Transgenic Mice Expressing Cre-Recombinase Specifically in M- or S-Cone Photoreceptors

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PURPOSE. To establish lines of transgenic mice that express Cre-recombinase in M- or S-cone photoreceptors for generating cone photoreceptor-specific (conditional) mutants.

METHODS. Five kilobases of 5′ upstream sequence of the mouse red-green (M) opsin gene or 0.5 kb of the mouse blue (S) opsin gene was cloned into a Cre-expression plasmid. Transgenic mice were generated and characterized, and appropriate lines were established. The Cre-transgenic mice were crossed with ROSA26-*lacZ* mice (containing floxed β-galactosidase gene) and analyzed to determine Cre-recombinase activity.

RESULTS. Immunofluorescence study showed successful targeting of Cre-recombinase expression to cone photoreceptors. Double staining with anti-Cre antibody and anti-M- or anti-S-opsin antibody revealed specificity of Cre expression in M-opsin– and/or S-opsin–positive photoreceptors. Mating with ROSA26-*lacZ* mice demonstrated that Cre-recombinase was functionally active in M- or S-cones.

CONCLUSIONS. Lines of transgenic mice that specifically express functional Cre-recombinase in M- or S-cones were established in this study. Because mutations in several widely expressed genes lead to photoreceptor degeneration, these transgenic mice should be valuable in generating conditional mutants to investigate the function of various genes specifically in cone photoreceptors. (*Invest Ophthalmol Vis Sci.* 2004;45:42–47) DOI:10.1167/iovs.03-0804

The retina of mammals has two kinds of photoreceptors: rods and cones. Rods contain the visual pigment rhodopsin and are responsible for vision under conditions of low ambient light. Cones bestow high visual acuity under bright-light conditions, and their different subtypes (each with unique visual pigment) constitute the basis of color vision. Rods dominate the mammalian retina. Cones represent only 3% to 5% of all photoreceptors in rodent and primate retinas.[1,2] Hence, despite the importance of cone function in vision, cone biology is greatly underexplored.

Defects in photoreceptor development and function are the major causes of inherited retinal degenerative diseases, which constitute a clinically and genetically heterogeneous group (RetNet; http://www.sph.uth.tmc.edu/Retnet/home.htm/ provided in the public domain by the University of Texas Houston Health Science Center, Houston, TX). In many instances, these diseases result from mutations in retina-specific genes; however, mutations in several widely expressed genes have also been identified in retinal disorders.[3–15] For example, the majority of patients with X-linked RP have mutations in RPGR or RP2, two ubiquitous proteins of unknown function.[3–11] One approach to delineating mechanisms of disease pathogenesis involves the generation and characterization of animal (particularly mouse) models. Gene targeting using homologous recombination (gene knockout strategy) offers a unique opportunity to produce mouse models of human disease.[16] Investigations of gene knockouts in mice have revealed significant insights into gene function during retinal differentiation and disease.[17–22] However, conventional techniques are generally not sufficient to evaluate cell type specific function of widely expressed genes. Embryonic lethality is often observed in mice harboring two copies of targeted nonfunctional alleles (or one copy on the X chromosome in males). In addition, the germ-line incorporation of the mutation may result in gene inactivation in all cells, leading to secondary, noncell autonomous phenotypes that may be difficult to distinguish from cell autonomous phenotypes. Moreover, complex systemic changes due to a targeted mutation may obscure more subtle phenotypes of the retina.

The Cre/loxP recombination system offers an opportunity to introduce the mutations in a tissue-specific or inducible fashion[16,23–25] (Fig. 1A). In mice, this approach has been successfully used to accomplish both cell-type–restricted activation of transgenes (TG) and generation of cell-type–restricted null alleles by the deletion of loxP flanked (floxed) gene segments.[16,23–25] Several lines of transgenic mice expressing Cre-recombinase in specific cell types (see http://www.mshri.on.ca/nagy/), including ocular tissues, have been established (Feiner L, et al. *IOVS* 2003;44:ARVO E-Abstract 3539).[26–31] As a prelude to cone photoreceptor-specific gene targeting, we have generated transgenic mouse lines that express Cre-recombinase under the control of mouse red-green (M) or blue (S) pigment gene promoters. These Cre-transgenic mouse lines will be valuable for studying the in vivo function of genes in cone photoreceptors using a conditional gene targeting strategy.

