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Primary structure of a visual pigment in bullfrog green rods

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Abstract In frog retina there are special rod photoreceptor cells ('green rods') with physiological properties similar to those of typical vertebrate rods ('red rods'). A cDNA fragment encoding the putative green rod visual pigment was isolated from a retinal cDNA library of the bullfrog, *Rana catesbeiana*. Its deduced amino acid sequence has more than 65% identity with those of blue-sensitive cone pigments such as chicken blue and goldfish blue. Antisera raised against its C-terminal amino acid sequence recognized green rods. It is concluded that bullfrog green rods contain a visual pigment which is closely related to the blue-sensitive cone pigments of other non-mammalian vertebrates.

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Key words: Visual pigment; Cloning; Immunohistochemistry; Green rod; Bullfrog (*Rana catesbeiana*)

1. Introduction

Vertebrate photoreceptor cells are generally classified morphologically into rods and cones, which mediate twilight and daylight vision, respectively. Cones are less sensitive than rods: in primates, cones require a 1000-fold greater number of photons than rods to achieve a half-maximal signal [1]. The light response of cones is faster, and terminated significantly more rapidly than that of rods [1], and light adaptation is much more pronounced in cones [2].

Photoreceptor cells of frogs can be classified into at least five types: red and green rods, principle and accessory members of double cones, and single cones [3,4]. Red and green rods are reported to have absorption maxima (λ_{max}) at about 500 and 430 nm, respectively, while the double cone principle members and single cones have a λ_{max} at about 560 nm, and double cone accessory members have a λ_{max} at about 500 nm [5]. Recently, short single cones in the frog retina have been reported to express a putative short-wavelength-sensitive pigment, similar to human blue-, chicken violet- and goldfish ultraviolet (UV)-sensitive pigments [6]. Red rods are one of the best characterized photoreceptors, both physiologically and biochemically, but little is known about green rods, except that their morphology is typical of vertebrate rods [3]. In the toad, the light response and sensitivity of green rods are similar to those of red rods [7,8].

In this paper, we report the isolation and characterization of a cDNA encoding the bullfrog visual pigment, RcVP-MS, which is expressed in green rods. Our data reveal that frog green rod RcVP-MS is closely related to chicken and goldfish blue-sensitive cone pigments.

2. Materials and methods

2.1. Isolation of bullfrog cDNA fragment encoding RcVP-MS

A cDNA fragment encoding a putative visual pigment (RcVP-MS) was amplified from a bullfrog (*Rana catesbeiana*) retinal cDNA pool with degenerate oligonucleotides (VVP-F1′ and R2′) as primers [6,9]. The 3′ end of the RcVP-MS cDNA was amplified with T-amp [6] and a gene-specific primer, RcVP-MS-F1 (5′-GGGATCCATGGTGATTATGATGATTGC-3′), and was used as a probe to screen a bullfrog retinal cDNA library. Construction of the retinal cDNA library was carried out as described by Hisatomi et al. [10]. Briefly, poly(A) RNA was isolated from the retinas of 10 bullfrogs (body length about 10 cm), using a Quick Prep Micro mRNA Purification Kit (Pharmacia), and double-stranded cDNA was synthesized with random hexa-oligonucleotides and oligo dT_{12-18} as primers (Pharmacia). cDNA was ligated with an *Eco*RI-*Not*I adapter, inserted into an *Eco*RI site of Lambda-ZAPII (Stratagene), and packaged with Giga-Pack II gold (Stratagene). Positive clones for a high stringency screening [6] were transformed into plasmids by an EXASSIST-SOLR system (Stratagene), and sequenced according to the cycle sequencing method (Applied Biosystems).

2.2. Data analysis

The deduced amino acid sequence of RcVP-MS was aligned with those of other vertebrate visual pigments reported previously. Amino acid identities were calculated for 290 amino acids from P32 to Q321 of RcVP-MS (corresponding to P23 and Q322 of human rhodopsin, respectively). Amino acid substitution rates, k (per site), were estimated using the amino acid difference, p (per site), with a correction for multiple substitutions of $k = -\ln(1-p-0.2p^2)$ [11]. An unrooted tree was constructed by the neighbor-joining (NJ) method [12] with an in-house program (ESAT) [13]. The mean clustering probabilities were obtained from five trials of 1000 bootstrap resamplings.

