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Genetic analysis of the homeodomain transcription factor Chx10 in the retina using a novel multifunctional BAC transgenic mouse reporter

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Abstract

Chx10 is a homeobox-containing transcription factor critical for progenitor cell proliferation and bipolar cell determination in the developing retina. Its expression in the retina has been reported to be restricted to these cell populations. To further understand Chx10 regulation and function, a multifunctional reporter construct consisting of GFP, alkaline phosphatase, and Cre recombinase was integrated into a BAC encoding Chx10. Stable lines of transgenic mice expressing this BAC were generated and analyzed. The reporter expression was faithful to the endogenous retinal Chx10 expression pattern and revealed a previously unappreciated locus of Chx10 expression in a subset of Müller glial cells. In addition, Chx10 reporter activity was identified in mature or^J-Chx10 mutant retinas, although these retinas lack Chx10-expressing bipolar cells. Reporter and molecular analysis showed that the reporter-expressing cells in the mutant had hallmarks of progenitor cells or partially differentiated Müller glial cells. These results strongly suggest that Chx10 promotes bipolar fate by affecting differentiation of late progenitor cells. Crosses of the Chx10 BAC reporter mice to R26R mice for fate-mapping experiments revealed that Chx10 reporter-expressing progenitor cells contribute to all mature cell types of the retina. These results demonstrate the utility of these lines for generation of mosaic or complete genetic manipulations of the retina.

Keywords: Retina; Chx10; BAC; Transgenic mouse; Reporter; Cre; Fate-mapping; GFP; Alkaline phosphatase; Ocular retardation

Introduction

The mammalian eye is an ideal organ for the genetic elucidation of the networks that control its development. Beyond its accessibility and attractive design, visual function is dispensable for viability and fertility. The retina has been particularly informative in the understanding of mechanisms of cell fate determination in the developing central nervous system (discussed in Desai and McConnell, 2000; Edlund and Jessell, 1999; Livesey and Cepko, 2001). The similarity between the genetic program of retinal development and that of other areas of the central nervous system (Livesey et al., 2004) further emphasizes the value of identifying genes affecting retinal development.

Mutant strains that exhibit microphthalmia/anophthalmia have been invaluable in the identification of important factors in retinal development, for example, Pax6, Rax/Rx, Lhx2, and Chx10 (reviewed in Chow and Lang, 2001). All of these transcription factors, except Chx10, are required for proper development of the optic vesicle, as nullizygous lead to an early arrest of retinal development. The naturally occurring Chx10 mutant, or^J (for ocular retardation), has three distinct aspects to its phenotype. Most prominently, or^J retinas are very small, correlating with a significant decrease in retinal progenitor cell proliferation (Burmeister et al., 1996). Additionally, abnormalities are observed in the ventral fissure, which leads to a failure of retinal ganglion cell axons exiting the eye (Burmeister et al., 1996; Robb et al., 1978; Theiler et al., 1976). Lastly, molecular analysis revealed a complete absence of bipolar cells, a late-born interneuron (Burmeister et al., 1996).

Consistent with these observed phenotypes, Chx10 is expressed within retinal progenitor cells, beginning in the optic vesicle (Liu et al., 1994). As progenitor cells differentiate and exit the cell cycle, Chx10 expression is lost in postmitotic cells except for bipolar cells (Burmeister et al.,

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1996; Liu et al., 1994). Furthermore, Chx10 is the earliest known marker expressed in bipolar cells and its levels peak during bipolar differentiation and persist through adulthood (Burmeister et al., 1996; Liu et al., 1994). Several unanswered questions remain about the functions of Chx10 in bipolar cell development. In particular, it is unclear what fate would be conferred to cells that should have become bipolar cells in or^J mutant mice. Mice nullizygous for both Math3 and Mash1 do not generate bipolar cells either, but retain expression of Chx10 in Müller glial cells (Hatakeyama et al., 2001; Tomita et al., 2000). In these studies, Chx10 misexpression on its own was not sufficient to direct rod bipolar cell fate (Hatakeyama et al., 2001). Therefore, the requirement of Chx10 for the generation of bipolar cells is complex and indicates that Chx10 may have roles in both progenitor cells that give rise to bipolar cells, and in specified bipolar cells.

We set out to investigate the function of Chx10 in retinal development with a further goal of developing a generally applicable tool for dissecting gene function in the developing retina. In particular, generation of reporter constructs expressing histochemical markers and Cre recombinase could allow generation of pan-retinal-specific knockouts, while offering highly sensitive tools to study Chx10-expressing cells in normal or mutant mouse strains. With these goals in mind, we turned to bacteria artificial chromosome (BAC) transgenic mice, carrying BACs modified using homologous recombination in bacteria (Muyrers et al., 1999; Zhang et al., 1998). Transgenic mice generated from BAC DNA have been shown to accurately recapitulate endogenous gene expression patterns and often escape positional effects of integration (Antoch et al., 1997; Chi et al., 2003; Gong et al., 2003; Heintz, 2001; Yang et al., 1997). A recent study demonstrated the overall utility of BAC transgenesis for the purposes of generating a gene expression atlas (Gong et al., 2003). These BACs were modified to express enhanced green fluorescent protein (GFP) in place of the endogenous gene products, and high throughput detection of GFP by antibody staining and immunohistochemistry was performed. Such studies could be augmented by adding multifunctionality to BAC reporters, as we have done here.

We created a multifunctional Chx10 BAC reporter by using a GFPCre fusion protein (Gagneten et al., 1997; Le et al., 1999) combined with an internal ribosome entry sequence-human placental alkaline phosphatase cassette (IRES-AP) (Bao and Cepko, 1997) under control of Chx10 enhancer elements. Characterization of the Chx10 expression pattern using GFP and AP revealed new insights into the cell types that express Chx10 and the morphology of these cell types. Analysis of the Chx10 BAC reporter in Chx10 mutant or^J mice revealed that Chx10 is not required for its own expression and that Chx10 may play an important role in the differentiation of progenitor cells into many cell types. We also show how the Cre component can be used to perform fate-mapping analysis and demonstrate that Chx10-expressing progenitor cells are multipotent. A separate use for generation of genetically mosaic tissue in the retina is discussed. These multifunctional transgenic mice offer a new tool for studies in retinal development and demonstrate the successful application of a strategy for reporters that is applicable to other developmental systems.

Materials and methods

Generation of Chx10 BAC transgenic mice

The targeting construct was constructed in pBluescript (Stratagene) and utilized PCR amplification to introduce homology arms at the 5′ end by subcloning into GFPCre and at the 3′ end by subcloning into FRT-Neo. PCR primers used were: 5′-GATCTCGAGCTTGGGGGAGCAAAAAGACCCGCGGCCTCAGCCCCTCCAAAGAACCGGGAG-**ATG**GTGAGCAAGGGCGAGGAG-3′, 5′-CACCGGCATCAACGTTTTCTTTTC-3′ for the 5′ homology arm and 5′-GAAGGTCGACTCTGCAAACCCTATGCTACTCCGTCG-3′, 5′-ATAAGAATGCGGCCGCCCCCGAGGTACTCTTGGCCACTGTCTCGGATTTGGGCTTGCTCAGCGCTTTCCCGGCGGATTTGTCCTACTCAGGAGAGCG-3′ for the 3′ homology arm. The Chx10 start methionine is shown bold and underlined. These homology arms lead to the deletion of 32 bp within the N-terminal Chx10 coding sequences.

The GFPCre construct was a gift of B. Sauer (Le et al., 1999) and the GFPCre insert was subcloned into pBluescript to remove the polyA sequences. PCR amplification led to introduction of a new *Eco*RI site in GFP, resulting in a silent mutation. The FRTNeo cassette was a gift from F. Stewart (Zhang et al., 1998). The IRES-AP construct was subcloned from the LIA murine retrovirus (Bao and Cepko, 1997). The final construct was linearized by digesting with *Xho*I and *Not*I, restriction sites outside the actual homology arms. Manipulation of the Chx10 BAC (isolated from a BAC library as described below) was performed according to the published methods using the pBADαβγ plasmid conferring an inducible RecET recombination machinery (gift from F. Stewart) (Muyrers et al., 2000). Successfully recombined colonies were first identified by screening for kanamycin resistance followed by PCR and Southern hybridization analysis to ensure homologous recombination. BAC DNA was prepared using a BAC purification kit according to manufacturer's instructions (Genome Systems Inc.) and purified DNA was digested with *Not*I to liberate the BAC insert, which was isolated by electroelution into dialysis tubing. BAC DNA was purified on a gel-purification column (Qiagen). DNA at 2–3 ng/μl was injected into male pronuclei of SJL/B6 fertilized eggs using standard protocols (Nagy, 2003).

