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Mouse cone arrestin gene characterization: promoter targets expression to cone photoreceptors

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Abstract Cone arrestin (CAR) is a novel member of the arrestin superfamily expressed in retinal cone photoreceptors and the pineal gland. To understand the regulatory mechanisms controlling its cone- and pineal-specific expression, and to facilitate further functional studies using gene knockout approaches, we characterized the genomic organization and the 5′-flanking region of the mouse CAR (mCAR) gene. The mCAR gene is comprised of 17 exons and 16 introns, encoding five alternatively spliced transcripts. A 215-bp proximal promoter fragment containing a TATA box, an Sp1 site and four cone-rod homeobox-binding sites is sufficient to direct expression in cultured retinoblastoma cells and in cone photoreceptors and the pineal gland in transgenic *Xenopus laevis*. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Cone arrestin; Gene structure; Alternative splicing; Promoter activity; Transgenic *Xenopus laevis*

1. Introduction

Arrestins comprise a superfamily of regulatory proteins that bind to activated, phosphorylated G-protein-coupled receptors (GPCRs), leading to uncoupling from G-proteins in a process termed desensitization [1,2]. The arrestin superfamily includes visual arrestins, β-arrestins and insect chemosensory arrestins. Two members of the visual arrestins have been identified in vertebrate photoreceptors: rod arrestin (also known as S-antigen or arrestin 1) and cone arrestin (CAR, X-arrestin or arrestin 4).

Rod arrestin was the first member in the arrestin superfamily to be molecularly characterized [3], and its function has been extensively studied in recent years. In vertebrate rod photoreceptors, rod arrestin quenches the light-induced phototransduction cascade by binding to light-activated, phosphorylated rhodopsin [4,5]. Recently, arrestin has been found to participate in a molecular pathway for light-induced photoreceptor apoptosis in *Drosophila* through the formation of stable rhodopsin–arrestin complexes that are recruited to the cytoplasmic compartment through clathrin-dependent endocytosis [6–8].

Unlike visual arrestins, β-arrestin 1 and β-arrestin 2 have a ubiquitous expression pattern [9,10]. Although β-arrestins have a similar function to rod arrestin in the termination of GPCR signaling [9,10], recent evidence indicates that they have additional roles in signaling to downstream effectors [11,12] because of their ability to act as adaptors to facilitate clathrin-mediated endocytosis [13,14]. β-Arrestins also recruit activated tyrosine kinase c-Src into complexes with the β2 adrenergic receptor, which is involved in the activation of extracellular signal-regulated kinases (ERK1 and ERK2), one of three groups of mitogen-activated protein kinases (MAPKs) [15–17]. Recently, β-arrestin 2 has been identified as a binding partner of c-Jun amino-terminal kinase 3 (JNK3) and was suggested to act as a receptor-regulated MAPK scaffold for the activation of JNK3 [18].

CAR was first cloned from a rat pineal library and was discovered to be highly expressed in retina and pineal gland [19]. Based on this observation, a human retina cDNA (hCAR) was identified, its mRNA was localized in cone photoreceptors and a subset of pinealocytes, and the gene was mapped to the X chromosome [19,20]. Subsequently, numerous CAR orthologs have been cloned from other vertebrates, including clawed frog [21], killifish [22], bullfrog [23], leopard frog [23], bovine [24] and salamander [25]. Its high sequence homology to other arrestins and its cone photoreceptor localization suggest that CAR may play as important a role in the modulation of phototransduction in cones as rod arrestin does in rods; however, its actual function is still unknown [19,21,26].

To facilitate the functional studies of CAR using transgenic and gene knockout technologies, and to delineate the regulatory mechanisms controlling cone photoreceptor- and pineal-specific expression, we isolated and characterized the mouse CAR (mCAR) cDNA and gene, as well as its 5′ regulatory region. We show that a 215-bp fragment containing the proximal promoter drives reporter expression to cone photoreceptors and the pineal gland in transgenic *Xenopus*.

