Structural characterization and transcriptional pattern of two types of carp rhodopsin gene

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Abstract

This work characterizes the genomic structures of two types of carp (*Cyprinus carpio*) rhodopsin (*cRh*) gene, i.e. type I (*cRh-I*) and type II (*cRh-II*). Two types of *cRh* gene share only 45.6% polynucleotide identity in the upstream region from nucleotide -3436 to $+97$. However, three conserved regions are found. Homologies to the consensus recognition sites for transcription factors, Crx and Nrl, which are involved in photoreceptor-specific expression, are also observed in *cRh* genes. With specific polymerase chain reaction (PCR) primers, the two types of *cRh* gene can be clearly discriminated from each carp genome. Most carps exhibit both types of *cRh* gene, however, there are still carps possessing either *cRh-I* or *cRh-II*. Both *cRh-I* and *cRh-II* mRNAs are expressed at an approximately equal level in both eyes extracted from a carp carrying both types of *cRh* gene. © 2000 Elsevier Science Inc. All rights reserved.

Keywords: Cyprinus carpio; Fish; Genomic structure; PCR; Photoreceptor; Rhodopsin; Sequence comparison; Type discrimination

1. Introduction

Eyes are photoreceptor organs that contribute to image formation. Photoreceptor cells consist of rods and cones. Rods are sensitive to dim light, while cones contribute to daytime vision and color perception. Each photoreceptor consists of a seven-transmembrane protein, opsin, to which its chromophore, 11-*cis*-retinal, is attached (Rose et al., 1989). Previous investigations have reported on the presence of rhodopsin cDNA in extremely broad varieties, such as bovine (Nathans and Hogness, 1983), humans (Nathans and Hogness, 1984), chickens (Takao et al., 1988), mice (Baehr et al., 1988), rats (Morabito et al., 1991), lizards (Kawamura and Yokayama, 1994), canines (Pe-

terson-Jones et al., 1994), old world monkeys (Nickells et al., 1995), salamanders (Chen et al., 1996), *Xenopus* (Saha and Grainger, 1993; Batni et al., 1996), squids (Hall et al., 1991; Hara-Nishimura et al., 1993), lamprey (Hisatomi et al., 1991), goldfish (Johnson et al., 1992), marine lamprey (Zhang and Yokoyama, 1997), skates (O'Brien et al., 1997), carps (Tsai et al., 1994; Lim et al., 1997) and marine fish, John Dory (Archer and Hirano, 1998). These studies include terrestrial animals, amphibians and aquatic animals. Nevertheless, genomic structure of rhodopsin was studied primarily in *Xenopus* (Batni et al., 1996), chickens (Takao et al., 1988) and mammals, such as bovine (Nathans and Hogness, 1983), humans (Nathans and Hogness 1984; Bennett et al., 1995), mice (Baehr et al., 1988), and rats (Morabito et al., 1991). Those investigations demonstrate that rhodopsin gene contains four or five introns. Interestingly, Fitzgibbon et al. (1995) indicated that

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the rhodopsin gene of bony fish is intron-free, although the genomic structures of those bony fish have not been completely characterized. Among all the species studied, only one type of rhodopsin gene has been reported except *Xenopus* and the one studied in a previous investigation, the common carps (Lim et al., 1997).

In light of above discussion, this study demonstrates the rhodopsin genomic structures of the most primitive vertebrate, fish. The entire molecular sequences corresponding to two types of carp rhodopsin (*cRh*) cDNAs are elucidated. In addition, type discriminations and transcription patterns of *cRh* genes are studied as well. Comparing the polynucleotide sequences of the upstream region and investigating the transcriptional patterns of type I *cRh* (*cRh-I*) and type II *cRh* (*cRh-II*) genes facilitate an understanding of the rhodopsin gene regulation of fish. More thoroughly understanding rhodopsin genes of diverse species will provide further insight into visual systems.