Southern blotting and filter hybridization

BAC filters containing 129/Sv mouse genomic DNA inserts were purchased from Genome Systems Inc. A full-length mouse Chx10 probe (corresponding to NM007701) was labeled with [α-^{32}P] dCTP (Amersham) using Redivue II random-primed labeling according to manufacturer's instructions (Amersham). BAC filters were hybridized overnight at 42°C in a formamide hybridization buffer, and post-hybridization washes were performed according to standard protocols (Ausubel et al., 2001). Two Chx10-containing BACS were identified. The Chx10 BAC targeted in this study contained the entire Chx10 locus and extended approximately 55 kb upstream of the Chx10 start methionine and 22 kb downstream of the Chx10 polyadenylation sequences (H. Kim, personal communication).

Transgenic and mutant mice

or^{J} mice were obtained from Jackson Laboratories and were maintained on a 129/Sv background. Animals were genotyped by eye size, except at E11.5, where PCR and RFLP analysis was carried out as described previously (Burmeister et al., 1996).

R26R mice (gift from R. Awatramani and S. Dymecki), created by Soriano (1999), were maintained on a C57/B6 background before being crossed to Chx10 BAC mice. The R26R allele was genotyped using the following primers to *lacZ*: Frtz1, 5′-GGCAGATGCACGGTTACGATGC-3′; Frtz2, 5′-CCATGCAGAGGATGATGCTCGTG-3′.

FLPeR homozygous mice (gift from F. Farley and S. Dymecki) (Farley et al., 2000) were obtained in a mixed 129/Sv and C57/B6 background before being crossed to Chx10 BAC mice.

Chx10 BAC transgenic mice were maintained on a mixed background containing FVB, 129/Sv, SJL/B6, and C57/B6. Transgenic mice were genotyped using the following primers: Chx10cDNA1, 5′-GGGCACCTGGGACCAACTTCACGA-3′; GFPas1, 5′-CGGCGGCGGTCACGAACTCC-3′.

Alkaline phosphatase and β-galactosidase histochemical detection

Embryos or retinas were stained as whole-mounts for alkaline phosphatase (Fekete and Cepko, 1993). The reaction was allowed to proceed from 15 min at room temperature to overnight at 4°C until judged complete or for 4–6 h at 37°C. Embryos or retinas were stained as whole-mounts for β-galactosidase using a very sensitive protocol that uses glutaraldehyde for tissue fixation (Kwan et al., 2001). Tissue was stained overnight at 37°C. For cyrosectioning, tissue was embedded in OCT (Tissue-Tek) or gelatin (Sigma) and 20-μm sections were collected.

Immunohistochemistry

For antibody staining, cryosections or dissociated cells were prepared and stained as described previously (Chen and Cepko, 2002). Antigen retrieval was performed using the Vector antigen retrieval solution according to manufacturer's instructions (Vector). Primary antibodies used were 1:5000 rabbit anti-Chx10 (polyclonal antisera produced against an N-terminal 139-amino-acid peptide; A. Chen and C. Cepko, unpublished data), 1:1000 rabbit anti-GFP (Molecular Probes), 1:1000 mouse anti-GFP clone 3E6 (Molecular Probes), 1:1000 rabbit anti-human PLAP (Biomeda), 1:2000 mouse anti-glutamine synthetase (Transduction Laboratories), 1:10,000 mouse anti-CRALBP (gift of Jack Saari), 1:500 mouse anti-Nestin (PharMingen), and 1:20 mouse anti-Pax6 (University of Iowa Developmental Studies Hybridoma Bank). Secondary antibodies used were 1:250 Cy2- or Cy3-conjugated goat anti-rabbit (Jackson Immunologicals). Following antibody staining, DAPI was applied to stain nuclei (Sigma), and the sections were coverslipped and mounted in Gel/Mount (Biomeda).

In situ hybridization

Section in situ hybridization was performed as previously described (Murtaugh et al., 1999) using 20-μm cryosections from OCT-embedded tissue. Riboprobes labeled with DIG were detected with NBT/BCIP (Sigma). Chx10 3′ UTR was isolated from an *Msc*I/*Xho*I fragment of full-length Chx10 (+19 splice form) subcloned into pBluescript.

Results

Construction and generation of Chx10 BAC transgenic mice

To create a multifunctional reporter under control of Chx10 regulatory elements, a targeting construct was made for use in homologous recombination (Fig. 1A). The reporter elements consisted of GFPCre, a translational fusion of enhanced green fluorescent protein (GFP) and Cre recombinase, and IRES-AP, an internal ribosome entry site (IRES) fused to human placental alkaline phosphatase (AP). For selection in bacteria, the targeting construct also contained a cassette of the neomycin resistance gene (Neo^{R}) conferring kanamycin resistance (Kan^{R}) in bacteria under a dual mammalian/prokaryotic promoter phosphoglycerate kinase mammalian promoter (PGK) and Tn5 transposon promoter (Tn5), flanked by Flp recombinase target sequences (FRT sites marked as green triangles). Homology arms (red bars) were designed within the first exon of Chx10 such that translation of the 5′-most reporter would utilize the Chx10 start methionine. Following successful homologous recombination in bacteria, transgenic mice were generated from the modified Chx10 BAC DNA. Two independent transgenic founders were obtained, re-

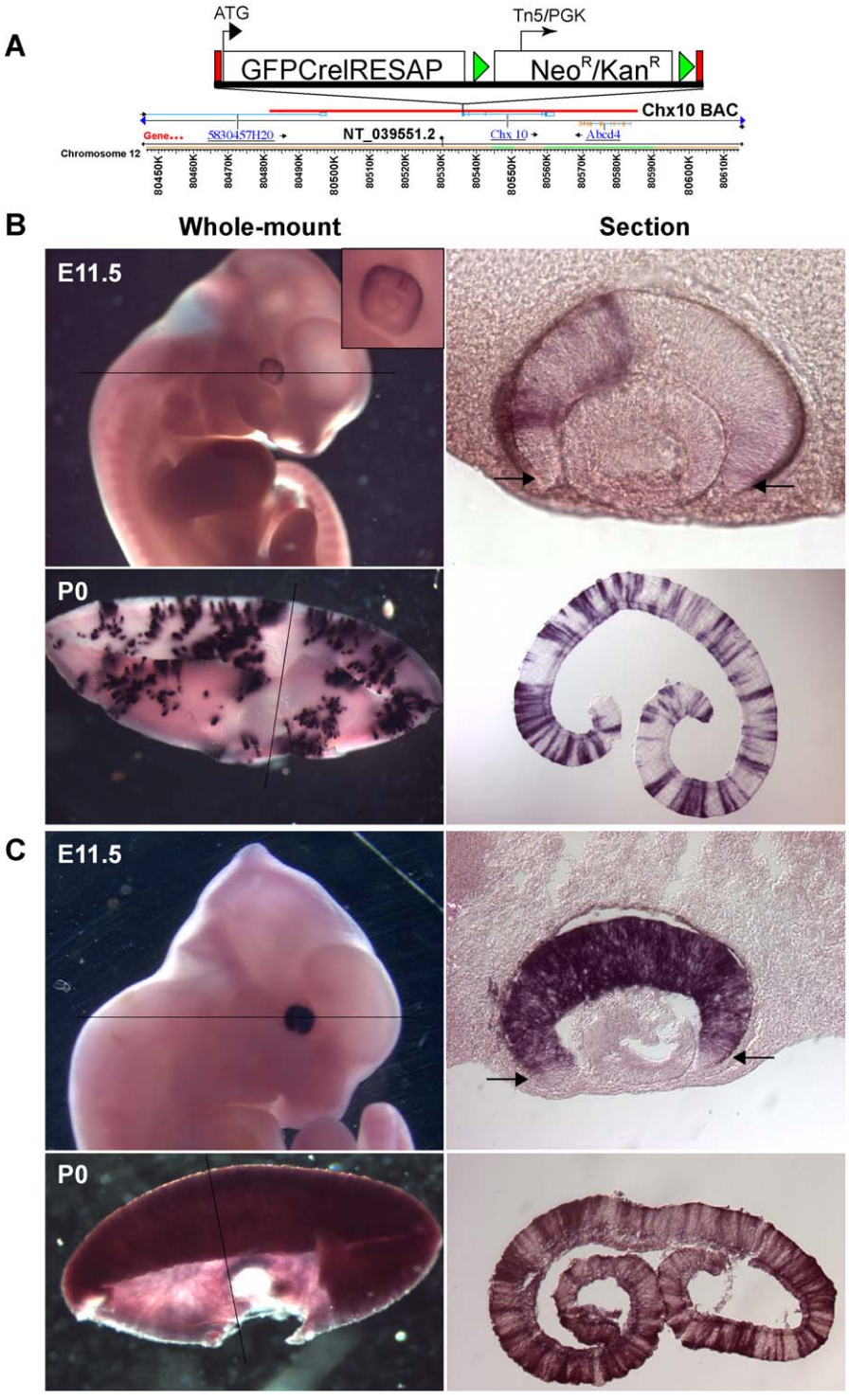

Fig. 1. Construction of the Chx10 BAC reporter and its expression in two independent transgenic lines. (A) shows a schematic of the modified BAC showing integration of the GFP Cre-IRES-AP cassette into the first exon of Chx10. The Chx10 BAC is outlined in red in reference to the location of the Chx10 locus on chromosome 12, shown below and adapted from the NCBI genome browser. Chx10 and its adjacent genes are indicated along with their intron/exon structure and directionality. Exons containing the 3′ untranslated regions are hollow bars. Homology arms within the targeting construct (top, red) and FRT sites (green) are shown. Location of the start methionine of Chx10 and Tn5/PGK promoters are indicated with arrows. (B, C) AP reporter activity in transgenic line 1 (B) or line 2 (C) mice in E11.5 embryos (top) or P0 retinas (bottom) whole-mount stained for AP (left) and subsequently sectioned (plane of section is indicated with a solid line) (right). (B) AP reporter activity in line 1 mice is observed in distinct areas in the retina (see inset for high-magnification image of E11.5 eye) and not elsewhere in the E11.5 embryo. Sections revealed these areas of AP reporter activity to be in retinal progenitor cells and not in the retinal pigmented epithelium, lens, or prospective pigmented ciliary body (indicated by arrow). (C) Expression in line 2 mice following removal of the FRTNeo cassette was broader and was well correlated with the Chx10 expression domain.

