Neuron, Vol. 26, 383–394, May, 2000, Copyright ©2000 by Cell Press

rax, *Hes1*, and *notch1* Promote the Formation of Müller Glia by Postnatal Retinal Progenitor Cells

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Summary

We are interested in the mechanisms of glial cell development in the vertebrate central nervous system. We have identified genes that can direct the formation of glia in the retina. *rax*, a homeobox gene, *Hes1*, a basic helix-loop-helix gene, and *notch1*, a transmembrane receptor gene, are expressed in retinal progenitor cells, downregulated in differentiated neurons, and expressed in Müller glia. Retroviral transduction of any of these genes resulted in expression of glial markers. In contrast, misexpression of a dominant-negative *Hes1* gene reduced the number of glia. Cotransfection of *rax* with reporter constructs containing the *Hes1* or *notch1* regulatory regions led to the upregulation of reporter transcription. These data suggest a regulatory heirarchy that controls the formation of glia at the expense of neurons.

Introduction

The diverse cell types found in the vertebrate central nervous system (CNS) can be broadly divided into two distinct classes: neurons and glia. Although the functions of glia are still being discovered, it is known that they play a number of roles in the support of neurons, such as uptake of neurotransmitters and insulation of axons. Very little is known about the molecular mechanisms underlying gliogenesis during development, although some genes that influence this process are beginning to be identified (Lillien, 1995; Bonni et al., 1997; Rajan and McKay, 1998; Morrow et al., 1999).

The vertebrate retina, which contains one glial (called Müller glia) and six neuronal cell types, has served as a model system for cell type specification in the CNS (Cepko et al., 1996; Harris, 1997). Two features regarding the genesis of Müller glia have been established. First, birth-dating studies demonstrated that each type of retinal cell is generated in a characteristic order with Müller glia, rod photoreceptors, and bipolar interneurons being born last (Young, 1985). Second, lineage analysis revealed that neurons and glia are derived from a common progenitor cell (Turner and Cepko, 1987). In a terminal cell division of a late progenitor cell, which results in only two daughter cells, a single rod photoreceptor and a Müller glial cell can be generated. In addition, no clones of >1 cell containing Müller glia only were detected. The data from lineage analysis have thus argued against the presence of a progenitor cell dedicated to gliogenesis alone, or the generation of glia by the division of preexisting Müller glia during normal perinatal development.

Some of the molecular signals that can influence the production of glia by retinal progenitor cells have been described. Previous studies have highlighted the role of one molecule, the basic helix-loop-helix gene, *neuroD*, in negatively regulating gliogenesis (Morrow et al., 1999). *neuroD* is expressed in a subset of developing and mature rod photoreceptors, as well as in cells that have recently exited the cell cycle and appear to be fated to be amacrine cells. Forced expression of *neuroD* in progenitor cells in vivo using a retrovirus vector resulted in the complete absence of Müller glia, along with a significant increase in the number of amacrine cells, a slight increase in the number of rods, and a decrease in bipolar cells. Retinas of mice homozygous for a deletion of *neuroD* had an increase in Müller glial and bipolar cells and a slight decrease in the number of rods. These lines of evidence have suggested that *neuroD* promotes the development and/or survival of rods and amacrine cells, while suppressing two of the last-born cell types, Müller glia and bipolar neurons.

Signaling through the receptor for epidermal growth factor, EGF-R, has been implicated in promoting the choice of glial cell fate. When progenitor cells were infected by a replication-incompetent virus expressing EGF-R and exposed to an EGF-R ligand, TGF-α, the number of clones containing Müller glia doubled (Lillien, 1995). Since the level of EGF family ligands gradually diminishes during retinal development, Lillien has argued that Müller glia are generated from the subset of retinal progenitor cells that are capable of responding to lower levels of the ligand. Cultured cells from the cerebral cortex respond to another ligand, CNTF, by producing glia (Bonni et al., 1997; Rajan and McKay, 1998). In contrast, retinal progenitor cells respond to CNTF in vitro by overproducing cells that express at least three markers of bipolar neurons at the expense of rod photoreceptors (Ezzeddine et al., 1997). However, overproduction of bipolar cells due to CNTF exposure did not significantly alter the number of glia produced.

