Retina-specific *cis*-elements and binding nuclear proteins of carp rhodopsin gene

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Abstract The upstream *cis*-elements controlling the retina-specific expression of carp rhodopsin gene were fully characterized in vivo. Transgenic studies demonstrated that both carp neural retina leucine zipper response-like element (cNRE, within nucleotides (nt) −63 to −75) and carp-specific element (CSE, nt −46 to −52) were crucial to reporter gene expression in medaka retinae. The retina-specific expression rates of embryos injected with nt −1 to −641 and longer fragments were much higher than those of embryos injected with nt −1 to −138 and shorter fragments, indicating that an enhancer is located in the nt −138 to −641 region. Retinal extracts and the probe BAT-1 (nt −90 to −120) formed two DNA–protein complexes, B1 and B2. Retinal extracts and the probes cNRE and CSE formed the complexes N1 and C1, respectively. The protein factors in B1 and C1 were mammal-like cone-rod homeobox proteins.

Key words: Carp; Photoreceptor; Regulatory element; Transgenic fish; Rhodopsin

1. Introduction

Rhodopsin (Rh) genes have been identified in several species, such as bovines, humans, *Drosophila*, mice, chickens, rats, *Xenopus* [1] and carp [2,3]. Only one, relatively conserved region, from nucleotides −10 to −180 (nt −10/−180), was found in the upstream sequences of the Rh genes of mammals, *Xenopus*, chickens and carp. The Ret 1 element (nt −110/−136) binds to retinal nuclear proteins during development, and is able to drive gene expression in rod photoreceptors in rats [4,5]. The Ret 4 element (nt −40/−54) is bound by both retina-specific protein and ubiquitously expressed protein factors [6]. Cone-rod homeobox (Crx) protein transactivates photoreceptor cell-specific genes and binds independently to three sites, Ret 1, Ret 4 and BAT-1, in the Rh upstream region of bovines, and to C/TTAATCC of other photoreceptor-specific genes [7]. Neural retina leucine zipper protein (Nrl), a member of the bZIP family, serves as a *trans*-acting regulator of Rh gene expression, and Nrl response element (NRE) helps stimulate Rh promoter activity [8,9]. The nucleotide sequences and positions of the *cis*-elements binding with Crx and Nrl are conserved among mammals, chickens and *Xenopus* [3]. Crx and Nrl binding sites in the proximal promoter are the primary *cis*-acting sequences regulating photoreceptor-specific gene expression [10].

Although Rh genes have been studied extensively, extremely little is known about the *cis*-elements controlling retina-specific expression in fish photoreceptor genes. In the two types of carp Rh (cRh) genes [2], the upstream regions having the highest identity (92.3%) are located at the region of nt −1/−166. Regions of cRh homologous to the binding core sequences of mammalian BAT-1 and NRE were observed in corresponding sites, named carp BAT-1-like element (cBAT-1, nt −90/−120) and carp NRE-like element (cNRE, nt −63/−75), respectively [3]. cBAT-1 has two potential binding sites for Crx: one at nt −96/−102 (CTAATAA) and one at nt −104/−110 (ATAATCT) on the template strand. cNRE contains 11 bases (TGCTGACAGCC) that are identical to 11 of the 13 bases of mammalian NRE (TGCTGAA/TTCAGCC), but cNRE lacks the central AT/TT bases. In addition, in cRh genes, a sequence homologous to C/TTAATCC is found at nt −46/−52. It is named carp-specific element (CSE), because this motif is located on the sense strand, rather than the template strand of all known Rh genes. In this study, we identified the proximal elements required for retina-specific expression of the cRh gene. Furthermore, we characterized the *cis*-elements and their retinal nuclear proteins bound to these motifs.

2. Materials and methods

2.1. Plasmid construction

A 6 kb *Bss*HII-digested fragment containing the upstream region of the type I cRh gene was inserted into pEGFP-1 (Clontech), resulting in pDel-6k. The nt −1261/+94 region was amplified by PCR using forward primer, 5′-AGCCCCACTGACATTCTGA-3′, and reverse primer EPA (5′-GGCTGCGGTTGGATGTTGTG-3′). The resultant 1.2 kb fragment was subcloned into pEGFP-1 to produce pDel-1.2k. A 4.9 kb fragment was obtained when pDel-1.2k was cut with *Hin*dIII and *Xba*I. This fragment was used to produce pDel-641, which contained the nt −641/+94 region. The nt −138/+94 region was also amplified by PCR using forward primer, 5′-CTGATAAGTGCAGATATGC-3′, and reverse primer EPA. The resultant 240 bp PCR product was subcloned into pEGFP-1 to construct pDel-138. Forward primer, 5′-GTGCTGACAGCCTGGAAACA-3′, and reverse primer EPA-B (5′-ATTGGATCCGGCTGCGGTTGGATGT-3′, nt +79/+94 with an extra *Bam*HI site) were used to produce a 179 bp PCR product of nt −76/+94. EPA-B was used with forward primer, 5′-CATCAGGTAATCCCAAGTGAGTCT-3′ to generate a 161 bp