ferred to henceforth as Chx10 BAC line 1 and line 2. Southern blotting analysis on both transgenic mouse lines revealed that the BAC transgenes had integrated in multiple copies and appeared to be intact in most or all copies (data not shown).

Analysis of early Chx10 BAC reporter activity in line 1 and line 2

To characterize the expression of the two Chx10 BAC reporter lines, crosses with animals from the two different lines were performed and litters were harvested at E11.5 or P0. Analysis of Chx10 BAC line 1 at both time points was carried out by either staining the entire embryo (E11.5) or dissected retinas (P0) for AP activity (Fig. 1B). At both time points, there was AP activity in subsets of progenitor cells. No staining outside of the eye could be observed at E11.5, although Chx10 is normally also expressed in interneurons in the spinal cord and in the limb. It is therefore likely that distal enhancers controlling these expression domains were not contained within the targeted Chx10 BAC. Sectioning of these retinas revealed that the radial dimension of the retina stained positive for AP, showing that progenitor cell processes spanned the entire retina. The mosaic nature of the expression of the Chx10 BAC reporter in line 1 was observed in every animal analyzed ($n > 50$). In general, there was consistency in the number of areas that were expressing between the different eyes, but high variability between littermates. Analysis of Chx10 RNA and protein suggested the mosaicism was not due to the intrinsic nature of Chx10 regulation, but rather a consequence of the integration site or chromatin configuration of the BAC DNA.

Analysis of Chx10 BAC line 2 revealed a different pattern of staining in terms of intensity and mosaicism relative to line 1 (Fig. 1C). Initial crosses were performed with mice that contained an intact FRT Neo cassette (see Fig. 1A), and in these animals, activity of all reporters was detected in the same tissues as line 1, but expression levels were faint and uniform (data not shown). Subsequent removal of the FRT Neo cassette by crossing transgenic mice to a germline FLP-expressing mouse line, the FLPeR line, enhanced the expression levels of the AP reporter significantly (Fig. 1C). This line did not always direct AP reporter activity uniformly within the Chx10-expressing domain, but demonstrated much less mosaicism than line 1. In some cases, greater than 95% of Chx10-expressing cells expressed the Chx10 BAC reporter [assessed by anti-GFP and anti-Chx10 antibody staining in tissue and dissociated cells (see Fig. 3 and data not shown)]. At E11.5, AP reporter activity was detected only in the retina, and analysis of sections revealed a central/peripheral boundary within the retina of Chx10 BAC reporter activity. Expression of the AP reporter was stronger in the center than periphery and ceased in the periphery of the retina, at the boundary of the presumptive pigmented ciliary body (Fig. 1C, arrows). In P0 retinas, AP reporter activity was more broadly detected than in line 1, but still showed some mosaicism.

Analysis of Chx10 BAC line 2 reporter activity in retinal progenitor cells

Although the AP staining shown in Figs. 1B, C showed reporter activity in whole-mounts and sections, it did not reveal sufficient cellular detail about progenitor cells because progenitor cell processes span the radial dimension of the retina, and thus obscure the location of cell bodies. To study the behavior of the Chx10 BACs in progenitor cells more precisely, GFP antibody staining was performed. Because the GFP was fused to Cre recombinase, it was targeted to the nucleus, although faint cytoplasmic staining was also observed. This allowed use of GFP as a fluorescent marker for Chx10-expressing cells. This was valuable as our anti-Chx10 antibody detects Chx10 in progenitor cells very weakly (see Fig. 3). Chx10 BAC line 2 was used for these experiments to see the broadest possible range of reporter expression. GFP antibody staining was performed at three time points, E14.5 (Fig. 2A), E17.5 (Fig. 2C), and P0 (Fig. 2E) to examine Chx10 BAC reporter activity in progenitor cells during development. These data were compared to in situ hybridizations for Chx10 RNA to assess the accuracy of the GFP reporter expression relative to the endogenous Chx10 expression (Figs. 2B, D, F).

GFP protein was detected in the outer neuroblastic layer (ONBL) at all stages (Figs. 2A, C, E). The ONBL contains a mixture of progenitor cells, newly postmitotic cells, and differentiating photoreceptors. At E14.5, most of the cells in the ONBL were GFP-positive, suggesting their identity as progenitor cells, as would be expected at this age (Fig. 2A). However, by P0, many of the cells near the outer part of the retina were GFP-negative and presumably were differentiating photoreceptor cells (Fig. 2E). At all time points analyzed, strong GFP expression was observed in the inner part of the ONBL, abutting the inner neuroblastic layer (INBL). In situ hybridization analysis of Chx10 RNA (Figs. 2B, D, F) showed a highly similar distribution of RNA in the ONBL, further suggesting that Chx10 BAC reporter activity accurately reflects the endogenous expression pattern of Chx10.

In addition to GFP reporter expression in the ONBL, GFP-positive cells and Chx10 RNA were detected in the periphery of the retina in the presumptive ciliary body (arrows in Fig. 2). These cells were always nonpigmented and likely define the presumptive nonpigmented ciliary body. The expression of Chx10 in this region has been previously described in chick (Kubo et al., 2003).

Analysis of Chx10 BAC line 2 reporter activity in the postnatal retina

Early bipolar cell development is a poorly understood process, in part because good markers and tools are not