MATERIALS AND METHODS

All reagents, buffers, or enzymes were purchased from Invitrogen (Carlsbad, CA), New England BioLabs (Beverly, MA), or Sigma-Aldrich (St. Louis, MO).

FIGURE 1. Cre/loxP strategy and the transgene constructs. (**A**) Schematic representation of the Cre/loxP system (see van der Weyden et al.[16] for a review). Cre-recombinase activity introduced in a cell-specific manner excised the region flanked by loxP sites. (**B**) Schematic representation of ROSA26-*lacZ* Cre reporter system. Cre-recombinase excises stuffer sequences, inducing β-galactosidase gene expression. (**C**) BP- and RGP-Cre transgenes. *White box*: 500 bp of blue pigment gene promoter and 5.0 kb of red and green pigment gene promoter; *black box*: the Cre-coding region; *gray box*: simian virus (SV) 40 polyadenylation signal; *Arrows*: PCR primers used for genotyping the mice; H: *Hinc*II sites. (**D**) Representative results of genotyping by Southern blot analysis. *Hinc*II digests at two adjacent sites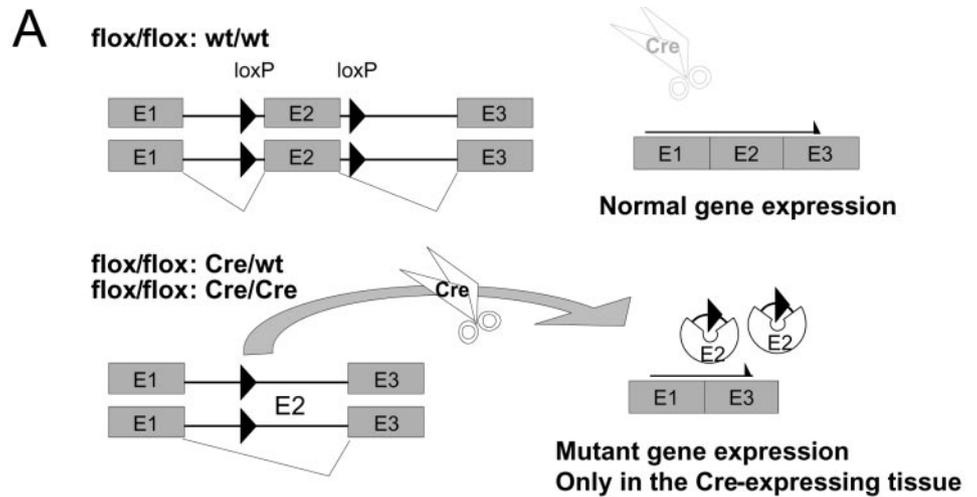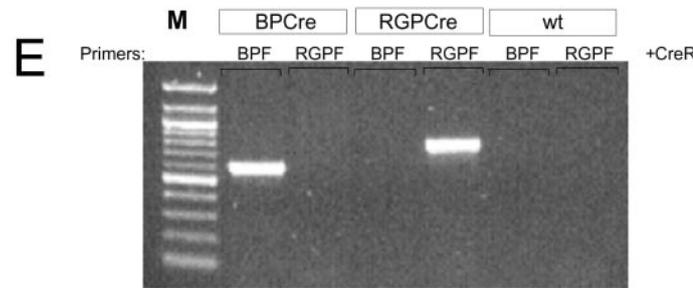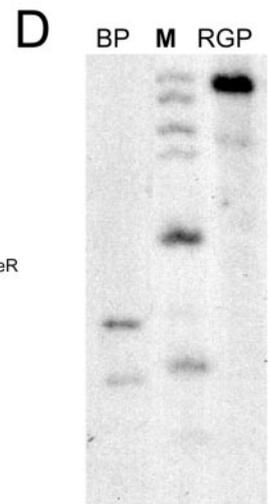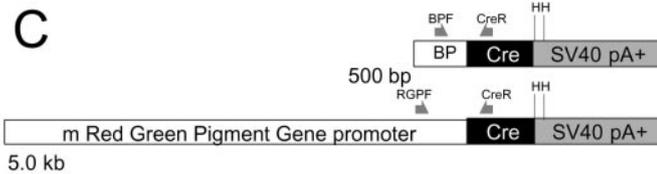 in the transgene constructs. The 2-kb band in BP-Cre and the 7-kb band in RGP-Cre represent the transgene fragment. *Lane M*: 1-kb ladder. (**E**) Representative results of genotyping by PCR. A 768-bp product was obtained for RGP-Cre and 600 bp for BP-Cre. *Lane M*: 100-bp ladder.