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product of nt −58/+94. Forward primer, 5′-GTGCTGACAGCCTG-GAAACATCAGCAAGTGAGTCTCTAT-3′ was used with EPA-B to generate a 172 bp PCR product of nt −76/+94 but without the CSE sequence. These PCR products were digested by *Bam*HI, and then inserted into pEGFP-1 to produce pDel-76, pDel-58 and pNCSE-76, respectively. To determine whether large DNA fragments affect the specific expression of transgenic green fluorescent protein (GFP), we made pDel-76-λh2. A 2 kb fragment, which was obtained from a *Hin*dIII-cut λ, and was unrelated to the regulatory element of the cRh gene, was added to the construct upstream of the −76 sequence. The polynucleotide sequences of all plasmids were confirmed, purified, linearized with *Xho*I and prepared for microinjection, except the pDel-641, which was used to inject directly after gel extraction. The 4.8 kb, pCMVm, containing GFP cDNA driven by human cytomegalovirus promoter, served as a negative control.

2.2. Gene transfer

Linearized plasmid was diluted to 10 ng/μl with phosphate-buffered saline (1.47 mM KH_2PO_4, 8.06 mM Na_2HPO_4, 137 mM NaCl, 2.68 mM KCl, pH 7.4) containing 0.1% phenol red. From 10 to 100 pl of DNA solution was microinjected into the cytoplasm of one-celled medaka (*Oryzias latipes*) embryos using the procedures described by Kinoshita and Ozato [11].

2.3. Fluorescence detection

Transgenic embryos developed from stages 35 to 39 [12] were examined under a fluorescence stereomicroscope (MZ FLIII, GFP-Plus filter set, Leica).

2.4. Immunohistochemical detection

Immunohistochemical detection in transgenic, adult medaka carrying the pDel-6k fragment was performed using standard procedures [13], except that the transgenic medaka was placed in a dark room for 2 h before the eyes were enucleated. Anti-GFP (Clontech) antibodies (1:200 dilution) and anti-rabbit IgG conjugated with alkaline phosphatase (1:2000 dilution) (Santa Cruz) were used. Positive signals were visualized using nitroblue tetrazolium and 5-bromo-1-chloro-3-indolylphosphate.

2.5. Nuclear extract preparations

Retinal, brain and spleen tissues were obtained from 30 adult carp. Nuclear extracts were prepared using the methods of Gorski et al. [14], except that 1% low-fat milk was added to the homogenization buffer.

2.6. Probe preparation

Several oligonucleotides, including mutated sequences, were designed based on cBAT-1, cNRE and CSE (Table 1). Each double-stranded DNA oligonucleotide was labeled with [α-^{32}P]dATP and [α-^{32}P]dCTP.

2.7. Electrophoretic mobility shift assay (EMSA) and supershift assay

EMSA was performed with radiolabeled oligonucleotides and carp nuclear extracts. The binding reaction was carried out in 20 μl with 0.8–12.8 μg of nuclear extracts, 0.8 ng (about 10^5 cpm) of DNA probe, 5 μg of poly(dI-dC) in a buffer containing 10 mM Tris–HCl (pH 7.5), 50 mM NaCl and 0.5 mM dithiothreitol. The reaction mixture was incubated for 60 min at 25°C and run on a 5% non-denaturing polyacrylamide gel in 0.5× TBE buffer at 4°C, 180 V for 3.5 h. For competition experiments, cold competitor oligomers were synthesized and added at graded concentrations to the reaction mixtures.

Anti-Crx antibodies were raised in a rabbit against a peptide corresponding to the Crx residue from 261 to 274, a region that is identical in all mammals. Anti-Nrl antibodies [8] were gifts from Anand Swaroop. After 30 min of EMSA reaction at 25°C, the antiserum was added for the 60 min binding reaction.

3. Results

3.1. Transgene expression

Non-specific GFP expression occurred in pCMVm-injected embryos (negative control). During stages 17–19 (the second day after microinjection), a very weak and sporadic pattern of early GFP expression was observed in the yolk sac and other somatic tissues in less than 10% of the surviving pDel-6k-, pDel-1.2k-, pDel-641- and pDel-138-injected embryos. About 30% of the surviving pDel-76- and pDel-58-injected embryos also exhibited early GFP expression. Early GFP expression decreased gradually as the embryos developed.

