CMLS, Cell. Mol. Life Sci. 58 (2001) 1583–1598
1420-682X/01/111583-16 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Review

Vision in the ultraviolet

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Received 22 February 2001; received after revision 11 May 2001; accepted 15 May 2001

Abstract. Sensitivity to ultraviolet light (UV) is achieved by photoreceptors in the eye that contain a class of visual pigments maximally sensitive to light at wavelengths <400 nm. It is widespread in the animal kingdom where it is used for mate choice, communication and foraging for food. UV sensitivity is not, however, a constant feature of the visual system, and in many vertebrate species, the UV-sensitive (UVS) pigment is replaced by a violet-sensitive (VS) pigment with maximal sensitivity between 410 and 435 nm. The role of protonation of the Schiff base-chromophore linkage and the mechanism for tuning of pigments into the UV is discussed in detail. Amino acid sequence analysis of vertebrate VS/UVS pigments indicates that the ancestral pigment was UVS, with loss of UV sensitivity occurring separately in mammals, amphibia and birds, and subsequently regained by a single amino acid substitution in certain bird species. In contrast, no loss of UV sensitivity has occurred in the UVS pigments of insects.

Key words. Opsin; visual pigment; evolution; ultraviolet; spectral tuning; G-protein receptor.

The electromagnetic spectrum forms a continuum from very longwave and low-energy radio waves to shortwave high-energy gamma rays. However, only a very small portion of this spectrum is visible to animals as light. Radiation below 320 nm [ultraviolet (UV)A] is largely screened out by the ozone layer in the Earth's upper atmosphere and is therefore unavailable to the visual system, but radiation above 320 and below 400 nm (UVB) can be perceived by many animal species. In humans, sensitivity in the shorter-wavelength region is truncated at around 400 nm by the high lens absorbance of wavelengths below 400 nm. This is not the case in many other species where the cornea and lens are transparent below 400 nm, and sensitivity in this region may be further enhanced by the presence of photoreceptor cells in the retina that are maximally sensitive below 400 nm, in the UV region of the spectrum. In fact, UV sensitivity is widespread in the animal kingdom. At the other end of the visible spectrum, sensitivity extends to around 700–750 nm.

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Spectral sensitivity of the vertebrate visual system

In vertebrates, sensitivity to light is achieved by the presence of rod and cone photoreceptors in the retina. Photoreceptors are composed of inner and outer segments connected by a cilium. Light sensitivity is conferred by visual pigment molecules embedded in the disc membranes of the outer segment; each type or class of photoreceptor contains a visual pigment differing from others in its peak of spectral sensitivity (λ_{max}). A single class of rod cells is usually present containing a pigment with a λ_{max} generally around 500 nm in the blue-green region of the spectrum. Rods are functional in dim light and form the basis of the scotopic system. In contrast, cones are largely responsible for vision at normal light levels (phototopic vision), and the presence of two or more different

cone classes each with a different λ_{max} enables the visual system to sample light levels at different spectral locations. Comparison of the photon catch by the different cone photoreceptors of a neural opponency system provides the basis for colour vision (fig. 1).

Photon capture within the photoreceptors is the role of the photosensitive visual pigments (fig. 2). These pigments are members of the superfamily of G-protein-coupled receptors which function through the activation of a guanine-nucleotide-binding protein (G-protein) and an effector enzyme which changes the level of a second messenger in the cell cytoplasm. Each visual pigment is based on the same basic structure of a chromophore attached to an opsin protein. In vertebrates, the chromophore is either 11-*cis*-retinal or 11-*cis*-3,4-dihydroretinal, the derivatives of vitamin A1 and A2, respectively, to give either rhodopsin or porphyropsin pigments. Porphyropsins are not found in either birds or mammals but may be present in fish, reptiles and amphibia. Rhodopsin and porphyropsin pigments differ in λ_{max}, the latter being longwave shifted, particularly at longer wavelengths [1–3]. With this exception, the differing spectral sensitivities of visual pigments is determined by the amino acid sequence of the opsin protein.

In all vertebrate taxa except mammals, up to four different cone visual pigments may be present, each belonging to a different cone class, as demonstrated by phylogenetic analysis (fig. 3). The four cone classes are distinguished

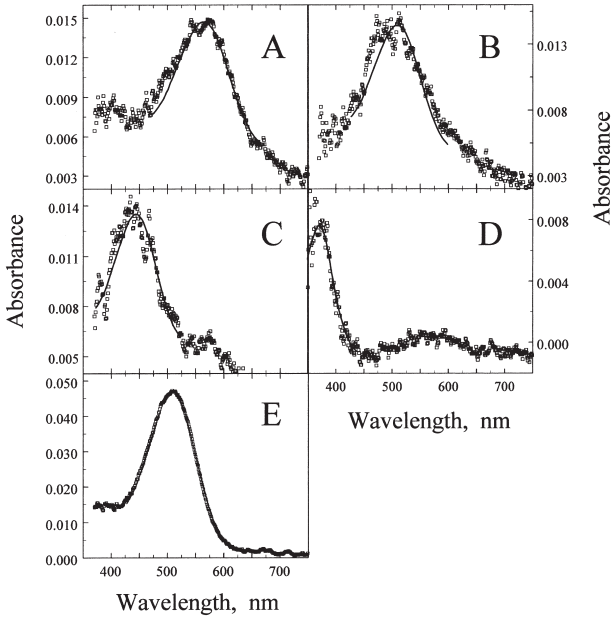

Figure 1. Spectra of visual pigments in rods and cones. The data shown here were collected by microspectrophotometry from the retina of a typical avian species, the budgerigar *Melopsittacus undulatus*, with a rod and four classes of cones. (*A*) LWS cones with λ_{max} 564 nm. (*B*) MWS cones with λ_{max} 508 nm. (*C*) SWS cones with λ_{max} 444 nm. (*D*) UVS cones with λ_{max} 371 nm. (*E*) Rod class with λ_{max} 509 nm.

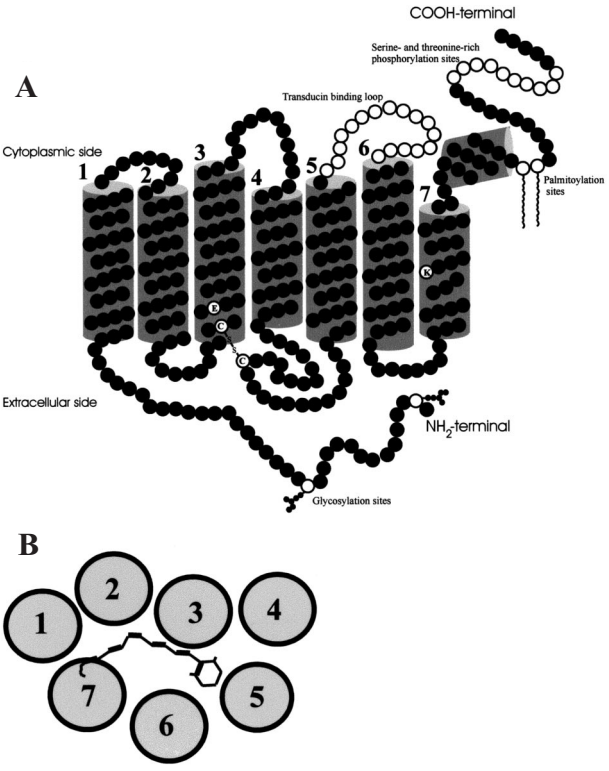

Figure 2. Structure of a visual pigment. (*A*) Diagram of an opsin molecule showing seven α-helical transmembrane regions connected by intra- and extra-cellular loops. The positions of key amino acid residues and protein domains are indicated [redrawn from ref. 52]. (*B*) Plan view of molecule as determined by Schertler and Hargrave [51], viewed from outside the cell, showing relative positions of the seven transmembrane regions forming a retinal-binding pocket.

