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Molecular cloning of the common carp (*Cyprinus carpio*) rhodopsin cDNA

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A recombinant phage clone containing a 1584 nucleotides rhodopsin cDNA was screened from a carp retinal cDNA library. The inserted DNA consisting of a single open reading frame of 1062 nucleotides at positions 72 to 1133 encodes a 354 amino acid polypeptide. The deduced amino acid sequence of carp rhodopsin showed 95.7, 85.5 and 74.4% identity with that of goldfish, sand goby and lamprey, respectively. The sites of palmitoylation, glycosylation, disulfide bond formation and Schiff base formation in the putative rhodopsin are all conserved.

Key words: Carp; PCR screening; Photoreceptor; Retinal cDNA library; Rhodopsin; Signal transduction; Transmembrane domain; Visual pigment.

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Introduction

The underwater environment limits both the intensity and the spectral bandwidth of ambient light for vision and aquatic survival (Lythgoe, 1980). Yet retina anatomy and photoreceptor proteins (opsins) show structural similarities between land and aquatic vertebrates (Yokote, 1982; Nathans *et al.*, 1986). Of the photoreceptor proteins, rhodopsin is the most completely investigated GTP binding protein in terms of structure and function relationship; it belongs to the family of seven-membrane-span receptors such as the β-adrenergic receptor (Ferretti *et al.*, 1986; Stryer, 1991; Khorana, 1992). Based on single cell electrophysiological measurements, a rod and three cone cell photoreceptors have been suggested (Tomita *et al.*, 1967). Recently several opsin cDNAs of the goldfish, a teleost that occupies a more surface habitat have been reported (Johnson *et al.*, 1993). In order to further understand the comparative aquatic visual physiology we have initiated a study on the opsins of the common carp, *Cyprinus carpio*, an important aquacultured species and a closely related teleost with goldfish but occupies a more bottom habitat.

In this paper, we report the isolation and characterization of a putative carp rhodopsin cDNA clone from the retina of the cultured carp. A comparison of the nucleotide sequence with those reported for goldfish, sand goby and lamprey indicates that factors other than phylogeny may be also important in determining sequence relatedness.

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Materials and Methods

Isolation of RNA and cDNA library construction

Retinae were freshly dissected from 25 carps (300–500 g each, a gift from Dr I-Chio Liao, and Fu-Guang Liu, Taiwan Fisheries Research Institute, Keelung). After grinding in a mortar in liquid nitrogen, the homogenate was suspended in prechilled denaturing solution containing 4 M guanidinium thiocyanate, 0.51% *N*-laurylsarcosine, 0.125 mM β-mercaptoethanol, 5 μg/ml polyvinyl sulfate, and 26.25 mM sodium citrate; this suspension was then drawn 5–6 times with a syringe fitted with an 18-gauge needle. Total retinal RNA was prepared by using the acid/phenol method described by Chomczynski and Sacchi (1987), with an additional gel filtration through a PD-10 column (Pharmacia) to remove melanin.

The poly(A)$^+$ RNA was isolated and the retinal cDNA library was then constructed in a lambda gt10 (Promega) using *Escherichia coli* VC257 as the host cells as previously described by Tsai *et al.* (1993).

Screening by polymerase chain reaction (PCR)

The initial screening of the cDNA library (430,000 phages divided into ten equal fractions) was achieved by PCR using a synthetic 27-mer, 5′-GGCTGGAGCAGGTATATCCCGGAGGGT-3′ as the forward primer. It corresponds to the nucleotide positions 731 to 757 of the *Drosophila* rhodopsin cDNA and is homologous with bovine sequence (Zuker *et al.*, 1985). This sequence mimics the conserved rhodopsin intradiscal segment involved in disulfide and tertiary structure formation (Khorana, 1992). The reverse primer, 5′-GAGGTGGCTTATGAGTATTTCTTCCAGGGTA-3′, was derived from lambda gt10 (Promega).