Table 1
Oligomer and mutated oligomer probes used in EMSA

Elements	Mutated Oligomers Symbol	Sequences, Location and Mutation Sites of Synthetic Primers
cBAT-1		-120 ATGATGGCTGAGATTATTTTATTAGCT ACCGACTCTAATAAAATAATCGACACC −90
	MBA	-120 ATGATGGCTGCTCGGCTTTTATTAGCT ACCGACGAGCCGAAAATAATCGACACC -90
	MBB	-120 ATGATGGCTGAGATTATTGGCGGCGCT ACCGACTCTAATAACCGCCGCGACACC -90
	MBAB	-120 ATGATGGCTGCTCGGCTTGGCGGCGCT ACCGACGAGCCGAACCGCCGCGACACC -90
cNRE		-83 TAGACTCGTGCTGA CAGCCTGGAA TGAGCACGACT GTCGGACCTTTGT −57
	MNA	-83 TAGACTCGGTAGTC CAGCCTGGAA TGAGCCATCAG GTCGGACCTTTGT −57
	MNB	-83 TAGACTCGTGCTGA ACTAAGGGAA TGAGCACGACT TGATTCCCTTTGT −57
	MNAB	-83 TAGACTCGGTAGTC ACTAAGGGAA TGAGCCATCAG TGATTCCCTTTGT −57
CSE		-58 CATCAGGTAATCCCAAGTGAGTCT GTCCATTAGGGTTCACTCAGAGAT −32
	MC	-58 CATCAGACGGCTTCAAGTGAGTCT GTCTGCCGAAGTTCACTCAGAGAT −32
non 30 (non-specific)		CACGTCACGAGCTATCGGTGATCATCTCTG GTGCAGTGCTCGATAGCCACTAGTAGAGAC

cBAT 1, cNRE and CSE are three putative *cis*-acting elements of the cRh gene. Numbers indicate the nucleotide position in the upstream region. Sequences in open boxes are designed for mutation. Shaded boxes contain the mutated sequences.

After stage 35, GFP was expressed specifically and exclusively in one or both eyes of embryos carrying transgenes pDel-6k, pDel-1.2k, pDel-641, pDel-138 and pDel-76, (Fig. 1). Embryos carrying transgenes pDel-58 and pCMVm did not exhibit this type of GFP expression. Transgenic GFP expression continued without degradation after the fish hatched. Retina-specific GFP expression occurred in about 50% or more of the surviving transgenic embryos injected with pDel-6k, pDel-1.2k, and pDel-641, but in less than 5% of the surviving embryos injected with pDel-138 and pDel-76 (Fig. 2).

All embryos injected with DNA fragments that included the −76 bp upstream sequence expressed GFP specifically in the retinae, but the shape and intensity of the green fluorescent signals differed. In pDel-6k-, pDel-1.2k-, and pDel-641-injected embryos, signals in the eye were usually prominent and broad. In contrast, in embryos injected with pDel-138 and pDel-76, the signals were faint and spotty and GFP expression was occasionally ectopic. The rate of early sporadic GFP expression was much lower in embryos injected with upstream fragments of 138 bp (nt −1/−138) and longer.

To prove that the differences in retina-specific expression rates for pDel-76- and pDel-1.2k-injected embryos were not due to differences in the molecular size of the injected DNA, pDel-76-λh2 was also injected. The specific-expression rate of