Recent work has focused on the role of $p27^{kip1}$, a cyclin-dependent kinase inhibitor, in the genesis of Müller glia. In *Xenopus*, overexpression of $p27^{Xic1}$, a *Xenopus* homolog of $p27^{kip1}$, resulted in an increase in Müller glia and a concomitant decrease in bipolar cells (Ohnuma et al., 1999). Moreover, the misexpression of *notch* in conjunction with $p27^{Xic1}$ resulted in an even greater increase in the number of Müller glia, suggesting that $p27^{kip1}$ may collaborate with the *notch* pathway. Levine and coworkers have analyzed the role of $p27^{kip1}$ in the

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mouse retina (Levine et al., 2000). When mice homozygous for the $p27^{kip1}$ deletion were analyzed, no decrease in the number of Müller glial cells was found, suggesting that $p27^{kip1}$ is not involved in Müller glial cell development in the mouse or that it is redundant for this function.

Several genes necessary for gliogenesis in *Drosophila melanogaster* have been cloned. The development of lateral glia in *Drosophila* depends on a master regulator gene, *gcm*. *gcm* activates other transcription factors required for normal gliogenesis, such as *repo*, *pointed*, and *tramtrack* (for review, see Granderath and Klambt, 1999). Although vertebrate homologs of *gcm* genes have been identified (Kim et al., 1998), there is no evidence that these homologs are involved in the production of glia.

We were interested in genes expressed in retinal progenitor cells that were downregulated in neurons but expressed in differentiating glia. Our initial studies revealed that a subset of previously identified genes fulfilled these criteria. *rax* is a paired-type homeobox gene that is expressed in retinal progenitor cells (Furukawa et al., 1997; Mathers et al., 1997). Since homozygous *rax* mutants fail to develop an optic vesicle, *rax* has been known to play a role in the initial formation of the vesicle, perhaps by promoting the division of progenitor cells early in development (Mathers et al., 1997). However, due to the absence of an eye in mice homozygous for a null mutation in *rax*, the role of *rax* in the development and differentiation of different retinal cell types has not been clarified.

Two other genes, *notch1* and *Hes1*, also are expressed in retinal progenitor cells and are downregulated in differentiating and mature neurons (Tomita et al., 1996; Bao and Cepko, 1997). Previously, we showed that forced expression of a constitutively activated *notch1* gene in rat retinal progenitor cells blocked the normal differentiation of the neuronal cell types and promoted formation of an unidentified cell type (Bao and Cepko, 1997). *Hes1* is a basic helix-loop-helix (bHLH) gene that is known to act downstream of *notch1* (for review, see Kageyama et al., 1997). Like *rax* and *notch1*, the role of *Hes1* in retinal cell fate choices has remained unclear due to the embryonic lethality of mice carrying a homozygous *Hes1* mutation. However, cultures of *Hes1* knockout tissue did not contain bipolar neurons, which could be due to survival problems or an effect on cell fate (Tomita et al., 1996).

The goals of this study were to: (1) determine the patterns of expression of *rax*, *notch1*, and *Hes1* during later stages of retinal development; (2) characterize the effect of forced expression of these genes on retinal cell fate choices in vivo; and (3) investigate the transcriptional links among the three genes.

Results

rax, *Hes1*, and *notch1* Are Expressed by Differentiating Müller Glia

A paired-type homeobox gene, *rax*, is expressed at high levels in the anterior neural folds of the mouse at embryonic day 7.5 and then is restricted to retinal progenitor cells after that point (Furukawa et al., 1997). At postnatal day 9 (P9) of murine retinal development, the generation of retinal cells is nearly complete and the population of mitotic progenitor cells drops markedly. At P9, we observed that the in situ hybridization signal for *rax* persisted in the middle of the INL (inner nuclear layer) (Figures 1G–1I), where the cell bodies of Müller glia are located (Figures 1A–1F).

To further characterize the *rax*-expressing cells, we used fluorescent in situ hybridization (FISH) to detect *rax* transcripts. FISH was followed by fluorescent immunohistochemistry with an antibody against CRALBP, a Müller glial marker, and a FITC-conjugated secondary antibody (Figure 1). Using confocal microscopy, an optical section thinner than a single layer of cells was visualized. *rax* expression, appearing as intense red spots of fluorescence, colocalized entirely within cell bodies in the middle of the INL, which were outlined in green by anti-CRALBP staining (Figures 1G–1I). There was very scant *rax* signal on CRALBP-negative cells in the INL. Although *rax* expression in other cell types within the INL cannot be ruled out, the in situ hybridization signal of *rax* did not localize to the area immediately abutting the OPL (outer plexiform layer), where the cell bodies of bipolar cells are located (Figures 1A–1C), nor adjacent to the IPL (inner plexiform layer), where amacrine cell bodies are found.