on the basis of the amino acid sequence of their respective opsins and roughly correlate with spectral sensitivity: longwave sensitive (LWS) with λ_{max} 500–570 nm, middlewave sensitive (MWS) with λ_{max} 480–520 nm, shortwave sensitive (SWS) with λ_{max} 415–470 nm, and violet/ultraviolet sensitive (VS/UVS) with λ_{max} lying between 435–355 nm. In mammals, this complement is reduced to only two classes, LWS and VS/UVS, an event believed to have resulted from a nocturnal life style that mammals went through during their evolution, and during which the other clone classes were discarded. This reduction has been partially reversed in Old World primates [4] and in the New World howler monkey [5, 6] by recent duplications of the LWS opsin gene that gave rise to different 'green' and 'red' sensitive variants of the LWS class pigment, resulting in trichromacy. The so-called 'blue' pigments of mammals are in fact from the VS/UVS class and are more accurately described, for example, as 'human violet' (λ_{max} 419 nm) or 'mouse UV' (λ_{max} 357 nm). In other mammalian species such as the African giant rats, *Cricetomys gambianus* and *C. emini*, and the earless seals, *Phoca hispida* and *P. vitulina*, VS/UVS pig-

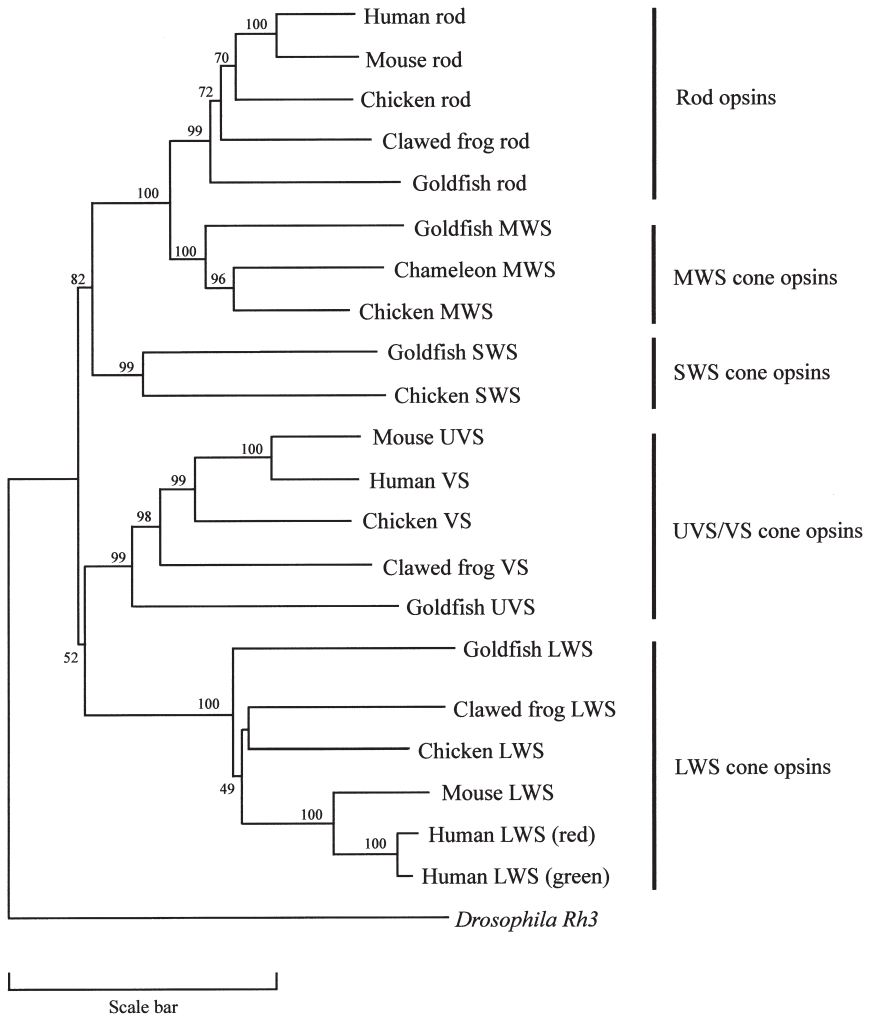

Figure 3. Phylogenetic tree of representative rod and cone opsins. The amino acid sequences of the opsin proteins were aligned by Clustal W and the tree was generated by the neighbour-joining method [129]. The bootstrap confidence values are shown for each branch. The *Drosophila Rh*3 opsin sequence was used as an outgroup. The scale bar is equal to 0.1 substitutions per site.

ments are totally lacking from the retina [7], and in the nocturnal owl monkey [8] and the dolphin *Tursiops truncatus* [9], their absence is explained by the accumulation of mutations in the VS/UVS opsin gene. Whether the loss of VS/UVS cones in these species confers any advantage has yet to be established.

Vision in the shortwave region of the spectrum is subserved therefore by the VS/UVS class of pigments and, as shown in figure 3, UVS or VS pigments are encoded by members of the same opsin gene family. In all species examined so far, the presence of a VS or UVS pigment is mutually exclusive. In primates, even though the pigment has a λ_{max} of around 420 nm, spectral sensitivity would extend into the UV if the cornea and lens transmitted light in this region of the spectrum. In other species such as the mouse, however, the VS/UVS gene specifies a true UVS pigment [10] which together with a UV-transparent cornea and lens provides for light sensitivity at wavelengths below 400 nm [11].

Function of UV sensitivity

The function of UV vision has been most clearly established in vertebrate taxa other than mammals. In birds, the presence of UVS pigments is relatively common, particularly amongst passerines, and an adaptive role in mate selection has been demonstrated in three species, zebra finch, *Taeniopygia guttata,* starling, *Sturnus vulgaris*, and blue tit, *Parus caeruleus* [12–14]; in all three species, UV reflectance from plumage has been shown to be an important factor in the selection of male partners by females. There is also evidence that UV sensitivity is important in some species for foraging for food, for example in prey detection by kestrels [15] and for nectar collection in humming birds [16]. Another example of the use of UV vision is communication in anoline lizards whose dewlap reflects UV strongly [17], and this is subserved by the presence of a UVS pigment in the retina.

UVS pigments are also present in many freshwater and marine fish species, with examples distributed across a number of the major orders such as the cyprinids [18–20], the beloniforms [21], the perciformids (cichlids) [22] and the salmonids [23]. A common feature of UVS cones is that they are present in young fish but may be lost in adults. In brown trout (*Salmo trutta*), for example, UVS cones can only be found in fish up to 1–2 years of age [23] and in the sockeye salmon (*Oncorhynchus nerka*), the number of UVS cones diminishes greatly when the fish transform from parr to smolts [24, 25], although in this case, they increase again in the adult [26, 27]. UV sensitivity is also seen in many species of marine reef fish [28]. The presence of UVS cones in young fish may be related to plankton feeding, with sensitivity to UV light enhancing the detection of zooplankton [23, 29, 30], whereas their loss in older animals may correlate with a change in feeding behaviour, because the larger fish move to deeper water where less UV light is available. A role in conspecific recognition may also be important [31, 32].