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Abbreviations: GPCR, G-protein-coupled receptor; CAR, cone arrestin; mCAR, mouse cone arrestin; CRX, cone-rod homeobox; ERK1 and 2, extracellular signal-regulated kinases 1 and 2; MAPKs, mitogen-activated protein kinases; JNK3, c-Jun amino-terminal kinase 3; EGFP, enhanced green fluorescent protein; RT-PCR, reverse transcriptase-polymerase chain reaction; RACE, rapid amplification of cDNA ends; MTS, minor transcription start site

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2. Materials and methods

2.1. cDNA library screen

An mCAR cDNA fragment of 550 bp was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) from mouse retinal total RNA using the published protocol [27] with the following primers designed based on the human CAR (hCAR) cDNA sequence (GenBank accession number U03626) [19]: +hCAR (171–194): 5′-TCGCTATGGCCGTGATGACTTGGA-3′, −hCAR (743–720): 5′-TTGGTGTACTTGTCTAGTGAATAC-3′.

The fragment was labeled with [α-^{32}P]dCTP and was used as a probe to screen a λZAP mouse eye cDNA library (gift from Hans-Jurgen Fulle, USC and David Garber, UT Southwestern Med. Ctr.) as previously described [28]. After three sequential rounds of plaque screening, the final isolates were excised and the isolated cDNAs were sequenced on both strands by automatic sequence analysis using the ABI Genetic Analyzer Model 310 (Perkin Elmer, Foster City, CA, USA) [29].

2.2. Determination of intron/exon junctions of the mCAR gene

A mouse ES 129/Svj BAC genomic library was screened with an mCAR cDNA probe by Genome Systems, Inc., according to their standard protocol. Southern blot analyses with a 5′ probe (the short promoter fragment p-215) and a 3′ probe (the cDNA fragment from the anchor to the stop codon) were used to map the positive clones. One clone (mCAR BAC16505) was identified to contain both the 5′-flanking region and the 3′ end of the cDNA and was used as a template in the PCR reactions to amplify overlapping genomic fragments for sequence analysis. Primers were designed according to the mCAR cDNA sequence to amplify overlapping genomic fragments with the high fidelity Turbo DNA polymerase (Stratagene). The amplified fragments were cloned into the pCR-Blunt II-TOPO vector (Invitrogen) and sequenced on both strands. Comparison of the genomic sequence and the cDNA sequence was used to confirm the identity of the clones and to ascertain intron/exon boundaries.

2.3. RT-PCR

To isolate the full-length mCAR cDNA without unspliced introns, a primer was designed at the predicted translation start codon ATG of the cDNA clones (5′-**ATG**/TCC/ACA/GTG/TTT/AAG/AAG-3′) and was paired with an antisense primer from the 3′ non-coding region of the rat pineal CAR (rpCAR) sequence (5′-GCT/TCC/ACA/CAG/AAT/TAT/CAG/AGA-3′) [19] for RT-PCR. RT-PCR was performed with 1 μg of mouse retinal total RNA as previously described [27]. The RT-PCR products were cloned into the PCR 2.1 vector (Invitrogen, Carlsbad, CA, USA) and sequenced on both strands.

2.4. 3′-Rapid amplification of cDNA ends (3′-RACE)

3′-RACE was performed using a 3′-RACE kit (Life Technologies, Gaithersburg, MD, USA) following the manufacturer's instructions. Mouse retinal cDNA was synthesized from 1 μg of total RNA using the adapter primer provided in the kit. The abridged universal amplification primer (AUAP) from the kit was paired with the arrestin anchor primer, +rpARR (878–898) (5′- CTC/AAG/CAT/GAA/GAC/ACA/AAT-3′) (amino acid: LKHEDTN) [19] to amplify a fragment that contained sequences from the conserved arrestin signature domain [19] to the poly(A) tail. The RACE products were cloned into the PCR2.1 vector (Invitrogen) and sequenced on both strands.

2.5. Isolation of the 5′-flanking region of the mCAR gene

To clone the promoter region of the mCAR gene, the Mouse GenomeWalker kit (Clontech, Palo Alto, CA, USA) was used according to the manufacturer's instructions. An mCAR gene-specific primer 1 (5′-AAAGGAGGTGGACTTAGTATGGGC-3′), which was derived from the first intron of the gene located within the 5′ untranslated region of the mCAR cDNA, was used in primary PCR using the five different mouse genomic libraries supplied by the kit as templates. Undiluted primary PCR products (1 μl) were used as templates for secondary PCR using an mCAR gene-specific primer 2 (5′-GAGGGAAGAGATGAAGCTCACATCAG-3′), derived from the 5′ untranslated region upstream of the first intron. The secondary PCR products were cloned into the pGlow-TOPO TA cloning vector (Invitrogen) and sequenced on both strands. The sequences were analyzed using on-line Promoter Scan II (Prestridge, 1995), Signal Scan (Prestridge, 1991), and TFSEARCH programs.

