.0) for 3 min at approximately 25 °C, followed by a 10 min rinse in 0.1 mol $l^{-1}$ triethanolamine with 0.25 % acetic anhydride. The tissue was dehydrated through an ethanol series and air-dried. About 150 $\mu$l of hybridization solution (0.3 mol $l^{-1}$ NaCl, 2.5 mmol $l^{-1}$ EDTA, 20 mmol $l^{-1}$ Tris–HCl, pH 8.0, 50 % formamide, 10 % dextran sulphate, 1 mg $ml^{-1}$ yeast tRNA, 1× Denhardt's medium), containing 0.5 $\mu$g $ml^{-1}$ probe cRNA, was placed on each slide, and a coverslip was applied. Slides were hybridized at 65 °C for 16 h. After hybridization, the coverslips were removed, and the slides were washed in 2× SSC (standard saline citrate) followed by a 2 h incubation in 50 % formamide in 2× SSC at 65 °C. The tissue was then washed in 2× SSC and treated with RNase A (20 $\mu$g $ml^{-1}$) at 37 °C for 1 h.

Digoxigenin-labelled RNA probes were detected, after hybridization to target mRNA, by enzyme-linked immunoassay using a DIG nucleic acid detection kit (Boehringer Mannheim, catalogue no. 1175 041). The digoxigenin probe was visualized with anti-digoxigenin antibody conjugated to alkaline phosphatase, and stained with Nitroblue Tetrazolium (NBT) and X-phosphate (5-bromo-4-chloro-indolyl phosphate). The tissue was observed by light microscopy.

### *Electrophysiology*

The spectral sensitivity of the compound eye was determined by recording the ERG. Crabs were made to autotomize their legs and fixed on an experimental stage. The eye stalk was glued with dental cement to prevent movement. The recording electrode was a chlorinated silver wire, painted with a trace amount of electric paste (Z-401CE, Nihon Kohden) and placed in contact with the corneal surface. Another chlorinated silver wire was inserted into the carapace as the indifferent electrode. The signals were led through a preamplifier (MEZ 8201, Nihon Kohden) and displayed on an oscilloscope (VC-11, Nihon Kohden) and a chart recorder. The animals were dark-adapted for at least 1 h before each recording.

The light source was a 500 W xenon arc lamp. The stimulating beam passed through a set of neutral-density filters, through one of the interference filters in the range 300–700 nm (in 20 nm steps) and was led into a Faraday cage by a quartz optic fibre. The tip of the optic fibre was placed 20 mm away from the eye. The maximum quantum flux of each monochromatic stimulus was adjusted to $8\times10^{11}$ photons $cm^{-2}$ $s^{-1}$ at the corneal surface. Flashes of light 200 ms in duration were used for stimulation. The interval between flashes was 20 s.

The spectral sensitivity was defined as the reciprocal of the relative number of photons per flash at each wavelength needed to evoke a constant peak amplitude in the ERG.

## Results

### *cDNA cloning*

To obtain cDNAs encoding the full length of opsins of the brachyuran crab *Hemigrapsus sanguineus*, we performed PCRs on four portions of cDNA which partially overlapped one another (Fig. 1; for details, see Materials and methods). First, PCRs were carried out using degenerate oligonucleotide primers corresponding to amino acid sequences highly conserved in invertebrate visual pigments. We then designed primers specific to the crab cDNAs and, using these, the 5′ and 3′ ends were amplified by the RACE PCR method. Two types of cDNA fragments were amplified for all portions of the cDNA. Since the nucleotide sequences of overlapping regions (at least 50 nucleotides, except for the primers) were in complete agreement, we could reconstitute two complete cDNAs from these cDNA fragments. Proteins encoded by these two cDNAs were named opsins BcRh1 and BcRh2 of the brachyuran crab.

### *Sequences of BcRh1 and BcRh2*

Fig. 2 shows the nucleotide and deduced amino acid sequences. The cDNAs of opsin BcRh1 and BcRh2 were 1391 bp and 1388 bp, respectively, and their sequences were

74% identical. Using cDNA probes to the two opsin cDNAs, northern blot analyses of crab retinal total RNA showed a single band corresponding to about 1.5 kb (data not shown). Each cDNA sequence revealed an open reading frame of 1131 bp starting from the first ATG and encoding 377 amino acid residues. The flanking region of the first ATG in each reading frame displays a Kozak consensus sequence for the initiation of translation (Kozak, 1981, 1984). The polyadenylation signal (AATAAA) was found in both cDNAs.

