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

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Abstract

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 3.5kb 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 350bp 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.

Keywords

Rds gene; promoter activity and specificity; cis-elements; *in vitro* transfection; gene therapy

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

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(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, Acland, et al., 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 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, Cai, et al., 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 3.5kb 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.

Materials and methods

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 Figure 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 3.5kb 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 3.5kb 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 (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 ratio of greater than 0.6. In all cases, the translation initiation site (ATG) was defined as +1.

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).

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 hours 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 CaPO₄ 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 hours 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.

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).

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).

Statistical Analysis

One-way ANOVA with Bonferroni's post-hoc test was used to test the null hypothesis in cases where there were three or more different groups. Two-tailed Student's t-tests were used in cases where there were only two groups to compare (Fig. 4B and 5A).

Results

Sequence analysis of the 5' flanking region

The 5' flanking region of murine RDS was sequenced and analyzed as described in the methods (Supplementary Fig. 2). The GC content in the 400 bp immediately 5' to the ATG initiation codon is 55%. The observed/expected CpG ratio was 0.49 indicating that the immediate upstream promoter region is not CpG rich. Sequence analysis of the flanking region showed the absence of typical TATA and CCAAT elements. Over 800 potential cis-regulatory elements were detected in the analyzed region. Those relevant to photoreceptor gene regulation are shown on the sequence in Supplementary Fig. 2, and schematically in Figure 1. At the -3 and +4 positions (see Supplementary Fig. 2), A and G residues were observed respectively, consistent with the proposed consensus sequence for initiation of translation in higher eukaryotes (Kozak, 1987). Transcription start sites are observed at -218 and -221. An OTX binding site was observed at position -237 to -253. AP1 elements were observed at -641 to -651 and -1707 to -1717. Several binding sites for RXR (Retinoid X receptor) family members including CAR, VDR, RAR, PXR and RTR were located throughout the promoter region as were putative Mef2C sites. Three Nr2e3 binding sites (PNR) were found, at -1424 to -1450, -3094 to -3118 and -3139 to -3163. Five E-boxes were located in the distal region of the fragment, with two additional E-boxes in the proximal region. Sites for Otx2 (-1836 to -1852), and NF1 (-1663 to -1684) were observed near the middle of the promoter region. Just upstream from these elements are one SP1 site (-2021 to -2035), one GAGA-box (-2075 to -2131) and four GC-boxes. One mammalian transcriptional repressor binding site was found located in the extreme distal part of the isolated flanking region (-3457 to -3471).

On the base pair level, the 5' flanking region is moderately conserved among the species we examined; 46% similarity between mouse and human, 46% between mouse and bovine, and 44% between mouse and *Xenopus Tropicalis*, and 39% between mouse and rat (Supplementary Fig. 3). Cis-element analysis of the 5' regions of each of these species was then undertaken. The only regulatory element that appears in the same region of all the mammalian species examined is the CRX binding site (OTX) at position -237 suggesting a prime regulatory role for this transcription factor. As shown in Table 1, additional cis-elements were conserved across species within regions of the promoter (as opposed to in exactly the same place). Those conserved in the proximal (0 to -500), medial (-500 to -1500), and distal regions (-1500 to -3500) are shown in Table 1. Cis-element analysis of the HPRT (housekeeping) gene and the non-ocular HSD17B (hydroxysteroid 17 β dehydrogenase) gene are included on table 1 as a reference for which cis-elements are found ubiquitously and which may be more retina-specific.

Promoter activity of the *Rds* gene *in vitro*

To test the promoter activity of the 5' flanking region of the *Rds* gene, transient transfection experiments were carried out *in vitro* using human Y-79 retinoblastoma cells. Although these cells have been shown to express both rod and cone specific genes, (Di Polo and Farber, 1995) it was necessary to confirm the presence of all basal and tissue-specific factors required for *Rds* expression. The most straightforward way to confirm that the cells possess the ability to express *Rds* was to examine *Rds* expression. RT-PCR across intron 1 revealed that Y-79 cells express *Rds* transcripts: a PCR product of the expected size (387 bp) was detected in both Y-79 and the positive control (mouse retina) samples (Fig. 2a). Immunocytochemistry with mAB 5H2 (against RDS) was performed and substantial specific immunoreactivity was observed (Fig. 2b) confirming that RDS is expressed in these cells.