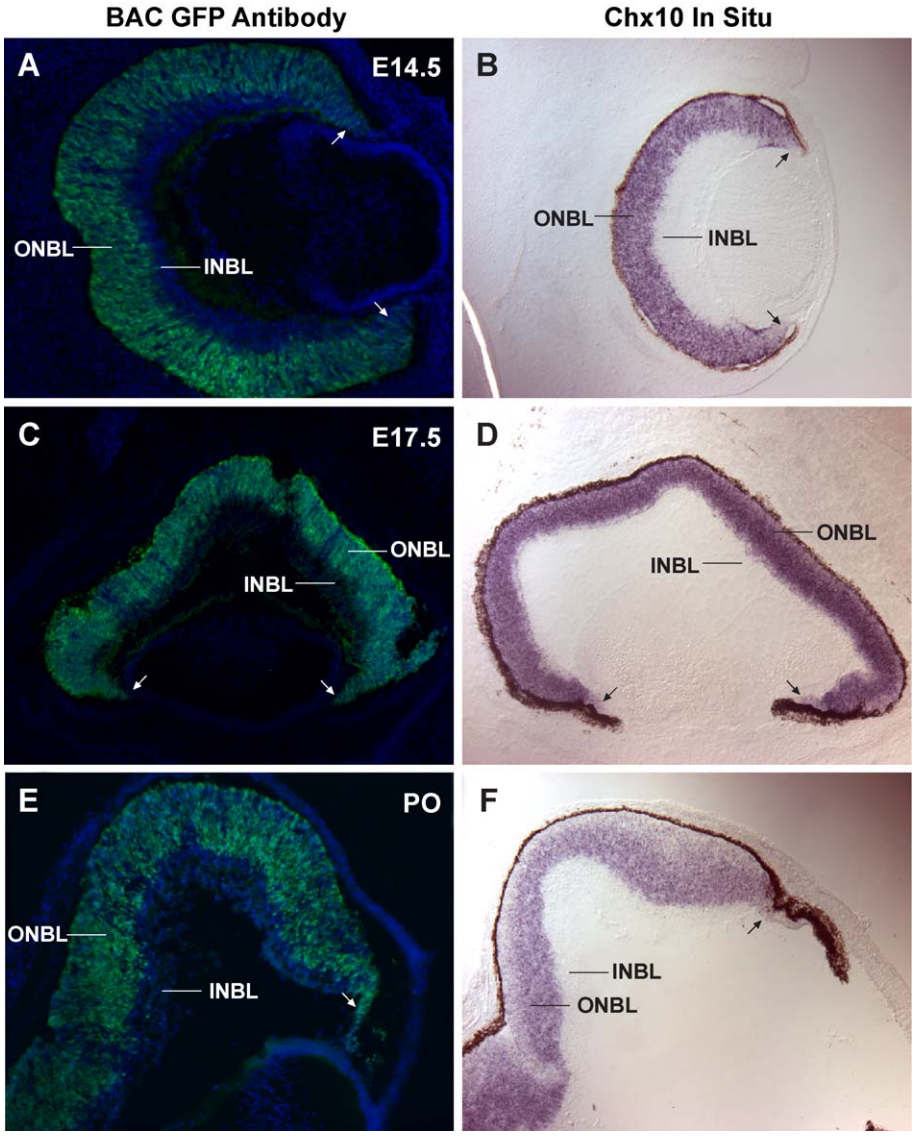

Fig. 2. Analysis of Chx10 BAC reporter activity in retinal progenitor cells. Transverse sections are shown for E14.5 embryos (A, B), E17.5 embryos (C, D), or P0 neonates (E, F). (A, C, E) Antibody staining to detect GFP reporter expression shows localization of GFP to cell bodies within the outer neuroblastic layer (ONBL) in presumptive progenitor cells and absent from the inner neuroblastic layer (INBL), containing differentiating early-born neurons. GFP reporter expression is also observed in the nonpigmented prospective ciliary body (arrows) but not in pigmented cells. Nuclei are counterstained by DAPI (blue). (B, D, F) Location of Chx10 RNA is demonstrated by in situ hybridization. Chx10 RNA is restricted to the ONBL and the nonpigmented prospective ciliary body (arrows).

available. Since Chx10 expression appears specific to, or is highly enriched in bipolar cells beginning at approximately P6, we were able to utilize the AP component of the Chx10 BAC reporter to examine the morphology of developing bipolar cells. In addition, the GFP component of the Chx10 BAC reporter was examined for detection of the location of bipolar cell bodies. Chx10 BAC line 2 transgenic retinas were AP stained or immunofluorescently stained with anti-GFP, anti-AP, and anti-Chx10 antibodies, and analyzed at three times in bipolar cell development, P6 (Figs. 3A–D), P10 (Figs. 3E–H), and P23 (Figs. 3I–L). Retinas from these time points contain newborn bipolar cells, differentiating bipolar cells, and mature bipolar cells, respectively.

At P6, AP staining was concentrated in the outer nuclear layer (ONL), although lighter staining was observed throughout the developing inner plexiform layer (Fig. 3A). Bipolar cell bodies could also be observed in the inner nuclear layer (INL). However, the AP staining did not show the precise location of cell bodies. This was determined by double immunofluorescent staining for AP and GFP protein (Fig. 3B). The majority of cell bodies had already migrated to the INL except in the periphery of the retina (Fig. 3B, left). The high concentration of AP protein throughout the ONL indicated dense ascending processes for immature bipolar cells as has been described by Ramâon y Cajal (1995) in classic cell labeling studies. Descending proces-

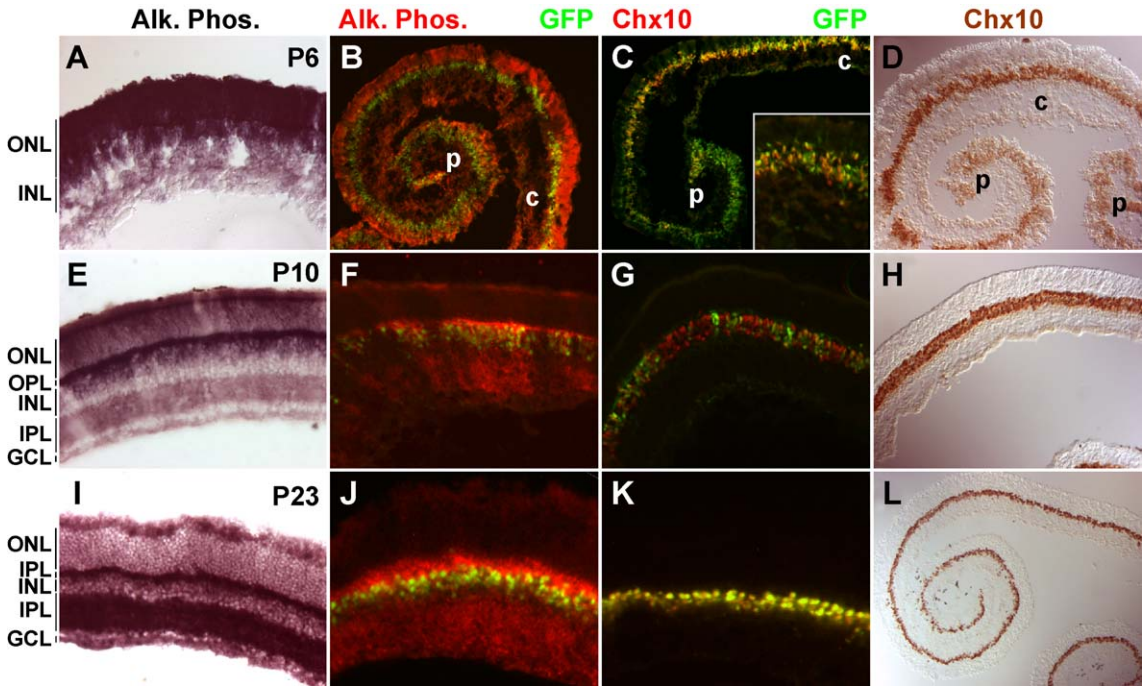

Fig. 3. Analysis of Chx10 BAC reporter activity in developing bipolar cells. Retinas were dissected and sectioned from P6 pups (A–D), P10 pups (E–H), or P23 pups (I–L). (A, E, I) Histochemical detection of AP reporter activity shows a transition of AP activity from being concentrated in ascending processes of developing bipolar cells at P6 (A) to cell bodies and developing dendrites of bipolar cells at P10 (E) to axons and dendrites of mature bipolar cells at P23 (I). Ascending processes are observed at all time points, although with decreasing intensity over time. Labels at left indicate layers of the retina: outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), and ganglion cell layer (GCL). (B, F, J) Sections immunofluorescently stained for AP (red) and GFP (green) reporter proteins. (B) Developing bipolar cell bodies (marked by GFP) are largely in the INL in the central retina (c) but not in the periphery (p). AP protein is largely concentrated in ascending processes although descending processes are also visible. (F, J) AP protein is concentrated in bipolar cell processes, mostly dendrites and axons, although weak staining in ascending processes is observed. (C, G, K) Sections immunofluorescently stained for Chx10 protein (red) and GFP reporter protein (green). (C) GFP protein and Chx10 protein colocalize in developing bipolar cell nuclei (yellow) except in the periphery. These cells do express Chx10 protein, detectable with sensitive antigen retrieval and immunohistochemistry (D, H, L). Inset shows a high-magnification image from the central retina. (G) Patches of non-reporter-expressing bipolar cells are observed as Chx10-expressing and GFP-negative regions interspersed among double-positive cells. (K) GFP reporter protein exclusively localizes to Chx10-expressing cells. (D, H, L) Detection of Chx10 protein following antigen retrieval and histochemical detection with DAB. (D) Chx10 localizes to the INL except in the periphery where it is observed throughout the ONBL in a similar manner to GFP reporter protein (C). (H, L) Chx10 is expressed exclusively in the nuclei in the outer half of the INL.

ses, presumably developing axons, also contained some AP protein. In the peripheral retina, GFP-positive cell bodies were observed dispersed throughout the ONL. This was likely due to the mixture of progenitor cells and newborn bipolar cells as the peripheral retina is delayed in differentiation relative to the central retina. Double immunofluorescent staining was performed for GFP and Chx10 to ensure that Chx10 BAC reporter-expressing cells reflected true Chx10-expressing cells (Fig. 3C). Cell bodies in the INL were positive for both markers except for the peripheral retina, where GFP-positive cells were observed dispersed throughout the ONL and appeared Chx10-negative. This staining pattern likely reflects Chx10-expressing progenitor cells as our antibody recognizes Chx10 in progenitor cells very weakly. To more sensitively assess Chx10 expression in the peripheral retina, P6 retinal sections were processed for antigen retrieval and Chx10 was detected using immunohistochemistry (Fig. 3D). This treatment revealed the Chx10-expressing cells in the peripheral retina. Thus, double staining transgenic retinal tissue for Chx10 and GFP can identify progenitor cells as those that have easily detected GFP protein and poorly detected Chx10 protein.