Transgene Vectors and Generation of Transgenic Mice

We generated a promoterless pCI vector (pCIpl) by excision of the cytomegalovirus (CMV) promoter from the pCI expression vector (Promega, Madison, WI) by *Msc*I and *Nhe*I endonuclease digestion, followed by blunt-ending and self-religation. A promoterless Cre vector (pCICre) was generated by insertion of a 1.1-kb *Mlu*I fragment from pMCCre[23] into the *Mlu*I site of pCIpl. We then amplified mouse red-green (GenBank accession S44742; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD) or blue (GenBank accession L27831) pigment gene promoter region by PCR using genomic DNA from the R1 mouse strain. The primers used were: forward 5′-CTAGCTAGCATACCTTGAAACCCACA-3′ and reverse 5′-CGCCTC-GAGGCTGTAGAAAACTG-3′ for the mouse red-green pigment gene (mRGP) promoter; and forward 5′-GGCAGGATGCAGTTGTTTCT-3′ and reverse 5′-TCCCGCTTGGGATGCCCCT-3′ for the mouse blue pigment gene (mBP) promoter. The 5.0-kb PCR product of the mRGP promoter and the 500 bp of the mBP promoter were subcloned into the pGEM-T easy vector (Promega, Madison, WI), digested by *Eco*RI, and then cloned into the pCICre vector. We excised the mRGP promoter driving Cre-recombinase or the mBP promoter driving the Cre-expression cassette from recombinant plasmids by *Nhe*I and *Nae*I digestion or *Xho*I and *Nae*I digestion, respectively (Fig. 1C). After purification (NucleoSpin; Clontech, Palo Alto, CA), each fragment was injected into the pronuclei of (C57BL/6 X SJL) F2 mouse eggs, which were implanted into pseudopregnant foster mothers using standard techniques. Transgenic founder mice and their progeny were identified by PCR using the following primers: forward RGPF: 5′-AATGG-GAACAGTGGTGTGTG-3′; BPF: 5′-AGGAGGGTGCTGTAGGGAAG-3′; reverse (CreR): 5′-GAACGAACCTGGTCGAAATC-3′. Southern blot analysis of *Bam*HI- or *Hinc*II-digested genomic DNA or dot blot analysis was performed by hybridization with a 1.1-kb Cre gene probe,
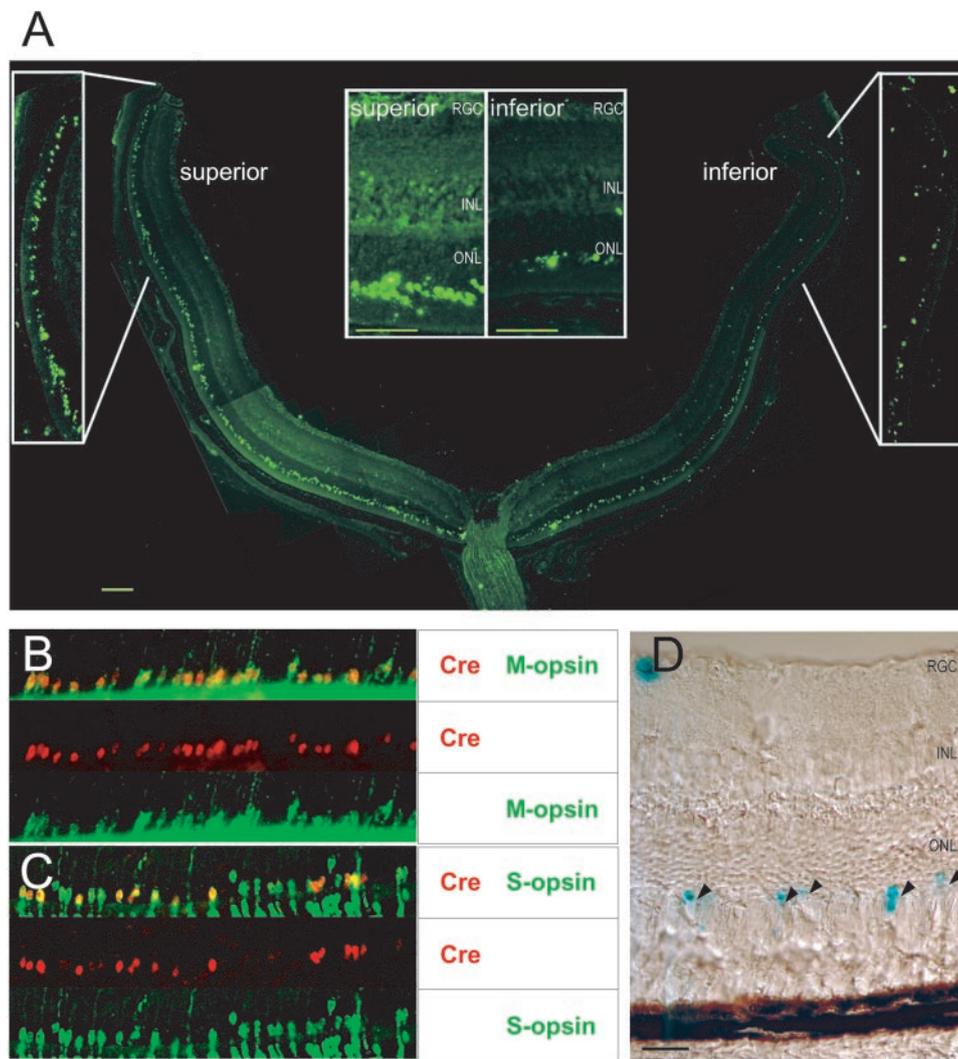
FIGURE 2. (A) Immunofluorescence of Cre immunoreactivity with rabbit anti-Cre polyclonal antibody in RGP-Cre transgenic mouse retina. *Insets*: immunostaining at higher magnification. RGC, retinal ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. (B, C) Double staining with mouse anti-Cre monoclonal antibody and rabbit anti-mouse-S-opsin or anti- mouse-M-opsin polyclonal antibody. (D) X-gal staining of retina from ROSA26-*lacZ* and RGP-Cre double-transgenic mice. *Arrowheads*: *blue* staining of cone photoreceptors. Scale bars, 50 μm.

excised by *Mlu*I from pMCCre, and copy numbers were estimated. Founders were bred to C57BL/6 mice to generate F1 progeny.

The research reported herein was performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the institutional review committee.