To evaluate the promoter activity and cell type specificity of the isolated flanking region, a series of deletion mutants (Fig. 3a) was generated by PCR or restriction digestion. Promoter fragments in the native orientation were inserted into the pGL2-Basic plasmid containing the luciferase reporter gene as follows: 1) pLUC468, [-154 to -468], 2) pLUC1084, [-154 to -1084], and 3) pLUC1439 [-154 to -1439], 4) pLUC2632 [-154 to -2632] and 5) pLUC3304 [-154 to -3304]. The tested fragments did not include the region downstream from positions -154 to +58. This region includes a modified start codon (ATG) and stop codon (TGA) and based on our initial tests it does not have any promoter activity (data not shown). The fragments were transfected into Y-79 retinoblastoma cells or COS-1 cells (as a non-ocular control) and luciferase activity was measured. Transfection with pGL2-Basic (no promoter) was used to determine the basal level of luciferase activity and transfection with pGL2-SV40 (pGL2-Basic with SV40 promoter) served as a positive control. As an internal control to evaluate the transfection efficiency, pCH110 containing the β -gal reporter gene driven by SV40 promoter was co-transfected with the luciferase vectors.

High levels of luciferase activity were observed when the pGL2-SV40 plasmid was delivered to Y-79 and COS-1 cells, indicating that these cells can be successfully transfected and the plasmid expressed (data not shown). In Y-79 cells, pLUC468, pLUC1084 and pLUC1439 drove significantly higher levels of luciferase expression than pGL2-Basic, although the level observed in pLUC1084 transfected cells was approximately 33% lower than that in pLUC468 (Fig 3b). The longer pLUC2632 construct promoted much lower level luciferase expression than other constructs; 79% reduction compared to the activity of pLUC468. This strongly suggests the presence of basic promoter activity in the 314 bp fragment (pLUC468), potential negative regulatory element(s) in the region from [-468 to -1084], and additional enhancer elements in the region between [-1084 and -1439]. pLUC2632 exhibited a modest but not statistically significant amount of activity, while pLUC3304 was virtually identical to non-expressing controls (pGL2-basic). The absence of detectable *in vitro* promoter activity in the longer pLUC3304 constructs suggests that additional elements in the distal regions of the 5' flanking region may also negatively regulate transcription. In COS-1 cells, only pLUC1439 showed a low level of tissue non-specific promoter activity (Fig. 3b).

Cell type specificity of pLUC468 mediated gene expression

In Y-79 cells, pLUC468 showed the highest promoter activity of all the fragments (12 fold higher than the promoterless construct) but it directed no detectable expression in COS-1 cells. To further explore the cell-type specificity of this region, two additional non-photoreceptor cell lines, NIH3T3 (mouse embryonic fibroblast derived) and MCF-7 (human breast cancer derived) and one additional photoreceptor cell line, 661W (cone photoreceptor derived (Tan, et al., 2004)) were transfected with pLUC468. None of the non-photoreceptor cell lines exhibited any gene expression above baseline when transfected with pLUC468 (Fig. 4a), while transfection in 661W cells resulted in a significant level of luciferase expression.

In addition to the cell-type specificity of the promoter activity, we were also interested in its directional specificity. To that end, we tested the promoter activity of the 314 bp fragment in Y-79 cells when it was inverted (pLUC468IV). As can be seen in Figure 4b, inversion of the promoter fragment abolished its ability to drive luciferase gene expression confirming that the promoter activity is directional.