Similar analyses as described above were performed on P10 Chx10 BAC line 2 transgenic retinas (Figs. 3E–H). At P10 in development, all cells have exited the cell cycle and bipolar cells are in the later stages of their differentiation. AP staining showed that the ascending processes of bipolar cells were still present, but contained significantly less AP relative to P6 ascending processes. Most of the AP was concentrated in the bipolar cell bodies within the outer half of the INL and in the outer plexiform layer (OPL), in the developing bipolar cell dendrites (Fig. 3E). AP staining was not detected in the inner half of the INL, in amacrine cell bodies, or in the ganglion cell bodies within the ganglion cell layer (GCL). Weaker levels of AP were observed throughout the inner plexiform layer (IPL) where bipolar cell axons extend and terminate. Weak AP staining was also observed in the vitreal surface below ganglion cells. This particular retina showed a high-degree mosaicism, indicated by regions of cells that were Chx10-positive but GFP-

negative (Fig. 3G). Antibody staining for AP and GFP (Fig. 3F) or GFP and Chx10 (Fig. 3G) indicated that all Chx10 BAC reporter-expressing cell bodies were restricted to the outer half of the INL.

Finally, P23 retinas were analyzed for AP and antibody staining to GFP, AP, and Chx10 (Figs. 3I–L). The AP staining pattern resembled that observed at P10 (Fig. 3E), except that more intense staining was observed in dendrites in the OPL and in axons in the IPL than in cell bodies in the INL or ascending processes through the ONL. The persistent weak AP staining in ascending processes (Figs. 3I, J) suggested that these represented either bona fide bipolar cell processes or Müller glial cell processes (discussed below). Comparison of Chx10 protein with GFP reporter protein revealed excellent correspondence (Fig. 3K). Notably, bipolar cells exhibited varying amounts of Chx10 antibody staining, depending on their location within the INL, and these differences were also observed in GFP antibody staining.

Morphological analysis of Chx10 BAC reporter-expressing cells

The AP staining in Figs. 3A, E, I showed that Chx10-expressing cells contain processes spanning the radial dimension of the retina throughout their differentiation. These results suggested the presence of previously unappreciated ascending bipolar cell processes, or alternatively that Chx10 BAC reporter activity was observed in some or all Müller glia. To more carefully examine the processes of single reporter-labeled cells, Chx10 BAC reporter line 1 mice were used to perform AP staining and examine reporter-expressing cells within small regions of expression.

Figs. 4A–J provide examples of regions with a few, or a single AP-positive bipolar cell, in an adult retina. AP-stained cells were compared to results obtained by injecting single bipolar cells with Lucifer Yellow and Neurobiotin that allowed classification of bipolar cells in the mouse retina (Ghosh et al., 2004). Figs. 4A–E show representative examples of rod bipolar cells (Figs. 4A, B), ON cone bipolar cells (Figs. 4B, C), and OFF cone bipolar cells (Figs. 4B, C, E). In some cases, mixtures of different classes of cone bipolar cells were observed within a given region of expression (Figs. 4C–E). In many regions, AP-stained cells morphologically resembling cone bipolar cells also appeared to contain an ascending process (Figs. 4F–H). Although such cells were generally unusual, on occasion, a large number of cells with ascending processes were observed (Fig. 4H). These ascending processes had clear terminations at the outer limiting membrane (asterisks) and resembled Müller glial processes. It could not be excluded that these ascending processes were Müller glial processes. However, in many cases, they appeared connected to the dendrites of cells that morphologically resembled bipolar cells, contained bipolar cell-like terminations in the IPL, and did not contain obvious Müller glial endfeet.

A small number of AP-stained cells morphologically resembled Müller glia (Figs. 4I, J). These cells contained processes that spanned the radial dimension of the retina, had clear terminations at the outer limiting membrane (asterisks), as well as prominent endfeet at the vitreal surface (arrowheads). Müller glial cells were frequently in regions containing other bipolar cells, and were difficult to distinguish from bipolar cells containing ascending processes (and vice versa).

To molecularly determine whether any Chx10 BAC reporter-expressing cells were Müller glia, dissociated retinal cells from P10, P14, and P21 Chx10 BAC line 2 were stained for Müller glial markers [Nestin, Cellular retinaldehyde binding protein (CRALBP), and Glutamine synthetase (GS)] and for GFP. At all time points analyzed, a small percentage of Müller glial marker-expressing cells were identified that contained GFP in their nucleus. Examples of such cells from P14 dissociated retinas are shown in Figs. 4K, L. Marker-positive cells that contained GFP in their nuclei are marked with arrows. In addition, many of these double-positive cells contained prominent endfeet (arrowheads), further suggesting a Müller glial identity. Despite the above observations, the majority of GFP-expressing cells did not co-express Müller glial markers, nor did the majority of Müller glia express GFP in their nuclei. Notably, the level of GFP antibody staining in these Müller glial-like cells was routinely lower than in bipolar cells, suggesting a different regulatory pathway for expression. This was consistent with the fainter AP staining in presumptive endfeet compared to the IPL in Figs. 3E, I. Chx10 protein was also stained and scored for expression in Müller glial cells. Weak anti-Chx10 immunoreactivity was detected in many Müller glial marker-expressing cells; however, the immunoreactivity was not specific to the cell nucleus as in bipolar cells (data not shown). RNA analysis of genes expressed in isolated Müller glial using microarrays also suggested that Chx10 RNA is expressed in some Müller glia (B. Sun and C.L. Cepko, unpublished results).

The Chx10 BAC reporter can mediate recombination at loxP sites in retinal progenitor cells

To investigate whether the Chx10 BAC can mediate recombination at loxP sites in the genome, Chx10 BAC line 2 mice were crossed with a reporter mouse line, the R26R mouse. R26R contains a floxed stop cassette immediately upstream of the *lac*Z gene within the Rosa26 locus and expresses β-galactosidase in cells and their progeny following Cre-mediated excision of the stop cassette (Soriano, 1999). Double transgenic mice were initially analyzed at E10–E10.5 in development to determine how early recombination could be detected. Chx10 RNA is first detectable at E9.5 in the distal optic vesicle and subsequently RNA levels increase (Liu et al., 1994). Due to the temporal delay expected between reporter expression, Cre-mediated excision, and detectable expression of β-galacto-