2. Materials and methods

2.1. Genomic library construction, screening and subcloning

Genomic DNA extracted from carp blood (Sambrook et al., 1989) was utilized to construct the genomic DNA library by using Lamda FIX® II/*Xho*I Partial Fill-In Vector Kit and Gigapack® III Gold Packaging Extract (Stratagene). Approximately 60 000 phages were hybridized by a radiolabeled probe (1.3 kb fragment of *cRh-I* cDNA) overnight at 42°C. The membranes were washed and then exposed to X-ray films. Each putative phage clone (100 μl) was heated and vigorously vortexed as a template for polymerase chain reaction (PCR). Type-specific primers were used to detect the *cRh-I* and *cRh-II* genes. PCR was performed for 25 cycles in a 20 μl solution containing 7 μl of phage lysate, 0.2 mM of dNTP, 0.5 μM of primers, 1 × buffer and 2.5 U *VioTaq* DNA polymerase (Viogene). Each amplification cycle consisted of denaturation at 94°C for 1 min, annealing at 62°C for 1 min, and extension at 72°C for 1 min.

Insert fragments were excised from recombinant phage DNA by *Sal*I digestion and purified by gel extraction. The 5′ and 3′ flanking regions of *cRh* genes were determined by PCR using the upstream- and downstream-specific primers (5′-GGCTGCGGTTGGATGTTGT-3′, 5′-CTGATAAGTGCCAGATATG C-3′, 5′-CCCCGGGAAAGAGCAACCA-3′ and 5′-CATGCCTCTACAATCACAACAAA-3′). Insert fragments were then subcloned into pBluescript® II SK (+/−) vector.

2.2. The primer extension assay

Total retinal RNA from adult carps dissected during the daytime was extracted with TRI Reagent (Molecular Research Center, Inc). The $[\gamma\text{-}^{33}p]$ATP-labeled primers were hybridized by retinal RNA at 50°C for 22 h, and then precipitated by ethanol. The pellet was resuspended in reverse transcription buffer and reversely transcribed with SuperScript™ II RNaseH⁻ Reverse Transcriptase (Gibco BRL). Finally, the extension reaction was loaded along with the sequencing reaction of the subcloned DNA sequenced with the same primer on a 6% polyacrylamide/8 M urea gel.

2.3. Sequence analysis

Sequencing was performed with Dye Terminator Cycle Sequencing Ready Reaction Kit (ABI) and analyzed on ABI PRISM™ 310 Genetic Analyzer. Polynucleotide sequences were read at both ends and analyzed with PC/GENE® Program (IntelliGenetics).

2.4. Type discrimination

Fifty nanograms of genomic DNA extracted from carp blood (Sambrook et al., 1989) was used as template to perform PCR reaction with type-specific primers as above description. The PCR products were digested by *Tfi*I for differentiating *cRh-I* and *cRh-II* genes. In total, 100 carps randomly selected from the local aquacultural ponds (Tzu-Pei, located in northern Taiwan and Lu-Kang, located in middle Taiwan) were analyzed for the distribution frequency of *cRh-I* and *cRh-II* genotypes.

2.5. Transcription pattern

Total retinal RNA of each eye of an adult carp carrying both *cRh-I* and *cRh-II* genes was extracted and reversely transcribed as above descrip-

A

Fig. 1. Comparison of the upstream (A) and downstream (B) polynucleotides of the type I (*cRh-I*) and type II (*cRh-II*) rhodopsin genes of carp. Numbers on the right side of each column represent the nucleotide position relative to transcription start site (+ 1), which is boxed. The transcribed region is underlined. TATA and CAAT boxes of *cRh-I* and *cRh-II* genes are indicated above and below, respectively. Identical bases are denoted by vertical bars. Gaps, denoted by broken lines, are introduced to maximize identities. Regions containing greater than 75% identity between the upstream region of two types of *cRh* genes are shaded. The polyadenylation signals found in cDNA clones are in boldface and italics.

tion. The RNA was removed by RNaseH digestion. PCR was performed with type-specific primers as described above except that retinal cDNA was used as a template.

3. Results and discussion

3.1. Isolation of recombinant phages containing cRh genes

A genomic DNA library of titer 10^{11} pfu/ml was constructed. Two *cRh-I* clones and three *cRh-II* clones were isolated from the library. Thereafter, recombinant phages contained 14 kb of *cRh-I* insert and 10 kb of *cRh-II* insert were obtained, respectively. After restriction mapping and subcloning, DNA fragments around 3.4 and 0.3 kb containing upstream and downstream flanking regions of *cRh* genes, respectively, were sequenced (Fig. 1). These sequences are submitted to the EMBL/GeneBank/DDBJ databases under Accession No. AJ 012013, AJ 012009, AJ 012014 and AJ 012010.