Effect of photoreceptor developmental transcription factors on *Rds* expression

Rds is expressed in both rod and cone photoreceptor cells. Sequence analysis of the 3.5kb 5' flanking region of the mouse *Rds* gene revealed the presence of several cis-elements known to bind key transcription factors in the development of these two divergent cell types, specifically Crx and Nr2e3. Crx and Nr2e3 are known to regulate transcription with the aid of a third photoreceptor-specific factor, Nrl (Peng, et al., 2005) and some ubiquitous transcription factors such as the Sp zinc finger proteins (Hennig, et al., 2008). Crx and Nr2e3 are known to regulate *Rds* transcription. Chromatin immunoprecipitation assays demonstrate that Nr2e3 binds to the *Rds* promoter region and in a mouse model lacking Nr2e3 (*rd7/rd7* mice), *Rds* transcript levels are several fold lower than in wild-type controls (Nystuen, et al., 2008). Similarly, *Rds* levels are decreased in mice lacking Crx (*Crx*^{-/-}) (Furukawa, et al., 1997, Livesey, et al., 2000). In spite of the observation that these photoreceptor transcription factors usually work as a group, our cis element analysis did not identify any Nrl sites in the *Rds* promoter region. To determine whether Nrl might therefore have an indirect regulatory affect on *Rds*, we co-transfected Y-79 cells with pLUC2632 or pLUC468 and a plasmid containing the *Nrl* (pNrl) gene under the control of the adenovirus major late promoter (pMT3 vector, a generous gift from Dr. Anand Swaroop, NEI). Co-transfection of the pNrl and pLUC468 resulted in significantly enhanced (~5 fold) promoter activity compared to single transfected controls (Fig. 5a). Co-transfection with pLUC2632 and pNrl also resulted in a ~2-fold increase in promoter activity (Fig. 5a). To determine whether these transcription factors might affect *Rds* expression *in vivo*, we measured *Rds* message levels by qRT-PCR in mice that overexpress Nrl (Crx-Nrl transgenics (Oh, et al., 2007)). As can be seen in Figure 5, overexpression of *Nrl* leads to enhanced expression of *Rds* transcript (~3 fold higher than in wild-type animals, Fig. 5b) and protein (higher in wild-type animals, actin shown to verify loading, Fig. 5c). Nr2e3 promotes rod development directly in concert with Nrl while simultaneously suppressing cone differentiation. In Figure 5b, we show that, consistent with previously published results (Nystuen, et al., 2008), the absence of Nr2e3 (*Nr2e3*^{-/-} mice) yields significantly decreased steady-state *Rds* transcript levels (~50% reduction compared to wild-type).

Discussion

In this study, we evaluated the 3.5kb flanking region of the *Rds* gene due to its structural importance in the photoreceptor outer segment, functional necessity for proper vision, and experimental value in the design of optimized gene therapy vectors. Unlike many other dual-photoreceptor specific genes, it lacks typical TATA and CAAT promoter elements but contains several other regulatory elements. This region is not CpG rich. Notably, several critical photoreceptor transcription factor binding sites were observed on the isolated 3.5kb fragment including OTX (Crx), PNR (Nr2e3), EsrrB, Mef2C and RXR family members. Additional, non-photoreceptor specific regulatory elements were detected including SP1 sites, several E-boxes, one GAGA-box, and several GC-boxes. *In vitro* analysis of the activity of various promoter fragments contained within the 3.5kb 5' flanking region indicated that the region from [-154 to -468] possesses the basal, direction-specific promoter activity in retina-derived cell lines (Y-79/661W) but not in any other non-ocular cell lines tested. Although two of the other promoter fragments possessed some activity (pLUC1084, pLUC1439), expression of longer constructs was significantly suppressed in Y-79 cells.

Photoreceptor gene regulation is controlled by a highly conserved network of transcription factors centered on Crx (Hennig, et al., 2008). Crx regulates expression of the *Nrl* gene which, among other things, regulates expression of *Nr2e3*. Crx plays a critical role in the differentiation and maintenance of photoreceptor cells, (Chen, et al., 1997, Furukawa, et al., 1997), regulation of tissue specificity, and levels of rod and cone gene expression and photoreceptor development (Boatright, et al., 1997, Kennedy, et al., 1998, Kennedy, et al., 2001, Mani, et al., 2001, Martinez and Barnstable, 1998, Morabito, et al., 1991, Tao, et al., 1993, Whitaker and Knox, 2004, Yu, et al., 1996). *Nrl* and *Nr2e3* work together to promote rod development although *Nr2e3* is considered a dual action transcription factor. In the absence of *Nr2e3*, cone genes are de-repressed and rod genes are suppressed resulting in an enhanced S-cone phenotype (Haider, et al., 2006, Haider, et al., 2009). All three (Crx, *Nr2e3*, and *Nrl*) can act together along with ubiquitously expressed transcription factors such as the Sp zinc finger proteins to co-regulate photoreceptor gene expression (Cheng, et al., 2004, Hennig, et al., 2008).

Binding sites for Crx (OTX), *Nr2e3* (PNR), and Sp1 were identified in the *Rds* promoter region however, the specifically modified AP1 sites (Rehemtulla, et al., 1996) to which *Nrl* binds were not found in the 5' flanking region of the *Rds* gene. In spite of this, *in vitro* results shown here demonstrate that reporter gene expression is increased in cells co-transfected with our pLUC468 *Rds* construct and pNrl, supporting a role for *Nrl* in the regulation of *Rds*. Although *Nrl* could possibly exert regulatory effects by binding to the various non-modified AP1 sites identified in the 5' flanking region, the absence of these sites in the region shown to be regulated by *Nrl* (pLUC468) suggests this is unlikely. It is possible that *Nrl* regulates *Rds* expression indirectly by enhancing expression of *Nr2e3* or Crx, or by participating in the Crx regulatory complex. It is likewise possible that *Nrl* regulates *Rds* expression by binding to an as yet unidentified sequence. The lack of a predicted cognate binding site does not preclude transcription factor binding; indeed many photoreceptor gene regulatory elements have been identified after being missed by standard sequence comparisons (Hsiau, et al., 2007, Peng and Chen, 2005). Support for the idea that *Nrl* regulates *Rds* comes from our observation that in the *Nrl* over-expressing all-rod retina (Crx-*Nrl*) *Rds* message levels are significantly increased by approximately 3 fold. As the murine retina is only 3% cones, this dramatic increase in *Rds* message (and protein) cannot be accounted for by the small increase in *Rds* which would be expected to accompany the conversion of cones to rods.