2.6. 5′-RACE

The transcription start sites of the mCAR gene were identified by 5′-RACE using the SMART RACE cDNA Amplification kit (Clontech) according to the manufacturer's instructions. Briefly, first-strand cDNA was synthesized from mouse retina and cerebellum poly(A)$^+$ RNA. An mCAR gene-specific primer (−mCAR (462–442), 5′-TTC-CTGAATCTTCAGGCCCAG-3′), derived from the mCAR cDNA sequence, and the universal primer mix from the kit were used in primary PCR using 2.5 μl of diluted cDNA as template. Gel analysis of 10 μl of primary PCR products revealed a smear. A secondary PCR was performed using a nested mCAR gene-specific primer (−mCAR (391–367), 5′-GGATAGGCATTGACCCCGAGCTTG-3′), and the nested universal primer from the kit with 2.5 μl of 1:50 diluted primary PCR products as template. The PCR products were cloned into the TOPO TA cloning vector (Invitrogen) and sequenced on both strands.

2.7. Primer extension

Primer extension reactions were carried out using the primer extension system (Promega, Madison, WI, USA) with 10 μg of total RNA and 0.1 pmol of γ-^{32}P end-labeled primer (−mCAR (36–15), 5′-CCC-ATTGGAGCTAGTCTTCTTA-3′) that is complementary to the sequence of nucleotides 15–36 relative to the translation start site, following the manufacturer's instructions. Half of the extended products were resolved on 8% denaturing polyacrylamide gels and exposed to a Phosphorimager screen (Molecular Dynamics).

2.8. Analysis of mCAR promoter–reporter constructs in cell culture

The p-215, p-523 and p-1003 mCAR 5′-flanking fragments in the pGlow-TOPO vector in the sense orientation were digested with *Spe*I and *Pst*I, subcloned into the pRL-null vector through its *Spe*I and *Pst*I sites, and analyzed for promoter activity.

Cell culture and transient transfection of Weri-Rb-1 retinoblastoma and COS-7 cells were performed as described previously [30]. Weri-Rb-1 and COS-7 cells grown in six-well plates were transiently transfected with 1.6 μg of mCAR promoter–luciferase reporter construct or the promoterless pRL-null vector, and 0.4 μg of the pGL3-C plasmid as an internal control of the transfection efficiency. For cone-rod homeobox (CRX) co-transfection, 1 μg of the CRX mammalian expression construct in the pcDNA3 vector was used with 0.8 μg of mCAR promoter–luciferase reporter construct and 0.2 μg of the pGL3-C plasmid. After 44 h, cells were harvested and assayed for luciferase activity [30]. *Renilla* luciferase activity was normalized to the firefly luciferase activity of the same sample and expressed as fold of the promoterless control.

2.9. Production of transgenic Xenopus laevis

The p-215, p-523 and p-1003 mCAR promoter fragments in the pRL-null vector were digested with *Hin*dIII and *Pst*I and subcloned into the pEGFP1 reporter vector (Clontech) through its *Hin*dIII and *Pst*I sites. Transgenic *X. laevis* embryos were produced as described [31]. The numbers of green fluorescent protein (GFP)-positive embryos out of the total that survived beyond 2 weeks were 19 of 40 for the p-1003 construct, eight of 21 for the p-523 construct and 11 of 28 for the p-215 construct. Tadpoles were photographed on a dissecting microscope equipped with fluorescence module (MZFl111, Leica, Inc.) and an RT Slider Spot CCD camera (Diagnostic Instruments, Inc.). Low power images were collected with both bright field and fluorescent illumination. GFP-positive transgenic tadpoles (stages 50–56) were fixed overnight in 4% paraformaldehyde in phosphate buffer and transferred to 20% (w/v) sucrose in phosphate buffer. Frozen sections of 10–15 μm thickness were prepared, and fluorescent images were obtained as described [32]. Figures were prepared with Adobe Photoshop.