The PCR procedure was carried out in a 100 μl solution containing 9×10^6 phage DNA as template, 20 pmol reverse primer, 6 pmol forward primer, 200 μM of each dNTP, 10 μg of bovine serum albumin, and 5 U Taq polymerase with 10 μl of 10× buffer (Promega). A DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT) was used for PCR amplification. The temperature profiles were: 94°C for 1 min, 68°C for 2 min, and 72°C for 3 min for the first two cycles; 94°C for 1 min, 65°C for 2 min, and 72°C for 3 min for a second three cycles; and 94°C for 1 min, 60°C for 2 min, and 72°C for 3 min for the final 30 cycles. The resulting PCR products were analyzed by 0.8% agarose gel electrophoresis.

Plaque hybridization screening

Those phage fractions generating the most intense PCR products of the predicted sizes were screened with a goldfish rhodopsin cDNA probe. This probe, molecular size 1.3 kb, was isolated from an amplified plasmid pRJ1 (a gift from Dr. Kathy Grant of the Department of Chemistry, Columbia University, NY) followed by *Eco*RI digestion. Approximately 75,000 recombinant phages were plated on three NZY (0.5% NaCl, 0.2% $MgSO_4 \cdot 7H_2O$, 0.5% yeast extract and 1% NZ amine) plates; they were then transferred to nitrocellulose membranes by blotting. Hybridization with the 1.3 kb ^{32}P-labeled goldfish probe was carried out in a buffer (10% dextran sulfate, 1% SDS, 1 M NaCl, 50 mM Tris–HCl; pH 7.5) at 42°C overnight. The membranes were washed twice (20 min each) with a solution containing 0.5% SDS and 6× (0.15 M NaCl, 1 mM EDTA, and 30 mM Tris–HCl; pH 8) at 27°C. Positive clones were identified via exposure to X-ray film (Kodak) at −70°C for two days, then amplified in a second screening.

The hybridized nitrocellulose membranes from the second screening were washed three times (20 min each) in 0.5% SDS and 6× SSC (1 × SSC: 0.15 M NaCl and 0.015 M sodium citrate) at 27°C, followed by another two washings in 0.1% SDS and 2× SSC at 42°C, plus a final washing in 0.05% SDS and 1× SSC at 65°C for 10 min.

Southern blotting

Recombinant DNA inserts prepared from the positive clones after *Eco*RI digestion were analyzed by Southern blotting (Southern, 1975). The probe used was the 1.3 kb goldfish rhodopsin cDNA radiolabeled by the random priming (Feinberg and Vogelstein 1983).

Subcloning

The cDNA insert isolated from the 0.8% agarose gel was ligated onto an *Eco*RI digested phagemid pGEM-7ZF(+), (Promega). The transformation of *E. coli* DH5α was carried out following the procedures described by Hanahan (1985). The transformants harboring the cDNA inserts were screened via plasmid DNA mini-preparation analysis (Sambrook *et al.*, 1987).

DNA sequencing

Our strategy for determining the nucleotide sequences of the isolated carp retinal cDNA is shown in Figure 1. We used the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977) with a Sequenase kit (US Biochemical Corp., Cleveland). In addition to the T7 and SP6 primers, four sequence extension oligomers were synthesized: primer CRH1, +188 5′-CCTGGCCGCGTACATGTTC-3′ +206 sense strand; primer CRH2, +1345 5′-GGGACATGGTCTGTC-3′ +1331 antisense strand; primer CRH3, +536 5′-GGGGGTTGTCTTCAC-3′ +551 sense strand; and primer CRH4, +1034 5′-CAGGGTGGTGATCATGC-3′ +988 antisense strand (numbers refer to the nucleotide position on the carp cDNA sequence).

Sequence analysis

Both the nucleotide and predicted amino acid sequences of the isolated carp rhodopsin cDNA were compared to those of other fishes deposited with the GenBank and National Biomedical Research Foundation data bases. To align these sequences, we introduced gaps to maximize identity via procedures described by the Genetic Computer Group (Higgins and Sharp, 1988). Hydropathicity profile of carp rhodopsin amino acid sequence was obtained according to analyses described by Kyte and Doolittle (1982). In addition, we used a Chargpro program from the PC Gene software package for estimating the isoelectric point (pI) of carp rhodopsin.