3.2. Characterization of both types of cRh gene

The primer extension assay revealed that the transcription initiation site ($+1$) of *cRh* was 94 bp upstream of the start codon (data not shown). A TATA box was observed at nucleotide (nt) $-33 \sim -36$ in both *cRh-I* and *cRh-II* genes. As generally known, this nt position is relatively conserved among all known species. Similar to the rhodopsin gene of mice (Baehr et al., 1988), the *cRh-II* gene had a CAAT box at nt -121 to -118. However, the *cRh-I* gene had a CAAT box at nt -235 to -238, which was 120 bp ahead of that of *cRh-II* gene (Fig. 1A). Both types of *cRh* gene were intronless. This finding correlates with Fitzgibbon et al. (1995), which contended that rhodopsin gene of bony fish lacks intron.

3.3. Upstream flanking sequences analysis

Compared to the *cRh-II* gene, the *cRh-I* gene shared only 45.6% polynucleotide identity in the upstream region ranging from nt -3434 to $+97$ of *cRh-I* or from nt -3435 to $+97$ of *cRh-II*. However, three relatively highly conserved regions were observed at nt -1262 to -614, -539 to -419 and -166 to $+97$, with 78.0, 79.3 and 92.3% identities, respectively (Fig. 1A).

B

Fig. 1. (*Continued*)

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cRh-I   GTCAGGTACAACCTTAATAA-CTTGATA-------AATT--GCCAGATATGCAGTGCCATGATGGCTGA-GATTATTTT-ATTAGCTGTGGCCTAAATAGA-CTC---G-   -76
cRh-II  TGCAGGTACAACC-TAATAA-CT-GAT---------AAGT--GCCAGATATGCAGTGCAATGATGGCTG--GATTATTTT-ATTAGCTGTGGCCTAAATAGA-CTC---G-   -76
Xen     GACAGGTGTAGACTTAATACGTTTAAGGG-AAGCCAATTA-ACACTTTG---CAATTT---AGC--TT-GGATTACAGTGATTAATAGTGCGCTAAATC---CTTT--GT   -69
Chk     AACAGGTGTCACCTTAATACCCACCCAGAAAAAGCCAATTA--CCC------C--T-------------GGATGATTATGATTAATAACTCTCTAAGCC---CTAATCG-   -69
Ham     ACCCGGTGTCACCTTGGC-CCCT-TTGC--AAGCCAATTAGGCCC------CAGT---GGCAGCAGTG-GGATTAGTATTCTTAGTATG-------ATAT-CTCCCAGA   -68
Rat     ACCTGATGTCACCTTGGC-CCCT-CTGC--AAGCCAATTAGGCCC-----CAGT--CGCAGCAGTG-GGATTAGT----ATTAGTGTG-------ATAT-CTCCCGGA   -67
Mus     ACCCGATGTCACCTTGGC-CCCT-CTGC--AAGCCAATTAGGCCC-----CAGT--CGCAGCAGTG-GGATTAGC---GTTAGTATG-------ATAT-CTCGCGGA   -68
Hum     TCCTAGTGTCACCTTGGC-CCCT-CTTAG-AAGCCAATTAGGCCC-T---CAGTTTCTGCAGC-G-G-GGATTAATATGATTATGAACACCCCC-A-AT-CTCCCAGA   -65
Bov     ACCTGGAGTCACCTTGGC-CCCACCTGG--AAGCCAATTAGGCCCT----C-GT---TGCAGCAGTGGAGGATTAATATGATTAATAACGCCCCC-A-AT-CTCCGAGG   -67

