TRANSGENIC MICE EXPRESSING CRE-RECOMBINASE SPECIFICALLY IN RETINAL ROD BIPOLAR NEURONS

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PURPOSE. To establish a transgenic mouse line that expresses Cre-recombinase in retinal rod bipolar cells for the generation of rod bipolar cell-specific knockout mutants.

METHODS. The IRES-Cre-eDNA fragment was inserted into a 173-kb bacterial artificial chromosome (BAC) carrying the intact Pcp2 gene, by using red-mediated recombineering. Transgenic mice were generated with the modified BAC and identified. The Cre-transgenic mice were crossed with ROSA26 and Z/EG reporter mice to detect Cre-recombinase activity.

RESULTS. X-gal staining showed that strong Cre-recombinase activities were present in retinal inner nuclear layers and cerebellar Purkinje cells. Double staining with an anti-GFP antibody and an anti-PKc antibody (specific for retinal rod bipolar cells) revealed that Cre-recombinase activity localized exclusively to the rod bipolar cells in the retina.

CONCLUSIONS. A mouse BAC-Pcp2-IRES-Cre transgenic line that expresses Cre-recombinase in retinal rod bipolar neurons has been established. Because mutations in some ubiquitously expressed genes may result in retinal degenerative diseases, the mouse strain BAC-Pcp2-IRES-Cre will be a useful new tool for investigating the effects of retinal rod bipolar cell-specific gene inactivation. (Invest Ophthalmol Vis Sci. 2005;46:3515–3520) DOI:10.1167/iovs.04-1201

The rod pathway in mammalian retina, consisting of rod photoreceptors and rod-connected neurons, is responsible for night vision and is most useful in scotopic conditions. In the outer plexiform layers (OPLs), rod photoreceptors form synapses with rod bipolar cells. In the inner plexiform layers (IPLs), rod bipolar cells make connections with amacrine cells, which spread out the rod signals before reaching the ganglion cells. Degeneration of rod photoreceptors leads to night blindness as in the familial inherited ocular disease retinitis pigmentosa (RP) in humans.1 Several animal models of rod photoreceptor degeneration have been established.2,3

The appropriate arrangement of different neurons during development depends on pre- and postsynaptic connectivity. In the absence of rod photoreceptors, rod bipolar cells form ectopic synaptic connections with cone photoreceptors,4,5 illustrating the capability for synaptic rewiring of a second-order neuron in response to deafferentation, the loss of pre-synaptic connections. Similarly, postsynaptic neurons may also play a crucial role in the establishment or refinement of projections and in the survival of afferent neurons.6,7 To our knowledge, it is not known whether ectopic synaptogenesis will occur in the rod photoreceptor and amacrine cells in the absence of rod bipolar neurons. It also remains unclear how the absence of rod bipolar cells contributes to retinal degenerative disease. We therefore sought to establish a Cre-transgenic mouse line in which rod bipolar cell-specific genetic manipulation can be performed. For example, these animals could be used to generate mice lacking rod bipolar cells by crossing with a toxic gene that would kill the cells expressing it.8 This model would allow an evaluation of aberrant synaptic connections that might form in the absence of rod bipolar cells. Furthermore, this mouse line can be used to investigate the roles of rod bipolar cells in retinal diseases and the functions of specific proteins in rod bipolar cells.

Disease studies based on gene-targeting approaches in the mouse have yielded remarkable advances in the understanding of roles played by specific gene products in mammalian development and adult physiopathology. The efficient introduction of somatic mutations in a given gene, at a given time, in a specific cell type, facilitates studies of gene function and the generation of animal models for human diseases. Strategies for conditional gene targeting in mice are based on cell type-specific expression of a bacteriophage P1 site-specific recombinase, Cre. Cre-recombinase can efficiently excise a DNA segment flanked by two LoxP sites (floxed DNA) in animal cells.9 Spatially and temporally controlled somatic mutations can be obtained by placing the Cre gene under the control of a cell-specific promoter. Through temporal control, there would be no effect during development from embryonic to adult stage until the gene is inactivated at the desired time. Through spatial control, only a specific cell lineage would be affected when cell-specific gene inactivation is achieved, without affecting multiple tissues.10,11