Results and Discussion

Identification of the putative rhodopsin clone

The initial PCR screening of the carp retinal cDNA library revealed bands of 0.4–0.6 kb in five of the 10 fractions on the agarose gel (Fig. 2). Since the forward primer corresponds approximately to the middle segment (see Materials and Methods) of the rhodopsin sequence, one fraction (lane 2, Fig. 2) containing the longest PCR product was chosen for plaque hybridization screening. Twenty-four positive recombinant phage clones were isolated following the first screening; seven clones were purified via a second plaque screening, and the inserted DNA from three of these clones was digested with *Eco*RI (Fig. 3A) and analyzed by Southern blotting (Fig. 3B). Two inserts of approximately 0.9 and 1.6 kb showed strong hybridization with the goldfish probe (lanes 2 and 3, Fig. 3B). The 1.6 kb insert (lane 3, Fig.3) was constructed in plasmid pGEM-7Zf(+) and transformed into host *E. coli*, then sequenced.

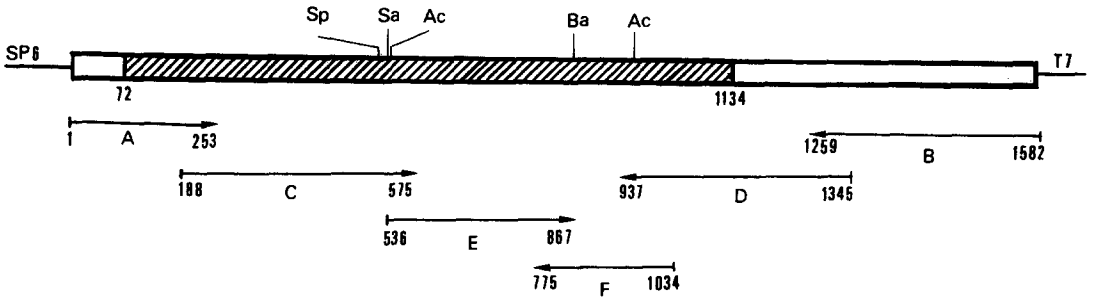

Fig. 1. A partial restriction map and sequencing strategy of carp rhodopsin cDNA. The untranslated region (empty box) and coding region (hatched box) of the cDNA are shown. DNA fragments A, B, C, D, E and F were sequenced by using primers SP6, T7, CRH1, CRH2, CRH3, and CRH4, respectively. See Materials and Methods for details. Ac, *Acc*I; Ba, *Bam*HI; Sp, *Sph*I; Sa, *Sma*I restriction site.

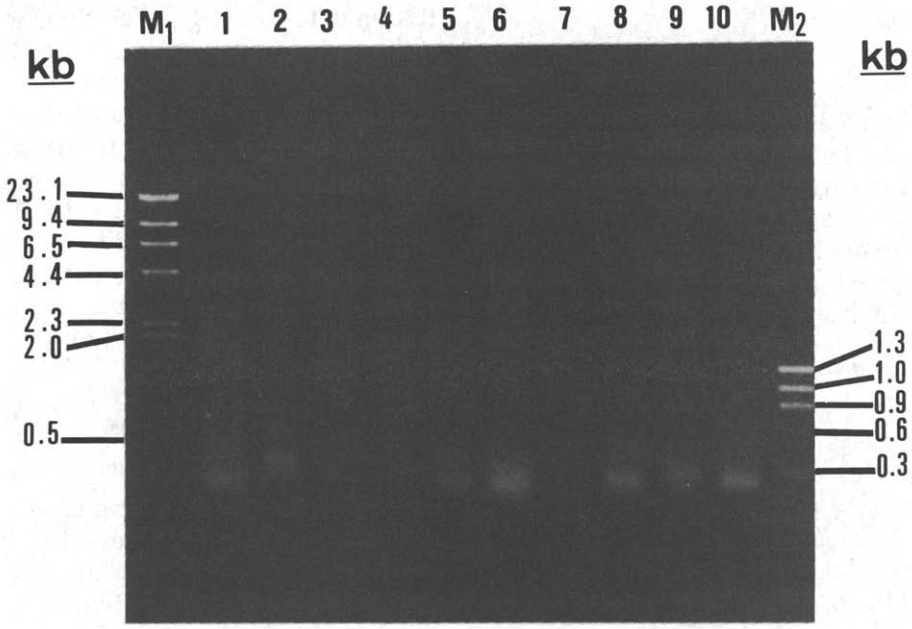

Fig. 2. Analysis of PCR products. PCR products from the carp retinae cDNA library divided into 10 43,000 phages fractions were shown in lanes 1 to 10. The molecular markers, M1 and M2, were *Hin*dIII-digested lambda genome and *Hae*III-digested ϕX-174-RF DNA, respectively.

