Experimental Eye Research 91 (2010) 186–194

Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

A 350 bp region of the proximal promoter of *Rds* drives cell-type specific gene expression

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A R T I C L E I N F O

Article history:
Received 16 December 2009
Accepted in revised form 26 April 2010
Available online 4 May 2010

Keywords:
Rds gene
promoter activity and specificity
cis-elements
in vitro transfection
gene therapy

A B S T R A C T

RDS (retinal degeneration slow) is a photoreceptor-specific tetraspanin protein required for the biogenesis and maintenance of rod and cone outer segments. Mutations in the *Rds* gene are associated with multiple forms of rod- and cone-dominant retinal degeneration. To gain more insight into the mechanisms underlying the regulation of this gene, the identification of regulatory sequences within the promoter of *Rds* was undertaken. A 3.5 kb fragment of the 5′ flanking region of the mouse *Rds* gene was isolated and binding sites for Crx, Otx2, Nr2e3, RXR family members, Mef2C, Esrrb, NF1, AP1, and SP1 in addition to several E-boxes, GC-boxes and GAGA-boxes were identified. Crx binding sequences were conserved in all mammalian species examined. Truncation expression analysis of the *Rds* promoter region in Y-79 retinoblastoma cells showed maximal activity in the 350 bp proximal promoter region. We also show that inclusion of more distal fragments reduced promoter activity to the basal level, and that the promoter activities are cell-type and direction specific. Co-transfection with *Nrl* increased promoter activity, suggesting that this gene positively regulates *Rds* expression. Based on these findings, a relatively small fragment of the *Rds* promoter may be useful in future gene transfer studies to drive gene expression in photoreceptors.

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1. Introduction

Rds is one of the most common ocular genes to carry pathogenic mutations. Over 80 different disease causing mutations in *Rds* have been identified and are responsible for a wide range of degenerative phenotypes including autosomal dominant retinitis pigmentosa (RP) and various forms of macular dystrophy (Berson, 1993; Keen and Inglehearn, 1996). We and others have successfully delivered wild-type murine *Rds* to the retinas of mice with an *Rds*-associated haploinsufficiency RP phenotype (the $rds^{+/-}$) and reported structural and functional rescue of the diseased retina (Ali et al., 2000; Cai et al., 2009b). For our studies we used both ubiquitous (chicken beta-actin, CBA) and photoreceptor specific (human interphotoreceptor retinoid binding protein, IRBP) promoters and while they represented satisfactory preliminary choices, further knowledge of the native *Rds* promoter will be a great advantage for our future studies. However thus far, no reports have specifically addressed regulation of this gene. Characterization of the *Rds* promoter region will give a better understanding of the native regulation of the *Rds* gene and may enable us to enhance our gene therapy studies by incorporating critical regulatory elements in our vector design.

Gene therapy has been a popular and promising therapeutic approach for the treatment of inherited retinal degenerations in various animal models and patients [rodents (Ali et al., 2000; Cai et al., 2009b; Weber et al., 2003), dogs (Acland et al., 2005, 2001; Le Meur et al., 2006), primates (Jacobson et al., 2006; Lotery et al., 2003; Weber et al., 2003) and humans (Bainbridge et al., 2008; Cideciyan et al., 2008; Hauswirth et al., 2008)], and optimization of ocular gene therapy by expanding promoter choices is advantageous. Due to the prevalence of inherited retinal degenerations associated with photoreceptor and retinal pigment epithelial (RPE) defects, these cell types have often been targets of gene delivery studies. While tissue-specific promoters like vitelliform

Abbreviations: RDS, retinal degeneration slow; RXR, retinoid X receptor; RTR, retinoid receptor-related testis-associated receptor; Crx, cone-rod homeobox; Nrl, neural retinal leucine zipper; Nr2e3, orphan nuclear receptor subfamily 2, group E, member 3; Otx2, orthodenticle homeobox 2; RxrG, retinoid X receptor γ; CAR, constitutive androstane receptor; VDR, vitamin D receptor; RAR, retinoic acid receptor; PXR, pregnane X receptor; PNR, photoreceptor cell-specific nuclear receptor; OTX, orthodenticle homeobox; Mef2c, myocyte enhancer factor 2C; Esrrb, estrogen-related receptor β; NF1, nuclear factor 1; AP1, activator protein 1; SP1, specificity protein 1.