Two other genes expressed in retinal progenitor cells also were found to have a pattern of expression similar to that of *rax* in the late postnatal retina. At P9, the signal from in situ hybridization for a negatively regulating bHLH molecule, *Hes1*, also was detected in the middle of the INL (Figures 1J–1L). *Hes1* expression (red fluorescence; Figures 1J–1L) localized to cell bodies outlined by CRALBP staining (green fluorescence; Figures 1K and 1L). Fluorescent in situ hybridization signal for *notch1* also persisted in the INL at P9 and localized to the cell bodies stained with the anti-CRALBP antibody (Figures 1M–1O).

Forced Expression of *rax* in Progenitor Cells Leads to Cells that Express Müller Markers

In order to determine if *rax* can influence the choice of cell fate in the retina, we created a replication-incompetent murine retrovirus coexpressing *rax* and *gfp* (*rax*-GFP) (Figure 2). *rax*-GFP virus and a control GFP virus, encoding GFP alone, were introduced into the retinas of rat pup littermates at P0 in vivo. After 4 weeks, 50 μm vibratome sections of infected retinas were visualized for the GFP signal to reveal the morphology of cells infected either by *rax*-GFP virus or the control GFP retrovirus. Clones derived from the control GFP virus infection contained primarily rod photoreceptors, as well as Müller glia and bipolar cells, as expected from previous studies (Figures 3A–3C). In contrast, only a small fraction of cells in *rax*-GFP-infected clones were rod photoreceptors. More than 90% of the *rax*-GFP-infected cells had their cell bodies in the INL, a process extending through the ONL (outer nuclear layer), and a terminus at the OLM (outer limiting membrane), a characteristic unique to the processes of Müller glia. Dense, bushy processes either terminating at the IPL, or extending to the ganglion cell layer (GCL) were also present in the *rax*-GFP virus–infected cells (Figure 3D).

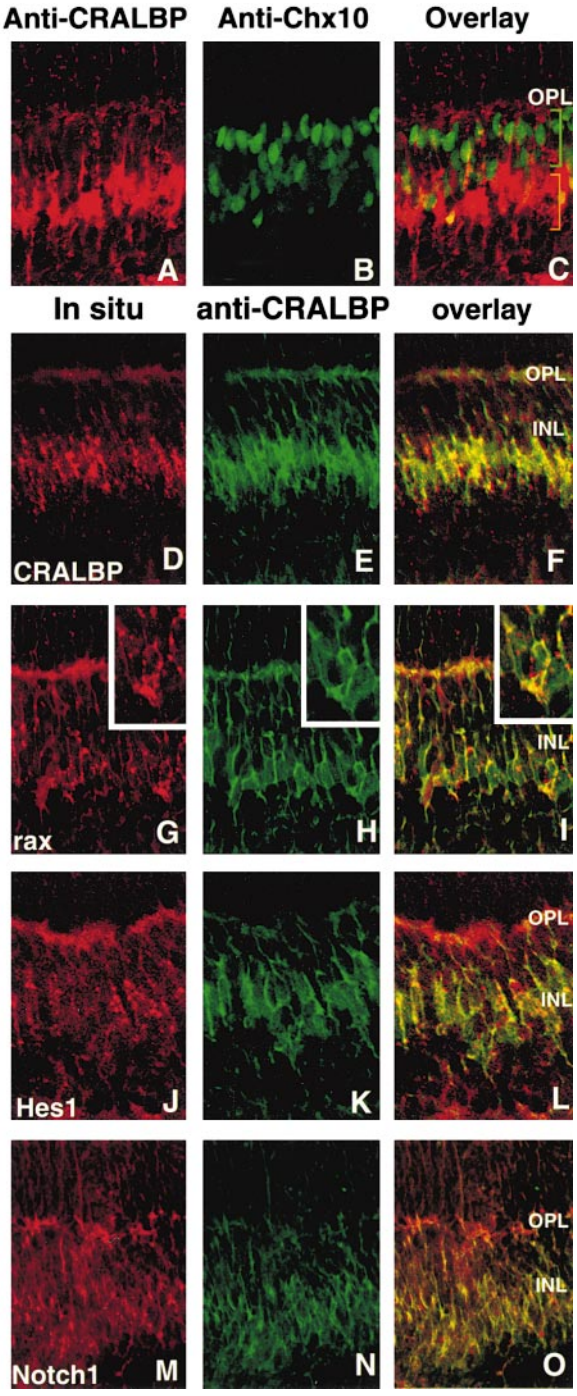

Figure 1. Immunohistochemical Analysis of Expression of *rax*, *Hes1*, and *notch1* in the Postnatal Retina