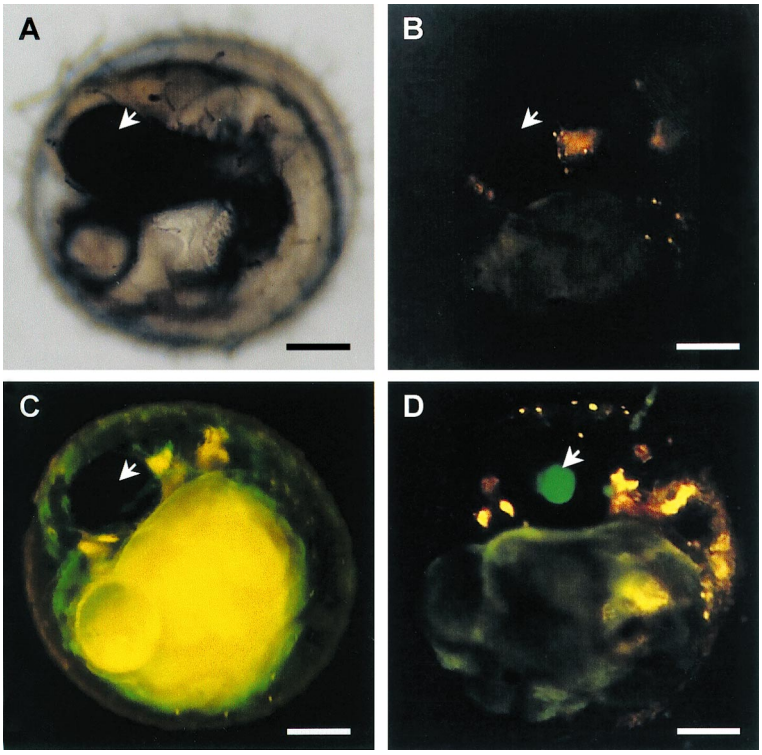

Fig. 1. GFP expression in transgenic medaka embryos. A: Bright field; B–D: fluorescent light. A,B: Untreated control embryos. The yellow and light green colors resulted from endogenous fluorescence. C: pCMVm-injected embryos. Green fluorescent signals appeared in the trunk and yolk sac. The bright yellow color in the yolk sac resulted from overexposure to GFP fluorescence. D: pDel-6k-injected embryos. GFP was expressed specifically and exclusively in the eyes. Arrows indicate the location of the eyes in stage 38 embryos.

pDel-76-λh2-injected embryos was about 5%, quite close to that of pDel-76-injected embryos (Fig. 2).

The CSE sequence was removed from the pDel-76 construct and the remaining DNA fragment (pNCSE-76) was transferred into fertilized eggs. No GFP expression occurred in the transgenic embryos (Fig. 2).

3.2. Location of transgenic GFP expression in the eye

Immunohistochemical analysis using GFP antibodies showed that the GFP-expressing cells in the retinae of medaka carrying pDel-6k were photoreceptors. The positive signals were strongest in the ellipsoids and nuclei of photoreceptor cells. Unexpectedly, GFP expression was also detected in cone cells (Fig. 3).

3.3. EMSA with three putative cis-regulatory elements

To define the DNA regulatory elements and protein factors involved in regulating cRh gene expression, EMSA was conducted with radiolabeled oligonucleotide probes of the three putative *cis*-elements (Table 1). Using carp retinal extracts, DNA–protein complexes B1 and B2 were formed with the cBAT-1 probe, N1 and N2 were formed with the cNRE probe, and C1 was formed with the CSE probe (Fig. 4). Comparing the DNA–protein binding patterns of the three putative elements with nuclear extracts from retina, brain and spleen, complexes B1 and C1 were exclusively observed with retinal extracts, while N1, N2 and B2 were observed with retinal and brain nuclear extracts (Fig. 5).

Except for N2, which was inhibited by both specific (cNRE) and non-specific (non30) competitors, the DNA–protein complexes were formed from specific *cis*-elements and extracts (Fig. 6). Using cold CSE and cBAT-1 oligomers to compete with radiolabeled CSE oligomer, both CSE and cBAT-1 competitors inhibited complex C1 (Fig. 6, right panel), suggesting that CSE and cBAT-1 oligomers are involved in the same DNA–protein complex formation. In addition, because CSE and cBAT-1 oligomers both exhibited putative Crx binding sites, it is highly likely that CSE and cBAT-1 bind the same nuclear protein and, probably, Crx.

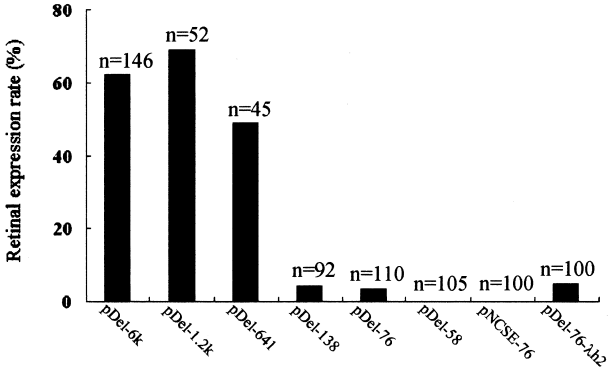

Fig. 2. Retinal expression rates in transgenic embryos. Embryos were microinjected and then examined from stages 35–39. The name of each construct includes the upstream position of the promoter. pNCSE-76 is the same as pDel-76 but lacks the CSE sequence; pDel-76-λh2 is also the same as pDel-76 but contains an additional 2 kb fragment from λ phage DNA cut with *Hin*dIII. The retinal expression rate is the percentage of all surviving, injected embryos that expressed GFP in their eyes. The number of embryos examined is indicated above the bar.