2.3. Preparation of antisera against RcVP-MS

Expression and isolation of the fusion peptide containing the RcVP-MS sequence was carried out as described by Hisatomi et al. [6]. The cDNA fragments encoding the C-terminal region (from M262 to A362) of RcVP-MS were amplified using RcVP-MS-F1 and RcVP-MS-R1 (5′-GAAGATCTTGCAGGAGCCACCTGGCT-3′) as primers. After confirming the sequence, the amplified fragment was inserted between *Bam*HI and *Bgl*II sites of a pQE-16 plasmid vector (Qiagen), and introduced into *Escherichia coli* cells (SG13009, Qiagen). The fusion protein (mouse dihydrofolate reductase, helices VI–VII and the C-terminal region of RcVP-MS, and a histidine hexamer tail) was isolated using an Ni-NTA column (QIA purification system, Qiagen), and used to immunize mice to produce antisera.

2.4. Western blot analysis

A bullfrog retina was immersed into a sodium dodecylsulfate (SDS) sample buffer and sonicated briefly. SDS polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the standard method [14] using 12.5% acrylamide mini-gels. Proteins were transferred to PVDF membrane (Bio-Rad) using a semi-dry transfer cell

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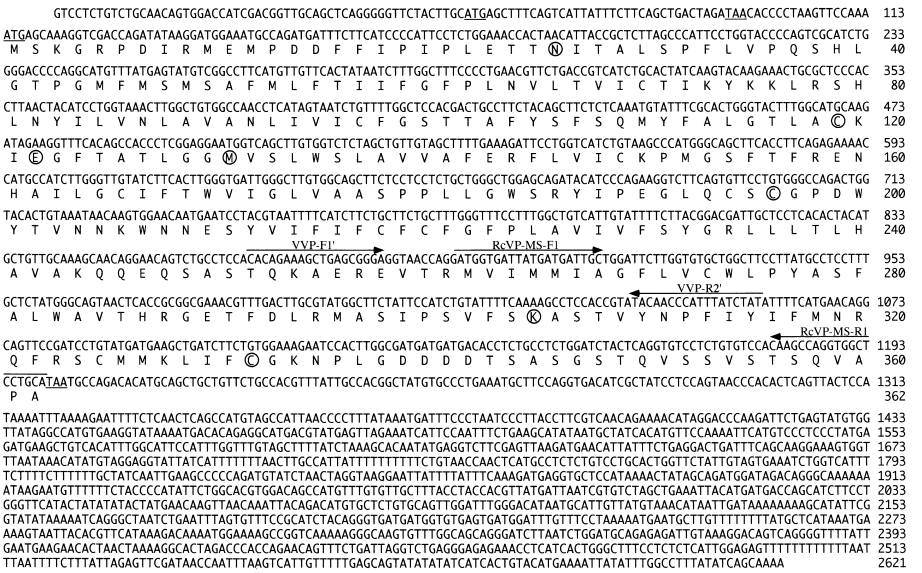

Fig. 1. The nucleotide sequence of cDNA and deduced amino acid sequence of RcVP-MS. Arrows mark the positions corresponding to the primers (as indicated) used for amplification of RcVP-MS cDNA fragments. Underlined nucleotides are the putative translational initiation and termination codons. Key amino acids enclosed in a circle (N26, C119, E122, M131, C196 and K305) are discussed in the text. The nucleotide sequence in this figure has been submitted to the EMBL nucleotide sequences database with accession number AB010085.

(Transblot SD, Bio-Rad) in the presence of 25 mM Tris, 92 mM glycine and 10% methanol at 100 mA for 1 h. Membranes were blocked with 3% bovine serum albumin in TBS buffer (200 mM NaCl and 2 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.4), and were incubated with a 1000-fold dilution of the antiserum in TBS buffer containing 0.1% Tween-20 at 37°C for 2 h. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG was reacted as recommended by the manufacturer (Vector Laboratories), and antibody binding was visualized with diaminobenzidine (DAB).

2.5. Immunohistochemistry

Immunohistochemical procedures were carried out as described by Hisatomi et al. [6]. Paraffin sections (4 μm) of the frog retina were incubated with a 100-fold dilution of the antiserum, washed with phosphate buffered saline, incubated with HRP-conjugated anti-mouse antibodies (Jackson) and developed with DAB solution. Localization of RcVP-MS was visualized using Nomarski optics (Olympus-BX50). For immunofluorescent observations, washed sections were reacted with fluorescein-conjugated anti-mouse IgG (Jackson), and counterstained with Evans blue (Wako).