The deduced amino acid sequences of BcRh1 and BcRh2 were 75% identical (284 of 377 amino acid residues). In both cases, the proteins had a calculated molecular mass of 42 kDa. Like other opsins, both deduced proteins were predicted to consist of seven transmembrane segments (from an analysis of hydropathicity and the alignment of their sequences with those of other opsins; data not shown) and both had appropriate conserved amino acid residues (Fig. 3). Lys-325 is probably the retinal binding site (Wang *et al.* 1980). The two cysteine residues which have been proposed to form a structurally important disulphide bond in bovine rhodopsin (Karnik *et al.* 1988) have equivalents (Cys-128 and Cys-205) in these proteins. Asn-3 in the N-terminal region is a potential *N*-glycosylation site (Struck and Lennarz, 1980). The cytoplasmic loops contained two series of residues which are thought to be responsible for the binding and activation of a G-protein (Franke *et al.* 1990, 1992): Asp-152 to Ile-157, and Ala-275 to Lys-281. The C-terminal regions of both opsins were rich in serine and threonine residues as potential

A

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GGAAGGAGCTTCATTCCACTTCTGTAGACTTAACAGTATTCAGAAGTTTTGGACAGAGGCCAACCTTACACTCCCTCACCATGGCTAACG     90
                                                                        M  A  N       3

TAACCGGACCCCAAATGGCGTTCTACGGATCTGGTGCGGCCACTTTTGGCTACCCGGAGGGGATGACCGTTGCTGACTTCGTCCCAGACC   180
V  T  G  P  Q  M  A  F  Y  G  S  G  A  A  T  F  G  Y  P  E  G  M  T  V  A  D  F  V  P  D     33

GAGTCAAGCACATGGTCCTCGACCATTGGTACAACTACCCTCCCGTGAATCCAATGTGGCACTACCTCCTAGGCGTTGTTTACCTTTTCC   270
R  V  K  H  M  V  L  D  H  W  Y  N  Y  P  P  V  N  P  M  W  H  Y  L  L  G  V  V  Y  L  F     63

                                                                  ------- IVRH-F2 -----
TTGGGGTTATCTCCATTGCGGGCAATGGCCTTGTCATCTACTTGTACATGAAAAGCCAGGCCCTCAAGACCCCCGCCAACATGCTGATTG   360
L  G  V  I  S  I  A  G  N  G  L  V  I  Y  L  Y  M  K  S  Q  A  L  K  T  P  A  N  M  L  I     93

--->                                                <------- Crab-R3 --------
TGAATCTTGCTCTTTCCGACCTGATCATGTTGACCACCAACTTCCCTCCCTTCTGCTACAACTGCTTCAGCGGTGGCAGGTGGATGTTCA   450
V  N  L  A  L  S  D  L  I  M  L  T  T  N  F  P  P  F  C  Y  N  C  F  S  G  G  R  W  M  F    123

GCGGCACCTACTGTGAGATCTATGCTGCTCTTGGTGCTATCACCGGTGTGTGCAGCATCTGGACGCTCTGCATGATCTCCTTCGATAGGT   540
S  G  T  Y  C  E  I  Y  A  A  L  G  A  I  T  G  V  C  S  I  W  T  L  C  M  I  S  F  D  R    153

ACAACATCATCTGCAACGGTTTCAACGGCCCCAAGCTGACCCAGGGCAAGGCCACCTTCATGTGCGGTCTTGCTTGGGTCATCTCTGTCG   630
Y  N  I  I  C  N  G  F  N  G  P  K  L  T  Q  G  K  A  T  F  M  C  G  L  A  W  V  I  S  V    183

GCTGGTCCCTTCCCCCCTTCTTCGGCTGGGGCTCCTACACCCTGGAGGGCATTCTGGATTCCTGCAGCTACGACTACTTCACCCGGGACA   720
G  W  S  L  P  P  F  F  G  W  G  S  Y  T  L  E  G  I  L  D  S  C  S  Y  D  Y  F  T  R  D    213

TGAACACCATCACCTACAACATCTGCATCTTCATCTTCGACTTCTTCCTCCCGGCCTCAGTCATCGTCTTCTCTTACGTCTTCATCGTGA   810
M  N  T  I  T  Y  N  I  C  I  F  I  F  D  F  F  L  P  A  S  V  I  V  F  S  Y  V  F  I  V    243

                        ---------- IVRH-F1 ---------->
AGGCTATCTTCGCCCACGAGGCCGCCATGCGCGCACAGGCCAAGAAGATGAACGTCACCAACCTCCGCTCCAACGAGGCCGAGACCCAGC   900
K  A  I  F  A  H  E  A  A  M  R  A  Q  A  K  K  M  N  V  T  N  L  R  S  N  E  A  E  T  Q    273

    ------- Crab-F1 ------->          <------- Crab-R2 --------
GCGCTGAGATCCGCATCGCTAAGACTGCCCTTGTCAACGTTTCTCTGTGGTTCATCTGCTGGACCCCCTATGCCGCCATCACCATCCAGG   990
R  A  E  I  R  I  A  K  T  A  L  V  N  V  S  L  W  F  I  C  W  T  P  Y  A  A  I  T  I  Q    303
                                                                              <--------
GACTGCTTGGTAACGCCGAAGGCATCACCCCTCTGCTGACCACTCTCCCCGCCCTGCTGGCCAAGTCCTGCTCTTGCTACAACCCATTCG  1080
G  L  L  G  N  A  E  G  I  T  P  L  L  T  T  L  P  A  L  L  A  K  S  C  S  C  Y  N  P  F    333