Nucleotide sequence of carp rhodopsin cDNA and comparison with that of other known genes

The nucleotide sequence of carp rhodopsin cDNA is shown in Fig. 4. The putative carp rhodopsin cDNA sequence contains a single open reading frame of 1062 nucleotides located between nucleotide positions 72 and 1133, which encodes 354 amino acid residues. The translation initiation codon (ATG) was observed at nucleotide positions 72 to 74; it was preceded by 71 untranslated leader sequences. The translation termination codon TAA, nucleotide positions 1134 to 1136, was followed by an untranslated region of 448 nucleotides at the 3′ end. An AATAAA hexanucleotides, the polyadenylation signal, starting at nucleotide position 1511, was 15 nucleotides upstream from the starting site of the poly (A) tail of 52 As.

The carp cDNA was identified as that of a putative rhodopsin sequence on basis of its 93.4% nucleotide homology with the coding region of that of the goldfish rhodopsin (Johnson *et al.*, 1993). In contrast, it showed a low respective homology of 56.0, 61.8, 63.4 and 65% with that of goldfish red, blue, green-1 and green-2 opsin. Compared with other known fish rhodopsins it exhibits 80.1 and 73.4% identity with that of sand goby (Archer *et al.*, 1992) and lamprey (Hisatomi *et al.*, 1991) respectively, suggesting a more distant phylogeny with the latter. It is interesting to note that the carp rhodopsin also showed 72.6, 72,8 and 74.3% identity with that of human (Nathans and Hogness, 1984), mouse (Al-Ubaidi *et al.*, 1990) and chicken (Takao *et al.*, 1988) respectively. These results suggest that other factor(s) in addition to phylogeny may also determine the extent of sequence similarity.

Comparison of the deduced amino acid sequences of carp rhodopsin with those of other known fish species

Figure 5 shows a comparison of our deduced amino acid sequence in carp rhodopsin with those reported for other fish species. Identity was found to be 95.7% with that of goldfish (Johnson *et al.*, 1993), 85.4% with the sand goby (Archer *et al.*, 1992) and 74.4% with the lamprey (Hisatomi *et al.*, 1991).

Membrane protein topology. The deduced amino acid sequence of the carp rhodopsin differs from that observed for goldfish in only 16 out of 354 residues; 10 of the 16 are homologous replacements for