The Purkinje cell protein2 (Pcp2, also known as L7) is expressed only in cerebellar Purkinje cells and retinal rod bipolar cells.12–14 The function of Pcp2/L7 remains unclear, though it has been suggested that bipolar cells may provide a trophic supply of the protein to other cells in the retina.14 Analysis of null mutations of Pcp2 mice and minigene transgenic mice also suggests that Pcp2 plays a role in the development and specific functions of Purkinje cells and bipolar neurons.15–17 Since Pcp2/L7 is one of the most restricted markers in rod bipolar cells, it is used in this study as the control element to direct expression of Cre-recombinase specifically to rod bipolar cells. We chose to direct Cre expression using a bacterial artificial chromosome (BAC) carrying the entire intact Pcp2 gene, since its large size may protect the transgene from

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To our knowledge, this is the first nase activities are present in the retinal bipolar neurons and
BAC-Pcp2
Gray boxes
Pcp2
BAC-Pcp2-IRES-Cre
XPA
Pcp2
Stxbp2
10
1
Cre-specific primers were used to detect the integration of the Cre gene into the mouse genome. Bottom: primers for an endogenous gene were used as an internal PCR control. Arrows: expected product sizes.

being influenced by a nearby locus. The Pcp2-containing BAC used in these experiments was estimated to be 173 kb, by BAC end sequencing with Sp6 and T7 primers. An IRES-Cre was inserted into Pcp2 exon 4, between the stop codon and poly(A) site of Pcp2 by red-mediated recombinering26 (Fig. 1A).

Using the BAC-Pcp2-IRES-Cre construct, we established a mouse transgenic line and showed that strong Cre-recombinase activities are present in the retinal bipolar neurons and cerebellar Purkinje cells. To our knowledge, this is the first mouse line that can be used to generate conditional knockout mice in rod bipolar cells. It will be an invaluable tool for future studies of retinal function.

**Materials and Methods**

All materials for general biochemical work, such as reagents, buffers, and enzymes were purchased from New England Biolabs (Beverly, MA), Sigma-Aldrich (St. Louis, MO), Invitrogen-Gibco (Carlsbad, CA), and Qiagen (Valencia, CA), unless otherwise indicated. All protocols involving the use of mice adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Mouse Lines**

The BAC-Pcp2-IRES-Cre transgenic mouse lines were established at the National Cancer Institute (Frederick, MD). The ROSA2620 and Z/EG20 mice used to monitor Cre expression were provided by Philippe Soriano.

To obtain embryos at different stages, male BAC-Pcp2-IRES-Cre transgenic mice were placed in the same cage with female ROSA26 or Z/EG reporter mice for mating on the first day. Vaginal plugs were observed the next morning and defined as 0.5 days postcoitus. The embryos were isolated from the uterus as described elsewhere.21

**BAC-Pcp2-IRES-Cre Construction and Generation of the Transgenic Mouse Lines**

BAC 467J9 containing the entire Pcp2 gene was purchased from Children’s Hospital Oakland-BACPAC Resources. The IRES-Cre-frt-Kan2frt fragment was obtained from plasmid pICGN21,22 but modified by removing the EGFP gene. Two homologous arms of 200 and 140 bp from exon 4 of Pcp2 were inserted into both sides of the IRES-Cre-frt-Kan2frt cassette in the pICGN21-noEGFP plasmid. The IRES-Cre-frt-Kan2frt cassette was introduced into the Pcp2 gene downstream of the Pcp2 stop codon and upstream of its poly(A) site by using red-mediated homologous recombination,18 followed by flp-mediated removal of the Kan2 selectable marker from the BAC-Pcp2-IRES-Cre construct (Fig. 1A). PCR, restriction digestion, Southern blot analysis, and sequencing were used to confirm the correct insertion of IRES-Cre gene into Pcp2 BAC (data not shown).

The BAC-Pcp2-IRES-Cre construct was purified with the BAC DNA purification method used for microinjection.27 The purified constructs were microinjected into the pronuclei of (C57BL/6NCr × C3H/HeN-Crt ON) F2 mouse zygotes, which were implanted into pseudopregnant foster mothers by using standard techniques. The transgenic founder mice and their progeny were identified by Southern blot analysis (data not shown) and PCR with Cre-specific primers (OY21-CRE-F: 5’-GGCAGTAAAAACTTACCG3’; and OY23-CRE-R: 5’TCCGGTATGAAAACTCCAGG3’). Primers MPG1 (5’-CACTTTGTTGGTCAAAAGCC-3’) and MPG2 (5’TCTCTGTTTAAGGATGAG-3’) were used as the PCR control. The expected PCR products were 650 bp with the Cre-specific primers and 180 bp with the PCR control primers (Fig. 1B).