                              Ret 1                                                  BAT-1


cRh-I   TGCTGA--CAGCCCT-----------------GGAAACATCAGGT--AATCCCAAGTGAGTCTCTATAAAG--CA-GGTTGCAGGCTCG--CCCCGTCAAGTCGTAGCAC   +8
cRh-II  TGCTGA--CAGCCCT-----------------GGAAACATCAGTT---AATCCCAAGCGAGCCTCTATAAAG--CCGTGGTG-CACGCTCG--CCCCGTCAAGTCGTAGCAC   +8
Xen     TCGTGA--C-G-CTGGGGGTTGCAAG-CTTACTCC------AGGTGGGACT-------------TAAAAAGGACGAGGGGACAGTGGGT--CATAC-----TGTAGAAC   +15
Chk     CGCTGA--CA-CCGATGCCCGG---GCTTACTGC---AGGGGGCACT--------TTATAAGGACGCCCTACAGCTGCTT--CTCTGC---GGGGCGAC   +14
Ham     TGCTGAATCAGCCCTCT-------GGCTTAGGGAGAGA--AGGTC-ACT-------TTATAAGGGTCTTGGGGGGTTGATG--CCTGG------AGTTGCGC   +14
Rat     TGCTGAATCAGCCCTCT-------GGCTTAGGGAGAGA--AGGTC-ACT-------TTATAAGGGTCTGGGGGGGTCAGTG--CCTG-------AGTTGTGC   +14
Mus     TGCTGAATCAGCCCTCT-------GGCTTAGGGAGAGA--AGGTC-ACT-------TTATAAGGGTCTGGGGGGGTCAGTG--CCTGG------AGTTGCGC   +14
Hum     TGCTGATTCAGCCAGGA--------GCTTA-GGAGGGGG-AGGTC--ACT-------TTATAAGGGTCTGGGGGGG-TCAGAA--CCCAG------AGTCATCC   +16
Bov     TGCTGATTCAGCCGGGA-------GCTTAGGGAGGGG--AGGTC--ACT-------TCATAAGGGCCTGGGGGGGGAGTTGGAGCCACG------AGTCGTCC   +17