---- IVRH-R1 ------
TGTACGCCATCAGCCACCCCAAGTTCCGTCTGGCCATCACCCAGCACCTGCCCTGGTTCTGCGTGCACGAGAAGGACCCCAATGACGTTG  1170
V  Y  A  I  S  H  P  K  F  R  L  A  I  T  Q  H  L  P  W  F  C  V  H  E  K  D  P  N  D  V    363

AGGAGAACCAGTCCAGCAACACCCAGACCCAGGAGAAGTCATAAGCAGTGAGGTCATGAGACGCCCCCTGCCGCTGTCTGGAGGTGTTGA  1260
E  E  N  Q  S  S  N  T  Q  T  Q  E  K  S  *                                                  377

GCCCCCTCCCTGGTGACACCTCCGCTCGTCTCGGCGAGCACACGACGCAACAACGAACTTTTTTCTCCTCATACCTTAAT    1340

TGACAGTCGAGGCCTCTGACTTTATGATCAATAAACAATCCTTGCAATTCC(poly(A))    1391
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Fig. 2. Nucleotide and deduced amino acid sequences of the brachyuran crab opsins BcRh1 (A) and BcRh2 (B). Consensus sequences for the polyadenylation signal (AATAAA) and for the initiation of translation (proposed by Kozak, 1981, 1984) are underlined. Horizontal arrows indicate the positions of PCR primers. The sequences have been deposited in GenBank under accession numbers D50583 (opsin BcRh1) and D50584 (opsin BcRh2).

phosphorylation sites for rhodopsin kinase (Wilden and Kühn, 1982). On the basis of the similarities with opsins of other animals, we concluded that these proteins were two visual pigment opsins of the brachyuran crab.

In vertebrate opsins, a glutamic acid in helix III (Glu-113 of bovine rhodopsin) is the Schiff base counterion (Sakmar *et al.* 1989; Nathans, 1990). By contrast, all invertebrate opsins (except the opsins in the ultraviolet visual pigments from flies) have a polar tyrosine in the position of the counterion of vertebrate opsins, and this tyrosine is thought to be the counterion in invertebrate opsins (Hall *et al.* 1991; Chang *et al.* 1995). Thus, the corresponding residue Tyr-131 of the brachyuran opsins is probably the Schiff base counterion (Fig. 3).

The amino acid sequences of the two opsins from the brachyuran crab were compared with those of other arthropod opsins [Table 1, using a computer program for the homology analysis (GENETYX; SDC Software Co.). The program is based on an algorithm proposed by Lipman and Pearson (1985), where gaps were allowed in the sequences to maximize alignment period]. Although the brachyuran crab opsin sequences showed a relatively high identity (43 %) with the *Drosophila melanogaster* opsin Rh1, the identities of the brachyuran opsins with other arthropod opsins were about 40 % (38–42 %). The opsins of *Limulus polyphemus* and the crayfish more resembled (over 50 % identity) the visible-wavelength opsins of *Drosophila* (Rh1 and Rh2, absorbing maximally at 480 nm and at 420 nm, respectively; Britt *et al.* 1993) than (about 40 % identity) the ultraviolet opsins of *Drosophila* (Rh3 and Rh4). The *Limulus* opsin is thought to be the visible-wavelength pigment (Smith *et al.* 1993). However, the brachyuran opsins showed only about 40 % identity either with the visible-wavelength opsins or with the ultraviolet opsins. The two opsins of the brachyuran crab may be somewhat different from the other arthropod opsins described here.

### *Tissue distribution of opsin transcripts*

The distribution of opsin mRNAs in the compound eye was examined by *in situ* hybridization using digoxigenin-labelled cRNA probes. Sense and antisense cRNA probes were

B

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GACGAAGGAGCTTCATTCCGTTTCGTCAGGAGTTCTGGACAGAGGCTAACCCTACGCTCAACCCTACATTCAACCCTTCACGATGACCAA    90
                                                                                  M  T  N     3

CGCTACAGGACCACAGATGGCTTACTACGGGGCTGCAAGCATGGACTTTGGATACCCCGAAGGAGTCAGCATTGTTGACTTCGTAAGGCC   180
  A  T  G  P  Q  M  A  Y  Y  G  A  A  S  M  D  F  G  Y  P  E  G  V  S  I  V  D  F  V  R  P    33

CGAAATCAAGCCGTACGTGCATCAACACTGGTACAATTACCCGCCCGTGAACCCCATGTGGCACTACCTCTTGGGTGTGATCTACCTGTT   270
  E  I  K  P  Y  V  H  Q  H  W  Y  N  Y  P  P  V  N  P  M  W  H  Y  L  L  G  V  I  Y  L  F    63