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doi:10.1016/j.exer.2010.04.017

macular dystrophy 2 (VMD2) and rhodopsin (MOP) and ubiquitously expressed promoters like chicken beta-actin (CBA) have been successfully used to direct expression in the retina (Allocca et al., 2007; Cai et al., 2009a, 2009b), strong promoters that can direct proper levels of gene expression in rods and cones have been lacking. The strongest currently used ocular promoter (the MOP promoter) is typically thought to be rod specific, or to drive very low levels of gene expression in cones (Glushakova et al., 2006). For the treatment of rod-based diseases, this promoter is a good choice, however, many diseases target both cones and rods. The most commonly used promoters to target both rods and cones have been the promoter for the photoreceptor transcription factor Crx and the IRBP (interphotoreceptor retinoid binding protein) promoter (Nour et al., 2004; Oh et al., 2007) although other promoters such as the rhodopsin kinase promoter, have also been studied for this purpose (Khani et al., 2007). To expand the available options for strong rod/cone promoters, we chose to characterize the promoter region for a gene that is expressed robustly in both photoreceptor types; *Rds* (retinal degeneration slow, also referred to as Peripherin/rds, P/rds, or Prph2). Our goals were first, to characterize a novel promoter that could be potentially used to direct high levels of expression of any gene (but particularly *Rds*) in rods and cones in gene therapy studies; and second, to study regulation of the *Rds* gene to better understand the expression and regulation of this key outer segment protein. We isolated a 3.5 kb fragment of the 5′ flanking region of the mouse *Rds* gene from wild-type C57BL/6 genomic DNA, identified regulatory factor binding sites in the promoter, and characterized the *in vitro* activity and cell-type specificity of various promoter fragments.

2. Materials and methods

2.1. Cloning of the 5′ flanking region and identification of regulatory sequences

3.5 kb of the 5′ flanking region of the murine *Rds* gene was isolated from C57BL/6 genomic DNA using the PromoterFinder™ DNA Walking kit (Clontech Laboratories, Inc., Palo Alto, CA, for details see Supplementary Methods and Supplementary Fig. 1). Products were cloned into the pBluescriptKS+ vector and sequenced. Sequences were blasted against the ensembl database (www.ensembl.org). Analysis for the presence of known transcription factor binding sites (cis-elements) in the 3.5 kb murine RDS 5′ flanking region was carried out by MatInspector version 7.0 using the Matrix Family library database version 7.0 (Genomatix, Munich, Germany). Similar assessment was carried out on 3.5 kb of the flanking region of bovine, rat, Xenopus, and human RDS promoter (using the ensembl sequences). For comparison sake, the same 5′ flanking region was analyzed from the mouse HPRT housekeeping gene and the liver specific HSD17b gene. Identity calculations and Clustal alignment of sequences from multiple species was carried out using Vector NTI 11 (Invitrogen, Carlsbad, CA). Analysis of CpG islands was carried out according to the method by Gardiner-Garden and Frommer (1987). Briefly, CpG rich areas were defined as regions at least 200 bp in length (starting from the transcription initiation site-TSS) that had a GC content above 50% and an observed/expected CG ration of greater than 0.6. In all cases, the translation initiation site (ATG) was defined as +1.