Immunohistochemical Analysis of Cre-Recombinase and β-Galactosidase

Mouse eyes were fixed for 1 to 2 hours with fresh 4% paraformaldehyde in 100 mM phosphate buffer (pH 7.2) and immersed overnight at 4°C in 20% sucrose in phosphate-buffered saline (PBS). Next day, the eye cups were infiltrated in a solution containing a 2:1 ratio of 20% sucrose-PBS and optimal cutting temperature compound (OCT; Tissue-Tek; Sakura Finetek, Torrance, CA), embedded in 100% OCT, and frozen. Sections (10 or 25 μm thick) were cut and mounted on silanized slides, dried, and kept in a freezer at −80°C until use. A polyclonal rabbit (at 1:500 dilution) or mouse (at 1:500 dilution) monoclonal anti-Cre antibody (Covance, Madison, WI) was used as the primary antibody. For double staining, a rabbit polyclonal M- or S-opsin antibody (at 1:500 dilution) was used.[21] Secondary antibodies were Alexa Fluor 488–conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR) and Cy3-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) antibodies.

To evaluate the activity of Cre-recombinase, Cre-mice were mated to ROSA26-*lacZ* mice,[32] in which the β-galactosidase gene is knocked in. In the resultant mice, cells with functional Cre-recombinase should express β-galactosidase driven by the ROSA26 promoter (Fig. 1B). 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining was performed by the following protocol.[33] Eyes were prefixed by immersion in 4% paraformaldehyde and PBS for 2 hours, washed in cold PBS, and incubated in PBS containing 1 mg/mL of X-gal, 0.01% sodium deoxycholate, 0.02% NP40, 2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, and 5 mM $K_4Fe(CN)_6$ overnight at 37°C. Each sample was embedded and sectioned and then observed by conventional microscopy.

RESULTS

Generation of Transgenic Mice

Eight founder animals of RGP-Cre and seven of BP-Cre were identified by PCR and Southern blot analysis. Each founder was bred to C57BL/6 mice to generate F1 progeny. Four of the eight lines of RGP-Cre and two of the seven lines of BP-Cre showed transgene expression. Typical results of genomic Southern blot analysis with *Hin*cII digestion are shown in Figure 1D. Genomic PCR generated the expected 768-bp band for RGP-Cre transgenic mouse and a 600-bp product for BP-Cre (Fig. 1E). Transgene inheritance was confirmed through more than five generations.

Immunostaining of Cre and M- or S-Opsin

The expression of Cre-recombinase protein was examined by immunohistochemical methods. Both polyclonal and monoclo-

Figure 3. (**A**) Immunofluorescence of Cre-immunoreactivity against rabbit anti-Cre polyclonal antibody in BP-Cre transgenic mouse retina. Background is higher than in Figure 2A because of low signal intensity. *Insets*: immunostaining at higher magnification. RGC, retinal ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. (**B**, **C**) Double staining with mouse anti-Cre monoclonal antibody and rabbit anti-mouse-S-opsin or anti-mouse-M-opsin polyclonal antibody. (**D**) X-gal staining of retina from ROSA26-*lacZ* and BP-Cre double-transgenic mice. *Arrowheads: blue* staining of cone photoreceptors. Scale bars, 50 μm.

nal anti-Cre antibodies revealed similar cone-specific staining. The retinas of RGP-Cre and BP-Cre transgenic mice show the inferior to superior gradient distribution of Cre-positive cells, which is similar to the distribution of normal M- or S-cone photoreceptors[34] (Figs. 2A, 3A). Higher background was consistently detected in BP-Cre mouse retina compared with RGP-Cre, due to differences in expression level. M- or S-cone specificity was examined by double staining with anti-Cre antibody and either M- or S-opsin antibody. In RGP-Cre transgenic mice, Cre and M-opsin immunostaining overlapped completely, whereas S-opsin staining demonstrated a partial overlap (Figs. 2B, 2C). In BP-Cre transgenic mice, Cre and S-opsin immunostaining overlapped completely, and M-opsin staining overlapped partially (Figs. 3B, 3C). This is consistent with previous findings that in mouse many cone photoreceptors express both M- and S-opsin.[35]

X-Gal Staining

To examine the enzymatic activity of Cre-recombinase in cone cells, Cre-transgenic mice were mated with ROSA26-*lacZ* mice, and the litters were analyzed. In both RGP- and BP-Cre mouse retinas, cone photoreceptors stained blue, demonstrating functional Cre-recombinase. Several cells in the retinal ganglion cell (RGC) layer and a few dispersed cells in the INL were also positive (Figs. 2D, 3D).