                                                                    ------ IVRH-F2 ----
CCTCGGGACCGTCTCCATCTTCGGCAATGGCCTAGTGATCTACCTTTTCAACAAATCCGCGGCCCTTAGGACCCCTGCCAACATCCTGGT   360
  L  G  T  V  S  I  F  G  N  G  L  V  I  Y  L  F  N  K  S  A  A  L  R  T  P  A  N  I  L  V    93

----->                                                  <------- Crab-R3 --------
GGTCAACCTCGCCCTGTCCGATCTCATCATGCTTACCACGAACGTTCCTTTCTTCACCTACAACTGCTTCAGCGGTGGTGTGTGGATGTT   450
  V  N  L  A  L  S  D  L  I  M  L  T  T  N  V  P  F  F  T  Y  N  C  F  S  G  G  V  W  M  F   123

CAGCCCCCAGTACTGTGAGATCTACGCCTGTCTCGGAGCTATCACCGGCGTGTGCAGCATCTGGCTGCTGTGCATGATCTCCTTCGACAG   540
  S  P  Q  Y  C  E  I  Y  A  C  L  G  A  I  T  G  V  C  S  I  W  L  L  C  M  I  S  F  D  R   153

GTACAACATTATCTGCAACGGTTTCAACGGCCCCAAGCTGACCACTGGCAAGGCCGTAGTTTTTGCCCTCATCAGCTGGGTCATTGCAAT   630
  Y  N  I  I  C  N  G  F  N  G  P  K  L  T  T  G  K  A  V  V  F  A  L  I  S  W  V  I  A  I   183

CGGTTGTGCCCTTCCCCCCTTCTTCGGCTGGGGCAACTACATCCTGGAGGGAATCCTGGACTCCTGCAGCTACGACTACCTCACGCAGGA   720
  G  C  A  L  P  P  F  F  G  W  G  N  Y  I  L  E  G  I  L  D  S  C  S  Y  D  Y  L  T  Q  D   213

CTTCAACACTTTTAGCTACAATATCTTCATCTTCGTCTTCGACTACTTCCTCCCAGCCGCAATCATCGTTTTCTCCTACGTCTTCATTGT   810
  F  N  T  F  S  Y  N  I  F  I  F  V  F  D  Y  F  L  P  A  A  I  I  V  F  S  Y  V  F  I  V   243

                        --------- IVRH-F1 ---------->
GAAGGCTATCTTCGCTCACGAGGCCGCCATGCGCGCACAGGCCAAGAAGATGAACGTCTCCACCCTCCGCTCCAACGAAGCCGATGCCCA   900
  K  A  I  F  A  H  E  A  A  M  R  A  Q  A  K  K  M  N  V  S  T  L  R  S  N  E  A  D  A  Q   273

     ------- Crab-F1 ------->         <------- Crab-R2 --------
GCGTGCCGAAATCCGCATCGCTAAGACTGCCCTCGTCAACGTTTCTCTGTGGTTCATTTGCTGGACCCCCTACGCCCTCATCAGTCTGAA   990
  R  A  E  I  R  I  A  K  T  A  L  V  N  V  S  L  W  F  I  C  W  T  P  Y  A  L  I  S  L  K   303

                                                                                  <------
GGGTGTAATGGGTGACACTAGTGGTATCACCCCTCTGGTTTCCACCCTGCCCGCCCTGCTGGCCAAGTCCTGCTCTTGCTACAACCCCTT  1080
  G  V  M  G  D  T  S  G  I  T  P  L  V  S  T  L  P  A  L  L  A  K  S  C  S  C  Y  N  P  F   333

----- IVRH-R1 -------
CGTGTACGCCATCAGCCACCCGAAGTACCGTCTGGCCATCACCCAGCACCTGCCATGGTTCTGCGTGCATGAGACGGAAACGAAGAGCAA  1170
  V  Y  A  I  S  H  P  K  Y  R  L  A  I  T  Q  H  L  P  W  F  C  V  H  E  T  E  T  K  S  N   363

CGACGATTCTCAGTCGAACTCTACTGTGGCCCAGGACAAGGCCTAAACCAGCGGACTAAGCGCCACAGAGATGCTTAACGAACTCCTGAC  1260
  D  D  S  Q  S  N  S  T  V  A  Q  D  K  A  *                                               377