3. Results and discussion

3.1. Gene structure and alternative splicing of mCAR

An adult mouse eye cDNA library was screened using an mCAR PCR fragment as a probe. Of 1×10^6 recombinants screened, four positive clones (411A, 422B, 511B and 1013B)

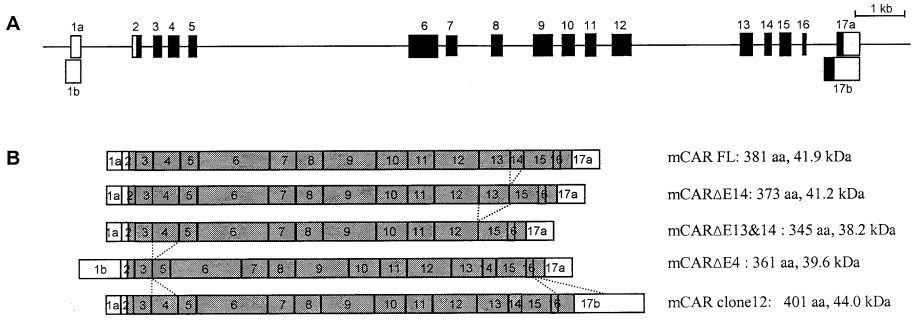

Fig. 1. Structure of the mCAR gene and its cDNA isoforms. The exon numbers are indicated. Filled and shaded boxes represent the coding sequence. A: Exon/intron organization of the mCAR gene. B: cDNA isoforms of mCAR obtained from an eye library screen, RT-PCR, 5′- and 3′-RACE of mouse retinal RNA. The length of the polypeptide and the predicted molecular weight for each isoform are given.

were obtained after the tertiary screen. Sequence analysis revealed that all four cDNA clones contained only partial coding regions with unspliced introns at various positions compared to the human gene [33], indicating incomplete splicing of the mRNA and a complex gene structure. The longest clone, 1013B, was 2.3 kb in length with a 5′ non-coding region of 48 bp, and its 3′ end was within intron 12. Clone 422B had an in-frame splicing out of exon 4.

The mCAR gene structure was determined by characterizing a BAC genomic clone containing the complete mCAR gene (mCAR BAC16505) isolated from a mouse ES cell library by Southern blot analysis (data not shown) and by sequencing overlapping gene fragments amplified with pairs of exon primers. A total of 17 exons and 16 introns were identified, spanning approximately 13 kb of genomic DNA (Fig. 1A). Exon sizes range from 10 to 315 bp, and intron sizes range from 104 to 4381 bp (Table 1). Both the organization and the exon/intron boundary sequences of the mCAR gene are in good agreement with those of the hCAR gene [33], and the splice junctions conform to the donor/acceptor consensus sequences [34] (Table 1). The whole gene sequence matches a contig (accession number AC091784) in the GenBank database except for a few nucleotides.

To isolate the complete coding region of mCAR, a sense primer containing the predicted first ATG and an antisense primer in the 3′ non-coding region were used for RT-PCR. Also, the 3′ non-coding sequence of mCAR was identified by 3′-RACE using the kit provided primer and the arrestin anchor primer [19] to amplify a fragment that contained mCAR cDNA sequence from the conserved arrestin signature domain to the poly(A) tail. The amplified products were cloned and sequenced. The sequences from 5′-RACE, RT-PCR and 3′-RACE were then assembled to generate the mCAR cDNAs with 5′ and 3′ non-coding regions. To our surprise, five different forms of cDNA were identified (Fig. 1B). The dominant species among the various isoforms corresponded to CARs from other species and was named mCAR full-length (mCARFL) (GenBank accession number AF156979). Clone 12 has an alternate exon 17 (E17b) that is produced by the use of a different splice acceptor in intron 16, which is 151 bp upstream of the splice acceptor for the original exon 17 (E17a). The existence of an in-frame stop codon in the 151-bp additional sequences in clone 12 results in a predicted protein with a different C-terminus than the other mCAR isoforms. The other three isoforms of mCAR each have an in-frame splicing out of one or two exons in exon 4 (mCARΔE4), 14 (mCARΔE14) or 13 and 14 (mCARΔE13 and 14). These results indicate that the primary mCAR transcript is processed into several mRNA variants, the major one being the mCARFL.