(A–C) Rat retinal sections at postnatal day 9 (P9) immunostained with (A) Anti-CRALBP/Texas red or (B) Anti-Chx10/FITC. (C) Overlay, showing bipolar cells (Chx10+ cells) lying immediately beneath the OPL (green bracket), and Müller glial cells positioned more vitreally within the INL (red bracket).

(D–O) Rat P9 retinal sections stained by FISH followed by immunostaining. In situ hybridization was carried out with probes against *CRALBP* (D–F), *rax* (G–I), *Hes1* (J–L), and *notch1* (M–O) (red fluorescence) and immunostaining by an anti-CRALBP antibody (green fluorescence). All optical sections were visualized by confocal microscopy.

(C, F, I, L, and O) Overlay images of each series. Overlapping staining appears yellow. OPL, outer plexiform layer; INL, inner nuclear layer. Insets in (G)–(I) show higher magnification of the sections.

Similar results were obtained when a virus, LIA, encoding *rax* and the human placental alkaline phosphatase gene as a marker was used to infect P0 rat retina. More than 90% of the *rax*-LIA-infected cells had also cell bodies in the INL, with a process extending to the OLM, and intense bushy, alkaline-phosphatase staining detected just beneath the cell body, occasionally extending to the GCL (data not shown).

Vibratome sections of *rax*-GFP-infected retina were stained with a panel of antibodies and Texas red (or Cy-3)–conjugated secondary antibodies. *rax*-GFP-infected cells were not stained by antibodies directed against characteristic markers for rods (Rho4D2 [Molday, 1989]), bipolar cells (Chx10 [Liu et al., 1994]), or amacrine cells (VC1.1 [Barnstable et al., 1985]). However, they were stained brightly by two markers found in adult Müller glial cells: CRALBP (Bunt-Milam and Saari, 1983) and cyclin D3 (C. Ma and C. L. Cepko, submitted) (Figures 3E–3G and 3H–3J, respectively).

To determine the fraction of *rax*-GFP-infected cells expressing the Müller marker, CRALBP, retinas from P0 rats were cultured as intact organ cultures ("explants") and immediately infected with the *rax*-GFP or control GFP retroviruses. After 14 days, the retinas were dissociated into single-cell suspensions. FACS was used to recover GFP-expressing cells from the whole population. The sorted cells were fixed and then stained with anti-CRALBP and Rho4D2 primary antibodies and appropriate Texas red–conjugated secondary antibodies. In retinas infected with the control GFP virus, 8% of the GFP-expressing cells stained positive for CRALBP, and 84% stained with the anti-rhodopsin antibody, Rho4D2. In contrast, while 72% of *rax*-GFP-infected cells stained positive for CRALBP, only 12% of the *rax*-GFP-infected cells were positive for Rho-4D2.

Quantification of clone size was difficult for *rax*-LIA- or *rax*-GFP-infected clones as the extensive processes of the labeled Müller-like cells surrounded some of the neighboring cell bodies, making it difficult to discern if they were expressing the marker. A retrovirus encoding *rax* and a nuclear β-galactosidase gene (*rax*-BIN) was thus created to allow for a clear quantitation of clone sizes. Rat retinas were infected with this virus in vivo. There was no difference observed in the clone size between control (BIN only) and *rax*-BIN (data not shown).