TCGCTACGATTGAATGAACCTTCGACAGAAGTACGATATTGAGTACGATCCCTCGACGAATTCCCTCAAA     1330

CTCCTTATAACGAGAGGCCTCTCGGTTATAAAGCACTCAATAAACCCCTGCAATTCCT(poly(A))    1388
```

Table 1. *The percentage of amino acid identity among several arthropod opsins*

| | | Brachyuran crab | | | *Limulus* | *Drosophila melanogaster* | | |
|---|---|---|---|---|---|---|---|---|
| | | BcRh1 | BcRh2 | Crayfish | *polyphemus* | Rh4 | Rh3 | Rh2 |
| *Drosophila* | Rh1 | 43 | 43 | 53 | 53 | 40 | 38 | 69 |
| | Rh2 | 41 | 42 | 51 | 51 | 39 | 37 | |
| | Rh3 | 38 | 39 | 36 | 41 | 73 | | |
| | Rh4 | 39 | 41 | 35 | 39 | | | |
| *Limulus* | | 42 | 42 | 57 | | | | |
| Crayfish | | 42 | 41 | | | | | |
| Brachyuran crab | BcRh2 | 75 | | | | | | |

The amino acid identities (percentage identity) between each pair of sequences were calculated using a computer program for the homology analysis (GENETYX; SDC Software Co.). The program is based on an algorithm proposed by Lipman and Pearson (1985), where gaps were allowed in the sequences to maximize alignment.

The references for the opsin sequences are as follows: *Drosophila* Rh1, Zuker *et al.* (1985); *Drosophila* Rh2, Cowman *et al.* (1986); *Drosophila* Rh3, Zuker *et al.* (1987); *Drosophila* Rh4, Montell *et al.* (1987); *Limulus*, Smith *et al.* (1993); crayfish, Hariyama *et al.* (1993).

The horseshoe crab *Limulus* has two opsins (Smith *et al.* 1993): one was isolated from the ocelli, the other from the lateral eyes. They are 99 % identical, so only values for the lateral-eye opsin are presented in this table.

transcribed from cDNA fragments BcP-1 and BcP-2 (see Materials and methods) of the brachyuran crab opsins BcRh1 and BcRh2, respectively. We verified that the control (sense) probes with sequences identical to the opsin mRNAs gave no hybridization signal (for example, see Fig. 4A,C). Using antisense cRNA probes, we obtained a clear hybridization signal only in the photoreceptor cells of the compound eye. In longitudinal sections, the hybridization signals of the BcP-1 and BcP-2 probes were restricted to the layer of photoreceptor cells forming the main rhabdom in the retina (Fig. 4B,D). In cross sections, both probes gave the signal exclusively in the cell bodies of all the photoreceptors surrounding the rhabdom in each ommatidium (Fig. 4E,F). We also confirmed that, on adjacent cross sections obtained from one tissue, both probes

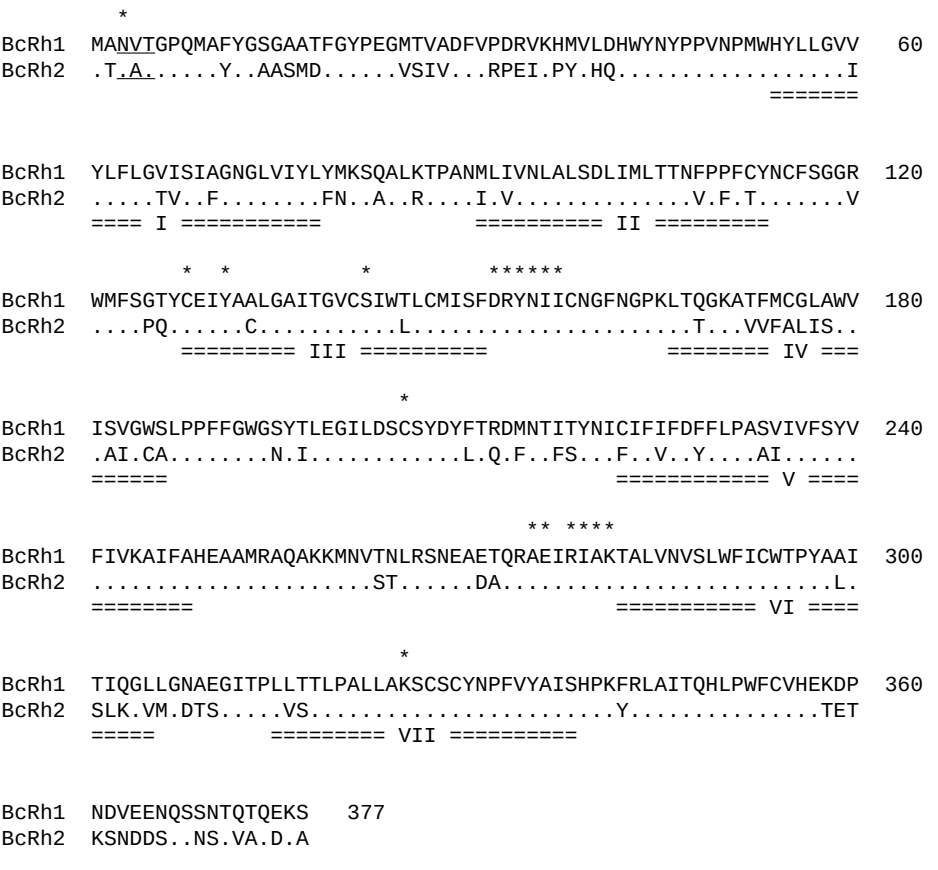


Fig. 3. Comparison of the amino acid sequences of the brachyuran crab opsins BcRh1 and BcRh2. Dots indicate sequence identity, and only where the two sequences differ is the amino acid indicated. Asterisks indicate the amino acid residues highly conserved in other opsins (for details see Results). The predicted transmembrane segments (I–VII) are indicated by double broken lines and putative glycosylation sites are underlined.

gave the hybridization signal in all the photoreceptor cells forming the main rhabdom in each ommatidium (as shown in Fig. 4E,F). The two probes showed similar patterns of hybridization, and the hybridization signal of each probe was observed in all regions of the compound eye. The two opsin mRNAs were expressed at similar levels.

There is a possibility that the two probes used for *in situ* hybridization cross-reacted. However, each of the cDNA fragments (BcP-1 and BcP-2) from which the two opsin probes were generated shared at most 40 % identity with the other opsin cDNA, and the probes did not cross-react on dot blots with the stringency conditions used for the *in situ* hybridizations (data not shown). Therefore, it is very unlikely that the probes cross-reacted on the sections used for the *in situ* hybridizations.