**Analysis of Cre-Recombinase Activities**

To evaluate the activity of Cre-recombinase, we mated the BAC-Pcp2-IRES-Cre transgenic mice to ROSA26 or Z/EG reporter mice and genotyped their offspring with Cre-specific primers. β-Galactosidase driven by the ROSA26 promoter should be expressed in the cells with functional Cre-recombinase.28 β-Galactosidase-expressing cells can be identified with Xgal staining. In the Z/EG reporter line, the Cre-mediated excision will activate the expression of enhanced green fluorescent protein (EGFP),29 which can be directly observed under a fluorescence microscope or identified with an anti-GFP antibody.

**Tissue Preparation**

Postnatal mice from postnatal day (P)2 to 5 months were anesthetized with an overdose of sodium pentobarbital (60 mg/kg body weight intraperitoneally) before death. Both the left and right eyelids were enucleated and placed in 2% paraformaldehyde (in 0.1 M phosphate buffer [pH 7.4]). The left retinas were carefully separated from the eyeballs. The wholemount retinas were postfixed in the same fixative for 1 hour at room temperature and then transferred to 0.1 M phosphate buffer (PB; pH 7.4) at 4°C until they were processed for Xgal staining. The corneas and lenses were removed from the right eyeballs.
Figure 2. Characterization of Cre activity in the retina of BAC-Pcp2-IRES-Cre×ROSA26 transgenic mice. (A, B) Wholemount X-gal staining of the retina of an 8-week-old BAC-Pcp2-IRES-Cre×ROSA26 transgenic mouse. (A) X-gal staining showed the distribution of Cre-positive cells. S, superior; I, inferior; N, nasal; T, temporal. The area within the box in (A) is shown at higher magnification in (B). The images in (A) and (B) are viewed from the inside/front of the retina. (C, D) Cross section of BAC-Pcp2-IRES-Cre×ROSA26 transgenic mouse retina. Cross-retinal sections from BAC-Pcp2-IRES-Cre×ROSA26 transgenic mice were subjected to X-gal staining, followed by counterstaining with neutral red. (D) A portion of (C) shown at higher magnification. Arrows: blue-stained areas showing Cre activity. The Cre positive cells are located in the outer margin of the inner nuclear layer (INL). OD, optic disc; GCL, ganglion cell layer; IPL, inner plexiform layer; OPL, outer plexiform layer; ONL, outer nuclear layer. Scale bars: (A) 300 μm; (B, D) 50 μm; (C) 200 μm.

The posterior eyecups were immersion fixed in 2% paraformaldehyde (in 0.1 M PB [pH 7.4]) for 4 hours at 4°C. After several washes in 0.1 M PB, the eyecups were transferred to 30% sucrose in 0.1 M PB (in 0.1 M PB [pH 7.4]) for 4 hours at 4°C. After several washes in 0.1 M PB, the eyecups were embedded in optimal cutting temperature compound (OCT; Tissue-Tek, Miles Inc., Elkhart, IN), frozen, and cut into 10-μm transverse sections with a cryostat. Sections were collected on gelatinized slides, air dried, and stored at −20°C until they were processed for X-gal staining and immunostaining.

X-gal Staining
To evaluate the activity of Cre-recombinase in the mouse retina, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining was performed. Sections were fixed in 4% paraformaldehyde and 0.2% glutaraldehyde in 50 mM EGTA, and 100 mM MgCl2 in PBS [pH 7.3] for 10 minutes at room temperature. After fixation, three 5-minute rinses with wash buffer (2 mM MgCl2, 0.02% NP-40, and 0.01% sodium deoxycholate in PBS) were performed at room temperature. Sections were stained using X-gal solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 0.5 mg/ml X-gal powder in a washing buffer) at 32°C overnight with protection from light. After they were stained, the sections were counterstained with 1% neutral red for better observation and then dehydrated and coverslipped (Permount; Fisher Scientific, Fair Lawn, NJ).