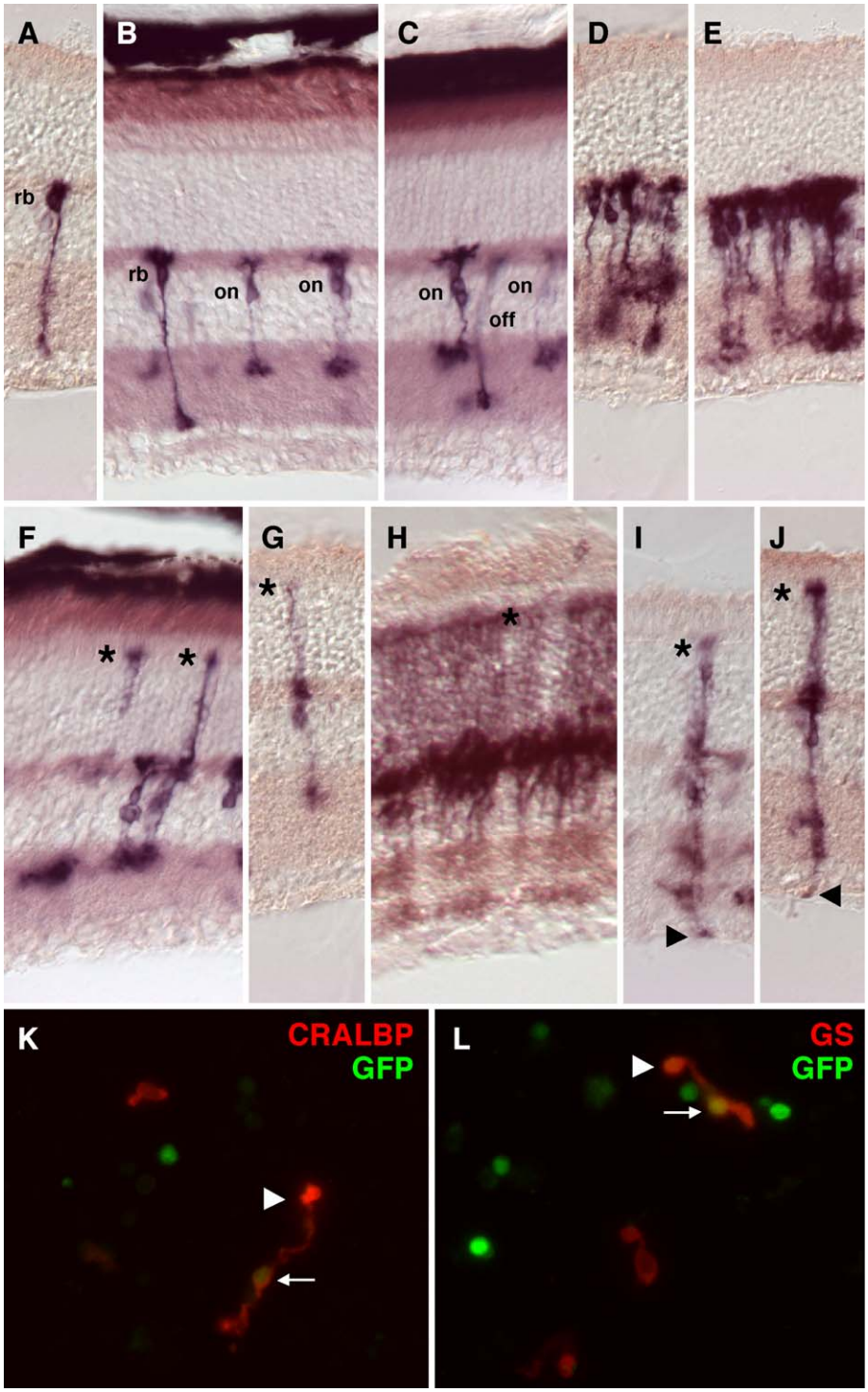

Fig. 4. Morphological identification of mature cells expressing the Chx10 BAC reporter. (A–J) AP-stained transverse retinal sections from adult BAC line 1 transgenic mice showing regions of AP reporter activity. (A) An isolated rod bipolar cell (rb). (B–E) Regions containing mixtures of rod bipolar cells, ON cone bipolar cells (on), and OFF cone bipolar cells (off). (F–H) Regions containing cone bipolar cells with ascending processes that terminate at the outer limiting membrane (asterisks). (I, J) Isolated Müller glial cells with ascending processes that terminate at the outer limiting membrane (asterisks) and defined endfeet at the vitreal surface (arrowheads). (K, L) Dissociated retinal cells from Chx10 BAC line 2 transgenic mice immunofluorescently stained for GFP (green) and cellular retinaldehyde binding protein (CRALBP) (red) (K) or glutamine synthetase (GS) (red) (L). Arrows indicate GFP-positive nuclei in CRALBP- or GS-positive cells. Arrowheads indicate distinctive Müller glial cell endfeet.

sidase, E10–E10.5 represent the earliest stages at which β-galactosidase activity should be detected.

Fig. 5A shows an E10–E10.5 R26R/Chx10 BAC embryo stained with X-gal. One retina contained some regions of β-galactosidase-expressing cells (Fig. 5A), although no staining could be observed in the other eye (data not shown). No other parts of the embryo showed any β-galactosidase staining. These β-galactosidase-positive cells likely resulted from the first recombination events mediated by GFPCre under Chx10 control. To assess the multipotency of Chx10 BAC reporter-expressing progenitor cells, fate mapping was performed in the mature retina at P23 (Fig. 5B). β-Galactosidase was detected in all cell types in the retina and in most cells in the retina. This required using the most sensitive methods for β-galactosidase histochemistry to observe photoreceptor staining and staining in all INL cell types. Because of the mosaicism exhibited in Chx10 BAC transgenic mice, small regions lacking β-galactosidase expression were observed, most notably in photoreceptor cell bodies, which have low levels of β-galactosidase driven by the Rosa26 promoter (Yang et al., 2003, and data not shown). Double labeling experiments using anti-β-galactosidase and anti-Pax6 immunostaining demonstrated that ganglion cells, horizontal cells, and amacrine cells were fate-mapped using Chx10 BAC Cre reporter expression (data not shown).

To compare the Chx10 BAC reporter activity to β-galactosidase generated by a completely activated R26R allele, a ubiquitous excision of the stop cassette in R26R was carried out by crossing R26R mice to β-actin Cre transgenic mice. R26R/β-actin Cre adult retinas were stained for β-galactosidase and sectioned (Fig. 5C). The fate-mapping results were very similar to those obtained with Chx10 BAC transgenic mice (Fig. 5B).

These BAC transgenic lines are useful tools for perturbing genes specifically in the retina; however, the mosaicism observed occasionally in BAC line 2 transgenic mice and always in BAC line 1 transgenic mice could complicate analyses. Fate mapping of expressing domains could determine if there is a lineal relationship between late-expressing areas and earlier ones. To address this question, Chx10 BAC line 1 and R26R transgenic mice were crossed, and retinas were analyzed for β-galactosidase (to mark cells that had undergone recombination) or AP (to mark cells currently expressing the reporter). Figs. 5D, E show a representative example of this analysis on a whole-mount-stained retina. The retina in Fig. 5D is a double transgenic P14 retina stained for β-galactosidase. As expected, only a few regions contained patches of β-galactosidase activity, corresponding to the progeny of retinal progenitor cells that underwent successful Cre-mediated recombination. In Fig. 5E, the same retina was additionally stained for AP activity. Because β-galactosidase largely localizes to the cytoplasm and AP is membrane-bound, AP activity could be detected in the processes and less so in the cell bodies of bipolar cells. Virtually all regions of the retina that had undergone

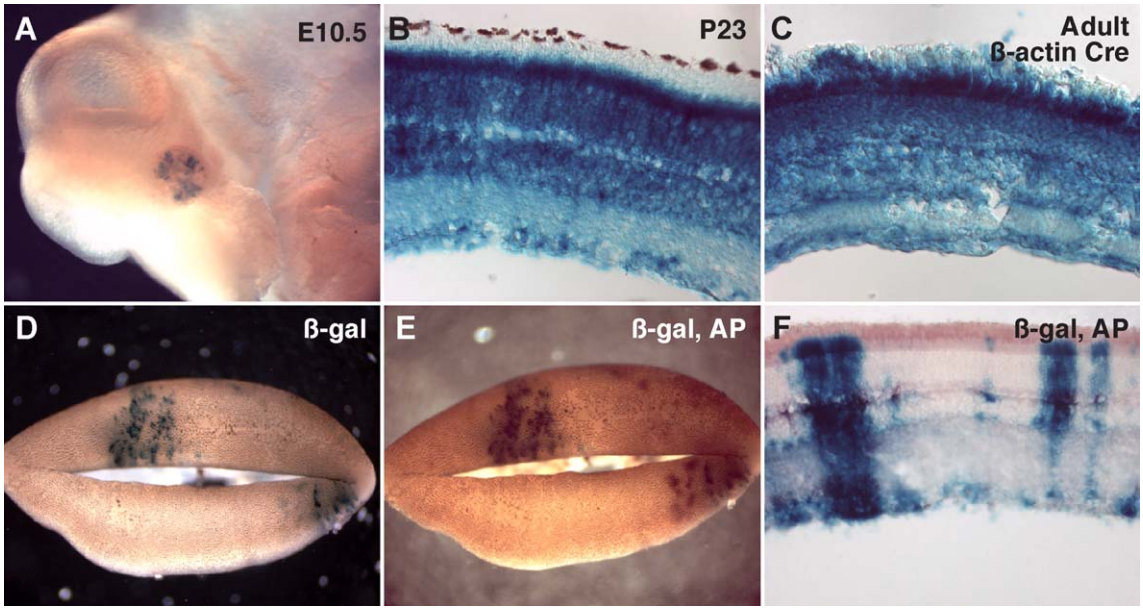

Fig. 5. Fate mapping of Chx10 BAC transgenic mice. (A) E10.5 double transgenic Chx10 BAC line 2 and R26R mouse whole-mount stained for β-galactosidase to indicate fate-mapped cells in the retina. (B) P23 double transgenic Chx10 BAC line 2 and R26R retinal transverse section stained for β-galactosidase indicating most or all cells in the retina express β-galactosidase. (C) Adult double transgenic β-actin Cre and R26R retinal transverse section stained for β-galactosidase showing the full range of retinal cells that express β-galactosidase. (D, E) P14 double transgenic Chx10 BAC line 1 and R26R retinas whole-mount stained for β-galactosidase (D) and subsequently stained for AP activity (E). Regions of fate-mapped cells (E) or Chx10 BAC reporter-expressing cells (F) are observed in the same location. (F) Transverse section taken through the retina shown in E. AP-positive cells (purple) are only observed in fate-mapped patches (blue).

recombination (marked by β-galactosidase) were also AP-positive, indicating that bipolar cells that expressed the reporter derived from progenitor cells that also expressed the reporter. In some places, there was prominent AP staining in regions that had very weak β-galactosidase staining. These likely are regions where recombination happened very late, possibly only in the bipolar cell, or its immediate parent. Sectioning revealed that different sizes of fate-mapped areas were obtained, ranging from large numbers of cells throughout the radial dimension of the retina (on left of Fig. 5F), to areas containing just a few cells (in middle and right of Fig. 5F). Importantly, AP-stained bipolar cells were not observed in regions lacking corresponding β-galactosidase-stained cells. These results indicate that genetically mosaic retinas can be obtained using Chx10 BAC transgenic mice, and that one can track where Cre-mediated excision occurred by finding the areas that express the Chx10 BAC reporter, even in the adult.

Analysis of Chx10 BAC reporter activity in a Chx10 mutant background

To address whether functional Chx10 protein is required for its own expression, and to evaluate the fate of incipient bipolar cells in the absence of Chx10 function, the Chx10 BAC line 1 reporter was studied in or^J mutant mice lacking Chx10 protein. BAC transgenic animals heterozygous or homozygous for or^J were analyzed at an early embryonic time point at E11.5 (Figs. 6A, B) or in maturing retinas at P17 (Figs. 6C, D) for AP reporter activity.