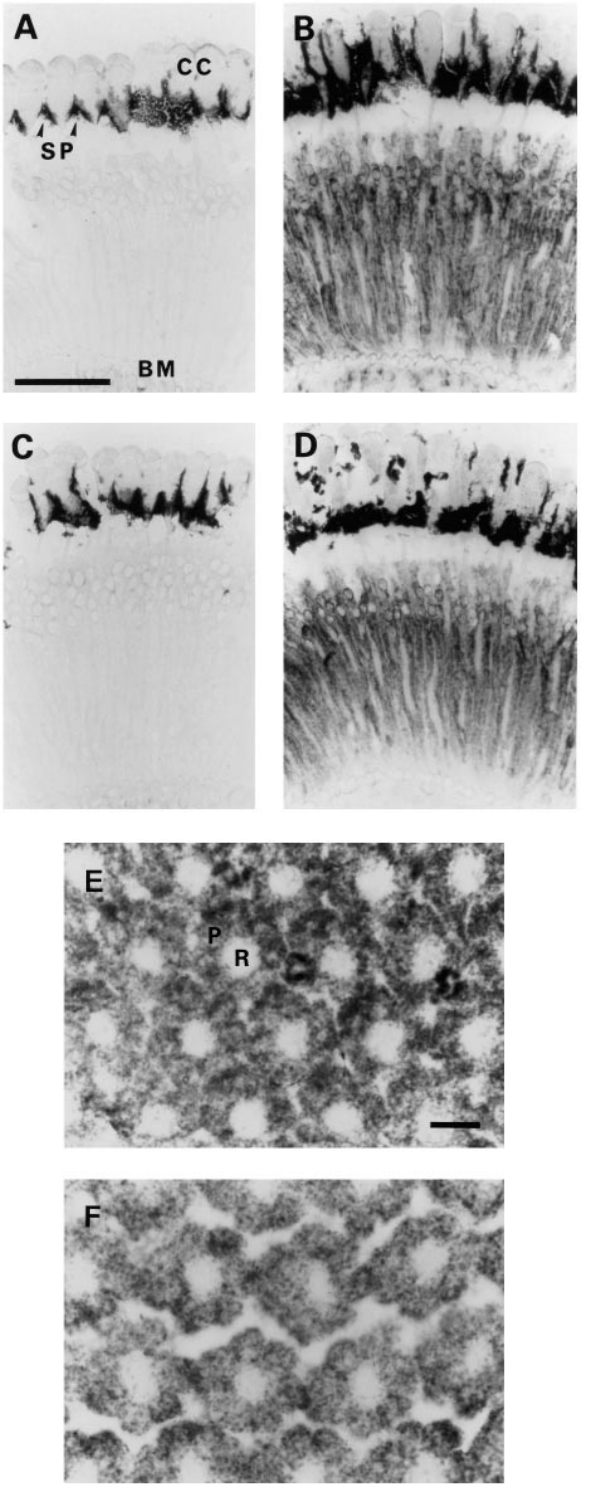


Fig. 4. Tissue distribution of the brachyuran crab opsin mRNAs examined by *in situ* hybridization. Sense and antisense cRNA probes were transcribed from cDNA fragments BcP-1 and BcP-2 of the crab opsins BcRh1 and BcRh2, respectively. The sense cRNA probes for BcP-1 (A) and BcP-2 (C) hybridized to longitudinal sections through retinular cells. The antisense cRNA probes for BcP-1 (B,E) and BcP-2 (D,F) hybridized to longitudinal sections through retinular cells (B,D) and cross sections through several ommatidia (E,F). The control (sense) probes gave no hybridization signal (A,C; the black matter under the crystalline cones was the screening pigment). On longitudinal sections, the hybridization signal from the antisense BcP-1 and BcP-2 probes was restricted to the layer of retinular cells (B,D). On cross sections, both antisense probes gave the signal exclusively in the cell bodies of all the photoreceptors surrounding the rhabdom in each ommatidium (E,F). BM, basement membrane; CC, crystalline cone; P, photoreceptor cell body; R, rhabdom; SP, screening pigments. Scale bars, 100 $\mu$m (A–D) and 10 $\mu$m (E, F).

As previously described in detail by Arikawa *et al.* (1987*b*), the ommatidial retina of *H. sanguineus* consists of seven regular retinular cells (R1–R7) forming the main rhabdom and a small distal retinular cell (R8), as in other crab eyes (Stowe, 1980; Shaw and Stowe, 1982). Therefore, these findings suggest that the mRNAs of the brachyuran opsins BcRh1 and BcRh2 were expressed simultaneously in all of the seven regular retinular cells (R1–R7). Two different visual pigments might be present in a single photoreceptor cell in the retina of this crab. We could not determine whether the hybridization signal was associated with the R8 photoreceptor in the distal retinular cell.

### *Spectral sensitivity*

The spectral sensitivity curve of the compound eye was determined by the ERG method (Fig. 5). The maximum sensitivity was at about 480 nm and there was a broad shoulder at 330–400 nm. This curve should reflect the absorption spectrum of the dominant visual pigment, except to the extent that it may be distorted by selective filtering by the dioptric apparatus and accessory pigments.

## Discussion

We isolated two opsin cDNAs from the compound eye of the brachyuran crab *Hemigrapsus sanguineus* (Fig. 2). Like other opsins, both deduced protein sequences were predicted to consist of seven transmembrane segments and had the appropriate conserved amino acid residues (Fig. 3). This crab probably possesses at least two visual pigments in its retina.

In crabs, one or two receptor systems have been described. Microspectrophotometric measurements have revealed only a

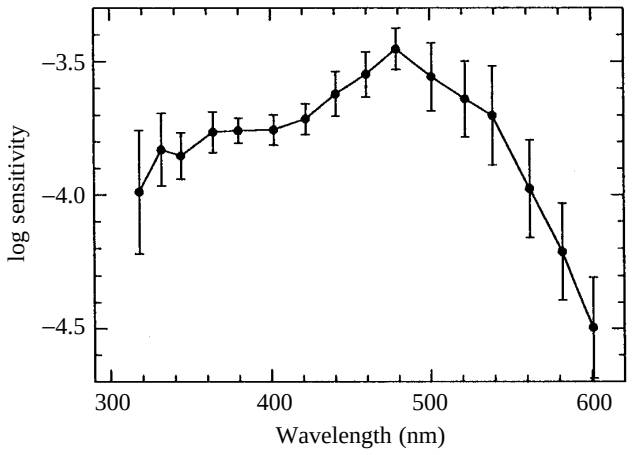


Fig. 5. Spectral sensitivity (defined in Materials and methods) of the compound eye determined by electroretinogram. The symbols indicate the mean values for 10 dark-adapted animals with standard deviations.