          NRE          Ret 4                              TATA box              +1


cRh-I   GGTCC----TGCCTCATTTCTCCACAGTCCTGCCGAGCCATCCAAACACTACCA---CAGAAAGGGGCTGAGACC--ACAACAAT-------CC---AA--CCGCAGCCATG   +97
cRh-II  GGTCC----TGCCTCGTTTCTCCACAGTCCTGCCGAGCCATCCAAACACTACTG---CAGAAAGGGGCTGATAC---ACAACA-T-------CC---AA--CCGCAGCCATG   +97
Xen     AGCTTCAGTTGGGATCACAGGCTTC--TAGGGATCCTTTGGGCAAAAAGAAACA--CAGAA-GGCATTCTTTCTATACAAGAAAGGGACTTTAT---AGAGCTGCTACCATG   +112
Chk     AGCAGAGGCTGGGCAGCG---GT-GCG--AGCCCAACTGCAGGGCGATTCCCCCATACAAAAACCGACGCTGAGTGCGACTGAGAGGC---GCCCCACAAACCGCAGCCATG   +102
Ham     TG------TGGGAGCTGTAGGTGGC-TGAGCTC--GCT-AGGCAGCCTCGGTCTCTATTGACGAACAGCCCCTGGGGC----------AGCCTCGAGGGCCACAGCCATG   +98
Rat     TG------TGGGAGCCGTAGGTAGC-TGAGCTC--GCC-AGGCAGCCTTGGTCTCTGTCTACGAACAGCCCGTGGGG----------AGCCTCAAGGGCCGCAGCCATG   +97
Mus     TG------TGGGAGCCGTCAGTGGC-TGAGCTC--GCC-AAGCAGCCTTGGTCTCTGTCTACGAAGAGCCCGTGGGGC---------AGCCTCGAGAGGCCGCAGCCATG   +98
Hum     AGC------T-GGAGCCCTGAGTGGC-TGAGCTCAGGCCTTCGCAGCAT---TCTTGGGTG-GGAGCAG-CCACGGGTC----------AGCCACAAGGGCCACAGCCATG   +98
Bov     AGCC-------GGAGCCCCGTGTGGC-TGAGCTCCGGCCTCAGAAGCAT---CCCCGGGTT-CGCCGCG-CCGGCGGGC----------AGCCGCAAGGGCCGCAGCCATG   +98
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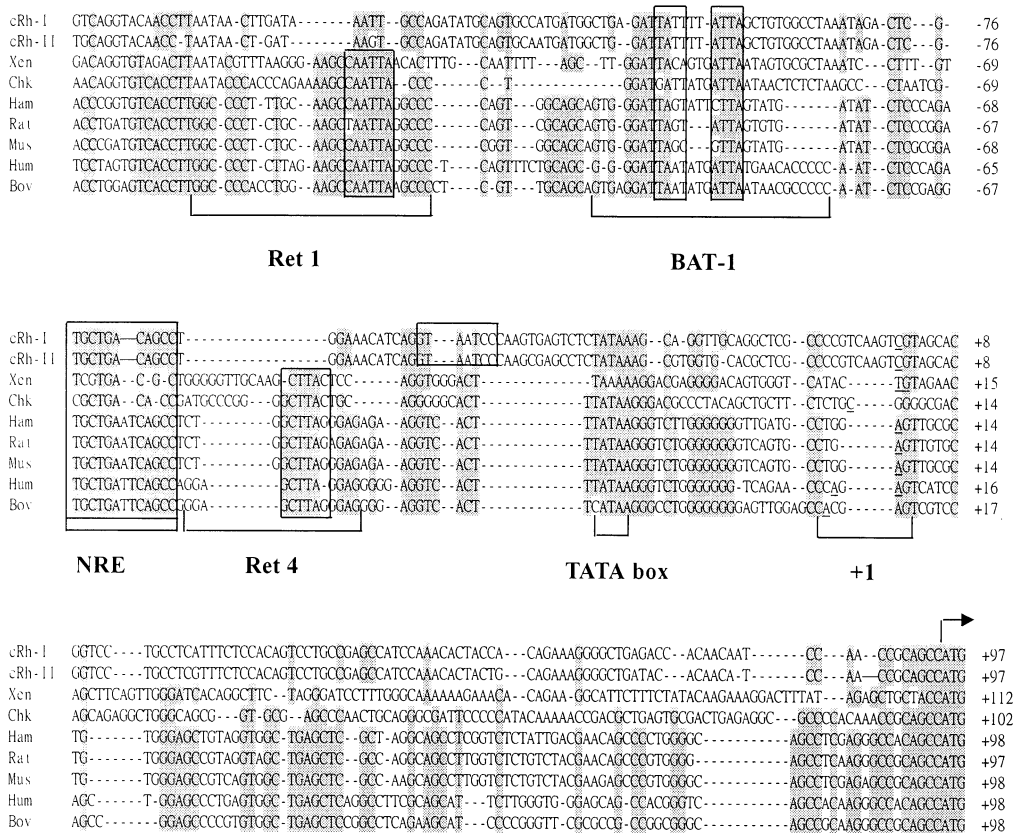
Fig. 2. Nucleotide identities among cRh and other known vertebrates rhodopsin upstream sequences. Sequence alignments of the ~290 immediate upstream nucleotides of cRh-I, cRh-II, Xenopus (Xen), chickens (Chk), Chinese hamsters (Ham), rats (Rat), mice (Mus), humans (Hum) and bovine (Bov) rhodopsin genes is shown. Numbers on the right side of each column represent the nucleotide position relative to transcription start site (+1), which is underlined. Gaps, indicated by broken lines, are introduced to maximize identities. Shaded areas represent sequences that are identical in all mammalian species; where the sequence of carp, Xenopus or chicken is identical, it is also shaded. Positions of the TATA box, Ret 4, NRE, BAT-1 and Ret 1 of mammals are indicated below the alignment. Boxed regions indicate consensus recognition sites of Crx, which binds to C/TTAATCC, Ret 4, BAT-1 and Ret 1 (Kumar et al., 1996), and Nrl, which binds to NRE (Chen et al., 1997; Rehemtulla et al., 1996). Arrow indicates the translation initiating codon.