We also addressed whether the high percentage of clones misexpressing *rax* comprised Müller glial cells due to negative selection against neuronal cell types. Typically, when P0 rat retinas are infected with a control retrovirus (LIA) encoding just the alkaline phosphatase gene as a histochemical marker, only 5%–8% of clones contain Müller glia (Morrow et al. 1999). Since only 5%–8% of the clones arising out of control retrovirus infection contain glia, if all the neuronal clones died as a result of the misexpression of a gene, approximately 92%–95% of the clones would be eliminated. If this were the case, then infection with an identical number of viral particles of control LIA and *rax*-LIA should lead to 10-

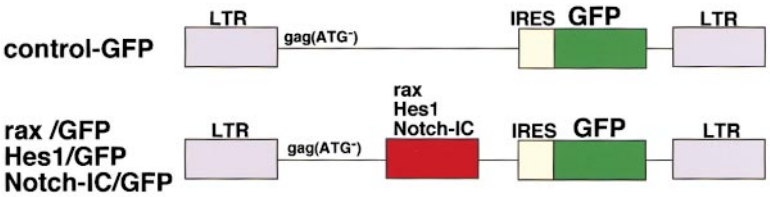

Figure 2. Virus Constructs Used to Express *rax*, *Hes1*, and *notch1-IC*

(A) The control GFP virus was derived from MMLV based viral vector called pBABE-MN-IRES-EGFP (gift of Dr. Gary Nolan, Stanford University). It is designed to express a marker gene, *GFP*, through an IRES sequence under the control of the LTR promoter.

(B) *rax*-GFP, *Hes1*-GFP, and *NIC*-GFP viruses each contain one of these genes cloned upstream of the IRES sequence. A bicistronic RNA containing one of these genes and the *GFP* gene is produced from the viral LTR promoter.

to 20-fold more clones for LIA than for *rax*-LIA, since all the neuronal clones infected with *rax*-LIA would die. To test this experimentally, we titrated the LIA and *rax*-LIA viruses in vitro on NIH 3T3 cells. An essentially identical number of viral particles was used to infect rat retinal explants at P0 with LIA and *rax*-LIA. We used two dilutions of virus, and, for each dilution, retinas were infected in triplicate. The number of clones per retina resulting from LIA and *rax*-LIA was within the same range for the higher dilution: 9.66 $\pm$ 5.88 versus 5.66 $\pm$ 1.52. Although the 5-fold lower dilution gave so few clones per retina that the data from this dilution were not statistically meaningful (1.5 $\pm$ 1.33 versus 1 $\pm$ 0.8), there was nonetheless again no great difference. *rax*-LIA infection did not result in 10- to 20-fold fewer clones as compared with LIA alone, as would be expected if there had been negative selection against neuronal cell types.

Forced Expression of *Hes1* in Progenitor Cells Leads to Cells that Express Müller Markers

To investigate whether *Hes1* can effect cell fate choices in the developing retina, a replication-incompetent murine retrovirus carrying the *Hes1* gene upstream of an IRES-GFP gene was created (*Hes1*-GFP) (Figure 2). P0 rat retinas infected with this virus were harvested at 4 weeks to analyze the effect on cell morphology and clone composition. Like the *rax*-GFP-infected cells, cells infected by the *Hes1*-GFP virus had a process extending from the cell body in the INL to the OLM (Figures 4A and 4B). In some cells, a bushy endfoot terminating at the GCL, characteristic of Müller glia, was observed, while in others, the endfoot was truncated at the INL/IPL border or within the IPL.

Vibratome sections of *Hes1*-GFP virus–infected retina were stained using the panel of cell type–specific antibodies. Analysis of these sections on the confocal microscope revealed bright staining of the infected cells by antibodies against CRALBP and cyclin D3, markers expressed by Müller glial cells in the adult retina (Figures 4C–4H). Staining by markers for other retinal cell types was undetectable (data not shown).

Expression of Activated Notch1 in Progenitor Cells Leads to Cells that Express Müller Markers

Like *rax* and *Hes1*, *notch1* is also expressed in differentiating Müller glial cells in the retina as well as in progenitor cells (Figure 1). We thus tested whether misexpression of activated Notch1 could also promote the choice of glial cell fate. The *NIC*-GFP retrovirus coexpresses a truncated Notch1 protein and GFP (Figure 2B). This truncated version of Notch1, consisting of only the cytoplasmic domain, has been shown to mimic constitutively active Notch1 in various systems (Coffman et al., 1993; Rebay et al., 1993; Nye et al., 1994). The *NIC*-GFP virus was introduced into the P0 rat retina in vivo. The infected retinas were harvested 4 weeks later, and vibratome sections were analyzed for GFP fluorescence by confocal microscopy. Similar to the findings in the previous study (Bao and Cepko, 1997), the clone size appeared to be substantially larger than in control viral infections and cell bodies appeared in large, abnormal clusters (Figures 5A and 5B). Many cell bodies were located in the INL, although the clusters also included cell bodies in the ONL. Some cells had the characteristic bushy Müller glial endfoot in the ganglion cell layer (Figures 5A and 5B).