single visual pigment (e.g. spider crab *Libinia emarginata*, Hays and Goldsmith, 1969; green crab *Carcinus maenas*, Bruno *et al.* 1973; blue crab *Callinectes sapidus*, Bruno and Goldsmith, 1974; fiddler crab *Uca pugnax*, Scott and Mote, 1974; 27 species of crabs from a variety of habitats, Cronin and Forward, 1988). The visual pigments found in these photometric measurements have absorption maxima at wavelengths of 470–520 nm. From electrophysiological measurements using electroretinographic (ERG) or intracellular recordings, some workers have suggested that there is only one colour type of retinular cell, maximally sensitive in the blue-green or green region of the spectrum (*Callinectes sapidus*, Goldsmith and Fernandez, 1968; *Libinia emarginata*, Wald, 1968; *Carcinus maenas*, Bruno *et al.* 1973; *Uca pugilator*, *Uca pugnax*, *Callinectes sapidus* and the grapsid crab *Sesarma reticulatum* and , Scott and Mote, 1974; the grapsid crab *Leptograpsus variegatus*, Stowe, 1980). However, other workers have found an additional shorter-wavelength receptor system in some crabs, including several of these species (*Carcinus maenas*, Wald, 1968; *Uca pugilator*, Hyatt, 1975; *Carcinus maenas* and *Callinectes sapidus*, Martin and Mote, 1982; the gecarcinid crab *Gecarcinus lateralis*, Lall and Cronin, 1987). It is therefore possible that crabs in general are dichromats. In any case, the sensitivity of crab compound eyes is dominated by a visual pigment with maximal absorption at wavelengths of 470–520 nm.

As in other decapod compound eyes (Shaw and Stowe, 1982), the ommatidial retina of crabs consists of seven regular retinular cells (R1–R7), forming the main rhabdom, and a small distal retinular cell (R8) (Stowe, 1980; Arikawa *et al.* 1987*b*). Main rhabdoms of decapod crustaceans usually have only a single visual pigment, absorbing at intermediate wavelengths (450–550 nm) in the visible spectrum (Cronin, 1985; Cronin and Forward, 1988). In crabs, these seven retinular cells (R1–R7) also have only one colour type of visual pigment maximally sensitive at 470–520 nm, when measured using intracellular recordings and microspectrophotometry (Stowe, 1980; Cronin and Forward, 1988). The present *in situ* hybridization experiments suggested that the two opsins of the crab *Hemigrapsus sanguineus* were expressed simultaneously in all of the seven retinular cells (R1–R7) in each ommatidium (Fig. 4). In addition, the spectral sensitivity curve of the dark-adapted eye, determined from the ERG, suggested that the sensitivity of the compound eye was dominated by a visual pigment maximally sensitive at about 480 nm (Fig. 5). Thus, the two opsin proteins of this crab might produce visual pigments with maximal absorption in the blue-green region of the spectrum.

Because main rhabdoms in crabs (formed from the seven retinular cells R1–R7) have only a single visual pigment maximally sensitive in the blue-green or green region of the spectrum, the shorter-wavelength sensitivity apparently resides in the rhabdomeres of the eighth retinular cell (R8) (Stowe, 1980; Martin and Mote, 1982), as in other crustacean species (Cummins and Goldsmith, 1981; Cummins *et al.* 1984; Cronin *et al.* 1994). The R8 cells are smaller, and there are fewer of them, than the seven regular cells (Shaw and Stowe, 1982). This may make it difficult to isolate different colour receptors in the retina using either microspectrophotometric or electrophysiological measurements. We could not determine using *in situ* hybridizations whether the hybridization signal was associated with the R8 cell of *H. sanguineus*. However, the spectral sensitivity curve of the crab had a shoulder at 330–400 nm that appears to be due to the shorter-wavelength receptor (Fig. 5). If the R8 cell is maximally sensitive at a shorter wavelength, the crab might have another opsin protein. Because only retinal has been found in the eye of *H. sanguineus* as the visual pigment chromophore (Arikawa *et al.* 1987*b*), the crab should produce different colour types of visual pigments from different opsin proteins. In this study, we could not amplify cDNAs other than the two opsins BcRh1 and BcRh2. This is possibly because the amino acid sequence of the putative shorter-wavelength opsin was rather different from those of other invertebrate visual pigments and thus the primers IVRH-F1 and IVRH-R1 used in PCRs did not hybridize to the opsin cDNA.