Historically, *Nr2e3* and *Nrl* were considered the only rod-specific transcription factors. However, recent evidence has suggested that two more photoreceptor transcription factors may be rod-enhanced: Mef2C and EsrrB (Hsiau, et al., 2007). Both of these transcription factors have binding sites in the 5' flanking region of *Rds* and may play a role in *Rds* expression in rods. The transcription factor RxrG, which interacts with the RXR cis-element (found in the 5' flanking region of *Rds*), is required for some cone gene transcription (Hennig, et al., 2008, Roberts, et al., 2006, Yoshida, et al., 2004), is upregulated in *Nrl*^{-/-} mice (Hsiau, et al., 2007) and may positively regulate *Rds* expression in cones. However, RXR/RAR cis elements, and Mef2C binding sites were found in all promoter regions examined, so it is difficult to make conclusions on their role in the regulation of *Rds* specifically.

For most photoreceptor genes, the maximal promoter activity and cell type specificity resides within the first 500 bp of the 5' flanking region (Fujimaki, et al., 2004, Mohamed, et al., 1998, Pittler, et al., 2004, Young, et al., 2003). This appears to be the case for the *Rds* promoter: we observed strongest activity with our pLUC468 construct. This region contains the only highly conserved cis-element among the species we examined, the OTX element (conserved in the *X. laevis* *Rds* promoter region (Moritz, et al., 2002)) which will likely be recognized by Crx thus transactivating the promoter activity. There appear to be suppressors in the regions between [-468 to -1084] and [-1439 to -2632] and a positive regulator or enhancer between [-1084 to -1439]. The pLUC1084 construct (which showed 33% less activity than pLUC468)

contains one AP1 site and one E-box. The increased promoter activity conferred by inclusion of the region between [-1084 and -1439] (i.e. pLUC1439 vs. pLUC1084) is likely due to positive regulation by Nr2e3 (PNR) and possibly Mef2C and CAR/RXR. However, additional PNR, RXR, and Mef2C sites are found more distally in regions of the 5' flanking sequence which demonstrated suppressed transcription (e.g. within pLUC2632 and pLUC3304), so regulation is clearly not straightforward and additional interpretation will require further experiments. In some cases, gene expression is inversely correlated with plasmid size (Kreiss, et al., 1999, Yin, et al., 2005), however not always (Maucksch, et al., 2009). In this case, however, lack of expression with the larger constructs is not likely to be due primarily to increased plasmid size. In experiments designed specifically to test the effects of plasmid size on gene expression, gene expression decreased in a consistent, near-linear fashion over the range of plasmid sizes comparable to what we use here (Yin, et al., 2005). In contrast, expression from our constructs does not steadily decrease as the plasmid size increases. In fact, expression is higher for pLUC1439 than the smaller pLUC1084 and then drops precipitously for pLUC2632.

Gene therapy studies require a well-characterized promoter fragment to reliably drive high levels of tissue specific gene expression. Several photoreceptor specific promoters have been studied including those for rod opsin, cone opsin, Crx, and IRBP. However, the preponderance of inherited retinal diseases associated with mutations in *Rds* coupled with RDS' unique expression pattern (expressed at high levels in rods, and substantial but lesser levels in cones (Farjo, et al., 2006)) makes characterization of the endogenous *Rds* promoter a useful step for our progress in developing gene based therapeutics. Furthermore, our experiments demonstrating that co-delivery of constructs containing positive regulators of transcription (including *Nrl*) and therapeutic constructs containing the *Rds* promoter may help increase expression of the therapeutic gene. Since *Nrl* is thought to have a positive regulatory role on the expression of many rod genes, it is also possible that delivery of *Nrl* alone could improve the phenotype of rod-dominant eye diseases that are associated with haploinsufficiency in various genes. Development of a genetic therapy that is not specific to any particular gene has been an area of interest and would be a great advantage for the treatment of multiple retinal degenerative conditions.