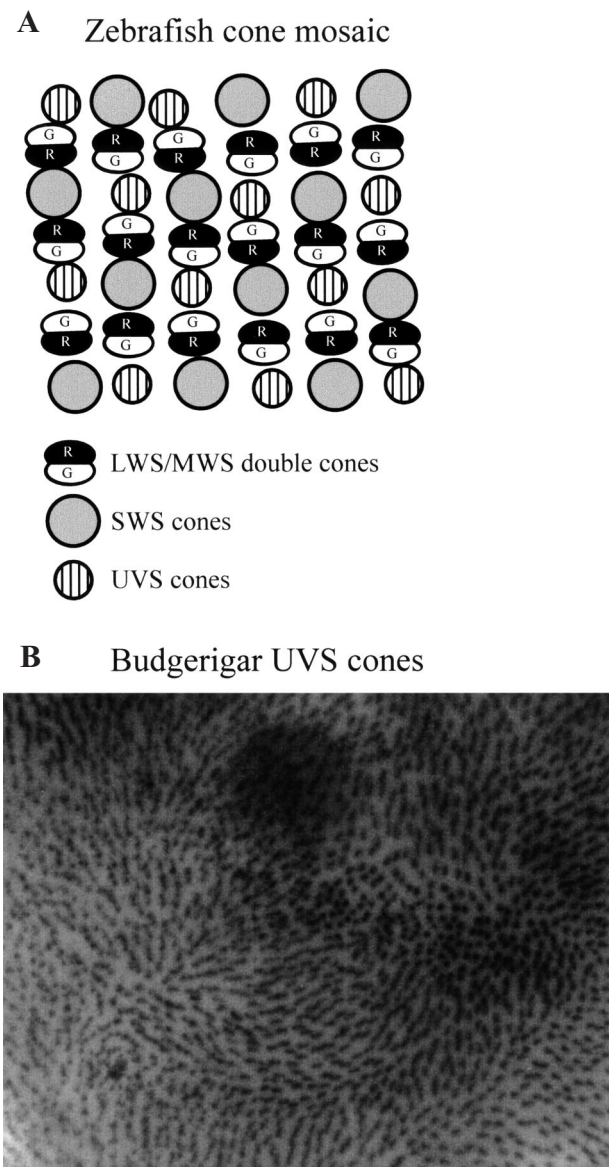

Figure 4. Retinal photoreceptor mosaics. (*A*) Zebrafish mosaic redrawn from Tohya et al. [130] (*B*) Semi-regular mosaic of UVS cones in whole mounts of the budgerigar retina [from ref. 40].

Distribution of VS and UVS photoreceptors

In mammals, the frequency of VS/UVS cones is generally less than that of the MWS or LWS cones, even in species such as the Californian ground squirrel, *Spermophilus beecheyi*, with a cone-rich retina [33]. They may also have a far less uniform distribution. For example, primates possess VS cones that are distributed throughout the retina (with the exception of the very central fovea in humans). In the mouse, the situation is more irregular, since all cones express LWS opsin and most co-express UVS opsin [34, 35]. The 'LWS only' cones are restricted to the dorsal retina where they predominate, whereas the cones that express both opsins show a gradation of increasing UVS opsin from dorsal to ventral. This phenomenon was first reported by Szél et al. [36], who demonstrated that all cones express the 'shortwave pigment' in a ventral region of variable width. The significance of these asymmetries is unknown.

In contrast, a mosaic of photoreceptors is seen in a number of fish species, consisting of regular alternating rows of double and single cones, although the specific pattern varies with species [23, 37]. UVS and SWS single cones frequently occupy a corner position in the mosaic such that there are twice the number of LWS and MWS cones as SWS and UVS (fig. 4A). The temporal expression of the different opsins in cone photoreceptors in the retina of the developing goldfish (*Carassius auratus*) follows a precise sequence of LWS : MWS : SWS : UVS and, in contrast to the mammalian retina, individual photoreceptors express only one type of opsin [38]. The distribution of VS/UVS cones has been reported for only two avian species, the chicken *Gallus gallus* [39] and the budgerigar *Melopsittacus undulatus* [40]. In both cases, the cells are evenly distributed across the retina and, at least in the budgerigar, appear to be arranged into an approximate mosaic (fig. 4B), although their positioning in relation to other cone classes has yet to be resolved. The UVS cones in this species represent around 9% of total cones, a frequency approximately equal to that of SWS cones [40].

VS/UVS visual pigments

The sequences of VS and UVS opsin genes have now been reported in over 20 species. These range from the

VS opsins of primates [41, 42] and chicken [43] to the true UVS pigments of several fish [21, 22, 44], birds [40, 45, 46], mammals [10], and a single reptilian species [47]. As described above, a molecular phylogenetic analysis of opsin sequences confirms that the VS and UVS pigments belong to a single opsin gene class, consistent with a single evolutionary origin. A more detailed phylogenetic analysis is shown in figure 5 where representatives of VS and UVS sequences from all four vertebrate taxa have been included. From this, it is not clear whether the ancestral pigment was VS or UVS. Teleost fish generally possess a UVS pigment (although it is not uncommon for a shortwave-shifted SWS pigment with λ_{max} of <430 nm to be present [48]), whereas all amphibia examined so far have only VS pigments. The American chameleon *Anolis carolinensis* has a UVS pigment [47], and both VS and UVS pigments are seen amongst the different avian and mammalian species. For reasons which will be explained later, the most likely scenario is that the ancestral pigment was UVS and, during evolution, spectral shifts into the violet and secondarily into the UV occurred separately in the different taxa. It would follow from this that the molecular basis for these spectral shifts may not be the same in fish, reptile, bird and mammalian UVS pigments.

Tuning of visual pigments

Opsins consist of a single polypeptide chain of 340–500 amino acids that form seven α-helical transmembrane (TM) regions connected by cytoplasmic and extracellular loops [49–50]. In the tertiary structure (fig. 6), the seven α helices form a bundle within the membrane creating a hollow cavity on the extracellular side, the chromophore-binding pocket [51, 52]. All visual pigments possess a Lys residue at site 296 (bovine rod opsin numbering) that is covalently linked to the chromophore via a Schiff base (SB). In vertebrates, this SB is generally thought to be protonated, with the negatively charged residue at site 113 (Glu113) acting as a counterion to stabilize electrostatically the proton of the SB [53]. Absorption of light causes the isomerization of the chromophore from 11-*cis*- to all-*trans*-retinal and this in turn causes major structural changes that include the displacement of the positively charged SB from its interaction with Glu113 [54, 55]. Low stability of the uncompensated, positively charged group in the hydrophobic environment of the retinal-binding pocket leads to deprotonation and the production of the photointermediate metarhodopsin II (MII). The λ_{max} of bovine rod opsin shifts from around 500 to 380 nm in the MII unprotonated state. These structural