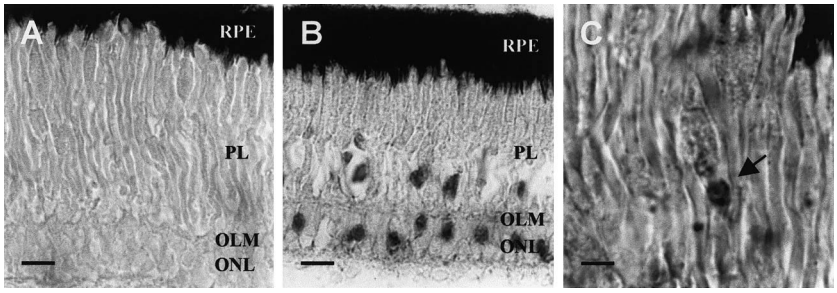

Fig. 3. Immunohistochemical detection of transgenic GFP genes in medaka embryos using anti-GFP polyclonal antiserum. A: Non-transgenic medaka. B: Retina-specific GFP expression in transgenic medaka carrying pDel-6k. GFP positive signals were detected in photoreceptors. C: Close up of (B). The arrow indicates the GFP signals in cone cells. Scale bars: A,B: 10 μm; C: 4 μm. RPE, retinal pigment epithelium; PL, photoreceptor layer; OLM, outer limiting membrane; ONL, outer nuclear layer (scale bars represent 250 μm).

3.4. EMSA with cis-elements containing mutated sequences

To determine whether retinal nuclear extracts bind to oligonucleotide probes at the predicted recognition sites, EMSA was conducted with mutated sequences of the cBAT-1, cNRE and CSE elements (Table 1). Formation of DNA–protein complexes B1 and B2 was greatly reduced when retinal extracts were incubated with probes having a mutation at binding site A of the cBAT-1 element (MBA) (Fig. 7A, left panel). Probes having a mutation at binding site B (MBB) slightly reduced formation of B1 and greatly reduced formation of B2. Unexpectedly, an extra band of higher molecular mass was also observed. It is possible that a SP-1 site was introduced to site B during mutagenesis. Probes with mutations at sites A and B (MBAB) prevented formation of DNA–protein complexes B1 and B2 (Fig. 7A, left panel). In addition, the mutated oligomer was studied in a cold competition EMSA with radiolabeled cBAT-1 oligomer (Fig. 7B, left panel). Like cold cBAT-1 oligomer, cold MBB oligomer reduced the radiolabeled signal that formed by extracts and the cBAT-1 oligomer probe. However, unlike cold MBB and cBAT-1 oligomers, MBA oligomer only slightly decreased the radiolabeled signal formed by B1 and B2. In addition, MBAB oligomer did not significantly decrease the B1 and B2 signals (Fig. 7B).

A similar strategy was used to analyze the DNA recognition site of complex N1. Oligomers with a mutation at binding site A (MNA) of the cNRE element did not reduce the signal of complex N1. However, oligomers with a mutation at binding site B (MNB) of the cNRE sequence reduced the N1 signal significantly. Mutations at both sites A and B of the cNRE oligomer (MNAB) prevented formation of the N1 signal (Fig. 7A, middle panel).

For the CSE sequence, the putative Crx recognition site was mutated to form oligomer MC. The signal formed by complex C1 was greatly reduced by the MC oligomer (Fig. 7A, right panel) and in a competition EMSA with a radiolabeled CSE probe binding was inhibited only slightly (Fig. 7B, right panel).

3.5. Carp DNA–protein complexes detected by supershift assay

Anti-Crx antibodies and the cBAT-1 and CSE probes formed supershifted bands whose intensity increased with the amount of antibodies (Fig. 8). This demonstrated that carp retinal nuclear protein, which bound to the cBAT-1 and CSE sequences, was recognized by anti-Crx antibodies. However, the DNA–protein complex N1, formed with the cNRE probe, did not exhibit a mobility shift in a supershift assay with anti-Nrl antibodies (Fig. 8).

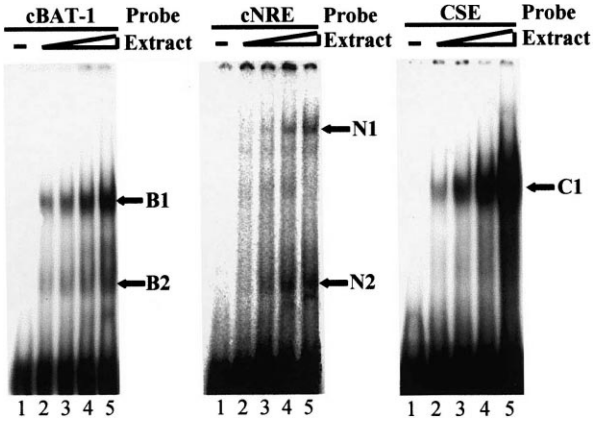

Fig. 4. EMSA of retinal nuclear extracts and three upstream *cis*-elements of the cRh gene. Each element is indicated along the top. The amounts of retinal nuclear extracts reacted with each probe were: cBAT-1 probe: 0, 0.8, 1.6, 3.2 and 6.4 μg (lanes 1–5); cNRE probe: 0, 0.9, 1.8, 3.6 and 7.2 μg (lanes 1–5); and CSE probe: 0, 0.4, 0.8, 1.6 and 3.2 μg (lanes 1–5). Arrows indicate the DNA–protein complexes formed by oligomer probes and retinal nuclear extracts.

