**Rlbp1 Promoter Drives Robust Müller Glial GFP Expression in Transgenic Mice**

Félix R. Vázquez-Chona, Anna M. Clark, and Edward M. Levine

**PURPOSE.** Müller glia are essential for maintaining retinal homeostasis and exhibit neuroprotective and deleterious responses during retinal degeneration. Having the ability to visualize and genetically manipulate Müller glia in vivo will facilitate a better understanding of how these cells contribute to these processes. The goal of this study was to determine whether regulatory elements of the retinaldehyde binding protein 1 (Rlbp1; formerly Cralbp) gene can drive robust Müller glial gene expression in vivo.

**METHODS.** Transgenic mice were generated by pronuclear injection of a construct carrying a 3-kilobase (kb) region of the Rlbp1 gene and 5′-flanking sequences linked to the enhanced green fluorescent protein (GFP) cDNA. GFP expression was analyzed by immunohistology in regions of the central nervous system in which RLBPI protein is expressed, in retinas from wild-type and retinal degeneration 1 (rd1) mice, and during retinal development.

**RESULTS.** Three transgenic lines were generated, and the one with the strongest and most consistent GFP expression was characterized further. Müller glia displayed robust GFP expression at all postnatal developmental stages and in the rd1 retina. Onset of expression occurred by birth in retinal progenitor cells.

**CONCLUSIONS.** Regulatory elements in a restricted region of the Rlbp1 gene are sufficient to drive GFP expression in vivo. This transgenic line provides robust GFP expression that can be used to visualize retinal progenitor cells during postnatal development and Müller glia during their differentiation and in the healthy or degenerating adult retina. (*Invest Ophthalmol Vis Sci.* 2009;50:3996–4003) DOI:10.1167/iovs.08-3189

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The role of Müller glia during retinal degeneration and trauma is complex. In response to environmental perturbations, mammalian Müller glia can react in a reactive state characterized by hypertrophy, proliferation, or inappropriate migration, the consequences of which are secondary neuronal cell death and retinal remodeling. However, Müller glia also protect retinal cells by releasing neurotrophic factors and antioxidative compounds and by maintaining the blood retinal barrier. Under the appropriate conditions, mammalian Müller glia exhibit neurogenic potential. However, our understanding of the cellular and molecular mechanisms governing these diverse properties is still rudimentary. One problem is that the ability to target gene expression specifically in Müller glia remains a challenge. A first step toward genetic targeting of this cell population is to identify a region of DNA that can drive robust Müller gene expression in vivo.

Regulatory elements of the retinaldehyde binding protein 1 (Rlbp1; formerly Cralbp) gene are candidate drivers for Müller glial targeting. RLBPI is a retinoid-binding protein highly expressed by Müller glia and by retinal pigment epithelial (RPE) cells. In Müller glia, RLBPI participates in the regeneration of cone visual pigment. RLBPI is highly expressed in Müller glial cytoplasm, including end feet, cell bodies, radial processes, and apical microvilli. In situ hybridization studies in cat, bovine, mouse, and zebrafish retina confirmed that Müller glia express RLBPI mRNA. Moreover, RLBPI is a classical marker for identifying Müller glia during homeostasis, postnatal development, early phases of retinal degeneration, and in culture. Various fragments of the 5′ region of the human RLBPI gene can promote robust luciferase reporter expression in cultured Müller glia, and a portion of the mouse Rlbp1 promoter can drive sparse reporter expression in rat Müller glia after in vivo electroporation. These findings suggest that regulatory regions of the Rlbp1 gene are ideal for driving Müller glial-specific gene expression in vivo. Here, we tested whether a 3-kilobase (kb) region of mouse genomic DNA incorporating a portion of the Rlbp1 gene is sufficient and specific to promote Müller glial expression of green fluorescent protein (GFP) in mice. Contained within this stretch of DNA are the putative Rlbp1 promoter and the first two exons and the first intron of the Rlbp1 gene (Fig. 1). These analogous regions of the human RLBPI gene can drive luciferase expression in cultured Müller glia, RPE cells, and ciliary epithelium cells.

We examined GFP expression in the adult retina and in other tissues known to express RLBPI protein and in mice harboring the retinal degeneration 1 (rd1) mutation, a missense mutation in the beta-subunit of the cGMP phosphodiesterase (*Pde6b*) gene. Mice harboring the rd1 mutation undergo rapid, early-onset degeneration of rod photoreceptors. Finally, we examined GFP expression during retinal postnatal development. The sum of our data shows that this construct has the necessary elements to drive gene expression in a robust manner in Müller glia and postnatal retinal progenitor cells in vivo.