3. Results

3.1. Isolation of RcVP-MS cDNA

The cDNA fragments encoding frog visual pigments were amplified using degenerate oligonucleotides (VVP-F1′ and -R2′) corresponding to the amino acid sequences conserved in vertebrate visual pigments [9]. Among the amplified fragments, we found a cDNA fragment resembling chicken and goldfish blue-sensitive cone pigments belonging to MS group [6]. We therefore named this cDNA RcVP-MS (*R. catesbeiana* visual pigment, MS group) cDNA. The complete coding region of RcVP-MS cDNA was isolated by screening a bullfrog retinal cDNA library (Fig. 1).

3.2. The deduced amino acid sequence of RcVP-MS

RcVP-MS cDNA consists of 2621 bases; an in-frame stop codon (TAA) is located just downstream of the first ATG. On assuming the second ATG as the translational initiation codon, this cDNA may encode 362 amino acids with a molec-

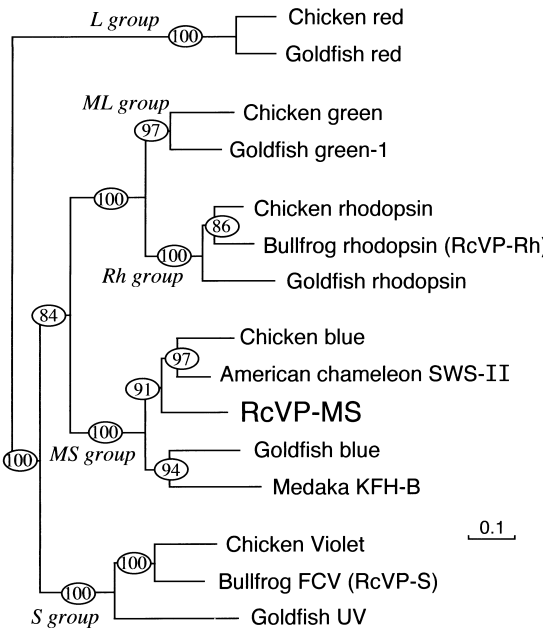

Fig. 2. An NJ tree calculated from the amino acid sequences of vertebrate visual pigments. Circled numbers indicate the mean clustering percentage obtained from 1000 bootstrap resamplings. The fluctuation of each percentage is less than 3% with different random series ($n = 5$). Bar indicates 10% replacement of an amino acid per site ($k = 0.1$, see Section 2). L, ML, MS, S and Rh group represent long, middle-long, middle-short and short wavelength-sensitive pigment, and rhodopsin group, respectively. The sequence data used in the present analyses were taken from EMBL and Swiss-Prot databases (accession numbers in parentheses), and from the literature: chicken rhodopsin (P22328), and red (P22329), green (P28683), blue (P28682) and violet (P28684) cone pigments; bullfrog rhodopsin (RcVP-Rh) (P51470) and FCV (RcVP-S) (AB001983); goldfish rhodopsin (P32309), and red (P32313), green-1 (P32311), blue (P32310) and ultraviolet (Q90309) cone pigments; American chameleon (*Anolis*) SWS-II [22]; and medaka KFH-B (P87365).