At E11.5, or^J mutant eyes (Fig. 6B) were already noticeably smaller than or^J/+ heterozygous littermates (Fig. 6A). AP staining was strong and restricted in the embryo to the retina. No significant differences were observed in AP staining between littermates. The same analyses were performed at P0 in late progenitor cells, and AP reporter activity was very similar between or^J/+ heterozy-

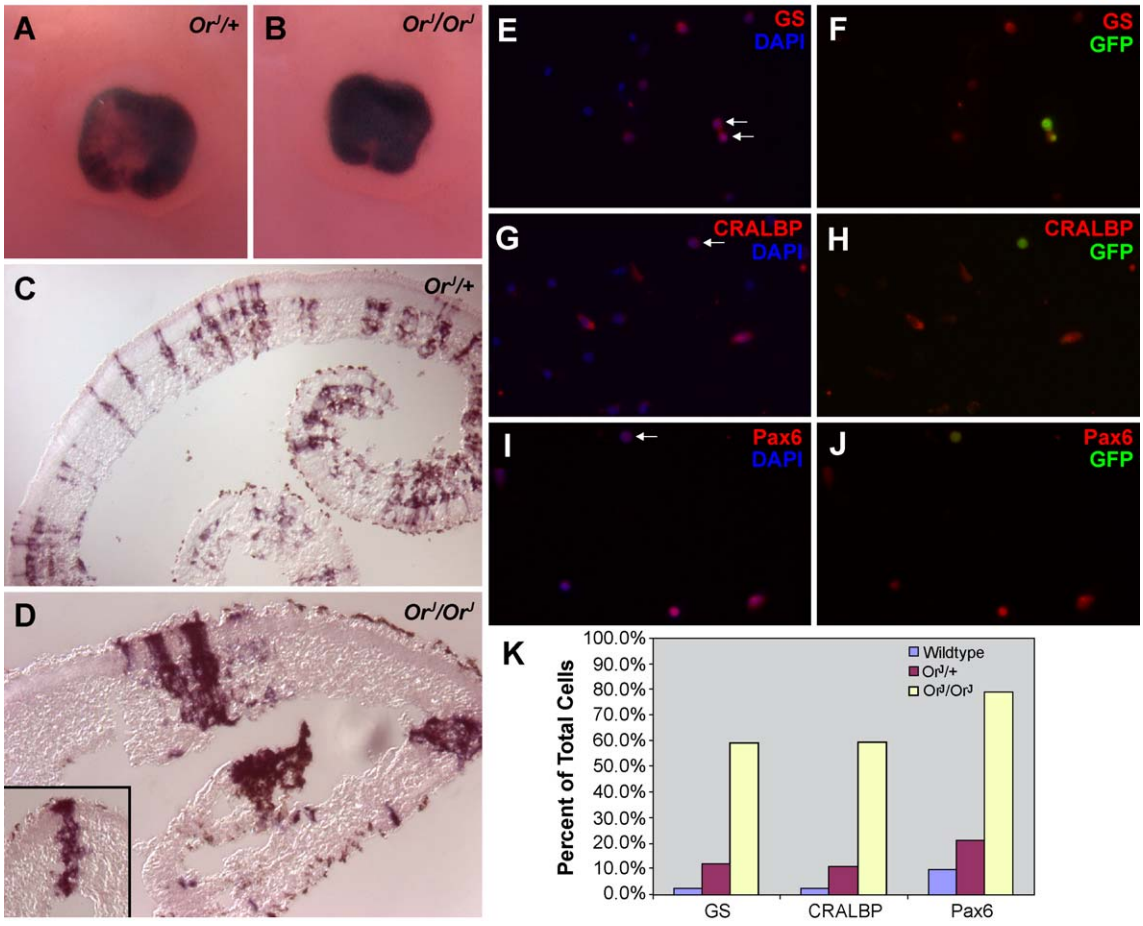

Fig. 6. Expression of Chx10 BAC reporter in or^J mutant retinas. (A, B) E11.5 Chx10 BAC line 1 transgenic mice whole-mount stained for AP reporter activity in or^J/+ (A) or or^J/or^J (B) littermates. AP reporter activity is observed in retinas of both genotypes. (C, D) P17 BAC line 1 transgenic retinal transverse sections stained for AP reporter activity in or^J/+ (C) or or^J/or^J (D) littermates. AP reporter activity is observed in bipolar cells and Müller glial cells in or^J/+ retinas (C) and cells that morphologically resemble Müller glial cells in or^J/or^J retinas (D). Inset in D shows a Müller glial-like cell in an adjacent section. (E–J) Dissociated cells from P14 Chx10 BAC line 1 transgenic and non-transgenic or^J/or^J retinas immunofluorescently stained for GFP (green) and GS (red) (E, F), CRALBP (red) (G, H), or Pax6 (red) (I, J). (E, G, I) Fields of cells showing respective antibodies without GFP and with DAPI (blue) counterstaining of nuclei. Arrows indicate cells that are GFP-positive (F, H, J). All GFP-expressing cells are positive for both markers. (K) Quantitation of dissociated P14 retinal cells from wild-type mice (blue), or^J/+ mice (red), and or^J/or^J mice (yellow) that also express GS (left), CRALBP (middle), or Pax6 (right).

gotes and or^J homozygotes (data not shown). These results demonstrate that Chx10 is not required for tissue-specific expression of its own transcript. Consistent with this finding, mutant Chx10 RNA was also detectable in or^J mutants and generally resembled the RNA pattern in or^J/+ heterozygotes (data not shown).

Bipolar cells have not been observed in or^J mutant retinas and therefore it was of interest to see whether Chx10 would be expressed in mature or^J mutant cells, and if so, what those cell types would resemble. In BAC line 1 transgenic or^J/+ heterozygotes, all AP-expressing cells were bipolar cells or Müller glial-like cells (Fig. 6C). A seemingly larger number of Müller glial-like cells were observed in the or^J/+ heterozygote than in wild-type transgenic mice, a result corroborated by quantitation of cell fates in this genetic background (Fig. 6K, see below). The majority of or^J mutant retinas did not have sufficient lamination to allow for morphological identification of cell types based on AP staining. However, one mutant retina had sufficient lamination to allow identification of a photoreceptor layer and thus permitted morphological identification of cell types (Fig. 6D). The AP staining pattern observed most closely resembled that of Müller glial cells in that the cells extended processes throughout the radial dimension of the retina had prominent ascending processes, and terminations resembling endfeet. It is not surprising that the cells did not exhibit the precise features of Müller glia due to the significantly altered morphology of the or^J retina. Other retinas were analyzed that had Müller glial-like cells, but because they did not have a distinct photoreceptor layer, it was more difficult to assess their identity, although they had prominent endfeet (data not shown). In addition to the Müller glial-like staining, the AP staining intensity in or^J mutants was significantly stronger than or^J/+ heterozygote controls.

To further investigate the identity of the AP-positive cells in the or^J mutant retinas, Chx10 BAC line 1 transgenic retinas from P14 or^J mutant or heterozygous mice were dissociated and stained with antisera to several retinal cell types. The staining was analyzed for co-staining with anti-GFP in the nucleus. Due to the very small size of or^J mutant retinas, an entire litter of or^J mutant retinas was combined together, regardless of BAC genotype. Consequently, the percentage of GFP-positive cells for a given retina could not be determined. Figs. 6E–J show examples of fields of dissociated cells stained for a retinal marker (Figs. 6E, G, I) or marker along with GFP (Figs. 6F, H, J) to assess colocalization. GS (Figs. 6E, F) and CRALBP (Figs. 6G, H) were chosen as markers of Müller glia or progenitor cells and Pax6 (Figs. 6I, J) as a marker of amacrine, horizontal, ganglion, and progenitor cells. GFP colocalized with all three markers (double-positive cells are marked by arrows in Figs. 6E, G, I). Notably, the expression of these markers was weaker in the GFP-expressing cells of the or^J mutants than in or^J/+ heterozygous or wild-type animals (data not shown). Additionally, all GFP-positive cells expressed each of these three markers ($n > 50$), implicating simultaneous expression of all three markers.

If, during differentiation in or^J mutant mice, the majority of Chx10-expressing cells remain progenitor cells or differentiate into Müller glia cells, one would expect to see an unusually high percentage of Müller glia/progenitor cell markers expressed in or^J mutant retinas. This was quantified by counting the above markers as a percentage of total retinal cells in wildtype, or^J/+ heterozygotes, or or^J homozygotes at P14 (Fig. 6K). As predicted, GS and CRALBP were increased from approximately 2.5% in wild-type retinas (18/703 GS-positive and 19/770 CRALBP-positive) to approximately 60% in or^J retinas (209/354 GS-positive and 97/163 CRALBP-positive) ($P < 0.01$). As was predicted from AP staining in Fig. 6C, there was a small increase of Müller glia cell markers in or^J/+ heterozygous mice (55/476 GS-positive and 4/36 CRALBP-positive) ($P < 0.1$) compared to wild-type mice. Pax6 was also expressed in a much larger percentage of cells in or^J homozygous retinas (180/228) than wildtype (112/1138) or or^J/+ heterozygous retinas (36/170) ($P < 0.01$) and showed a small increase in or^J/+ heterozygous retinas compared to wild-type retinas. Markers of photoreceptors, including rhodopsin and recoverin, were also analyzed in sections and by staining dissociated cells. The large variation in lamination within different or^J mutant littermates made determination of a representative photoreceptor composition very difficult. The percentage of rhodopsin-expressing cells among dissociated cells from or^J mutants was approximately 10–25% of all cells compared to over 70% of all wild-type retinal cells (data not shown).