Our results suggest that the 314 bp or 1,285 bp regions contained in pLUC468 and pLUC1439 drive the best expression *in vitro* and are likely the best choices for incorporation into photoreceptor-targeting vectors. Often promoter activity *in vitro* does not correlate well with activity *in vivo*, and future studies will involve the use of these constructs *in vivo*. Our data suggest that *Rds* is regulated by the same core Crx-based photoreceptor regulatory network as many other photoreceptor specific genes. The presence of binding sites for both cone and rod transcription factors may help explain the differential expression pattern of *Rds* in rods and cones.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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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

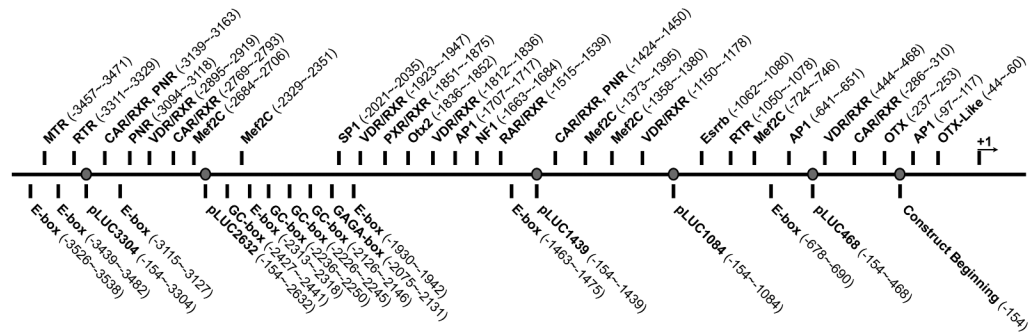


Figure 1. Schematic distribution of relevant regulatory sequences in the 5' flanking region of *Rds* gene

Relevant putative transcription factor binding sites or other regulatory regions are highlighted (as identified using the procedures found in the Materials and Methods section). The relative location of start/end sites for the *in vitro* expression constructs is also noted (grey circles).