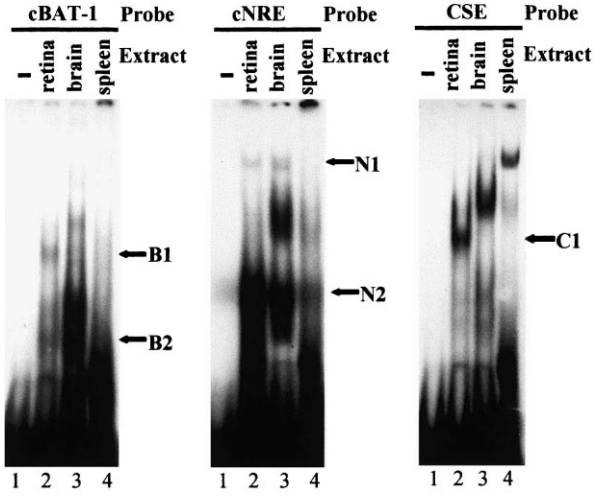

Fig. 5. EMSA with nuclear extracts isolated from different tissues. The amounts of retinal, brain and spleen nuclear extracts reacted with each probe were: cBAT-1 and CSE: 0.8, 1.6 and 1.6 μg, respectively; cNRE: 7.2 μg of each. Arrows indicate the DNA–protein complexes formed by oligomer probes and nuclear extracts.

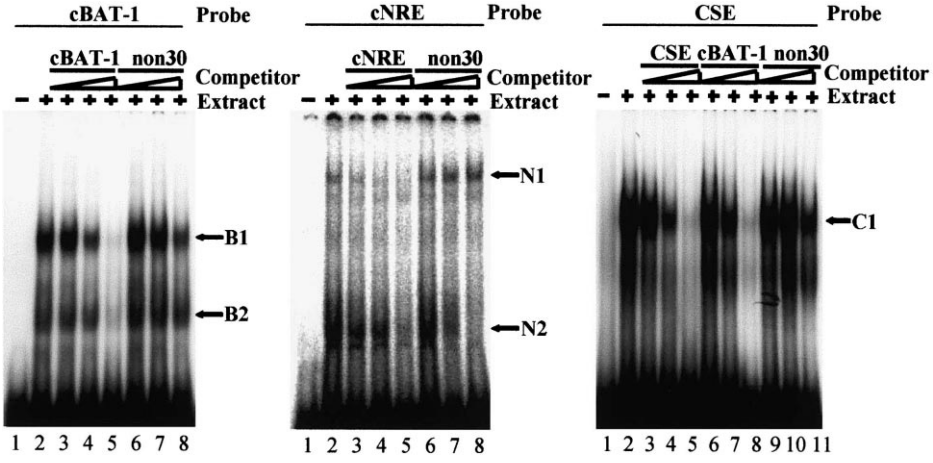

Fig. 6. Competitive inhibition analysis by EMSA. The cold competitors used in each reaction with the cBAT-1, cNRE and CSE oligomers are indicated. The molar ratios of cold competitor to labeled probe were 1:1, 10:1, and 100:1 for the cBAT-1 and CSE motifs. For the cNRE motif, the molar ratios for each reaction were 2:1, 20:1, and 200:1. non30 was a non-specific oligomer. B1, B2, N1, N2 and C1 are the compounds formed by oligomer and nuclear extracts isolated from carp retinae.