Alternative splicing is a common feature for the arrestin gene family [35–38], and for many other photoreceptor-specif-

Table 1
Exon/intron organization of the mCAR gene

Exon number[a]	Exon size (bp)	Intron size (kb)	Splice acceptor	Splice donor
1a	39	1.016		CTTCCCTCAG**gt**aatattct
1b	89	1.016		CTTCCCTCAG**gt**aatattct
2	28	0.239	ctcatctc**ag**GAAAGATTCA	GCATGTCCAC**gt**aagaatcc
3	31	0.150	caccccac**ag**AGTGTTTAAG	CAATGGGAAG**gt**aagagaac
4	61	0.197	tccacaac**ag**TTTTCCATCT	GAGCCCATTG**gt**tagtgagt
5	45	4.381	ctctctgc**ag**ATGGAGTCGT	GGTCGAAAGT**gt**aagtagaa
6	200	0.104	ttgccagc**ag**TGTTTGTCAG	TACACTTCAG**gt**actgacct
7	60	0.677	gatttcct**ag**ATGGTTGCTA	TTCAGGAAAG**gt**aaggaaga
8	68	0.636	ttttttat**ag**CCCTGTGGGG	TCCCTAAGAG**gt**agtctttg
9	136	0.195	ctttactc**ag**TGATTCTGTG	GGACAGGGAG**gt**tggtagca
10	85	0.216	cctgcttc**ag**GTTCATTACC	AAGATTGCAG**gt**aagcttct
11	73	0.398	acttttttc**ag**TTGTCCAGAC	AGGAGTTCAC**gt**gagctggg
12	138	2.160	tctttcaa**ag**AGAGACAGTA	CTAGCACAAT**gt**aagctcat
13	84	0.247	taatgtca**ag**TCTTCGACCT	CCTATGGGGG**gt**aagtgagg
14	24	0.171	ttattcac**ag**CATTCTAGGA	TGCCAGCCAG**gt**gagaagct
15	65	0.229	atgcttgc**ag**TGATGTTGGT	GAGAGAGCAG**gt**gaggctac
16	10	0.601	cttgccac**ag**TGGCTACCAG	TGGCTACCAG**gt**aagtcagc
17a	164		ctcctgaa**ag**CTCGGAGGAC	
17b	315		cattatct**ag**ACAGATTCCG	

[a]Exon numbering is according to Fig. 1.

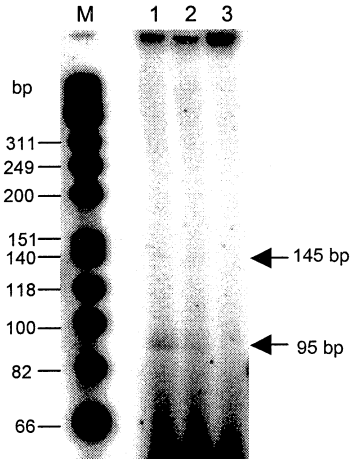

Fig. 2. Primer extension of total RNA (10 μg) from light-adapted mouse retina (lane 1), dark-adapted mouse retina (lane 2), and mouse cerebellum (lane 3). The extended products are identified with arrows, and the sizes of the extended products are indicated. M, [γ-^{32}P]ATP end-labeled ϕX174 *Hin*fI DNA size markers. The length in bp for each DNA *Hin*fI fragment in the marker lane is indicated next to the corresponding band.

ic genes [27,39–42]. It has been suggested that P44, the truncated form of bovine rod arrestin lacking the C-terminus generated by splicing out exon 16 of the bovine rod arrestin gene, is the major form to bind phosphorylated, activated rhodopsin in vivo [43]. The mRNA for the human splice variant of rod arrestin, which is present at approximately 1/20 the level of the human rod arrestin mRNA, was produced by splicing out exon 12 of the human gene [38]. The splice variant lacking E14 (8 aa) has been reported both in β-arrestin 1 [35] and β-arrestin 2 [36].

Although different splice variants have been reported for both rod arrestin and β-arrestins in various species, this is the first report of alternative splicing of the CAR gene. Moreover, isoforms lacking E4 (ΔE4), lacking E13–14 (ΔE13 and 14), and having an alternate E17 (clone 12), have not been reported for any members of the arrestin family. Ongoing experiments are being directed to determine the relative amount and the functional significance of each alternatively spliced form.