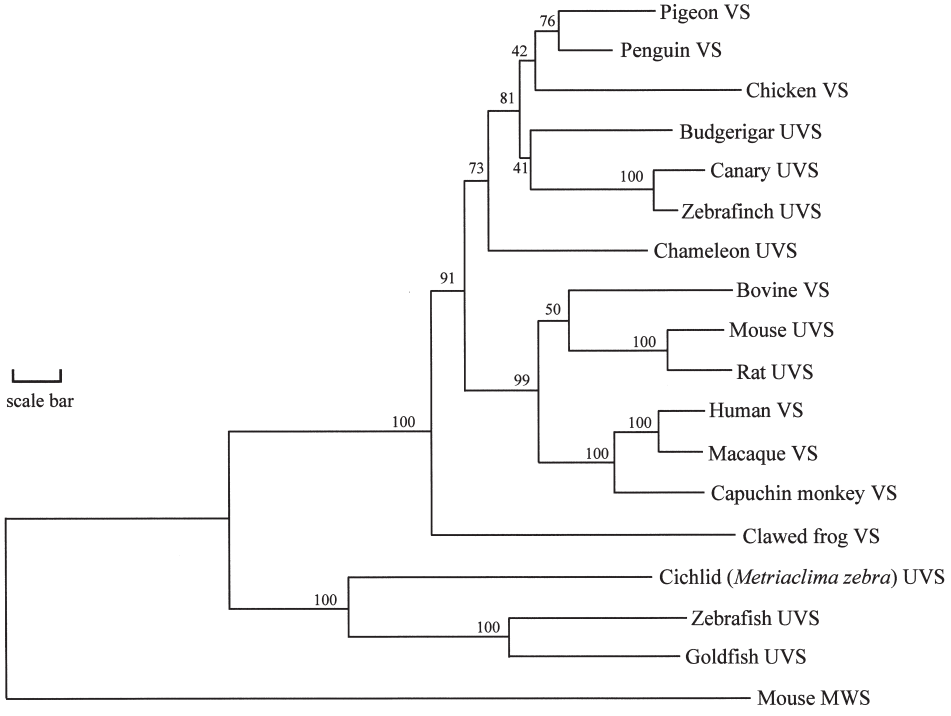

Figure 5. Phylogenetic tree of VS and UVS opsins. Details of analysis are as given in the legend to figure 3. GenBank accession numbers and references: pigeon VS, AJ238856; penguin VS [81]; chicken VS, M92039; budgerigar UVS, Y11787; canary UVS, AJ277922; zebrafinch UVS, AF222331; chameleon UVS, AF134192; bovine VS, U92557; mouse UVS, AF190671; rat UVS, AF051163; human VS, NM001708; macaque VS, AF158976; *Cebus olivaceus* VS, AF039422; clawed frog VS, U23463; *Metriaclima zebra* UVS, AF191219; zebrafish UVS, AF109373; goldfish UVS, D85863; mouse MWS, AF191085. The mouse MWS opsin sequence was used as an outgroup. The scale bar is equal to 0.01 substitutions per site.

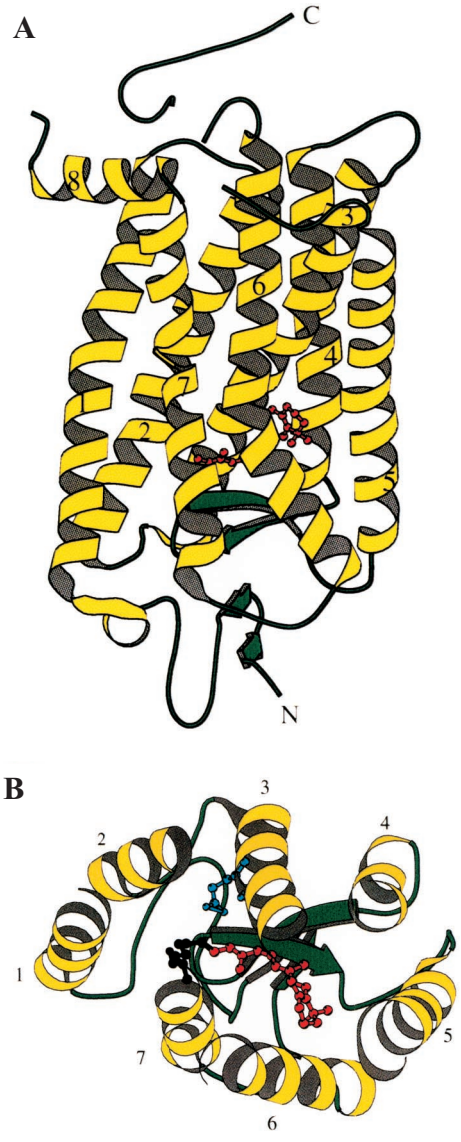

Figure 6. Ribbon drawings showing the crystal structure of bovine rhodopsin; view parallel to the plane of the membrane (*A*) and view into the plane of the membrane from the cytoplasmic side (*B*). Helices 1–7 are transmembrane and helix 8 is cytoplasmic. The retinal chromophore is shown in red bonded by a Schiff base linkage to Lys296 (black) with the Glu113 counterion shown in pale blue. These figures are derived from crystallographic data [52].

changes enable MII to activate the G-protein transducin. Protonation of the SB and its subsequent removal is not a prerequisite, however, for the structural changes that lead to the production of MII. Removal of the Glu113 counterion by replacement with uncharged Gln in site-directed mutagenesis experiments with bovine rod opsin [56] resulted, after regeneration with 11-*cis*-retinal in vitro, in a pH-dependent equilibrium mixture of unprotonated and protonated pigments with λ_{max} values of 380 and 490 nm respectively [56]. At a pH of 8.8 and above, only the unprotonated SB was present, whereas at pH 3.3, this was replaced by a protonated species. However, both forms were capable of activating transducin after illumination [57].

The particular λ_{max} of a visual pigment is thought to depend on a number of interactions [58], although their relative importance may vary from pigment to pigment. The strength of the interaction between the Glu113 counterion and the protonated SB (PSB) is critical, since a strong interaction will prevent delocalization of the charge on the PSB along the chromophore, thereby stabilizing the ground state and resulting in a shorter-wave-sensitive pigment. Photoexcitation of 11-*cis*-retinal induces a significant increase in π electron delocalization, and a corresponding change in its dipole moment [59, 60]. Interactions of charged, polar or polarizable residues that alter delocalization will lead to a change in the energy difference between ground and excited states. Increases in delocalization will result in longwave shifts in the absorbance spectrum, whereas decreases will lead to shortwave shifts. Constriction of the chromophore-binding pocket by bulky residues particularly at site 121 may also affect spectral tuning by planarization of the polyene chain of the chromophore due to stearic interactions with the opsin [55].

The opsin shift

Solvated retinal has a λ_{max} at 380 nm which when combined with an amino-group-containing compound into a simple PSB chromophore is longwave shifted to 440 nm. The λ_{max} values of visual pigments however range from 360 to above 600 nm, depending on the specific opsin involved. This shift in λ_{max} is termed the 'opsin shift' and its molecular basis has been examined largely in primate red LWS and green LWS cone opsins [61, 62] and in bovine rod opsin [63]. In the former case, the spectral shift from around 530 nm of the green pigment to around 560 nm of the red pigment is largely the result of substitutions at site 164 in TM 4, and sites 261 and 269 in TM 6. The location of these sites within the retinal-binding pocket was first established by reference to a model based on conserved residues across more than 500 G-protein-linked receptor proteins [64, 65] and refined by crystallographic studies [51, 66]. In each case, the sites are orientated towards the interior of the central hydrophobic pocket and involve the replacement of non-polar by polar amino acids. The three sites involved are adjacent to the polyene chain of retinal; the polar substitutions would serve to increase charge delocalization, reducing the energy required for the transition from ground to excited state and thereby producing a longwave shift.