Retinal explants prepared from the P0 rat pups were infected with the *NIC*-GFP virus and the control GFP virus and cultured for 10 days. Vibratome-cut sections were stained with antibodies specific for various cell types in the retina. Similar to the *NIC*-GFP in vivo infection, the majority of the cell bodies were found in the INL (data not shown). Most of the *NIC*-GFP-infected cells stained positively for the Müller glia markers CRALBP (Figures 5C–5E) and cyclin D3 (Figures 5F–5H), regardless of the location of the cell bodies. In contrast, most of the control GFP virus–infected cells were localized to the ONL and were positive for Rho4D2 staining (data not shown), indicating that they were normal rod photoreceptor cells. Further analysis was performed by immunofluorescent labeling with the cell type–specific antibodies on the dissociated cells from the *NIC*-GFP-infected retinal explants. About 90%–95% of *NIC*-GFP-infected cells expressed the Müller markers CRALBP and cyclin D3, compared with only 8% in the control GFP-infected cells.

Hes1 and *notch1* Are Upregulated in *rax*-Expressing Cells

Since transduction of *rax*, *Hes1*, or *notch1* via a retrovirus vector led to a similar phenotype, we wanted to investigate whether these genes were in a transcriptional cascade. To investigate whether the introduction of *rax* led to an increase in *notch1* and/or *Hes1* RNA levels, the retina of P0 rat pups was infected with *rax*-GFP or control GFP retrovirus. After 4 weeks, the retinas from these rats were harvested and dissociated into a single-cell suspension (Figure 6A). Live, dissociated

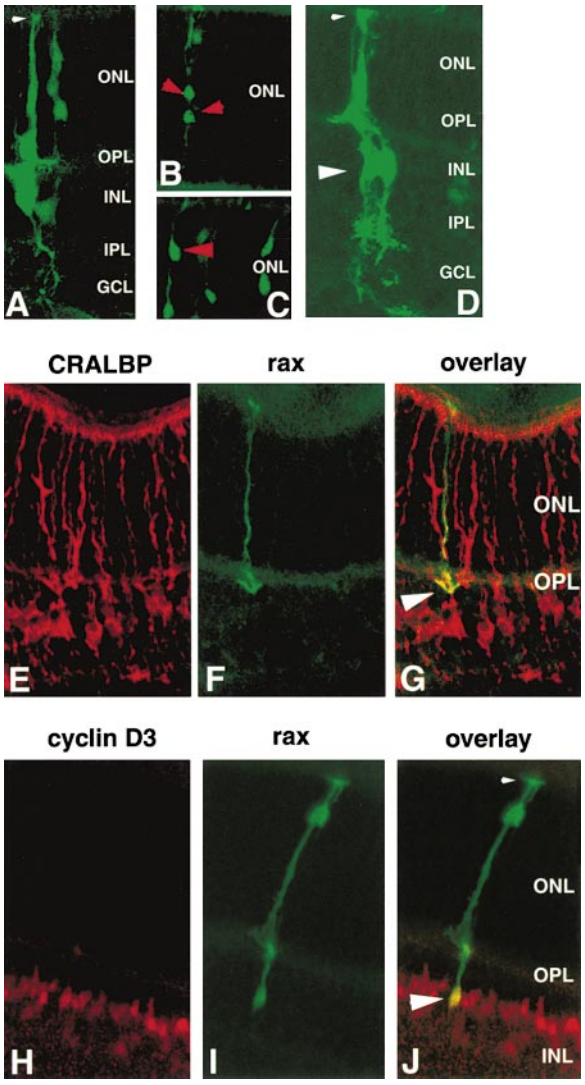

Figure 3. Morphology of Cells Transduced by *rax*-GFP Virus

(A–C) Rat retina (infected at P0, harvested at 4 weeks) infected by control GFP virus.

(A) Clone transduced with the control GFP virus containing one Müller glial cell and three rod photoreceptors.

(B and C) Clones containing rod photoreceptors.

(D) Rat retina showing the morphology of a two cell clone transduced by the *rax*-GFP virus. Both cells have processes terminating at the outer limiting membrane (small arrowhead), and one contains a characteristic endfoot in the ganglion cell layer. The cell bodies of *rax*-GFP-infected clones are located in INL (large arrowhead).