3.2. Structural analysis of the mCAR 5′-flanking region

A 5′-RACE was performed to characterize the transcription start site of the mCAR mRNA. After the second round of amplification, the RACE products from the retinal RNA turned out to be a doublet of ∼500 bp on an ethidium bromide-stained agarose gel, but no amplified products were obtained from the cerebellum RNA (data not shown), confirming the retina-enriched expression, and suggesting two potential transcription start sites of mCAR. The RACE products were cloned into the pCRII-TOPO cloning vector, and 31 clones were randomly picked for sequence analysis. The majority of the clones (19 out of 31) started 59 bp upstream of the predicted translation start codon, 11 bp longer at the 5′ end than the cDNA from library screening (1013B). This transcription start site is confirmed to be the major transcription start site by primer extension (see below) and is designated +1 (Fig. 3). The remaining clones (12 out of 31) started 50 bp further upstream, and the location is marked MTS as minor transcription start site (Fig. 3). Interestingly, none of the clones that had the additional 50-bp 5′ non-coding region had exon 4, while all the clones that started from the major transcription start site had exon 4, suggesting that the cDNA isoform of mCARΔE4 is the one that has a longer 5′ non-coding region because of the use of the MTS (Fig. 1B). A PCR analysis was performed with a sense primer within the additional 50-bp 5′ non-coding region and an antisense primer within exon 4 using the 5′-RACE cDNA as template. As expected, no specific products were amplified. However, a specific band was amplified when a sense primer in the 5′ non-coding region downstream of the major transcription site was used.

To confirm the transcription start sites, we performed primer extension with an antisense primer in the third exon, which is complementary to 74–95 of the cDNA sequence relative to the major transcription start site decided by 5′-RACE. As shown in Fig. 2, two extended bands were obtained from the retinal RNA but not from the cerebellum RNA, with the major extended product being 95 bp and the minor one being 145 bp, exactly matching the 5′-RACE results. The major extended product was about 10 times more intense than the minor one, suggesting that the major transcription start site is used 10 times more. We did not see this difference

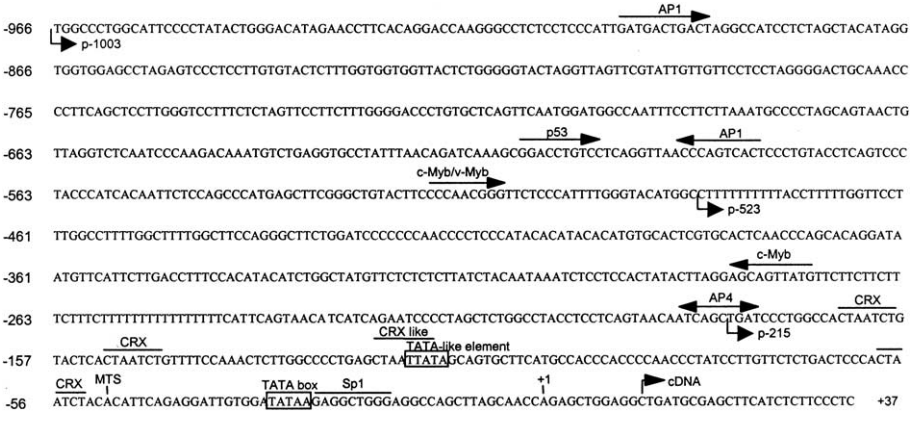

Fig. 3. Sequence analysis of the mCAR 5′-flanking region. The nucleotide sequence of the mCAR 5′-flanking fragments is shown with the potential transcriptional regulatory elements identified by sequence comparisons. The major transcription start site is indicated +1, and the minor one is marked MTS. The first base of the mCAR cDNA identified by retinal cDNA library screening is marked cDNA. The TATA box and the TATA-like element are boxed. Other promoter elements and their direction are also indicated.

with the 5′-RACE experiment because 5′-RACE was a PCR-based experiment that was not quantitative since we amplified for 30 cycles, which was already beyond the linear phase of the PCR reaction. No significant difference was seen in RNA from retinas between day (light) and night (dark) (Fig. 2), suggesting that mCAR mRNA expression may not be regulated by light exposure as in the case for rod arrestin [44,45].

To amplify the 5′-flanking region of mCAR, nested gene-specific antisense primers were designed according to the sequences of intron 1 and exon 1 for use with the GenomeWalker kit as described in Section 2, resulting in the generation of three overlapping PCR fragments of 215, 523 and 1003 bp. Analysis of the 1003-bp fragment, p-1003, with online promoter analysis programs, identified numerous potential *cis*-elements (Fig. 3). The proximal promoter region is highly conserved between hCAR [33] and mCAR, both carrying a TATA box, a TATA-like element and at least three potential binding sites for CRX, a retinal/pineal-specific transcription factor [46–49]. The second transcription start site (MTS) of mCAR, which is 50 bp upstream of the major one, is probably due to the use of the upstream TATA-like element at position −119 (Fig. 3), 63 bp upstream of the MTS. Although this TATA-like element is conserved between mouse and human, it is unclear if a second transcription start site is also present in the human gene.