Discussion

Diseases exhibiting clinical phenotypes of the retina can result from mutations in genes that are expressed in multiple tissues and cell types. The conditional gene inactivation or activation strategy using the Cre/loxP system offers an attractive method to investigate gene function and/or disease pathogenesis focusing on cell-type–specific effects. One excellent example of this is the elucidation of KIF3A function in photoreceptors.[27] Our goal in this study was to create Cre-recombinase–expressing mouse lines that could be used to conditionally knockout (or activate) the expression of loxP-modified genes in only M- or S-cones to understand their specific function in cone photoreceptors. Herein, we describe the successful generation of RGP-Cre and BP-Cre transgenic mice, where Cre-recombinase is under the transcriptional control of the mouse red-green (M) or blue (S) opsin gene promoter, respectively.

Our data support previous studies showing that the 6.5-kb upstream region of the human red-green visual pigment gene can direct transgene expression specifically to M-cone photoreceptors,[36,37] and that the 500-bp upstream region of the human blue pigment gene can target expression to S-cone photoreceptors.[38,39] The human S-opsin promoter also directed the reporter gene expression to bipolar cells in addition to S-cones,[38,39] though the 6.4-kb upstream region of mouse S-opsin promoter was specific for S-cones.[40] In our studies, the

mouse 500-bp promoter directed reporter gene expression primarily to S-cones; hence, this sequence in mouse but not human promoter may include the minimal S-cone–specific element. The detection of Cre-transgene expression in S-opsin–positive cells of RGP-Cre– and M-opsin–positive cells of BP-Cre mouse retina is consistent with a previous study that demonstrated coexpression of both M- and S-opsins in many cone photoreceptors in mice.[35]

Retina from both RGP-Cre and BP-transgenic mice showed *lacZ* expression in a subset of cells in the ganglion cell layer, even though Cre immunoreactivity was not observed. This discrepancy may result from the difference in expression levels and the sensitivity of detection. Even a low level of Cre expression, undetectable by immunofluorescence, may excise the floxed region and activate β-galactosidase expression. A recent study demonstrated the existence of a subpopulation of photoreceptor and cone bipolar cells displaced to the ganglion cell layer.[41] We have not characterized X-gal–positive cells in the RGC layer in Cre-transgenic mice as yet, but these cells may represent displaced photoreceptors and/or cone bipolar cells. Alternatively, these cells may indicate residual background staining as a previous report implied.[42] Whatever the cause, the small amount of ectopic expression should not affect the usefulness of the Cre-transgenic mice.

The Cre-transgenic mouse lines reported herein will permit specific deletions of loxP-harboring genes in M- or S-cone photoreceptors, overcoming problems encountered in conventional gene-knockout techniques. Several examples of the potential use of these Cre-transgenic mice can be illustrated. Mutations in the *RPGR* and *RP2* genes are the primary cause of X-linked retinitis pigmentosa, a relatively severe form of retinal degenerative disease.[3–11] Although mouse *Rp2*-knockout has not yet been generated, some of the human *RPGR* mutations[6,7] and the only published mouse *Rpgr* knockout model[22] demonstrate cone photoreceptor degeneration. Conditional disruption of *Rp2* and *Rpgr* in cone photoreceptors will therefore provide significant new insights into their role in cone biology. Similarly, many transcription factors, including several homeodomain proteins and nuclear receptors, exert a major influence on retinal development. The RGP- and BP-Cre mice that we have generated should be valuable for delineating the specific function of these and other widely expressed proteins in cone photoreceptors.

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