(E–J) CRALBP (E and G) or cyclin D3 (H and J) immunostaining of *rax*-GFP-infected cells at 4 weeks visualized by optical thin section confocal microscopy. GFP signal for infected cells is in green (F and I), while immunostaining is in red (E and H). Overlapping signal appears in yellow (G and J) and is marked by a large arrowhead. Optical thin sections reveal only a *part* of the morphology of the entire cell; the whole cell occupies and arborizes in multiple layers and is depicted fully in (D) above. Note that CRALBP gives cellular staining and cyclin D3 gives nuclear staining.

ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

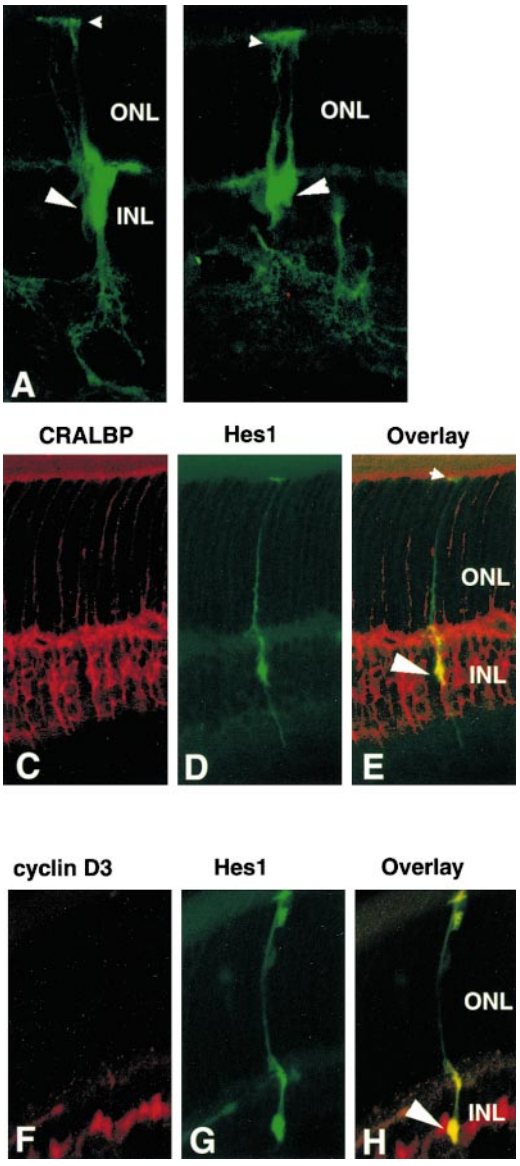

Figure 4. Morphology of Cells Transduced by *Hes1*-GFP Virus

(A and B) Cells infected by the *Hes1*-GFP virus at P0 in vivo and harvested at 4 weeks have a terminus at the outer limiting membrane (OLM) where the termini of Müller glial cells are found ([A and B], small arrowheads) and cell bodies located in the INL ([A and B], large arrowheads). Bushy endfeet terminate at the INL or extend to the ganglion cell layer (GCL).

(C–H) *Hes1*-GFP-infected cells immunostained with anti-CRALBP and anti-cyclin D3. Green signal indicates GFP fluorescence, while red indicates CRALBP (C and E) and cyclin D3 (F and H) immunostaining on the same section. Overlapping signal is indicated in yellow and by a large arrowhead.

ONL, outer nuclear layer; INL, inner nuclear layer.

cells from four rat retinas were sorted by FACS, and about 2000 GFP-positive cells of each population (*rax*-GFP-infected and control GFP-infected) were recovered (Figure 6B). Nested RT-PCR using intron-spanning primer pairs for *Hes1* and *notch1* was performed on the cDNA synthesized from the FACS-sorted cells. In three separate trials, bands of appropriate size for *notch1* and *Hes1* were found in the *rax*-GFP-infected, but not in the control GFP-infected, population of cells, showing that cells misexpressing *rax* also expressed *notch1* and *Hes1* (Figure 6C).