2.2. Animal care and use

C57/Bl6 and Balb/C mice were maintained in the breeding colony under cyclic light (14L:10D) conditions; cage illumination was ~7 foot-candles during the light cycle. All procedures were approved by the University of Oklahoma Health Science Center Institutional Animal Care and Use Committee (IACUC) and adhered to the *ARVO Statement for the Use of Animals in Ophthalmic and Visual Research* (http://www.arvo.org/). Genomic DNA was harvested from tail cuts as described previously (Naash et al., 1993).

2.3. Plasmid construction, cell transfection and luciferase assay

Rds promoter fragments were inserted upstream of the luciferase reporter gene in the multiple cloning site of the pGL2-Basic plasmid (Promega, Madison, WI) using standard techniques. For cell transfections, plasmids were prepared using the Endofree Plasmid Maxi Kit (Qiagen, Chatsworth, CA). COS-1 cells, low passage (<40) human Y-79 retinoblastoma cells (Di Polo and Farber, 1995), and in some experiments NIH3T3, 661W, and MCF-7 cells were seeded in six well plates at 3.2×10^5 cells/well 24 h prior to transfection. Y-79 cells were cultured/transfected in suspension, in RPMI-40 media containing 15% FBS (Invitrogen/Gibco, Carlsbad, CA) while adherent 661W, COS, MCF-7 and NIH3T3 cells were cultured/transfected in DMEM containing 10% FBS. All media contained standard antibiotics (penicillin 100 U/ml and streptomycin 100 μg/ml-Invitrogen/Gibco). Adherent cells were ~70% confluent at the time of transfection. 15 μg of each plasmid (pLUC468, pLUC468IV, pLUC1084, pLUC1439, pLUC2632, pLUC3304, pGL2-Basic, and pGL2-promoter) was used for standard $CaPO_4$ transfection carried out according to the manufacturer protocol using the CalMaximizer (Clontech) kit. As an internal control for transfection efficiency, 5 μg of pCH110 plasmid (Amersham GE, Piscataway, NJ) was co-transfected. Cells were harvested 48 h after transfection in 250 μl of 1× lysis buffer containing 5% (v/v) Triton X-100. Luciferase activity was assayed by mixing 20 μl of this lysate with 100 μl of luciferase assay reagent (Luciferase Assay System, Promega) and light emission was measured immediately in a spectrophotometer. As described previously (Rosenthal, 1987), β-gal activity was measured by *ortho*-Nitrophenyl-β-galactoside (ONPG) cleavage assay using the same cytosolic extract. The promoter activity of each construct in each cell line was defined as Relative Light Unit/β-gal activity. Each data point was the average of three readings and each experiment was repeated three times.

2.4. mRNA collection and real-time PCR

Murine eyes from P30 WT, CRX-NRL and $nrl^{-/-}$ animals were collected (5–6 eyes per group), and total RNA was isolated using Trizol (Invitrogen) as per the manufacturers instruction. DNase treatment and cDNA synthesis were performed as described previously (Cai et al., 2009b; Farjo et al., 2006). qRT-PCR for Rds was performed in triplicate for each sample using a Bio-rad iCycler single color system and the previously published primers (Farjo et al., 2006). The HPRT housekeeping gene was used as an internal control as previously described (Farjo et al., 2006).

2.5. Immunocytochemistry and western blotting

Cells were fixed with 4% paraformaldehyde at room temperature (RT) for 20 min. The monoclonal anti-RDS antibody 5H2 (1:2, a generous gift from Dr. Robert Molday, University of British Columbia) was applied overnight at room temperature. The cells were rinsed with PBS and covered with FITC-anti-rabbit IgG (1:100) for 30 min. After three washings in PBS, cells were mounted in Vectashield with DAPI (Vector Laboratory Inc, Burlingame, CA) and viewed under an epifluorescent microscope (Zeiss USA, Thornwood, NY). Western blotting was performed using the RDS-CT polyclonal antibody and HRP conjugated secondary (as previously described) (Farjo et al., 2006) and actin-HRP (Sigma–Aldrich, St. Louis, MO).

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