Immunofluorescent Double Staining of EGFP and PKCa
For immunofluorescent double staining, the retinal transverse sections were blocked in 10% normal donkey serum (Chemicon, Temecula, CA), 1% bovine serum albumin, and 0.3% Triton X-100 in 0.01 M PBS (pH 7.4) for 1 hour at room temperature. After blocking, the sections were incubated overnight at 4°C with a mixture of primary antibodies (the rabbit anti-GFP polyclonal antibody [Abcam, Cambridge, UK]) diluted 1:200 and the mouse monoclonal antibody against PKCa (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:800. After several washes in 0.01 M PBS, the sections were incubated for 2 hours at room temperature with a mixture of secondary antibodies (Alexa Fluor 488-conjugated donkey anti-rabbit IgG diluted 1:800 and Alexa Fluor 594-conjugated donkey anti-mouse IgG diluted 1:800; both from Mo-
RESULTS

Generation of Transgenic Mice

A total of 16 BAC-Pcp2-IRES-Cre transgenic mice from 4 independent BAC founder lines (TG3551, TG3555, TG3557, and TG3531) were collected and backcrossed to C57B6 mice for six generations, to confirm transgene inheritance before analysis of the Cre expression pattern. They were then mated to ROSA26 reporter mice. The genotypes of the progeny were determined by Southern blot analysis, with a 1-kb Cre fragment used as a hybridization probe (data not shown) and by PCR with primers OYY21-CRE-F and OYY23-CRE-R, which amplify a 650-bp fragment of the Cre gene. Primers MPG1 and MPG2, which amplify a 180-bp fragment of the myogenin gene, were used as the PCR control (Fig. 1B). Offspring from all the lines displayed Cre activity, as determined by X-gal staining (data not shown).

Detection of Cre Activities in Retina by X-Gal Staining

The mouse line TG3555 was selected for further analysis because it was the first to produce progeny. TG3555 mice were mated to ROSA26 reporter mice. Cre-recombinase activities were detected by X-gal staining and observed with conventional microscopy of wholemount and transverse sections of retinas from the progenies carrying both the BAC-Pcp2-IRES-Cre transgene and the ROSA26 reporter. Cre and ROSA26 double transgenic mice were identified by PCR. Littermates lacking the Cre transgene served as the control.

From X-gal staining of wholemount retinas, our results showed that strong Cre activities were present in the retinas with a salt and pepper pattern (Figs. 2A, 2B). To determine in which layer the Cre-positive cells were located, transverse sections of the eyeball were stained by X-gal histochemistry followed by counterstaining with 1% neutral red. The results showed that blue X-gal-positive cells were located in the outer margin of the inner nuclear layer (INL) of the BAC-Pcp2-IRES-Cre mouse retina (Figs. 2C, 2D).

Because the Pcp2 gene is known to express in cerebellar Purkinje cells, Cre activity was also examined in the cerebellum of the BAC-Pcp2-IRES-Cre transgenic line. Cre expression was detected in Purkinje cells, but not in any other tissues examined, including liver, heart, cornea, and kidney (data not shown).

Localization of Cre Activities in Rod Bipolar Cells

In the retina, the Pcp2 gene is known to express only in the rod bipolar cells. To determine whether the Cre-recombinase activities are also restricted to rod bipolar cells, we used the progenies from BAC-Pcp2-IRES-Cre×Z/EG mating, and the rod bipolar cells were identified selectively with the antibody against protein kinase C (anti-PKCα). An array of green fluorescent signals characteristic of the EGFP protein were detected in the inner retina by fluorescence microscopy (Fig. 3A'). Higher-magnification images showed the colocalization of EGFP and PKC (Fig. 3B). To confirm the EGFP signal further, we stained the retina with anti-GFP and anti-PKCα antibodies. Colocalization of EGFP and PKC was again observed, demonstrating that Cre activities were localized to retinal rod bipolar cells (Figs. 3C–F). Nontransgenic mice were used as the negative control (Fig. 3E). The retinal pigment epithelial layer was nonspecifically stained by the anti-GFP antibody in both transgenic and nontransgenic animals (Figs. 3C–E), whereas the INL was specifically stained in the transgenic mice only.