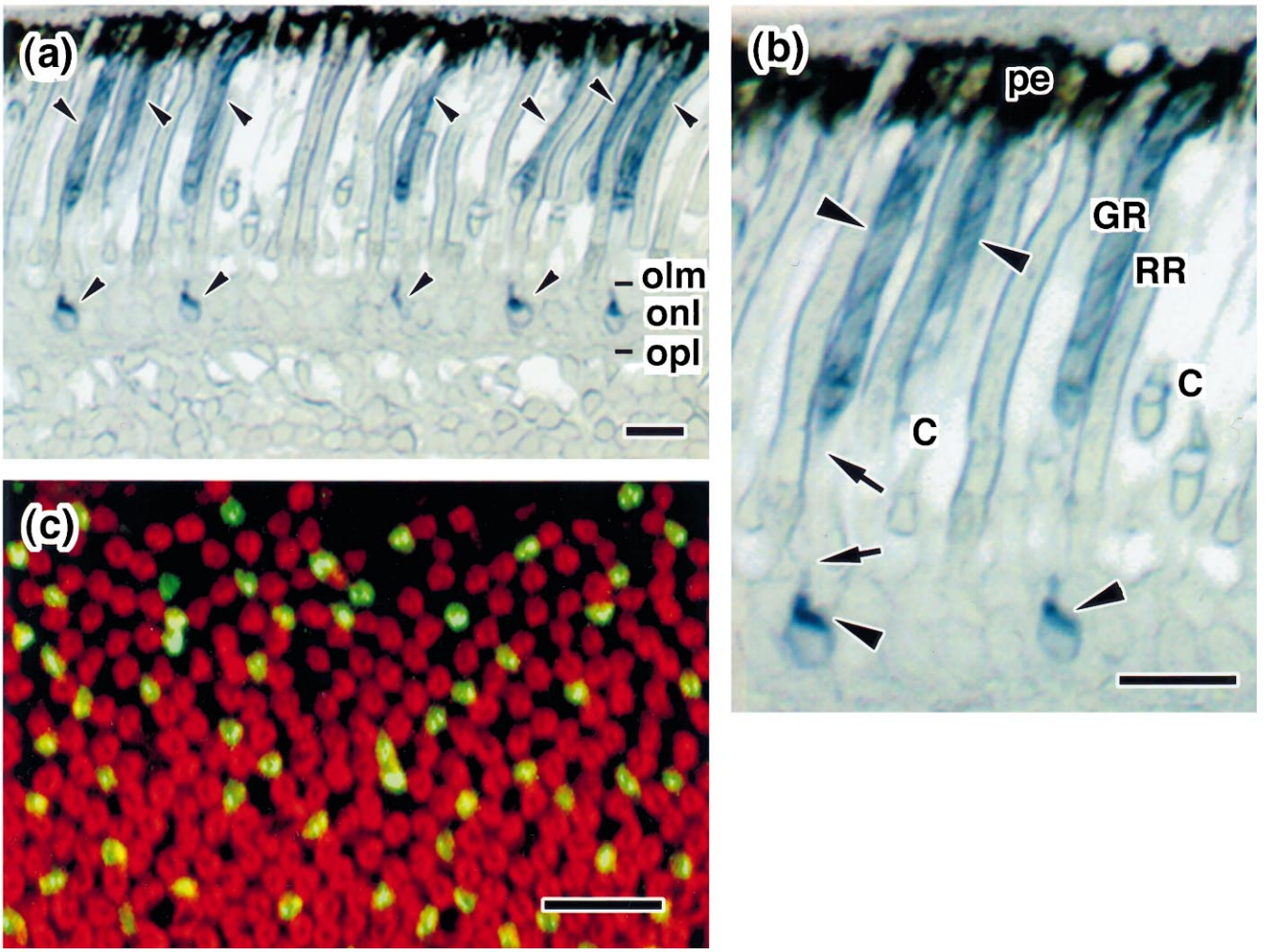

Fig. 3. Localization of RcVP-MS in bullfrog retina. a: Radial section, at higher magnification in b. Anti-RcVP-MS recognizes the outer segments and cell bodies of green rods (arrowheads). Arrows in b indicate the myoid of a green rod. c: Immunofluorescent image (counter-stained with Evans blue) showing anti-RcVP-MS antiserum reactivity in a tangential section. Green and red rods are indicated by green/yellow and red fluorescence, respectively. C, cones; GR, green rod; RR, red rod; olm, outer limiting membrane; onl, outer nuclear layer; opl, outer plexiform layer; pe, pigment epithelium. Scale bar=20 μm.

ular mass of about 40 kDa (Fig. 1). Its deduced amino acid sequence shows more than 40% identity with those of vertebrate visual pigments, including typical visual pigment features: a Schiff base linkage (K291) for the chromophore [15], a counter-ion of the protonated Schiff base (E108) [16–18], sites for a disulfide bond (C105 and C182) [19], an *N*-glycosylation site (N12), and possible phosphorylation sites near the C-terminal [20]. Therefore, this cDNA seems to encode a functional visual pigment. RcVP-MS shows high identities throughout the amino acid sequence with MS group pigments, such as chicken blue (74.8%) [21], American chameleon SWS-II (79.0%) [22] and goldfish blue (69.7%) [23]. However, there is less identity (41–56%) with visual pigments in other groups (L, ML, S and Rh groups) [6,9]. An NJ tree indicates that RcVP-MS apparently belongs to the MS group (Fig. 2).

3.3. *Localization of the RcVP-MS pigment in the bullfrog retina*

In paraffin sections, antisera against RcVP-MS fusion peptide (mouse DHFR, helices VI–VII with the C-terminal region of the RcVP-MS pigment, and a histidine hexamer tail) recognized only the outer segments and cell bodies of a small fraction of rods (Fig. 3a), which have long myoids and shorter outer segments than other rods (Fig. 3b). The morphological features of these immuno-positive cells correspond to those of green rods [3,4]. RcVP-MS signals were not observed in the outer segments of red rods, principle or accessory members of double cones, single cones or short single cones, indicating that the RcVP-MS pigment exists only in green rods. A similar pattern of reactivity of the antiserum was observed using fluorescein-conjugated secondary antibodies (Fig. 3c). It has been reported that green rods constitute about 8% of the rod population in the *R. catesbeiana* retina [24], which is in agreement with our results (see, for example, Fig. 3c).