3.3. Promoter activity of the mCAR 5′-flanking region

To delineate the sequences that drive photoreceptor-specific expression of mCAR, the p-215, p-523 and p-1003 5′-flanking fragments in the pGlow-TOPO vector were subcloned into the *Renilla* luciferase reporter vector pRL-null. As shown in Fig. 4, each of these promoter constructs was able to drive luciferase expression in Weri-Rb-1 retinoblastoma cells, which have been shown to express CRX at a high level [30,50], but none of them was able to drive reporter expression in COS-7 cells. Co-transfection of a CRX expression construct with the promoter–reporter constructs in COS-7 cells reveals luciferase expression (Fig. 4), suggesting that CRX is partially responsible for retina-specific expression of mCAR. CRX-binding sites have been localized to the promoter regions of many retina-enriched genes, including rhodopsin, rod arrestin

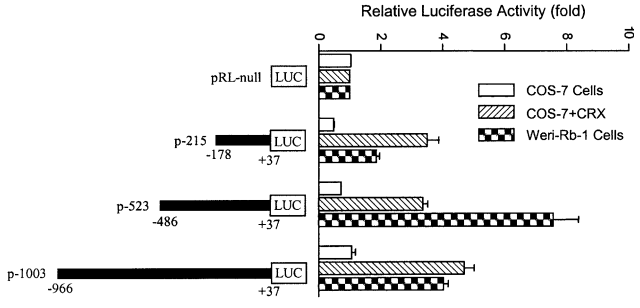

Fig. 4. Transient transfection analysis of the mCAR promoter–luciferase constructs in Weri-Rb-1 and COS-7 cells. The three different 5′-flanking fragments (p-215, p-523 and p-1003) in the pRL-null vector were transfected into either COS-7 or Weri-Rb-1 retinoblastoma cells with or without CRX co-transfection. The cells were processed and assayed for luciferase activity 44 h after transfection. The promoterless pRL-null vector was used as a control for basal luciferase activity of the cells and its luciferase activity was set at 1. Values are means+S.E.M. of results from three independent experiments performed in duplicate.

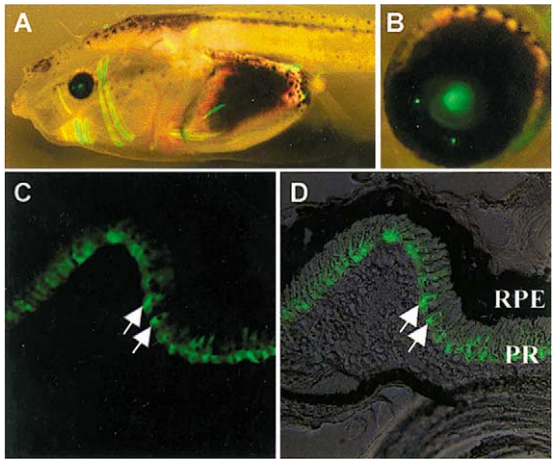

Fig. 5. mCAR upstream sequences direct cone-specific expression of EGFP in transgenic *Xenopus* tadpoles. A: Bright field and fluorescent lighting conditions showing EGFP expression in the eye, pineal and other tissues of the tadpole. B: EGFP expression in the eye. C: Fluorescent image of a radial cross-section of the retina of a transgenic tadpole generated using the pmCAR523-EGFP showing EGFP expression in the cone photoreceptors. D: Image of the same section under bright field and fluorescent lighting conditions showing EGFP expression limited to the cone photoreceptors. Arrows identify cone photoreceptor cells expressing high levels of EGFP. RPE, retinal pigment epithelium; PR, photoreceptors.

and interphotoreceptor retinoid-binding protein (IRBP) [32,33,46,50,51]. Thus, the cone photoreceptor-specific expression of CAR must involve other transcription factors in addition to CRX. The p-523 fragment has the strongest promoter activity in Weri-Rb-1 cells, indicating the existence of negative regulating elements in the p-1003 fragment. Interestingly, all three fragments show comparable promoter activities in CRX-co-transfected COS-7 cells, confirming that other transcription factors in Weri-Rb-1 cells participate in regulating the promoter activities through binding sites located upstream of the p-215 fragment.