The anti-GFP immunofluorescent staining pattern was similar to that of the X-gal staining except for the nonspecific epithelial staining (Figs. 2C, 3D), showing that Cre-recombinase was restricted to a single cell layer (Figs. 3A, 3A', 3C) within the inner nuclear layer (INL). More specifically, the Cre-positive cell bodies formed a tightly packed row one to two cells thick and situated in the outer region of the INL, with short wispy projections extending into the outer plexiform layer (OPL) and longer tapering projections extending toward the inner plexiform layer (IPL; Figs. 3F). All the Cre-positive neurons were double-labeled with anti-PKCα (Figs. 3B, 3F), demonstrating that Cre-recombinase was expressed in rod bipolar cells. However, it is observed that not all the PKC-positive cells are EGFP positive (Figs. 3B, 3F). Rod bipolar cells with Cre activities extended their dendrites into the invaginations of the rod terminals, forming synapses in the OPL as shown in Figures 3B and 3F. It is also clear that the rod bipolar axons terminate at the far periphery of the IPL, adjacent to the GCL (Figs. 3B, 3F), presumably forming synapses with all or other amacrine cells.

Expression of Cre Activities in BAC-Pcp2-IRES-Cre Transgenic Mice during Development

To determine the onset of Cre activities during bipolar cell development, we examined embryos at embryonic day (E)17.5, neonates, and mice from P2 to adult, by using X-gal staining. No blue staining was observed from E17.5 to P0 from the BAC-Pcp2-IRES-Cre×ROSA26 mating. Cre-positive cells were first observed in the retina from P7, and the number of Cre-positive rod bipolar cells increased gradually with development. Approximately 1%, 5%, 10%, 80%, and 90% of rod bipolar cells were observed to be X-gal positive at 1, 2, 3, and 8 weeks and 4 months of age, respectively (Figs. 2, 4).

DISCUSSION

We have created a BAC-Pcp2-IRES-Cre transgenic mouse line, in which Cre recombinase is under the transcriptional control of the mouse Pcp2 gene. Our results show that Cre activities were restricted to the cerebellar Purkinje cells and retinal rod bipolar neurons. These data are consistent with those in previous studies that showed that the Pcp2 gene is expressed only in cerebellar Purkinje cells and retinal rod bipolar neurons.12-14,25 Previously, a short Pcp2 DNA fragment was used to direct Cre expression in a mouse transgenic line, L7.17 However, few bipolar neurons were shown to be Cre positive in L7 mice. Furthermore, significant nonspecific staining was detected in various tissues other than the cerebellum.12,13,15-17 In the transgenic mouse line described herein, we chose to direct Cre expression using a bacterial artificial chromosome (BAC) carrying the entire Pcp2 gene, because its large size may protect the transgene from being influenced by a nearby locus. The extraregulatory sequences carried on the BAC may help ensure that Cre will be expressed in the same manner as the endogenous gene, in this case Pcp2. Indeed, a high level of Cre activities was detected only in Purkinje and rod bipolar cells. This work has further demonstrated the use of BAC-Cre transgenes generated by red-mediated recombineering.25,26

During development, retinal cells are born in sequence. The order is defined by the day when the different types of cells undergo their last S-phase, when examined by [3H]thymidine labeling and autoradiography.27,28 Immunolabeling with PKC, the specific cell marker of rod bipolar cells,29 has also provided data about the number and position of rod bipolar cells at different developmental stages. In the mouse retina, it has been
found that PKC-immunoreactive bipolar cells develop postnata-
tally, becoming distinguishable at P7. In the adult mouse
retina, anti-PKCo-immunoreactive cells were present in the
INL with projections extending into the outer and inner plex-
iform layer. In BAC-Pcp2-IRES-Cre transgenic mice, α-galactosidase
positive cells increased gradually un-
til adulthood, when most of the rod bipolar neurons were
to become EGFP positive, as was observed in β-galactosidase-
positive cells. We cannot rule out that the observed differences
between ROSA26 and Z/EG mice is due to the use of promot-
ers of different strength to drive the reporter genes. It is also
possible that the nature of the reporter proteins, such as
different sensitivities and half-lives, gave rise to the observed
variations.

This BAC-Pcp2-IRES-Cre transgenic mouse line can be used to
generate rod bipolar cell–specific conditional knockout
mice for the study of gene functions in postnatal rod bipolar
cells and to activate the expression of a cytotoxic protein to
 ablating the rod bipolar neurons in an age-dependent manner.
This transgenic line will be a valuable tool for the study of
the development, function, and physiology of retinal rod bipolar
cells.

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