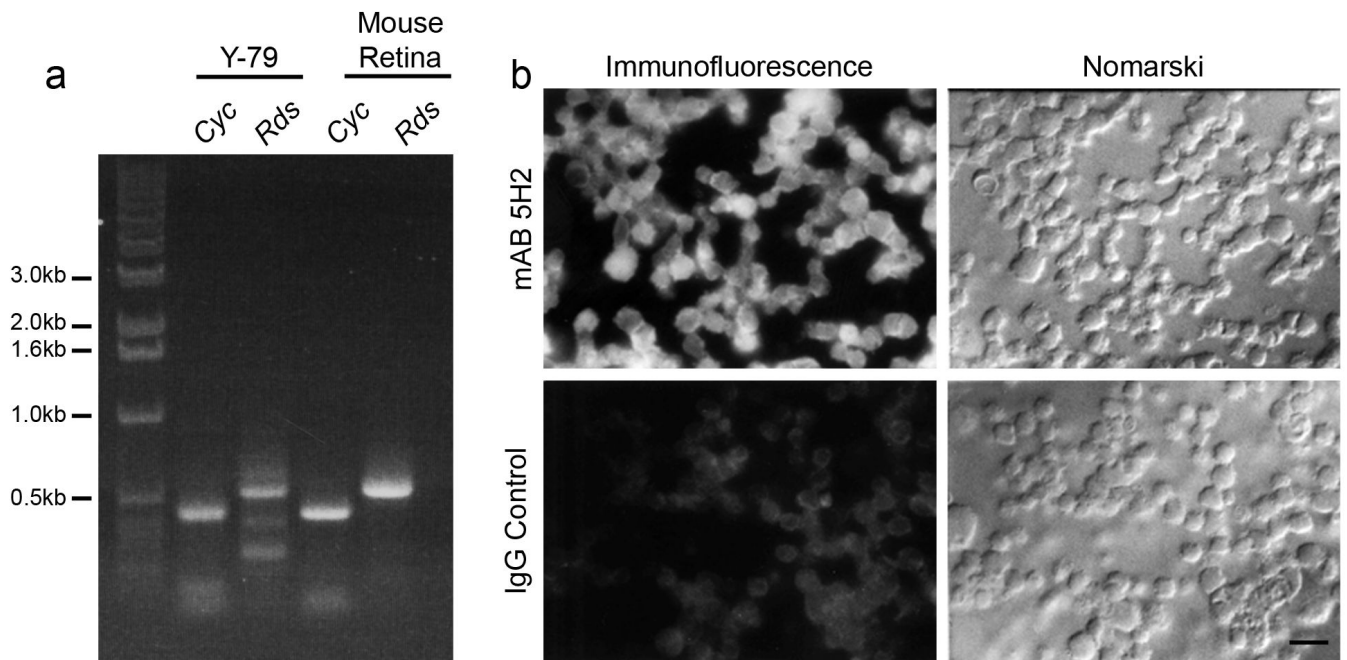


Figure 2. Expression of the mouse *Rds* gene in Y-79 cell lines

a. Expression of the *Rds* gene detected by RT-PCR. Primers used for each PCR reaction are indicated on top of each lane (*Rds*, *Cyc*). The size of the products from *Rds* and cyclophilin gene (control) are also labeled. **b.** Expression of the *Rds* gene detected by immunocytochemistry using mAB 5H2 against RDS. Immunostaining was observed in the majority of the Y-79 cells (top left) while no staining was seen in IgG stained control cells. Scale bar 25 μ m.