3.4. mCAR 5′-flanking sequence directs expression in cone photoreceptors and the pineal gland in transgenic X. laevis

The cone-specific expression of color opsins [52], cone transducin α subunit [53], and cone cGMP-phosphodiesterase [54,55] has been found to be tightly regulated. A conserved sequence was reported in the locus control region, upstream of the red and green opsin genes, that is required for cone photoreceptor-specific expression in transgenic mice [56]. Further functional analysis of the human red and green opsin gene promoters revealed that the cell-specific expression of these genes is controlled mainly by positive-acting elements in the proximal region between −130 and the TATA box [57]. Transgenic studies show that a 277-bp 5′-flanking fragment from the human cone transducin α subunit gene coupled with a 214-bp IRBP enhancer can direct cone-specific expression of the reporter gene in mouse retina [58]. In our studies, we show that the proximal promoter region of the mCAR gene has high sequence homology with that of its human counterpart, suggesting that the elements controlling the cone-specific expression of CAR are most likely within this region.

To elucidate this hypothesis, we developed transgenic *Xenopus* using the various mCAR 5′-flanking sequences to drive

an enhanced GFP (EGFP) reporter gene. Expression from the longest construct (p-1003) was weak in all tadpoles and was detectable in the eye, forebrain, heart and at lower levels throughout the nervous system (data not shown). Expression from the shorter promoter constructs (p-523 and p-215) was significantly higher, and readily detected early in development, by stage 42. These data confirm the promoter activity analyzed in Weri-Rb-1 retinoblastoma cells. In transgenic tadpoles harboring either of the two shorter constructs, prominent expression was seen in the eye, pineal gland, heart, individual nerve fibers in the jaw and head, and at lower levels throughout the nervous system (Fig. 5A). The non-specific expression in tissues other than retina and pineal has been noticed in other laboratories with other retina-specific promoters from species other than *Xenopus* to drive reporter expression in *Xenopus* retina. However, the reason for this non-specific expression is unclear (personal communications with other researchers at ARVO and with Dr. Barry E. Knox).

In frozen sections of tadpole eyes, EGFP expression was observed prominently in cone photoreceptors, identified by the colorless oil droplet in the myoid region (Fig. 5C,D). The EGFP expression was seen in the abundant cone photoreceptors throughout the retina, but it was not possible to distinguish cone subtypes in these animals. No EGFP expression was observed in other cell types of the retina. These experiments demonstrate that the proximal promoter of the mCAR gene is capable of driving cone-specific expression in the retina. Within this region, the TATA box, Sp1 site and two CRX-binding sites are highly conserved between the mCAR and the hCAR genes. However, the Ret-1 site in the proximal hCAR promoter is replaced by a CRX-binding site in the mCAR gene, and the most distal CRX-binding site and the PCE1-like element in the hCAR gene are absent in the mCAR gene (Fig. 3) [33]. Comparison of the mCAR gene 5′-flanking region with that of the red, green and blue opsin genes and the cone transducin α subunit gene [59] did not reveal any highly conserved regions except the TATA box region (data not shown), consistent with the hCAR gene. It is noteworthy that all cone-specific genes have a typical TATA box in their proximal promoter regions, but most of the rod-specific genes lack the TATA box. It is thus possible that the combination of a TATA box with CRX-binding elements is necessary for cone-specific expression. However, we cannot exclude the possibility that unidentified *cis*-elements within the proximal promoter region of the CAR gene might also contribute to its cone-specific expression. Further deletion and mutagenesis studies of the 215-bp 5′-flanking region of mCAR are being directed to elucidate such cone-specific elements.

Taken together, we have characterized the gene structure, multiple transcripts, and the 5′-flanking region of mCAR to facilitate further functional study using a CAR knockout mouse model. Using the new technology of examining promoter activity with EGFP reporter in transgenic *Xenopus*, we have also demonstrated that a 215-bp fragment containing the proximal promoter region of mCAR is sufficient to drive cone photoreceptor- and pineal-enriched expression, providing a useful molecular tool for targeting expression in cone photoreceptors and pinealocytes.

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