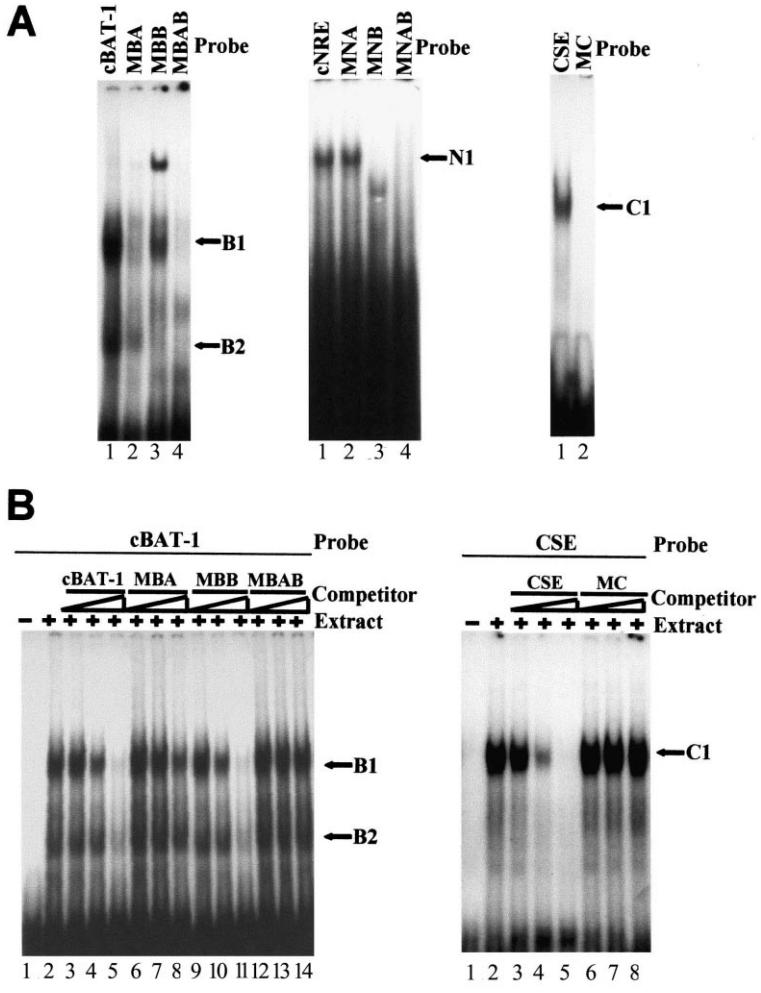

Fig. 7. EMSA with original and mutated cBAT-1, cNRE and CSE probes. A: ^{32}P-labeled probes with a variety of nucleotide mutations (see Table 1) of cBAT-1 (MBA, MBB and MBAB), cNRE (MNA, MNB and MNAB) and CSE (MC) reacted with carp retinal nuclear extracts. B: Competitive inhibition analysis with mutated cBAT-1 and CSE probes. Competitors used in the cBAT-1 and CSE reactions are indicated. The molar ratios of competitor to labeled probe for each reaction with cBAT-1 and CSE were 1:1, 10:1, and 100:1. Arrows indicate the DNA–protein complexes formed in EMSA.

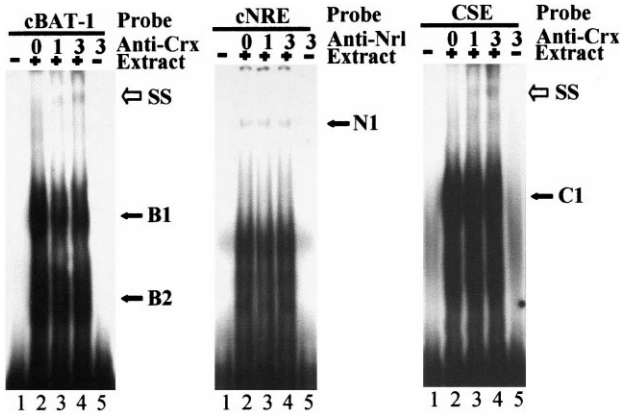

Fig. 8. Anti-Crx and anti-Nrl supershift EMSA with carp retinal nuclear extracts. ^{32}P-labeled probes of cBAT-1, cNRE and CSE, and different amounts (0, 1 and 3 μl) of anti-Crx (0.5 μg/μl) and anti-Nrl antibodies (10 μg/μl) were added, as indicated, in the presence (+) or absence (−) of retinal nuclear extracts. Boxed arrows indicate the supershifted bands (SS).

4. Discussion

The Rh genes of bony fish lack introns [15], making them ideal for studying the upstream regulatory regions that control tissue-specific expression of photoreceptor genes. The cRh gene is excellent for studying gene regulation because the common carp is the only bony fish in which the upstream regions of two types of cRh gene can be compared [2,3]. To determine the minimal regulatory elements controlling the specificity of the cRh gene, we generated transgenic fish with a variety of deletions in the upstream region of the cRh gene fused with GFP gene. Transferred upstream DNA fragments of −6 kb, −1.2 kb, −641 bp, −138 bp and −76 bp were also able to direct retina-specific expression of transgenic GFP. However, with the −58 bp upstream fragment, retina-specific expression of GFP on transgenic medaka was totally lost. Therefore, the upstream nt −1/−76 region contains the regulatory sequence that controls the retina-specific expression of the cRh gene. To date, this is the shortest, known regulatory sequence with this function. The regulatory sequences for bovine Rh, nt −222/+70 [16], mouse Rh, 500 bp of 5′ flanking sequence [17], and *Xenopus* Rh, nt −508/+41 [18], are considerably longer.