Spectral shifts into the violet region of the spectrum

The opsin shift from the 500 nm of the bovine rod to ~420 nm of the human VS pigment has been examined in detail using site-directed mutagenesis of bovine rod opsin to introduce residues present in human VS [67]. To short-wave shift rod opsin by around 60 nm and generate a VS pigment analogue, six substitutions were required: Gly90Ser, Ala117Gly, Glu122Leu, Trp265Tyr, Ala292Ser and Ala295Ser. The conformation of the chromophore was examined by resonance Raman vibrational spectroscopy by probing the SB base (C=N) stretch, the ethylenic stretch across C7–C15 of the polyene chain, the fingerprint stretch around C8 and C9, and the hydrogen out-of-plane wag of C11 and C12 [58] (fig. 7). Comparison of these vibrational modes of the VS analogue pigment with those of a simple PSB chromophore in methanol revealed a surprisingly high degree of similarity. This suggests that the λ_{max} of this analogue is not determined by strong perturbations of the chromophore structure by the protein. Instead, the protein environment solvates the PSB in a similar manner to methanol. Further comparisons with the vibrational spectrum of the human green LWS pigment showed a significant shift in the C=N stretch from 1660 cm^{-1} in the VS analogue to 1641 cm^{-1} in the LWS green pigment and an increase in the shift induced by transfer from H_2O to D_2O. These shifts correlate with an increase in the strength of both the hydrogen bonding of the SB proton and the electrostatic interaction between the SB proton and its counterion. Thus, the introduction of three Ser residues at position 90 in TM 2, and 292 and 295 in TM 7 in the vicinity of the PSB serves to generate a more polar, methanol-like environment, resulting in a dielectric stabilization of the ground state of the chromophore. The Ala117Gly and Glu122Leu substitutions in TM 3 would appear to act synergistically with the TM 2 and TM 7 substitutions by generating a slight movement of the counterion towards the SB, thereby increasing the strength of the electrostatic interaction. In contrast, the effect of the Trp265Tyr substitution in TM 6 was attributed to a decrease in solvent polarizability close to the β-ionone ring of the chromophore, destabilizing the excited state and further increasing the energy gap between ground and excited states. The recent crystal structure of rhodopsin [52] places sites 122 and 265 within the chromophore-binding pocket near the β-ionone ring, with site 117 providing one of the side chains lining the pocket near the polyene chain. Furthermore, TM 2 and TM 7 are distorted around site 90 and the SB linkage point (Lys296), respectively, with the result that residue 90 lies extremely close to the SB counterion.

Interestingly, the reverse mutations at sites 90 and 292 in the human VS pigment were found to have either no effect or an effect opposite to that expected of [68]. This demonstrates how context sensitive these substitutions may be and highlights the possibility that pigments from different opsin classes may have important structural differences.

Old World primates show significant differences in the λ_{max} of VS pigments: the human pigment peaks at around 419 nm [69, 70] whereas that of cercopithecoid monkeys is much closer to 430 nm [71–73]. Comparison of the amino acid sequences of human VS opsin [41] with that of the talapoin monkey *Miopithecus talapoin* [42, 74] indicates that a single amino acid substitution at site 292 in TM 7 may be responsible for the 10 nm difference. In the talapoin pigment, the residue is Ala, but in humans it is replaced by polar Ser. Since site 292 is close to the SB, the effect of polar serine in the VS opsins of cercopithecoids may again be to expose the PSB chromophore to a much more polar methanol-like environment.

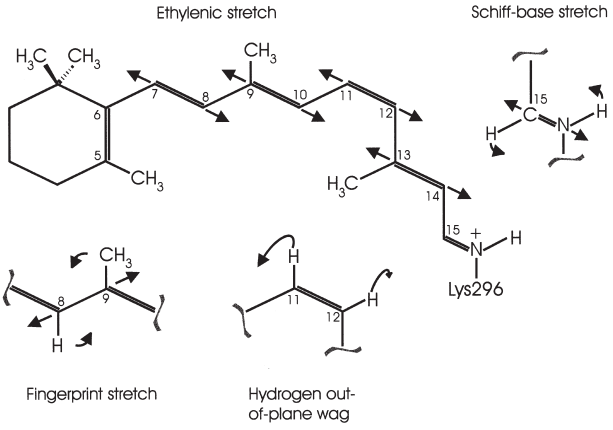

Figure 7. Structure of 11-*cis*-retinal showing nature of vibrational modes in the molecule after Raman spectroscopy at different laser excitations [re-drawn from ref. 58].

Tuning into the UV

Since solvated retinal has a λ_{max} at around 380 nm, one possibility for tuning into the UV is to maintain the SB base in an unprotonated state. Evidence that this might be the case has come from the demonstration of differences between the batho-intermediates of *Xenopus laevis* VS and mouse UVS pigments [75], indicating that the chromophore state or binding-site environment may be very different in the two pigments. However, the difficulty with this interpretation is that all UVS opsins sequenced so far have a charged Glu residue at site 113 [10, 40, 43, 45, 47, 76, 77], and there is no evidence for another neutralizing amino acid in the vicinity. Moreover, acid titration of the mouse UVS pigment to low pH shifts the λ_{max} from 357 nm to 440 nm, representative of a protein-bound PSB retinal in solution, with no intermediate protonated state with a λ_{max} between 357 and 440 nm generated before protein denaturation [75].

Table 1. Candidate spectral tuning sites for avian UVS/VS pigments.

Species	λ_{max} (nm)[a]	Amino acid sites[b]				
		86	90	93	118	298
Canary	366	Cys	Cys	Thr	Ala	Ser
Budgerigar	371 (365)	Ala	Cys	Thr	Ala	Ser
Pigeon	409 (393)	Ser	Ser	Thr	Ala	Ser
Penguin	403	Ser	Ser	Thr	Ala	Ala
Chicken	418	Ser	Ser	Val	Thr	Ala

[a] λ_{max} values were obtained from in vivo measurements by microspectrophotometry [40, 45, 78, 79]. Values in parentheses were obtained from in-vitro-expressed and -regenerated pigments [10, 40].
[b] Bovine rod numbering.

To identify the amino acid differences responsible for tuning into the UV, comparisons can be made between the sequences of VS and UVS opsins. However, when this is done across the combined pigments from teleosts, reptiles, birds and mammals, no obvious candidate substitutions emerge. One explanation for this is that the amino acid substitutions responsible for the violet to UV shifts may differ in the pigments from the different vertebrate taxa. Comparisons within a single taxon, the birds, have identified sites that are consistently substituted across the spectrally different pigments. Avian pigments have the added advantage that, in contrast to teleost fish and mammals where the λ_{max} values of the pigments cluster either at 420–430 nm or around 360 nm, their VS and UVS pigments show a greater range of λ_{max} values (table 1). For example, the Humbolt penguin *Spheniscus humboldti* has a pigment with a λ_{max} around 403 nm [78] while that of the pigeon *Columba livia* has a λ_{max} of 404–409 nm, as determined by in situ microspectrophotometry [79], or 393 nm when determined as a recombinant opsin regenerated in vitro [80]. This ambiguity highlights the problems encountered in determining λ_{max} values from native and recombinant forms of these shortwave pigments. Nevertheless, by comparing the amino acid sequences of the canary, budgerigar, penguin, pigeon and chicken, Wilkie et al. [81] were able to identify five candidate tuning sites which differ in a manner consistent with the differing λ_{max} values of the five species (table 1). In situ mutagenesis and regeneration of the recombinant opsins in vitro with 11-*cis*-retinal demonstrated that two of these sites were without effect whereas substitution at the other three sites produced LW shifts of varying magnitudes (table 2). In particular, the replacement of Cys by Ser at site 90 in TM 2 produced a spectral shift of about 35 nm from 363 to 398 nm [81]. The tuning of avian pigments into the UV has also been examined by Yokoyama et al. [46]. In this study, the reverse substitution of Cys inserted into site 90 of the VS opsin of pigeon and chicken was made and this produced a shortwave shift from 393 to 358 nm in pigeon and from 415 to 369 nm in chicken. These data clearly establish, therefore, that in avian species, the major spectral difference between VS pigments with λ_{max} values greater than about 395 nm and UVS pigments with λ_{max} values close to 360 nm depends on whether Ser or Cys is present at site 90.

Table 2. Spectral shifts of recombinant budgerigar UVS pigments.

Site-directed mutations	λ_{max} (nm)	Shift from wild-type pigment (nm)
Wild type	363	–
Cys90Ser	398	+ 35
Thr93Val	366	+ 3
Ala118Thr	366	+ 3

Data from Wilkie et al. [81].