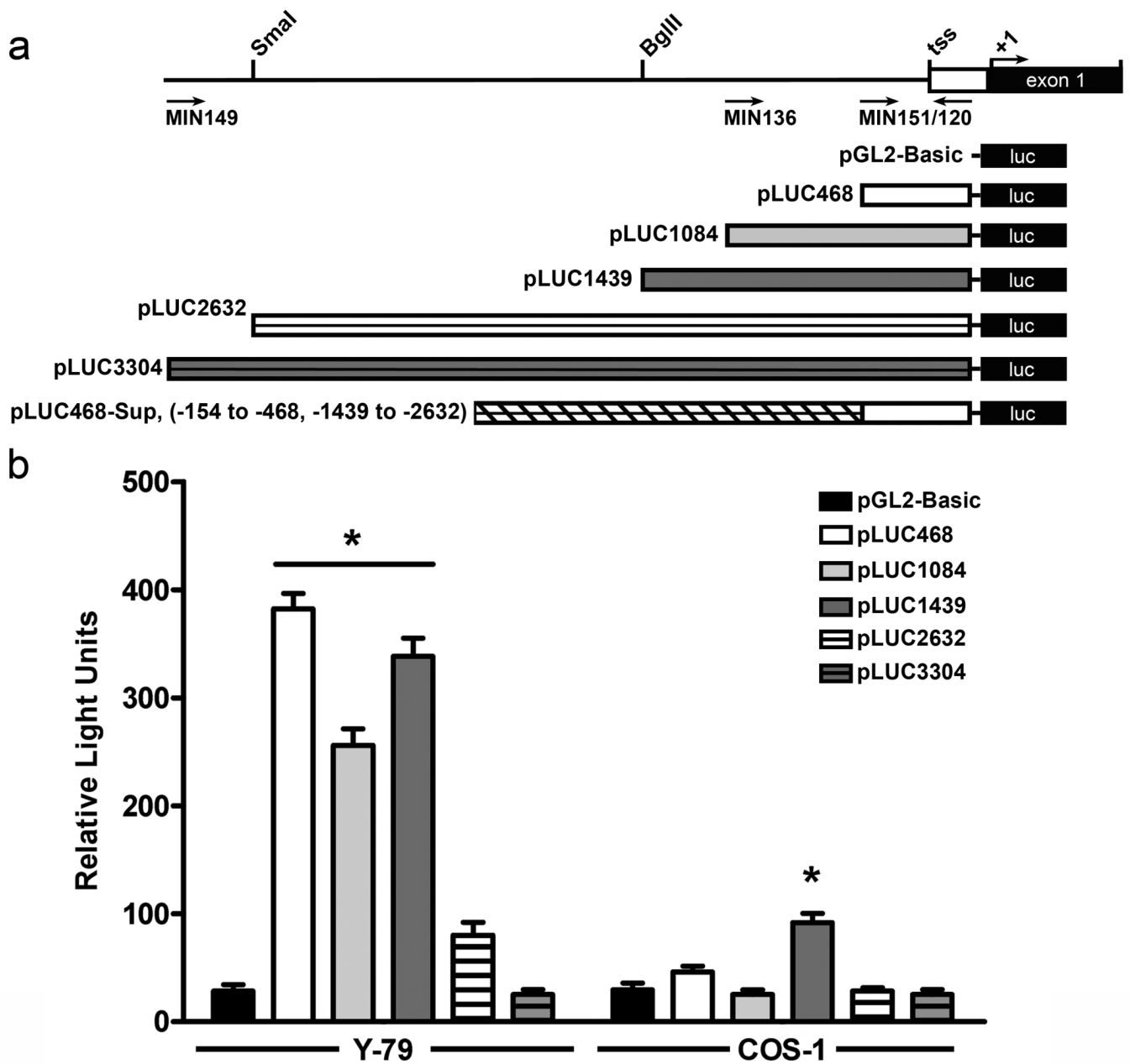


Figure 3. Promoter activity of the 5' flanking region of the mouse *Rds* gene

a. Schematic showing the construction of the luciferase reporter constructs. **b-c.** Luciferase activity (normalized to β -gal) after co-transfection of 15 μ g of the indicated luciferase construct (containing different *Rds* promoter fragments) and 10 μ g of pCH110 (used as an internal control) into Y-79 and COS-1 cells. **b.** Three of the constructs drove significant expression after transfection into human Y-79 retinoblastoma cells (left) while little expression was detected after transfection into COS-1 cells (right). The data shown is an average of three independent experiments, * $p < 0.01$.

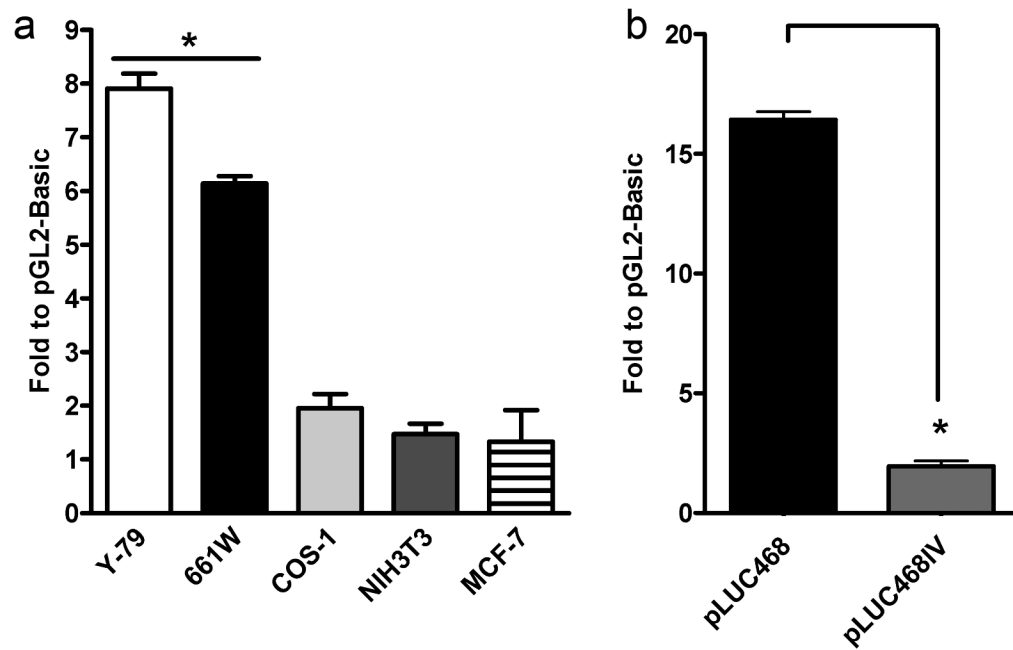


Figure 4. Tissue and direction specificity of *Rds* promoter activity

a. pLUC468 (with pCHIP110 as a control) was transfected into several different ocular and non-ocular cell lines. Significant expression (i.e. above levels detected in pGL2-Basic transfected cells) was only detected in Y-79 and 661W cells. **b.** pLUC468IV transfection did not direct significant promoter activity indicating that the *Rds* promoter is direction specific. The activity presented is an average of three independent experiments. * $p < 0.01$