The upstream −1/−76 region encompasses two retinal nuclear protein binding sites, the cNRE and CSE, which are bound by unknown and mammal-like Crx nuclear proteins, respectively. However, neither the cNRE alone (pNCSE-76) nor the CSE alone (pDel-58) could control the retina-specific expression of the transgenic reporter gene. The cNRE and CSE should co-exist to drive the retina-specific expression of the cRh gene. Thus, Crx contributes significantly to the activity of the cRh promoter. Our results concur with those from Crx gene knockout mice, which demonstrated that Crx is necessary for the expression of some photoreceptor genes in mammals [19]. Our results also support those from in vitro and in vivo studies demonstrating that bovine Nrl and Crx work synergistically to control tissue-specific expression [7,20,21]. Thus, the nuclear protein in DNA–protein complex N1 and the carp Crx protein might act synergistically to elevate GFP expression to the detectable level. The nuclear protein which binds cNRE might function as a mammalian Nrl homolog. Finally, the mechanism for regulating Rh gene expression might be conserved in mammals and in fish.

The retina-specific expression rates for embryos injected with linearized pDel-6k, pDel-1.2k, and pDel-641 were significantly higher than for embryos injected with linearized pDel-138 and pDel-76. These differences did not result from differences in the molecular size of the injected DNA fragments. Signal appearance and specificity rates for pDel-76-λh2-injected embryos were the same as that for pDel-76-injected embryos. Therefore, it is highly likely that a *cis*-element within −138/−641 enhances cRh specificity. The −6 kb upstream region of the cRh gene directs retina-specific GFP expression.

Immunohistochemical analysis using GFP antibodies showed that GFP-expression signals occurred mainly in photoreceptor cell bodies in the outer nuclear layer. However, GFP-positive signals were also detected in cones. Thus, it would be worthwhile to study the regulatory element that controls rod-cell-specificity of the cRh gene.

cBAT-1 and CSE both exhibited putative Crx binding sequences. The competition EMSA also demonstrated that both CSE and cBAT-1 competitors reduced the radiolabeled signal formed by complex C1. In addition, anti-Crx antibodies and the cBAT-1 and CSE probes formed supershifted bands, though the signals were faint. Taken together with the transgenic evidences, we strongly suggest that cBAT-1 and CSE oligomers bind with the same nuclear protein, Crx, and we think cBAT-1 functions like mammalian BAT-1. The cRh gene lacks a DNA element homologous to mammalian Ret 4 [6]. However, CSE is located between the TATA box and cNRE, and it binds with Crx, suggesting that CSE functions like mammalian Ret 4. Thus, carp have a Crx transcription factor whose binding sites are conserved among mammals, chicks, *Xenopus* and cRh genes.

The protected sequence of BAT-1 is a P3-type homeodomain consensus site [22] containing two potential binding sites with dyad symmetry. We designated the more 5′-site of the mutated oligomer, A, and the more 3′-site, B. Competitive inhibition EMSA using mutated oligonucleotides showed that cBAT-1 element sequences A and B both contribute to the formation of B1 and B2. However, the cBAT-1 binding site A sequence may be more important to the formation of complex B1 than the binding site B sequence. The CSE probe results showed that the mutated sequence contributed to the formation of complex C1. Thus, binding site A of cBAT-1 and the binding site of CSE are bound specifically by B1 and C1, the Crx protein. Interestingly, we found that a second nuclear protein, which forms complex B2, also binds cBAT-1; this has not been reported previously. For the cNRE element, the B site may play an important role in binding of the nuclear extract to form N1.

In the analysis of the cNRE sequence, we found that 11 cNRE bases were identical to 11 out of the 13 bases of mammal NRE. However, cNRE, like that from chicks and *Xenopus*, lacked the central AT/TT bases. Although cNRE probes were bound to concentrated carp retinal nuclear extracts at the predicted recognition site, the intensity of the shifted band was relatively faint compared with those generated by cBAT-1 and CSE probes. Supershift EMSA with anti-Nrl antibodies demonstrated the absence of mammal-like Nrl in carp retinae.

Furthermore, immunohistochemistry using anti-Nrl antibodies failed to detect positive signals in either carp or me-

daka retinal tissue via immunohistochemistry using anti-Nrl antibodies (data not shown). Thus, carp nuclear protein, which binds to the cNRE recognition site to regulate cRh gene expression, may differ antigenically from mammalian Nrl.

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