The Ser90Cys substitution in TM 2 does not amount to a substantial change in polarity, although the larger size of the sulphur compared to the oxygen atom may allow the thiol group to approach the PBS more closely, thereby increasing its effect. TM 2 of bovine rod opsin is kinked around residues 89 and 90 and this places the latter residue particularly close to Glu113 [52], raising the possibility that the local environment of Cys90 may serve to reduce its pKa such that a thiolate ion is present under neutral conditions. Such an ion could then serve to further stabilize the proton on the SB in the ground state, with a consequent shortwave spectral shift into the UV (fig. 8). In this context, it is interesting to note that the substitution of Gly90Asp in human rod opsin results in night blindness [82], presumably as a result of the destabilization of the salt-bridge between Glu113 and the SB by the presence of an additional negatively charged residue [56, 83]. A somewhat different interpretation has been proposed by Yokoyama et al. [46] who suggest that the hydrophobicity of Cys90 removes a water molecule from the vicinity of the SB, thereby displacing its positive charge. Under these conditions, therefore, the SB would be effectively unprotonated. The evidence for the presence of water molecules in the vicinity of the SB comes from resonance Raman studies [84] and from the study of ^{15}N-lysine- and ^{13}C-glycine-labelled opsin in angle spinning

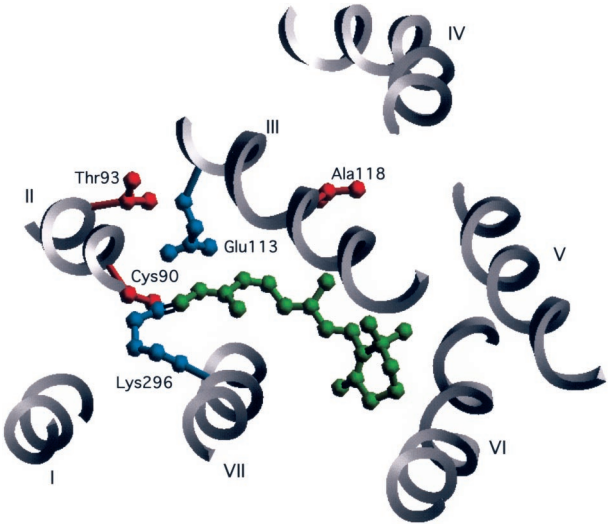

Figure 8. Structural model of budgerigar UV pigment viewed from the cytoplasmic side of the membrane. The retinal chromophore is shown in green with Lys296 and the Glu113 counterion in blue. The three residues Cys90, Thr93 and Ala118 that have been shown to be involved in spectral tuning into the UV [81] are shown in red. The model was built in Swiss Model and is based on the crystal structure of bovine rhodopsin.

NMR [85]. In these latter experiments, the estimated SB to counterion distance of 4.1 and 4.4 Å and the high C=N frequency can be reconciled by the inclusion of a structural water molecule into the retinal binding site (fig. 8), in tight association with the counterion and SB proton [86–89]. The recent crystal structure of bovine rhodopsin sets the SB to counterion separation at rather less than this (3.3 to 3.5 Å) although it remains uncertain whether a water molecule is included into this site [52].

The full shift from the λ_{max} of chicken VS pigment to that of the canary and budgerigar UVS pigments is around 46 nm, greater, therefore, than that achieved by the Ser90Cys substitution alone. Wilkie et al. [81] identified two other substitutions with smaller effects, Val93Thr and Thr118Ala, that may account for the additional 10 nm required. Site 93 is also in the vicinity of the SB/counterion, and the Thr substitution introduces an additional polar group that could further stabilize the PSB. Site 118 on the other hand lies towards the luminal side of TM 3 and is adjacent to the polyene chain of the chromophore. The Thr118Ala substitution results in the loss of a polar group in the shortwave-shifted pigments; the effect may be therefore to reduce π electron delocalization, with the consequent increase in energy difference between ground and excited states leading to a shortwave spectral shift.

The structure of other opsins can be modelled onto the crystal structure of bovine rhodopsin [52] using the Swiss Model program [90] and this has enabled us to obtain an estimate of the SB to counterion distances in avian pigments with reducing λ_{max} values and different amino acids at site 90 (table 3). The results indicate that there is indeed a gradual reduction in this distance of separation between the SB and counterion with reducing λ_{max}. This structural effect of amino acid substitution at site 90 may, therefore, be an important mechanism for shortwave tuning.

Table 3. Schiff base-counterion separation in rod and cone pigments.

Pigment	λ_{max}	Residue at site 90	Schiff base-counterion separation (Å)
Bovine rhodopsin	498	Gly	3.22
Chicken SWS	455	Gly	3.20
Chicken VS	418	Ser	3.17
Budgerigar UVS	371/365	Cys	3.08

Amino acid sequences of the chicken and budgerigar opsins were modelled onto bovine rhodopsin [52] using the Swiss Model program [90]. The separation between the SB base nitrogen atom and the proximal oxygen of Glu113 was estimated using Swiss-Pdb Viewer [128].

UV tuning in other vertebrate taxa

The central role of Cys90 for tuning into the UV would appear to be unique to birds since all UVS pigments of the other vertebrate taxa, from teleosts such as the goldfish [77] and zebrafish [91], reptiles such as the American chameleon *A. carolinensis* [47, 92], to mammals such as the mouse [93] and rat [94], retain Ser90. A different mechanism for spectral tuning into the UV must be present therefore in these species; as mentioned previously, this is not inconsistent with the evolutionary pattern revealed by phylogenetic analysis of this group of pigments (see fig. 5).

A recent study by Yokoyama and Shi [95] has gone some way to determining the mechanism of spectral shifts in mammalian VS and UVS pigments. A chimaeric opsin comprising TM 1–3 from human VS and TM 4–7 from mouse UVS, when expressed and regenerated with retinal, produced a pigment with λ_{max} very close to the native human pigment. This identifies, therefore, the same region of the opsin protein (TM 1–3) as important for UV/violet spectral shifts in mammals as in birds. Sequence comparison across VS/UVS opsins in other non-avian species (table 4) identifies five sites (Phe52Thr, Leu86Phe, Pro93Thr, Gly114Ala and Thr118Ser) which when simultaneously substituted into human violet opsin, produced a UV shift of around 40 nm. Substitution at site 52 was shown to have a small effect by itself, whereas single substitutions at the other four sites did not alter the λ_{max} of the expressed pigment, indicating that the spectral effect of these substitutions depends on synergistic inter-

Table 4. Amino acid substitutions implicated in spectral shifts of UVS/VS pigments.

	Type of pigment	Amino acid sites				
		52	86	93	114	118
Mammals						
Human	VS	Phe	Leu	Pro	Gly	Thr
Chimpanzee	VS	Phe	Leu	Pro	Gly	Thr
Macaque	VS	Phe	Leu	Pro	Ala	Thr
Squirrel monkey	VS	Leu	Leu	Pro	Gly	Thr
Capuchin monkey	VS	Leu	Leu	Pro	Ala	Thr
Marmoset	VS	Leu	Leu	Pro	Gly	Thr
Bovine	VS	Thr	Tyr	Ile	Ala	Cys
Porcine	VS	Thr	Tyr	Ser	Ala	Ser
Mouse	UVS	Thr	Phe	Thr	Ala	Ser
Rat	UVS	Thr	Phe	Thr	Ala	Ser
Clawed frog	VS	Thr	Met	Pro	Ala	Thr
American chameleon	UVS	Thr	Phe	Thr	Ala	Ser

[Data from refs 81 and 95 plus, for porcine VS pigment, ref. 96].

actions across these sites [95]. Other combinations of substitutions were not, however, tested, so whether substitution at all five sites is indeed required remains untested. Sites 52, 86 and 93 are in the vicinity of the PSB and, with the presence of polar Thr at sites 52 and 93 in the UVS pigment, could act to stabilize the SB-counterion interaction through the generation of a more polar environment. The role of sites 114 and 118 is less certain. Site 118 is involved in the tuning of avian pigments although, unlike birds where Ser is replaced by Ala, the replacement in mammals of Thr by Ser would not result in the loss of a polar group in the shortwave-shifted pigments and the substitution is not always associated with a spectral shift. Both sites provide side chains for the binding pocket around the polyene chain [52] and are distant, therefore, from the PSB. The particular substitutions of Gly114Ala and Thr118Ser would not be expected to generate any change in π electron delocalization and thereby any change in the energy difference between ground and excited states.