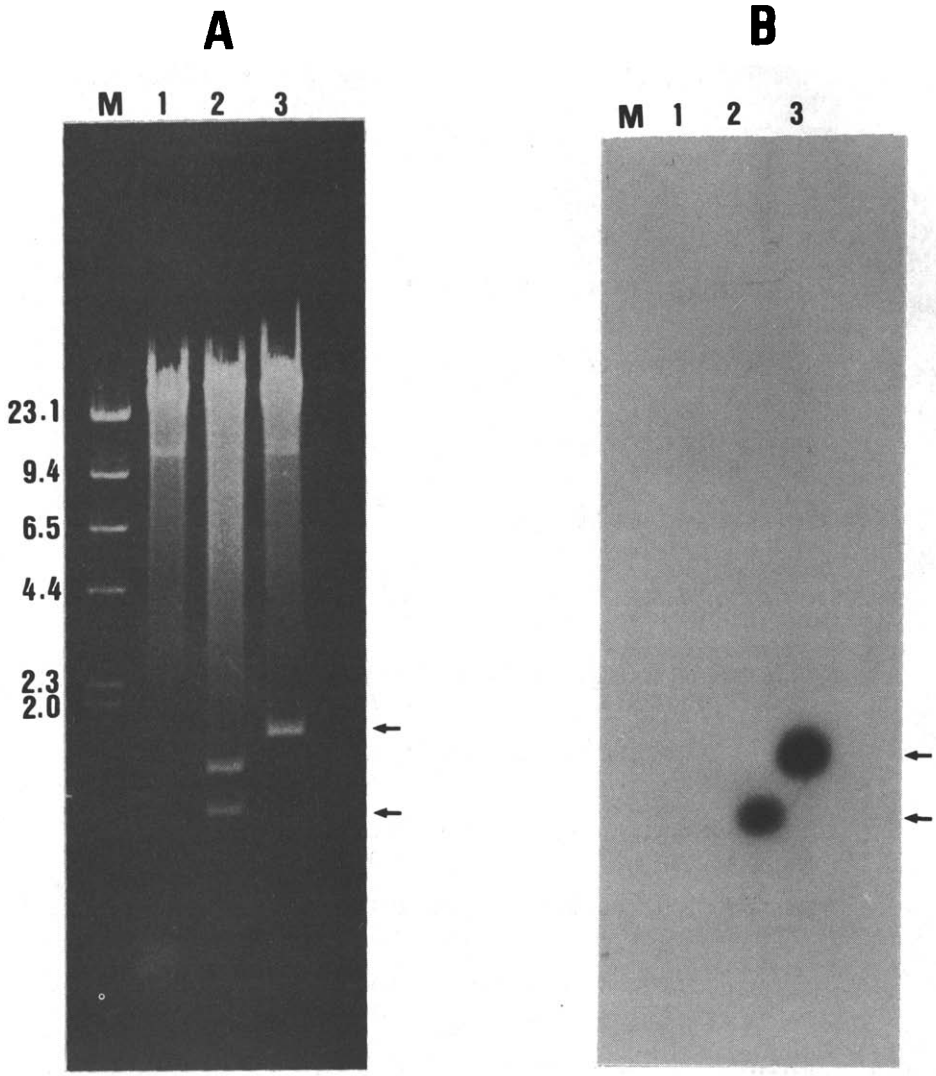

Fig. 3. Southern blot analysis of the DNA insert isolated from the lambda gt10 clones. (A) Ethidium bromide staining of genomic DNA from three positive clones extracted and digested with *Eco*RI; (B) autoradiogram of the nitrocellulose membrane hybridized with a ^{32}P-labeled goldfish rhodopsin cDNA probe. Lane M shows the molecular markers of *Hin*dIII-digested lambda genome in kb, and lanes 1–3 show the recombinant phages. Arrows indicate the positively hybridized 0.9 and 1.6 kb bands. In this study, the 1.6 kb clone was used for sequencing.

residues having similar properties. The observed carp sequence encodes a largely hydrophobic polypeptide with a predominantly hydrophobic N-terminal, while the C-terminal loop contains a 12 Ser/Thr segment which is essential for the phosphorylation control of visual signal transduction. The similarities observed in the hydropathy plots (data not shown) of carp and bovine rhodopsin suggest similarities in the transmembrane segmentation, i.e., repeating segment of 19–27 residues (Helix I–VII in Fig. 5), as well as in high proline and aromatic amino acid contents.