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**METHODS**

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A 4.7-kb fragment encompassing 1650 nucleotides (nt) of the 5’ intergenic region and 3117 nt of the Rlbp1 gene (nt 38–4805 in accession number AF084658) was amplified from genomic DNA isolated from 129/SvJ mice using two overlapping primer sets. Primer set 1 consists of (forward) TGGTTGAGGACCCCTTTAACCATA and (reverse) GGATGAAGGACTTTTGGAGAGGAAACTAGGAAA. Primer set 2 consists of (forward) CCCAAGAAAGAGCTGTCAGGTAA and (reverse) GGATGAA-CCCAAGAAAGAGCTGTCAGGTAA. Sequence-verified PCR clones werecarried through multiple cloning steps to yield a single contiguous gene (nt 38–4036 in accession number AF084638) was amplified from genomic DNA isolated from 129/SvJ mice using two overlapping primer sets. Primer set 1 consists of (forward) ATGTTGAGGACCCCTTAACCATA and (reverse) GGACCTTTGAGAGGAAACTAGGAAA. Primer set 2 consists of (forward) CCCAAGAAAGAGCTGTCAGGTAA and (reverse) GGATGAA-GAGCCCAAGATACTCACC. Sequence-verified PCR clones were carried through multiple cloning steps to yield a single contiguous gene fragment encompassing 1650 nt of the 5’ intergenic region and 2348 nt of the Rlbp1 gene (nt 38–4036 in accession number AF084658) placed into the pEGFP-1 vector (accession number U55761; Clontech, Palo Alto, CA). In preparation for the DNA injection, a 4.31-kb linear fragment encompassing 1650 nt of the 5’ intergenic region and 3117 nt of the Rlbp1 gene was inserted into the pEGFP-1 vector (accession number U55761; Clontech, Palo Alto, CA). In preparation for the DNA injection, a 4.31-kb linear fragment encompassing 1650 nt of the 5’ intergenic region and 3117 nt of the Rlbp1 gene was inserted into the pEGFP-1 vector (accession number U55761; Clontech, Palo Alto, CA). In preparation for the DNA injection, a 4.31-kb linear fragment encompassing 1650 nt of the 5’ intergenic region and 3117 nt of the Rlbp1 gene was inserted into the pEGFP-1 vector (accession number U55761; Clontech, Palo Alto, CA). In preparation for the DNA injection, a 4.31-kb linear fragment encompassing 1650 nt of the 5’ intergenic region and 3117 nt of the Rlbp1 gene was inserted into the pEGFP-1 vector (accession number U55761; Clontech, Palo Alto, CA). In preparation for the DNA injection, a 4.31-kb linear fragment encompassing 1650 nt of the 5’ intergenic region and 3117 nt of the Rlbp1 gene was inserted into the pEGFP-1 vector (accession number U55761; Clontech, Palo Alto, CA). In preparation for the DNA injection, a 4.31-kb linear fragment encompassing 1650 nt of the 5’ intergenic region and 3117 nt of the Rlbp1 gene was inserted into the pEGFP-1 vector (accession number U55761; Clontech, Palo Alto, CA). In preparation for the DNA injection, a 4.31-kb linear fragment encompassing 1650 nt of the 5’ intergenic region and 3117 nt of the Rlbp1 gene was inserted into the pEGFP-1 vector (accession number U55761; Clontech, Palo Alto, CA). In preparation for the DNA injection, a 4.31-kb linear fragment encompassing 1650 nt of the 5’ intergenic region and 3117 nt of the Rlbp1 gene was inserted into the pEGFP-1 vector (accession number U55761; Clontech, Palo Alto, CA). In preparation for the DNA injection, a 4.31-kb linear fragment encompassing 1650 nt of the 5’ intergenic region and 3117 nt of the Rlbp1 gene was inserted into the pEGFP-1 vector (accession number U55761; Clontech, Palo Alto, CA).
and share regions of conservation in their DNA sequences. There are inconsistencies, however, with exon assignments and nucleotide numbering among different studies. Using the Rlbp1 gene structure reported in the Ensembl genome browser (version 50; available at http://www.ensembl.org) as our reference, Figure 1A shows how the previously reported gene organization and numbering schemes for the mouse Rlbp1 gene correspond to each other through exon 3. Whereas Vogel et al. are generally consistent with this genomic DNA in cell lines, Kennedy et al. as their study marks the adenosine in the first translated codon (initiator methionine) in exon 3 as nucleotide +1. In contrast, Kennedy et al. marked nucleotide +1 as the first transcribed nucleotide in what they predicted to be exon 1 (also adopted by Matsuda and Cepko). However, based on RNA analysis and expressed sequence tag (EST) alignments, Vogel et al. reassigned this exon as exon 2, which is in agreement with Ensembl version 50. Similar discrepancies exist for the human Rlbp1 gene. For this study, we adopted the organization and nucleotide numbering reported in Ensembl version 50.