The *in situ* hybridization experiments suggested that two different visual pigments were present in one photoreceptor cell in the retina of *Hemigrapsus sanguineus* (Fig. 4). In vertebrate eyes, although it is generally accepted that there is one visual pigment per photoreceptor cell, cone cells of certain mammalian species have recently been shown to express different opsins simultaneously under natural conditions (Röhlich *et al.* 1994). However, although the existence of two different spectral functions in one visual cell is not uncommon in invertebrates (Nolte and Brown, 1969, 1972; Minke *et al.* 1973), to our knowledge there are no reports showing that two types of visual pigments coexist in one photoreceptor cell in invertebrate eyes. As described above, both visual pigments of the crab opsins were likely to have the same maximum sensitivity. However, we cannot assert that the two pigments had exactly the same absorption maxima. Intracellular

recordings of photoreceptor cells could answer this question to some extent. The absorption maxima of the visual pigments of the crab may eventually be obtained using opsins produced in tissue culture. To characterize these visual pigments, further studies are needed.

In the brachyuran opsins, the polar Tyr-131 seems to be the Schiff base counterion (see Results). However, these opsins have another polar residue (Ser-142, Fig. 3) in helix III, which is also conserved in all invertebrate opsins except the ultraviolet opsins. According to the model proposed by Chang *et al.* (1995) for blue shifts in opsin wavelength regulation, these two polar residues near the protonated Schiff base end of the chromophore are likely to stabilize the protonated Schiff base. As described by Chang *et al.* (1995), instead of having one charged amino acid residue in the position of the counterion to stabilize the protonated Schiff base, as in vertebrates (Glu-131 of bovine rhodopsin, Sakmar *et al.* 1989; Nathans, 1990), invertebrate opsins appear to have two polar residues. The invertebrate ultraviolet opsins are generally thought to have an unprotonated Schiff base chromophore without a positive charge (Kropf and Hubbard, 1958; Sakmar *et al.* 1989; Zhukovsky and Oprian, 1989; Nathans, 1990; Lin *et al.* 1992) and seem to have no polar residue near the Schiff base (Chang *et al.* 1995). In respect of the counterion, the brachyuran opsins are not ultraviolet but visible-wavelength visual pigments. The present findings suggest that the two opsins of the brachyuran crab are maximally sensitive in the blue-green region of the spectrum. However, the brachyuran opsins show only about 40 % identity (at the amino acid level; see Table 1 and Results) either with the visible-wavelength opsins or with the ultraviolet opsins of arthropods. The two opsins of the brachyuran crab may be somewhat different from other arthropod opsins.

In summary, the present findings suggest that the brachyuran crab *Hemigrapsus sanguineus* has two opsins which produce visual pigments with maximal absorption in the blue-green region of the spectrum and that both opsins are simultaneously expressed in a single photoreceptor cell.

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