Conserved amino acid residues. Lys-296 and Glu-113 are conserved for the retinal binding and counterion interaction required for Schiff base formation (Nathans, 1987). Disulfide between Cys-110 and Cys-187 is required for the formation of the retinal pocket from the seven membrane helical segments. The N-terminal Asn-2 and Asn-15, corresponding to the glycosylation sites of the Asn-X-Thr sequences, and the C-terminal Cys 322-Cys 323, the palmitoylation site essential to membrane anchoring, are completely conserved.

```
CTCCACAGTCTTGCCGAGCCATCCAAACACTACCACAGAAAGGGGCTGAGACACAACATC    60
CAACCGCAGCCATGAACGGTACAGAGGGACCTATGTTCTACGTGCCTATGTCCAATGCGA   120
          M  N  G  T  E  G  P  M  F  Y  V  P  M  S  N  A  T     17

CCGGCGTTGTTAAGAGCCCGTACGACTATCCCCAGTACTACCTGGTGGCGCCATGGGCAT   180
 G  V  V  K  S  P  Y  D  Y  P  Q  Y  Y  L  V  A  P  W  A  Y     37

ACGGCTGCCTGGCCGCGTACATGTTCTTCCTCATTATCACTGGCTTCCCTATCAACTTCC   240
 G  C  L  A  A  Y  M  F  F  L  I  I  T  G  F  P  I  N  F  L     57

TCACTCTGTACGTCACCATCGAGCACAAGAAGCTGCGTACACCTCTCAACTACATTCTGC   300
 T  L  Y  V  T  I  E  H  K  K  L  R  T  P  L  N  Y  I  L  L     77

TGAACCTCGCCATTTCCGACCTCTTCATGGTGTTCGGTGGCTTCACCACGACGATGTACA   360
 N  L  A  I  S  D  L  F  M  V  F  G  G  F  T  T  T  M  Y  T     97

CGTCGTTGCATGGCTACTTTGTTTTTGGACGCATTGGCTGCAACCTCGAAGGCTTCTTCG   420
 S  L  H  G  Y  F  V  F  G  R  I  G  C  N  L  E  G  F  F  A    117

CAACCCTGGGTGGTGAAATGGGCCTTTGGTCCTTGGTGGTGCTGGCCTTCGAGAGGTGGA   480
 T  L  G  G  E  M  G  L  W  S  L  V  V  L  A  F  E  R  W  M    137

TGGTTGTCTGTAAGCCCGTGAGCAACTTCCGCTTCGGAGAGAACCACGCCATCATGGGGG   540
 V  V  C  K  P  V  S  N  F  R  F  G  E  N  H  A  I  M  G  V    157

TTGTCTTCACCTGGTTCATGGCCTGCACCTGCGCCGTGCCTCCCCTGGTCGGCTGGTCCC   600
 V  F  T  W  F  M  A  C  T  C  A  V  P  P  L  V  G  W  S  R    177

GTTACATCCCCGAGGGCATGCAGTGCTCGTGCGGAGTCGACTATTACACTCGCGCCCCTG   660
 Y  I  P  E  G  M  Q  C  S  C  G  V  D  Y  Y  T  R  A  P  G    197

GCTACAACAATGAGTCCTTTGTCATCTACATGTTCCTTGTCCACTTCATTATTCCATTAA   720
 Y  N  N  E  S  F  V  I  Y  M  F  L  V  H  F  I  I  P  L  I    217

TCGTCATATTCTTCTGCTACGGCCGTCTCGTCTGCACCGTCAAAGATGCCGCTGCCCAGC   780
 V  I  F  F  C  Y  G  R  L  V  C  T  V  K  D  A  A  A  Q  Q    237

AGCAGGAGTCTGAGACCACCCAGAGGGCTGAGCGTGAGGTCACCCGCATGGTCGTCATCA   840
 Q  E  S  E  T  T  Q  R  A  E  R  E  V  T  R  M  V  V  I  M    257

TGGTCATCGGCTTCTTGATTTGCTGGATCCCATATGCCAGCGTGGCCTGGTATATCTTCA   900
 V  I  G  F  L  I  C  W  I  P  Y  A  S  V  A  W  Y  I  F  T    277

CCCACCAGGGAAGCGAATTTGGGCCTGTCTTCATGACCGTGCCAGCCTTCTTTGCCAAGA   960
 H  Q  G  S  E  F  G  P  V  F  M  T  V  P  A  F  F  A  K  S    297

GTGCTGCTGTCTACAACCCATGCATCTACATCTGCATGAACAAGCAGTTCCGTCACTGCA  1020
 A  A  V  Y  N  P  C  I  Y  I  C  M  N  K  Q  F  R  H  C  M    317

TGATCACCACCCTGTGCTGCGGCAAGAACCCCTTCGAGGAGGAAGAGGGCGCCTCCACTA  1080
 I  T  T  L  C  C  G  K  N  P  F  E  E  E  E  G  A  S  T  T    337

CTGCATCCAAGACCGAGGCTTCGTCCGTGTCTTCCAGCTCCGTGTCCCCTGCGTAAACAG  1140
 A  S  K  T  E  A  S  S  V  S  S  S  S  V  S  P  A  *          354

TTGTCCGTGACACAGAATAAGCAGTGACATGCACTGGGCTTCAACGGCAACCGACGACAC  1200
AGAGACCACAAAGTGTTCAGCCCCGGGAAAGAGCAACCACTACCACTTGCAGAAAAAAAT  1260
GTCTGTCAGTTTTCCTTTTTGTATTTTCACAAAACCCAATTGGTTCAACCAAAAGACAGT  1320
TTTGAGAGAGGACAGACCATGTCCCAGTTTCAGTACATCCAGCGAGTCCAGCGTAACGGT  1380
GCATAAGATTTTTTTTAATTTTTTCTTCCTAAAATGCAGCAAAAGGAAAAATATCTTAAC  1440
TCTCTTACTGTTGGACTCCTTATACTGGCTTTGTTGTGATTGTAGAGGCATGTATTCAAG  1500
GCAACGTAACAATAAAAAGCACTTTGCAAATTAAAAAAAAAAAAAAAAAAAAAAAAAAAA  1560
AAAAAAAAAAAAAAAAAAAAAAAA                                      1584
```