**Rlbp1 Promoter Drives GFP Expression in Müller Glia**

Reporter assays using human Rlbp1 genomic DNA in cell lines of ocular origin reveal transcriptional regulatory elements close to or within the first exon and intron (see the description of the clarification of the genomic organization at the Rlbp1 locus). A VISTA genome browser (available at http://genome.lbl.gov/vista/index.shtml) alignment of human and mouse genomic DNA at the Rlbp1 loci reveals multiple regions of sequence identity greater than 50% within 1 kb of the transcriptional start site in the 5' intergenic region and in the first intron (Fig. 1B). To assess whether these regions are sufficient to drive gene expression in vivo, GFP cDNA was cloned adjacent to genomic DNA encompassing these conserved regions, and transgenic mice were generated by pronuclear injection.

Three transgenic founder lines were obtained, and two revealed detectable GFP expression in the retina. One of the GFP-expressing lines showed weak and sparse expression in Müller glia and no expression in RPE cells (data not shown). This line was not maintained. The remaining line, Rlbp1-GFP, exhibited robust expression and was characterized further. Retinal cross-sections show radially oriented GFP processes extending from the outer limiting membrane to the inner limiting membrane (Fig. 2A). These GFP processes also express classic Müller glial markers, including glial fibrillary acidic protein (GFAP), astrocyte and reactive Müller glia, and ganglion cell subclass (GOA; Fig. 2B). The expression of GFAP is not limited to Müller glia and is detected in 8-month-old mice (Fig. 2C). GFP fluorescence reveals a close anatomic proximity between photoreceptors and Müller glia; however, when cells are cut at an optimal plane, a space filled by GFP fluorescence is discernible along the entire central and peripheral extent of the retina and is detected in 8-month-old mice (Figs. 2D and 2E).

### Table 1. Primary Antibodies

<table>
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<th>Antigen</th>
<th>Target</th>
<th>Host</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td>Cyclin D1 (CCND1)</td>
<td>RPCs</td>
<td>Rabbit</td>
<td>Lab Vision, Fremont, CA</td>
</tr>
<tr>
<td>Cyclin D1 (CCND1)</td>
<td>RPCs</td>
<td>Mouse</td>
<td>Santa Cruz Biotechnology, Santa Cruz, CA</td>
</tr>
<tr>
<td>Cyclin D3 (CCND3)</td>
<td>Müller glia and RPE cells</td>
<td>Mouse</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Cyclin D3 (CCND3)</td>
<td>Müller glia and RPE cells</td>
<td>Rabbit</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Cyclin-dependent kinase inhibitor 1B (CDKN1B/p27KIP1)</td>
<td>Müller glia and RPE cells</td>
<td>Mouse</td>
<td>BD Transduction Laboratories, Franklin Lakes, NJ</td>
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<tr>
<td>Glial fibrillary acidic protein (GFAP)</td>
<td>Astrocytes and reactive Müller glia</td>
<td>Mouse</td>
<td>Eldon E. Geisert, The University of Tennessee College of Medicine, Memphis, TN</td>
</tr>
<tr>
<td>Glial fibrillary acidic protein (GFAP)</td>
<td>Astrocytes and reactive Müller glia</td>
<td>Rabbit</td>
<td>Lipshaw Corporation, Detroit, MI</td>
</tr>
<tr>
<td>Green fluorescent protein (GFP)</td>
<td>Green fluorescent protein</td>
<td>Chicken</td>
<td>Millipore, Billerica, MA</td>
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<tr>
<td>Green fluorescent protein (GFP)</td>
<td>Green fluorescent protein</td>
<td>Rabbit</td>
<td>Torrey Pines Biolabs, East Orange, NJ</td>
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<tr>
<td>Glutamine synthetase (GLUL)</td>
<td>Müller glia</td>
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<td>BD Transduction Laboratories</td>
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<tr>
<td>Paired box gene 6 (PAX6)</td>
<td>RPCs, ganglion, amacrine, horizontal cells</td>
<td>Mouse</td>
<td>DSHB, University of Iowa, Iowa City, IA</td>
</tr>
</tbody>
</table>