Discussion

Analysis of Chx10 using a multifunctional BAC reporter

The results presented here demonstrate the many uses of multifunctional reporters for genetic analyses. The single cassette of GFPCre-IRES-AP as a reporter allowed for simultaneous reporter activity in both the nucleus and membrane of progenitor and bipolar cells. Nuclear-localized GFP was particularly useful in performing marker analyses that helped reveal the presence of Müller glia cell markers in a very small number of Chx10-expressing cells and the identification of a progenitor expression profile in Chx10-expressing cells in the or^J mutant retina. Membrane-localized AP helped elaborate the processes of progenitor cells, bipolar cells, and the Chx10-expressing cells in or^J retinas.

Chx10 BAC reporter activity in Müller glial cells

Prior characterization of Chx10 expression in postmitotic cells, using antibody staining, suggested that Chx10 was expressed exclusively in all bipolar cells (Burmeister

et al., 1996). However, other data in the literature hinted at a role of Chx10 in Müller glial cells. Overexpression of Chx10 alone in mouse embryonic explants caused an increase in cells in the INL with Müller glial cell-like characteristics (Hatakeyama et al., 2001). Additionally, mice deficient for two transcription factors, Math3 and Mash1, did not generate bipolar cells, yet maintained Chx10 expression in the INL, presumably in Müller glial cells (Hatakeyama et al., 2001).

Analysis using the Chx10 BAC reporter revealed reporter activity in a subset of Müller glial cells. These cells morphologically resembled Müller glia in their ascending processes, endfeet, and marker expression. These results strongly suggest that endogenous Chx10 is also expressed in Müller glia, and likely defines a distinct subset of Müller glia. Although Müller glia show heterogeneity in their morphology as well as their density across the retina, molecular differences have not been reported (Sarthy and Ripps, 2001). It is tempting to speculate that a small of population of Chx10-expressing Müller glia may be more progenitor-like than non-Chx10-expressing Müller glia and may have distinct cellular properties, including possibly the injury response. Chx10 reporter-expressing cells in the mature or^J retina resembled Müller glial cells, both morphologically and based on marker expression studies. In many respects, these resembled Chx10-expressing cells in mice deficient for Mash1 and Mash3, lacking bipolar cells (Hatakeyama et al., 2001). These above mouse models may be interesting contexts in which to study possible functions of Chx10-expressing Müller glial cells.

Do bipolar cells contain ascending processes in mice?

AP staining analysis in mature retinas revealed that subsets of cone bipolar cells appear to have an ascending process that terminates at the outer limiting membrane. Such processes resemble the Landolt's Club process in lower vertebrates, including reptiles, amphibians, and some birds (Ramâon y Cajal, 1995). Although ascending processes have not been reported in the mouse, either from Cajal's cell labeling studies (Ramâon y Cajal, 1995), or more modern studies using injections of Lucifer Yellow and Neurobiotin (Ghosh et al., 2004), Golgi staining in the chimpanzee or rhesus macaque has revealed a highly analogous structure to those described in Figs. 4G–H (Polyak, 1941). Such bipolars were observed infrequently and in the near peripheral retina. Furthermore, like the ascending processes described here, their processes tended to be thin and extended only from a single dendritic branch. However, because of Chx10 reporter activity in both bipolar and Müller glial cells, this present study cannot exclude the possibility that the observed ascending processes are from Müller glial cells elsewhere in the retina. Future cell labeling studies in isolated single cells should resolve whether some bipolar cells have ascending processes in the mouse.

Differentiation in the or^J mouse

Previous characterization of the or^J mouse suggested that generation and at least partial differentiation of most retinal neurons occurred normally in the absence of Chx10, although retinal lamination was altered and bipolar cells were not generated (Burmeister et al., 1996). The reporter analysis presented here suggests that differentiation in the or^J retina did not occur normally. First, greater than half of the cells in the or^J retina expressed markers for progenitor cells/Müller glial cells. Second, Pax6 was expressed in approximately 80% of all cells in the or^J retina. Normally, the most abundant cell type is the rod photoreceptor, which constitute approximately 70% of all cells. We and others (Burmeister et al., 1996; Green et al., 2003; Rutherford et al., 2004) have found evidence for photoreceptors in or^J mice, but at highly reduced numbers.

The above data are consistent with a model where there are three distinct populations in the orJ retina: Pax6/GS-negative cells, representing differentiated photoreceptors; Pax6-positive/GS-negative cells, representing differentiated early-born cell types including amacrine cells, ganglion cells, and horizontal cells; and Pax6/GS-positive cells, representing a progenitor cell/abnormal Müller glial cell type. Such cells appear to express low levels of GS, CRALBP, and Pax6, similar to normal progenitor cells. Importantly, all Chx10 reporter-expressing cells scored in BAC transgenic or^J mutant retinas corresponded to these kinds of cells. Abnormal Müller glial cells resembling progenitor cells have been observed in studies that overexpress Notch1, Hes1, or Rax/Rx, all key genes involved in progenitor cell regulation, further suggesting a link between progenitor cell identity and Müller glial cell determination (Furukawa et al., 2000).

The analysis of the or^J retina using the BAC reporter lines strongly suggests a previously unappreciated role of Chx10 in differentiation of late-born cell types. A sole function for Chx10 in fate determination in postmitotic cells is inconsistent with our data since normal ratios of all the non-bipolar cell types should be present if Chx10 only functioned postmitotically. Additionally, this model does not account for the large presence of progenitor cell/abnormal Müller glial cells. The small total percentage of photoreceptors in the or^J retina also argues against Chx10 primarily functioning to repress photoreceptor development. The data presented here suggest a model in which Chx10 is required in late progenitor cells to repress the progenitor cell gene program. Only then could the program(s) of cell fate determination assign the correct identities to postnatally generated cells. Bipolar cells may be most affected since they are all late-born cells (as opposed to rod photoreceptors which are generated earlier but continue to be generated late), and their differentiation is likely blocked by expression of progenitor cell genes like Pax6. It should be pointed out that this model does not preclude additional roles for Chx10 in bipolar cell fate determination. Very recently, a

study analyzing photoreceptor differentiation in *or*^{J} mice also came to the conclusion that late-born cell types appeared to be preferentially affected in the *or*^{J} mutant (Rutherford et al., 2004).

Retina-specific knockouts can be generated using the Chx10 BAC

The BAC transgenic mice presented in this study represent a highly accurate and sensitive reproduction of the native Chx10 expression pattern in the eye throughout ocular development. Reporter activity was not detected outside of the eye in crosses with R26R mice, suggesting that some Chx10 enhancers are not present within the BAC. Within the retina, both transgenic lines expressed in the correct cell types, but not always in all of the Chx10-expressing cells. This phenomenon of mosaicism, also called position-effect variegation, has been observed in many transgenic mouse lines and varies enormously among different animals and different genetic backgrounds (Alami et al., 2000; Martin and Whitelaw, 1996; Wilson et al., 1990). Despite the presence of mosaicism, we did not see ectopic expression of the reporter, using AP staining or crosses to R26R mice. Furthermore, the mosaic nature of BAC reporter expression allows one to create and mark small mutant clones.

Many Cre-expressing mice have been reported to mediate excision in retinal progenitor cells. However, only two transgenic mice have been successfully used for these purposes: a Pax6 enhancer that mediates excision in the periphery of the retina (Kammandel et al., 1999; Marquardt et al., 2001) and a Foxg1-Cre knock-in mouse that mediates excision in the early telencephalon, head structures, and anterior optic vesicle (Hebert and McConnell, 2000). Chx10 BAC reporter transgenic mice complement and extend this repertoire by mediating retinal-specific excision as early as E10–E10.5. These lines exhibit excision in virtually all progenitor cells of the retina in non-mosaic animals and mark sites of recombination by expression of GFP or AP. The usefulness of Chx10 BAC transgenic mice in performing retinal knockouts has recently been validated in generation of a retinal Retinoblastoma (Rb1) knockout (Zhang et al., 2004). The data presented here illustrate the value of multifunctional BAC reporters for a large number of developmental and genetic studies.

Note added to proof

A new lacZ reporter line for Cre activity has been developed by Anna Farago and Susan Dymecki (personal communication). Although this line is in early stages of characterization, a cross of Chx10 BAC line 2 to this reporter revealed more lacZ-positive cells within the CNS than our previous cross to R26R, allowing for the possibility that there is Cre activity at a low level in some other CNS locations.

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