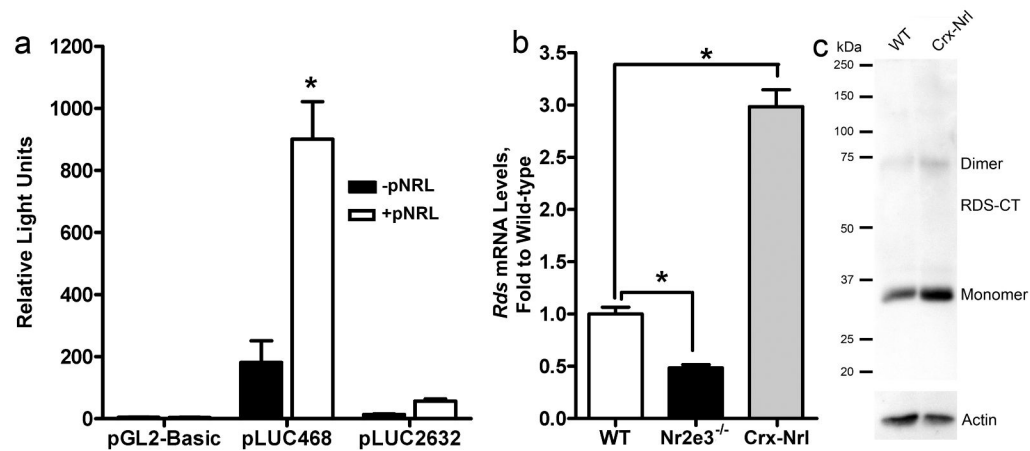


Figure 5. *Nrl* and *Nr2e3* regulation of *Rds* expression

a. Y-79 cells were co-transfected with the luciferase construct (and pCHIP110) and, as indicated, pNrl. Expression of *Nrl* led to a significant increase in *Rds* gene expression. **b.** *Rds* mRNA levels (qRT-PCR) were significantly reduced in *Nr2e3*^{-/-} mice and significantly increased in Crx-Nrl mice. * p<0.01. **c.** Western blots on 5 µg of total retinal extract from WT or Crx-Nrl mice were probed with polyclonal antibodies against the RDS C-terminal (RDS-CT) and with antibodies against actin. RDS protein levels (when normalized to actin) were increased by approximately 2-fold.

Table 1

Preservation of transcription factor binding sites across species

Transcription factor binding sites in the 3.5kb promoter region of the Rds gene from multiple species (with a non-tissue specific [HPR1] and non-photoreceptor gene [HSD17b] used as controls) were identified as described in the methods. The analyzed regions were divided into proximal (within 500 base pairs of the translation start site), medial (from 500-1500 base pairs) and distal (1500-3500 bp). An X indicates that the listed binding site was found in that region.

CREs (Trans factor)	Mouse Rds	Bovine Rds	Human Rds	Rat Rds	Xenopus Rds	Mouse HPR1	Mouse HSD17B	Role	References
Proximal									
API	X		X	X	X			Not Nr1 response element	(Rehemtulla, et al., 1996)
OTX (Crx)	X	X	X	X				Core activator of photoreceptor genes	(Chen, et al., 1997, Furukawa, et al., 1997, Kennedy, et al., 1998, Moritz, et al., 2002, Whittaker and Knox, 2004)
CAR/RXR (RXR γ)	X	X		X	X			Regulation of M- vs. S-cones	(Hennig, et al., 2008, Roberts, et al., 2006, Yoshida, et al., 2004)
Medial									
API	X	X	X	X				As above	
Mef2c (Mef2c)	X	X	X	X	X		X	Rod enhanced	(Hsiau, et al., 2007)
Esr1b (Esr1b)	X			X				Rod-enhanced orphan nuclear receptor	(Hsiau, et al., 2007)
VDR/RXR (RXR γ)	X	X	X	X	X		X	As above	
CAR/RXR (RXR γ)	X	X			X			As above	
PNR (Nr2E3)	X	X	X	X	X			Dual activity rod/cone transcription factor	(Cheng, et al., 2006, Cheng, et al., 2004, Peng, et al., 2005)
Distal									
OTX2 (OTX2)	X			X		X		Activator of Crx regulatory network	(Koike, et al., 2007, Rath, et al., 2006)
PXR (RXR γ)	X	X	X		X			Regulation of M- vs. S-cones	(Hennig, et al., 2008, Roberts, et al., 2006, Yoshida, et al., 2004)
SP1 (SP1)	X				X		X	Ubiquitous transcription factor	(Hennig, et al., 2008)
Mef2c (Mef2c)	X	X	X	X	X		X	As above	

CREs (Trans factor)	Mouse <i>Rds</i>	Bovine <i>Rds</i>	Human <i>Rds</i>	Rat <i>Rds</i>	Xenopus <i>Rds</i>	Mouse <i>HPRT</i>	Mouse <i>HSD17B</i>	Role	References
CAR/RXR (RXR γ)	X	X		X	X			As above	
VDR/RXR (RXR γ)	X	X	X	X	X	X	X	As above	
PNR (Nr2e3)	X	X	X	X	X	X		As above	
RTR (RXR γ)	X			X	X	X		As above	