RPCs, retinal progenitor cells.
2A-I) and in mice older than 12 months (data not shown). These results demonstrate that Rlbp1-GFP expression in the retina is uniform, robust, and long-lasting to Müller glia.

To further determine the specificity of the Rlbp1-GFP transgene, we examined GFP expression outside the neural retina. RLBP1 is expressed by RPE cells, ciliary epithelial cells, and optic nerve oligodendrocytes. 16,36,37 Surprisingly, GFP is not detectable in RPE cells or optic nerve oligodendrocytes (Figs. 2J–O). However, ciliary epithelial cells expressed GFP at low levels. Rlbp1 mRNA is also expressed in other neural regions though at lower levels than in the eye, including olfactory bulb and forebrain (GNF Expression Atlas [available at http://ex-
expression.gnf.org] on Mouse Affymetrix [Santa Clara, CA] U74A Chip; probe set 92435_at). Consistent with this, we detected GFP in a limited cohort of oligodendrocytes and astrocytes in olfactory bulb and forebrain (data not shown). The sum of these observations indicates that robust GFP expression in this line is highly selective for Müller glia.

**Rlbp1-GFP Expression in the rd1 Retina**

To determine whether expression of the transgene is maintained in the degenerating retina, we examined GFP expression in the homozygous rd1 mouse, which carries a recessive mutation in the β-subunit cGMP phosphodiesterase gene and causes a complete loss of rod photoreceptor cells by 1 month of age.31,32 In these mice, GFP expression in Müller glia is detected during the early phases of degeneration (3- to 4-week-old rd1 mice; Fig. 3B) and late phases of degeneration (2- to 6-month-old rd1 mice; Fig. 3C). In 3-week-old rd1 mice, GFP is detected at the outer limiting membrane, nucleus, and endfeet and colocalizes with phosphorylated MAP kinase 1/3 (pMAPK1/3; Fig. 3E), an early marker of glial reactivity.38 As degeneration progressed, pMAPK1/3 expression was limited to the apical GFP branches (Fig. 3F). In 6-month-old rd1 mice, Müller glia displayed increasingly hypertrophic GFP branches toward the interface with RPE cells (Fig. 3C, arrows), where the outer limiting membrane is replaced with a glial seal.39 Furthermore, GFP is colocalized with glial fibrillary acidic protein (GFAP; Figs. 3H-I), a classic marker of glial reactivity.40 GFP expression during late stages of retinal degeneration is consistent with lower levels of endogenous RLBP1 expression in 6- and 12-month-old rd1 mice (data not shown).31 Together these data suggest that Müller glia express GFP in the absence of photoreceptors. Therefore, our observations demonstrate the usefulness of the Rlbp1-GFP line to visualize morphologic changes in Müller glia during degeneration.

**Rlbp1-GFP Expression during Postnatal Retinal Development**

Examination of retinas from several developmental stages revealed that GFP is expressed in the neuroblast layer (NBL) as early as postnatal day (P) 0 (Figs. 4A–C; green channel), which is consistent with endogenous RLBP1 expression in the developing retina.20,36,42 Given that most Müller glia do not differentiate until after P0,43 we sought to determine whether the reporter was expressed in retinal progenitor cells. At P0, GFP is colocalized with the glutamate transporter SLC1A3 (formerly GLAST; Fig. 4A) and is expressed in the same laminar positions as the retinal progenitor markers PCNA (Fig. 4B), SOX2 (Fig. 4C), CCND1 (data not shown), and OLIG2 (data not shown). From P5 through P11, GFP and retinal progenitor markers gradually become restricted to a band of cells in the inner nuclear layer, which are presumed to be the last remaining retinal progenitor cells and newly differentiated Müller glia (Figs. 4D–L). The GFP+, SOX2+ cells are presumed to be amacrine cells, as indicated by their laminar position, rounded nuclear morphology, and strong SOX2 expression (Das et al., manuscript submitted).44 Elsewhere in the developing retina, we did not detect GFP expression in RLBP+ RPE cells or in RLBP+ astrocytes at the optic nerve (data not shown).40 A
small cohort of astrocytes (GFAP<sup>+</sup> and RLBP1<sup>+</sup>)<sup>20</sup> at the ganglion cell layer displayed GFP fluorescence, but this decreased as development progressed (data not shown). These observations indicate that the Rlbp1-GFP transgene is continuously expressed in postnatal retinal progenitor cells and during their transition to Müller glia.