In Fig. 2, the proximal upstream flanking sequences of cRh-I and cRh-II genes are compared with those of known vertebrates (Batni et al., 1996; Chen et al., 1996, 1997). Consensus recognition sites for Crx and Nrl are observed in the corresponding regions of cRh genes. Crx and Nrl are trans-factors that work synergically to bind to and transactivate the genes for several photoreceptor-specific proteins (Chen et al., 1997). Crx binds independently to three sites in the rhodopsin proximal promoter region of bovine, which are Ret 1, Ret 4 and BAT-1, and C/TTAATCC of other photoreceptor-specific genes (Chen et al., 1997); and Nrl binds to NRE (Kumar et al., 1996; Rehemtulla et al., 1996). In cRh genes, homologies of the binding core regions of BAT-1 and NRE were observed in corresponding sites. In addition, sequence sharing homology to C/TTAATCC was also found at nt −46 ~ −52 in cRh genes (Fig. 2). These findings suggest that Crx and Nrl may also regulate cRh genes.

Ret 1 core region (CTAATTAG) and its facilitated flanking sequences (GGCCCC) (Morabito et al., 1991; Yu et al., 1996) and Ret 4 (Chen and Zack, 1996) have been proven to play a critical role in driving photoreceptor-specific gene expression in rats. Nevertheless, no corresponding sequence was observed in either cRh-I or cRh-II gene. In addition, no significant matches were found in cRh genes with the known rhodopsin enhancer region, which is a highly conserved region located at 1.5 ~ 2 kb upstream of mammalian rhodopsin genes (Nie et al., 1996).

Except for the consensus recognition sites for Crx and Nrl, the upstream flanking regions of both types of *cRh* gene shared relatively low identity with those of other terrestrial vertebrates. This evidence implies the existence of other regulatory elements in *cRh* genes. It is speculated that those potential regulatory elements might locate within the well-conserved upstream regions between *cRh-I* and *cRh-II* genes. According to transgenic studies of rhodopsin upstream regulatory regions, the proximal upstream portion ($\sim$ 300 bp) is prerequisite and sufficient for photoreceptor-specific expression of the reporter gene. Meanwhile, sequences of more distal regions are responsible for accurate expression patterns and higher expression levels (Batni et al., 1996; Knox et al., 1998; Nie et al., 1996; Zack et al., 1991). Hence, it was postulated that the most proximally conserved region of *cRh* upstream sequence may play a prominent role in controlling the photoreceptor-specific expression of fish rhodopsin. And, the other two more distally conserved regions may regulate the spatial or temporal expression patterns or function as an enhancer.

3.4. Downstream flanking sequences analysis

The downstream untranscribed regions of *cRh-I* and *cRh-II* genes were sequenced from nt 1555 to 1915 and from nt 1653 to 1885, respectively. Sequence alignment of the downstream region and a portion of cDNA sequences between the two types of *cRh* gene revealed a highly conserved region with a 86.1% polynucleotide identity, which is located at nt 1466–1753 of *cRh-I* gene or nt 1476–1763 of *cRh-II* gene. Three of the four polyadenylation signals of *cRh-II* cDNA and one of the *cRh-I* cDNA were also indicated (Fig. 1B). Interestingly, corresponding sequences of the two excess polyadenylation signals of *cRh-II* cDNA were found in the downstream flanking region of *cRh-I* genomic DNA. In addition, the excess 88 bp of *cRh-II* cDNA shared 83% identity with a corresponding region of *cRh-I* gene. This finding suggests that the polyadenylation signals may be alternatively chosen while transcribing rhodopsin mRNA.

3.5. Type discrimination of cRh in genome

Two types of *cRh* gene were differentiated using *Tfi*I to restrict PCR products generated by type-specific primers (Fig. 3A). Type I specific primers generated a *cRh-I* PCR product of 272 bp. Notably, this fragment was not cut by *Tfi*I. However, a *cRh-II* PCR product of 281 bp generated by type II specific primers was cleaved by *Tfi*I into two fragments of 168 and 113 bp (Fig. 3B,C). Ninety carps from the local pond (Tzu-Pei) were examined for the distribution of two types of *cRh* gene. The results showed that only two fish (2.2% of total samples) exhibited merely *cRh-I* genotype, and 19 fish (21.1%) exhibited merely *cRh-II* genotype. However, most of them (76.7%) carried both *cRh-I* and *cRh-II* genotypes. Similar results were also observed with carps from a geographically different pond (Lu-Kang). Furthermore, the distribution of two types of *cRh* gene was irrelevant to the sex or size of fish. And there seemed to be no noticeable differences in terms of morphological appearance between carps showing only one *cRh* type and the ones showing both. Since not every carp carries both *cRh-I* and *cRh-II* genes, the two types of *cRh* gene are less likely to be two distinct genes but two rhodopsin alleles in the carp population.