Fig. 4. Nucleotide sequence and deduced amino acid sequence of carp rhodopsin cDNA. The nucleotide was numbered beginning with the first nucleotide at the 5′ end. Numbers on the second line of each row indicate the amino acid sequences. The polyadenylation signal (AATAAA) is boxed; the stop codon is marked with an asterisk.

```
                                                                I
                                                                ___________________
CP  MNGTEGPMFYVPMSNATGVVKSPYDYPQYYLVAPWAYGCLAAYMFFLIITGFPINFLTLY  60
GF  ......D...........I.R................A...............V......  60
SG  .......F..I..V.T..I.R...E.......N.A..AA........L............  60
LA  ......DN....F..K..LAR...E......AE..K.SA.........LV...V.....F  60

                        II
    ___     ___________________________                  _____
CP  VTIEHKKLRTPLNYILLNLAISDLFMVFGGFTTTMYTSLHGYFVFGRIGCNLEGFFATLG 120
GF  ...............................................V...P........ 120
SG  ..L.......A..L......VA................M.....L..L...V........ 120
LA  ..VQ................MAN....LF...V.....MN......PTM.SI........ 120

        III                                   IV
    ____________                  ____________________________
CP  GEMGLWSLVVLAFERWMVVCKPVSNFRFGENHAIMGVVFTWFMACTCAVPPLVGWSRYIP 180
GF  ............................................................ 180
SG  ..IA........V...V.....IS....T........A.S.I..A............... 180
LA  ..VA........I..YI.I...MG.....NT......A...I..LA..A........... 180

                                  V
                        ____________________________
CP  EGMQCSCGVDYYTRAPGYNNESFVIYMFLVHFIIPLIVIFFCYGRLVCTVKDAAAQQQES 240
GF  ..............PQA...........I......................E....HE.. 240
SG  ...............E.F..........I...LA............L.A..E...A.... 240
LA  ........P....LN.NF....Y.V...V...LV.FVI........L....E...A.... 240

                        VI                          VII
                    ____________________________  ____________________
CP  ETTQRAEREVTRMVVIMVIGFLICWIPYASVAWYIFTHQGSEFGPVFMTVPAFFAKSAAV 300
GF  ...................................................L......T... 300
SG  ..............I.......TS.L..............T....L...I.......S.L 300
LA  AS..K..K.......L......V..V......F........D..AT...L.......S.L 300

    ___________
CP  YNPCIYICMNKQFRHCMITTLCCGKNPFEEEEGASTTASKTEASSVSSSSSVSPA 354
GF  ........................................................ 354
SG  ...M.................................--................ 352
LA  ...V...L......N............LGDD.SGAS.-....V....T.P.... 353
```

Fig. 5. Comparisons of the predicted amino acid sequences of carp (CP) rhodopsin with that of goldfish (GF) (Johnson *et al.*, 1993), sand goby (SG) (Archer *et al.*, 1992) and lamprey (LA) (Hisatomi *et al.*, 1991). Numbers begin at the first amino acid residue; identical amino acid residues are indicated by dots. Dashes represent gaps created to maximize the degree of identity among all compared sequences. The seven transmembrane domains are labeled I to VII above the sequence.

*Net charges and p*I. The charge characteristics of the putative rhodopsin were estimated based on the following p*K* values: arginine, 12.48; lysine, 10.79; histidine, 6; asparatate, 3.65; glutamate, 4.25; cysteine, 8.35; and tyrosine, 10.13. A value of 6.3 was obtained for pI of the predicted carp rhodopsin. This is in the range of 5.9–6.3 observed for bovine and human rhodopsin (Okano *et al.*, 1992), but one

which differs markedly from the alkaline pI values of approximately 9.5 observed for color and/or photopic cone pigments (Okano *et al.*, 1992).

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