DISCUSSION

We found that a 3-kb region of mouse genomic DNA encompassing a portion of the Rlbp1 gene is sufficient to promote robust GFP expression in Müller glia. In transgenic mice, the temporal and spatial patterns of GFP<sup>+</sup> cells correspond to those of the endogenous Rlbp1 gene in the developing and adult retina. GFP expression is also maintained in Müller glia after photoreceptor degeneration. These features demonstrate the usefulness of using this portion of the Rlbp1 locus for driving gene expression in postnatal retinal progenitor cells and Müller glia.

In addition to Rlbp1, regulatory regions from other genes can also drive Müller glial gene expression in vivo. In transgenic mice, Gfap and Slc1a3 promoters drive expression in a subpopulation of Müller glia,<sup>45,46</sup> whereas the Pdgfra promoter drives expression in most, if not all, Müller glia.<sup>18</sup> Cd44 and Vim promoters can target transgene expression in Müller glia using lentiviral vectors.<sup>47</sup> However, Gfap, Slc1a3, Pdgfra, Cd44, and Vim promoters also drive expression in other neural cell types, including retinal astrocytes and optic nerve oligodendrocytes. Thus, a distinct feature of the 3-kb 5′ Rlbp1 fragment in transgenic mice is its homogeneous and robust activity in Müller glia.

Previous studies of RLB1 transcriptional regulation identified regions of human genomic DNA that are sufficient to drive reporter expression in cell lines derived from Müller glia, RPE cells, and ciliary epithelial cells.<sup>24,25,30</sup> Kennedy et al.<sup>24</sup> suggest that RLB1 transcription in RPE and ciliary epithelial cells require similar regulatory elements distinct from those important for Müller glia expression. Interestingly, the mouse
genomic DNA construct used in this study contained all the analogous regions tested by Kennedy et al., but we did not observe detectable GFP in RPE cells. Several possibilities could account for the difference between our results and those of previous studies. First, our study was performed in vivo, whereas other studies used cell culture paradigms. Second, the location in which the Rlbp1-GFP transgene integrated into the genome might have influenced its expression characteristics. Third, previous studies used human DNA, whereas we used mouse genomic DNA, and the required regulatory elements might differ between the two species. Fourth, our transgene contained a portion of the 5′ intergenic region that was not used in the previous studies. This region, located approximately 550 to 800 nucleotides upstream of the transcriptional start site, is conserved between human and mouse, as revealed by a VISTA alignment (Fig. 1B). Whether this region contains elements that repress in vivo expression in RPE cells will require further study.

Transcriptional activity of the 3-kb 5′ Rlbp1 fragment during development is consistent with findings that postnatal retinal progenitor cells and Müller glia have common attributes. Transcriptome-wide studies of postnatal retinal progenitor cells and Müller glia reveal a high degree of overlap in gene expression profiles and include genes such as Rlbp1, Slc1A3, Ca2, Dkk3, Slc38a3, and Clu. Finally, a growing body of evidence indicates that a population of mammalian Müller glia have neurogenic potential under certain conditions. After retinal injury, adult rodent Müller glia can reenter the cell cycle and can be induced to dedifferentiate and to express neuronal precursor markers. In this study, postnatal GFP + cells colocalized with proliferative markers (PCNA and CCND1) and neuronal progenitor markers (SOX2 and OLIG2). Similarly, in vivo electroporation of a 4-kb 5′ Rlbp1 fragment drove expression in rat retinal progenitor cells. The transcriptional activity of the 3-kb 5′ Rlbp1 fragment in cells that are dividing and in cells that display neurogenic potential makes this promoter a valuable tool to test and exploit the neurogenic potential of Müller glia. In sum, the identification of a Müller-specific regulatory sequence represents a step toward future targeted transgene expression in Müller glia of normal and diseased retinas.

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