The common carp is a phylogenetically tetraploid fish. In addition to the fact that there is only one rhodopsin gene in diploid vertebrates, the existence of two types of *cRh* gene may be attributed to tetraploidy, which is a similar event of the two *myc* genes of carp (Zhang et al., 1995). Subsequent to the original tetraploidization event, one of the two duplicate genes could have been silenced in their expression (Ferris and Whitt, 1977). In this study, RT-PCR was performed using retinal total RNA to determine whether or not one of the duplicated genes encoding rhodopsin has been silenced in a carp carrying both *cRh-I* and *cRh-II* genes. These results demonstrated that both *cRh-I* and *cRh-II* mRNAs were expressed at an approximately equal level in both eyes (Fig. 4). Related investigations have indicated that the tetraploid *X. laevis* has two types of rhodopsin corresponding to two morphologically distinct rod cells (Rohlich et al., 1989; Batni et al., 1996). In contrast, the rod cells of carp carrying both types of *cRh* gene were not morphologically differentiated into two types under light microscopic observation (data not shown).

A

| RHO1-f | 5'-CC**CC**GGG**G**AA**A**GAGC**A**ACC**A**-3' |
| RHO2-f | 5'-CC**A**GGG**A**AA**C**GAGC**G**ACC**G**-3' |

| RHO1-r | 5'-CATGCCTCTA**C**AATCACAACAA**A**-3' |
| RHO2-r | 5'-CATGCCTCTA**A**AATCACAACAA**C**-3' |

B

cRh-I

272 bp

1244   1515 (bp)

cRh-II

168 bp   113 bp

1244   *Tfi* I   1525 (bp)

C

*Tfi*I

I   II   I   II   M

(bp)
1353
1078
872
603
310
261

282
272
168
113

Fig. 3. Determination of two types of *cRh* gene via polymerase chain reaction (PCR) and restriction analysis. (A) Sequences of type-specific primers. Specific primers for *cRh-I* gene: forward RHO1-f and reverse RHO1-r; for *cRh-II* gene: forward RHO2-f and reverse RHO2-r. The different nucleotides between the two types of primers were underlined and in boldface. (B) Schematic of the expected molecular sizes of PCR products generated by type-specific primers. (C) Agarose gel electrophoresis of PCR products of *cRh-I* (I) and *cRh-II* (II) genes, and their *Tfi*I restriction patterns. Lane M: molecular weight marker, *Hae*III-digested φX174.

L   R

A   B   A   B

I   II   I   II   I   II   I   II   M

(bp)
1353
1078
872
603
310
261

Fig. 4. Transcriptional patterns of *cRh-I* and *cRh-II* genes of a carp carrying both types *cRh* gene. Retinal RNAs extracted from left (L) and right (R) eyes of a carp were subjected to RT-PCR in the presence (A) and absence (B) of reverse transcriptase using *cRh-I* (I) and *cRh-II* (II) specific primers. Lane M: molecular weight marker, *Hae*III-digested φX174.

4. Conclusions

(1.) Molecular structures of two types of carp rhodopsin gene, namely *cRh-I* and *cRh-II*, have been determined. Comparison of the upstream flanking sequences of the two types of *cRh* gene reveals three highly conserved regions, which are at nt -1262 to -614, -539 to -419 and -166 to $+97$, with 78.0, 79.3 and 92.3% identities, respectively.

(2.) The sequence of the proximal upstream flanking region of *cRh* genes shows homologies to consensus recognition sites for transcription factors, Crx and Nrl, which are involved in photoreceptor-specific expression.

(3.) Two types of *cRh* gene are clearly discriminated from each carp via PCR and restriction analysis. Most carps carry both types of *cRh* gene, however, there are still carps possessing either *cRh-I* or *cRh-II* genotype.

(4.) In a carp carrying both types of *cRh* gene, none of the genes is silenced but are expressed equally in both eyes.

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