The antiserum also recognized cell bodies of certain photoreceptors in the outer nuclear layer (Fig. 3b). Autoradiographic studies have shown a dynamic pathway of opsin biosynthesis initially involving rough endoplasmic reticulum and the Golgi complex [25,26]. By immunocytochemical experiments using frog (*R. pipiens*) photoreceptor cells, it has been found that opsin is localized in the Golgi zone near the nu-

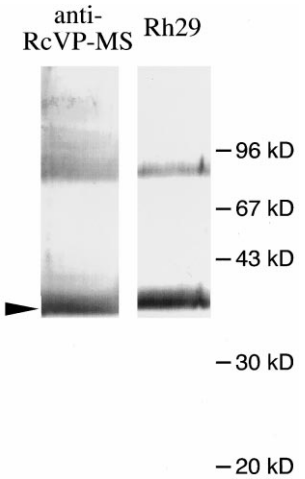

Fig. 4. Western blot analysis of bullfrog retina homogenate. Western blots using the anti-RcVP-MS antiserum (left lane) and using an anti-bovine rhodopsin antibody, Rh29 (right lane) which recognizes the bullfrog rhodopsin. The arrowhead indicates the band of RcVP-MS with a molecular mass of about 37 kDa.

cleus [27]. Newly synthesized RcVP-MS opsins in green rods may therefore accumulate in the Golgi complex, for subsequent transportation to the outer segments through the narrow myoid region. The immuno-positive cell bodies are localized vitreally in the outer nuclear layer, the red rod nuclei are located in outer side of outer nuclear layer (Fig. 3). This is in agreement with early studies that the nuclei of green rod cells are located among the cone nuclei [3,4].

3.4. Western blot analysis

Western blot analysis of the retinal homogenate demonstrated that the anti-RcVP-MS antiserum recognizes a major band of about 37 kDa with a minor band of about 80 kDa (Fig. 4, left lane). The latter band is probably a dimer of RcVP-MS. The 37 kDa band shows slightly greater mobility than that of bullfrog rhodopsin (Fig. 4, right lane) recognized by Rh29, a monoclonal antibody of bovine rhodopsin [28,29]. The mobility of RcVP-MS shows an agreement with the previous report for purified green rod pigment [30]. These results confirm that RcVP-MS is the green rod pigment.

4. Discussion

MS group pigments are expressed in cone photoreceptors of teleosts, a reptile and a bird, but in rods in the bullfrog. Green rods in other amphibia are likely to contain pigments phylogenetically closely related to RcVP-MS.

Recently, Imai et al. [31] have reported that E122 of bovine rhodopsin is a determinant for its efficiency to activate transducin (Gt), that is, E122I and E122Q mutants activate Gt with similar efficiencies to chicken red and green cone pigments, respectively. A methionine residue is located at a position in RcVP-MS corresponding to that in the MS group pigments, and is generally considered to be more similar to isoleucine than to glutamic acid. Also, the overall amino acid sequence of RcVP-MS is more similar to those of MS group than Rh group pigments, so it is likely that its primary structure is of a typical cone type.

By investigations of reptilian photoreceptors, Wald [30] proposed the transmutation theory: that is, that transmutation from cones to rods has taken place when nocturnal habits have been acquired by forms whose ancestors were diurnal with pure-cone retina. For example, the rod-like photoreceptor cells of the nocturnal gecko appear to have transmutated from the cone cells of a diurnal lizard. The primary structures of visual pigments contained in the rod-like photoreceptors of the nocturnal gecko, *Gekko gekko*, are similar to red- and green-sensitive cone pigments in other vertebrates [32]. This provides evidence for this theory from the molecular standpoint. However, the transmutation theory lead logically to the conclusion that it was practically inevitable that visual-cell transmutation should occur in reptiles, but it is improbable that many cases will be found outside the reptilian group [33].

In the case of the nocturnal gecko, all photoreceptor types have enlarged and become of rod-like. In contrast, frog green rods have a typical rod morphology even though other cones still remain as typical cones. Have green rods transmuted from an ancestral blue-sensitive cones? It has been reported that green rods are similar to red rods in all respects except spectral sensitivity, and no evidence has been found to support the assertion that green rods are 'cone-like' [7]. Why do green rods generate a light response similar to those of red rods? Further detailed analysis at the molecular level will be necessary to reveal the answers to these questions and to fully understand the diversity of vertebrate photoreceptor cells.

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