When the substitutions at these five sites (plus site 90) are placed onto a phylogenetic tree generated from the sequences of VS and UVS opsins from representative species of the five vertebrate taxa (fig. 9), two features become apparent. First, the residues at sites 114 and 118 show substantial variation across species with similar λ_{max} values, casting further doubt on their involvement in spectral tuning. Second, in primate pigments, Leu86 is always linked with Pro93 whereas the bovine and porcine [96] pigments have Tyr86 and either polar Ser93 or nonpolar Ile93. In the former case, synergism between Leu86 and Pro93 may be the key factor in the violet spectral shift, but Ser93 and Ile93 are unlikely to show a similar interaction with Tyr86. In this case, therefore, the key substitution for the violet shift in bovine and porcine pigments may be the replacement of Phe by Tyr at site 86. This substitution was not made by Yokoyama and Shi in their site-directed mutagenesis experiments [95] and verification, therefore, awaits further experimentation. However, if this is the case, this implies that the evolution of VS pigments in mammals occurred separately in the primate and bovine/porcine lineages.

Ancestral vertebrate UVS pigment

A further feature that emerges from the phylogenetic tree in figure 9 is the prediction that the sequence of the ancestral opsin pigment would have been Thr52, Phe86 and Thr93 and would therefore have shown UV sensitivity. A potential complication with this interpretation is that a number of teleost fish possess a violet-sensitive pigment with λ_{max} in the 400–420 nm range [28, 97–99]. However, in all species where gene sequencing has been carried out, such violet-sensitive pigments turn out to be members of the SWS class of pigments [21, 44, 91]. UV sensitivity may be universally retained therefore by VS/UVS pigments in teleosts. UV sensitivity is however lost in VS/UVS pigments of amphibia, birds and the nonrodent mammals by substitution at one or more of the above sites. In the clawed frog and in primates, the key substitutions for violet shifts may be Met/Leu86 with Pro93, whereas bovine and porcine may require Tyr86. Finally, in avian vision, the subsequent substitution of Cys90 resulted in the 're-invention' of UV sensitivity in passerine species such as the budgerigar, canary and zebrafinch.

UV sensitivity in invertebrates

Although cephalopod molluscs (octopus, cuttlefish and squid) have a well-developed visual system, they do not

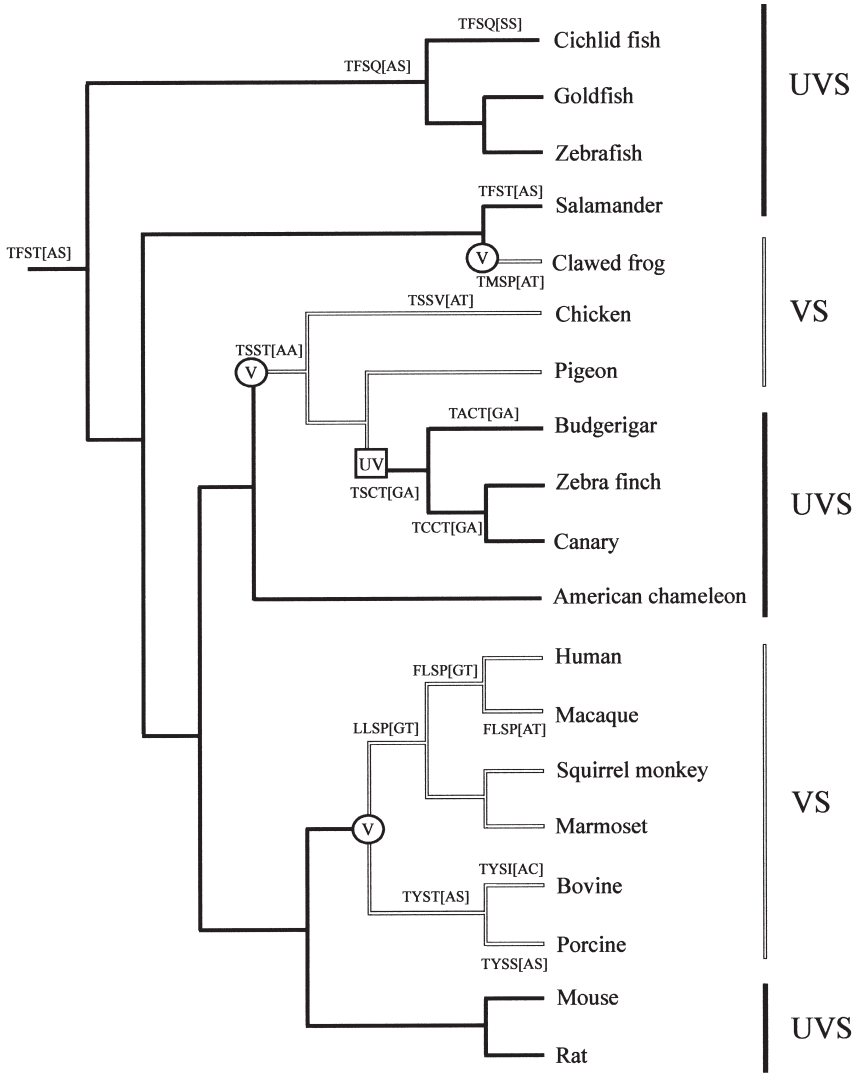

Figure 9. Phylogeny of UVS/VS opsins showing the pattern of amino acid substitutions at sites 52, 86, 90, 93, 114 and 118. At each point in the tree where substitution is inferred to have occurred, the residues at these six sites are shown in numerical order and with the residues at sites 114 and 118 in brackets (re-drawn with the addition of the porcine [96] and salamander sequences (accession number AF038948) from Yokoyama and Shi [95]).

appear to have evolved pigments that are sensitive in the UV [100]. In fact, vision in cephalopods is generally dependent on a single visual pigment with λ_{max} around 480 nm [101–105]. UV vision is, however, present in insects where it has been studied extensively. In insects, the different visual pigments are segregated into different sub-sets of cells that form the ommatidium. In the fruitfly *Drosophila*, seven genes encoding different opsins have been identified and sequenced. The *Rh1* gene (the *ninaE* locus) encodes the major opsin present in the *Drosophila* compound eye. The corresponding pigment is blue sensitive and is expressed in the R1–R6 class of photoreceptor cells [106, 107]. *Rh2* encodes a violet-sensitive pigment that is expressed in the simple eyes or ocelli on the vertex of the head [108, 109], and *Rh3* and *Rh4* encode UVS pigments that are expressed in non-overlapping sets of R7 cells [110–113]. UV sensitivity has been reported in the majority of insect species [114]. Examples where UVS opsin genes have been cloned and sequenced include the honey-bee *Apis mellifera* [115], the butterfly *Papilio xuthus* [116], and the moth *Manduca sexta* [117] although, unlike *Drosophila*, only a single UVS opsin has been identified in each case. These UVS opsin sequences, together with sequences from other insect opsins, have been used to generate the phylogenetic tree shown in figure 10. The striking feature of this tree is that all the UVS opsins form a single clade, even though the insect species involved are only distantly related. This implies that the UVS opsins not only appeared very early in the evolution of the insects but, in contrast to the vertebrate UVS pigments, have remained entirely UV sensitive.

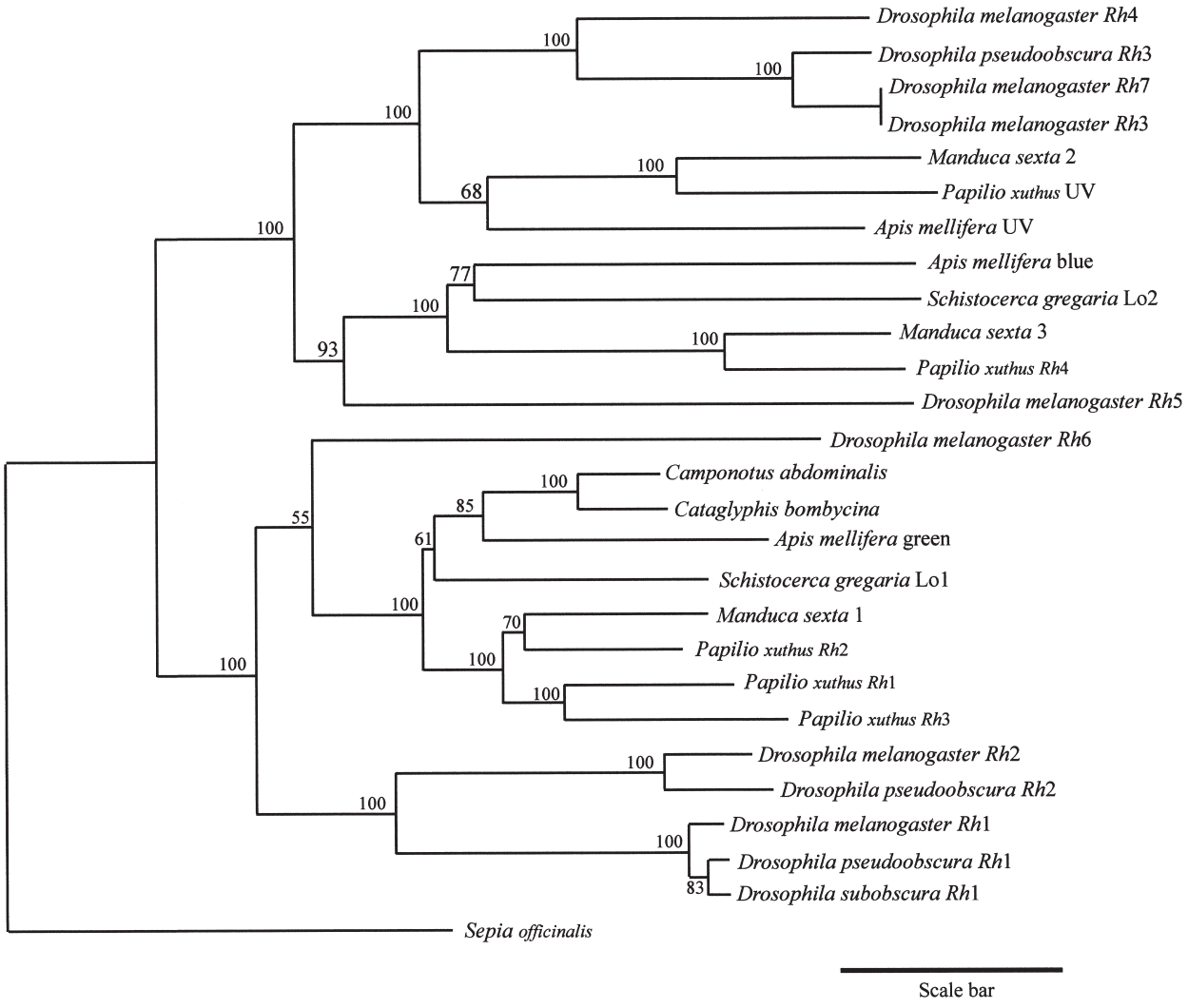

Figure 10. Phylogenetic tree of insect opsins. Details of analysis are as given in the legend to figure 3. GenBank accession numbers: *Drosophila melanogaster Rh*4, M17719; *D. pseudoobscura Rh*3, X65879; *D. melanogaster* Rh7, Y00043; *D. melanogaster Rh*3, M17718; *Manduca sexta* 2, L78081; *Papilio xuthus* UV, AB028218; *Apis mellifera* UV, AF004169; *A. mellifera* blue, AF004168; *Schistocerca gregaria* Lo2, X80072; *M. sexta 3,* AD001674; *P. xuthus Rh*4, AB028217; *D. melanogaster Rh*5, U80667; *D. melanogaster Rh*6, Z86118; *Camponotus abdominalis*, U32502; *Cataglyphis bombycina*, U32501; *A. mellifera* green, U26026; *S. gregaria* Lo1, X80071; *M. sexta* 1, L78080; *P. xuthus Rh*2; AB007424; *P. xuthus Rh*1, AB007423; *P. xuthus Rh*3, AB007425; *D. melanogaster Rh*2, M12896; *D. pseudoobscura Rh*2, X65878; *D. melanogaster Rh*1, K02315; *D. pseudoobscura Rh*1, X65877; *D. subobscura Rh*1, AF025813; *Sepia officinalis*, AF000947. The rod opsin sequence from the cuttlefish, *Sepia officinalis* [103], was used as an outgroup. The scale bar is equal to 0.1 substitutions per site.

The equivalent site in invertebrate pigments to the Glu113 counterion of vertebrate pigments is occupied by Tyr in all pigments with λ_{max} values > 400 nm, and by Phe in all UVS pigments. In the longerwave-sensitive pigments, a PSB is stabilized by polar Tyr113, together with polar residues at other sites in the vicinity of the retinal attachment site [118]. Phe113 will not provide this stabilization and insect UVS pigments are generally thought to have an unprotonated SB [53, 56, 59, 83, 119].

The only other group in which UVS pigments have been described in some detail are the Crustacea. In general, Crustacea possess a relatively simple visual system comprising two visual pigments, but in the stomatopods or mantis shrimps, the compound eye contains a mid-band region that contains up to 14 photoreceptor types [120–124], 12 of which are used in colour vision and sample the spectrum from below 300 nm to above 700 nm [120]. What is even more surprising is that there may be as many as 16 different visual pigments in a single retina [125]. All use the same chromophore [126], with at least four sampling in the UV from below 300 to 400 nm [127]. At present, the molecular basis of the UV sensitivity of these pigments is unknown, but it will be interesting to establish whether they also possess an unprotonated SB.

Summary and conclusions

UV vision is widespread throughout the animal kingdom where it is used for communication, in foraging for food and in mate selection. Amongst the vertebrates, UV sensitivity is conferred by a single class of cone pigments in which the SB is most likely protonated. In evolutionary terms, this class of pigments was originally UV sensitive, and this sensitivity has been retained by teleost fish. However, UV sensitivity by this class of pigments has been variously lost in the other vertebrate taxa. For example, it has been lost by amphibia and by certain mammals, most notably the primates; in both cases, the accumulation of amino acid substitutions in the opsin protein has served to longwave shift the λ_{max} of the pigments to the violet region of the spectrum. Phylogenetic reconstructions of avian evolution indicate that UV sensitivity may have been entirely lost in this lineage but subsequently regained in certain species through a single amino acid substitution at site 90 in the opsin protein. UV vision is also widespread in insects where it is again conferred by a single class of opsin-based pigments although, in this case, the SB is most likely unprotonated. Unlike the vertebrates, however, the UV sensitivity of this class of insect pigments has been retained throughout evolution.

Acknowledgements. We are indebted to Professors Martin Warren and Richard Pickersgill, Queen Mary College, University of London, for their invaluable help with the molecular modelling and use of computer graphics packages. This work was supported by a BBSRC grant.

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