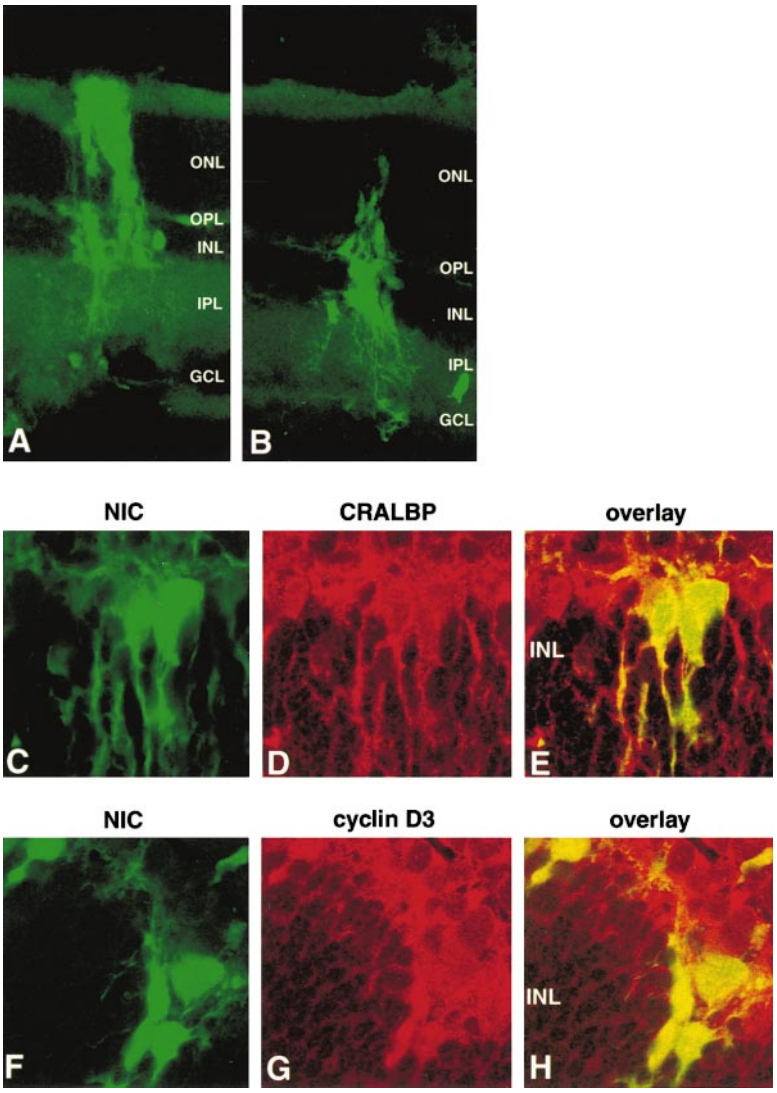

Figure 5. Morphology of Cells Transduced by *NIC*-GFP Virus

(A and B) Cells infected by the *NIC*-GFP virus at P0 in vivo and harvested at 4 weeks have cell bodies in the INL as well as the ONL with bushy processes extending from the ganglion cell layer to the outer segment layer.

(C–H) Rat retina (explanted at P0, harvested at P10) showing the GFP label of the *NIC*-GFP virus–infected cells (in green) and the immunostaining of anti-CRALBP (C–E) or cyclin D3 (in red) (F–H). Overlapping signal is indicated in yellow.

ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

We next analyzed whether *rax* could lead to the transcriptional activation of *notch1* and *Hes1* promoters in a heterologous system using a transient transfection method (Figure 6D). A CAT reporter plasmid containing mouse *Hes1* genomic sequences from −2000 to +46 appended upstream of the CAT gene (Takebayashi et al., 1994) was cotransfected with a plasmid expressing *rax* into NIH 3T3 cells. Increasing the amount of the cotransfected *rax* plasmid led to an increase of CAT activity in a dose-dependent manner, up to 5-fold relative to a control with no cotransfected *rax* (Figure 6D). Another CAT reporter plasmid containing approximately 11 kb of the promoter region of mouse *notch1* (Lewis et al., 1998) also was tested for transcriptional activation by *rax*. *rax* also led to transcriptional activation of the *notch1* promoter region in a dose-dependent fashion to a similar degree as activation of *Hes1*-CAT (Figure 6D).

Since some genes involved in transcriptional activation have reciprocal relationships with each other (Pignoni et al., 1997), we also examined whether Hes1 and Notch1 may have any activity on the *rax* promoter. In order to test this, we cloned a 7 kb region upstream of the mouse *rax* gene. This fragment was cloned upstream of a CAT gene and a CAT assay was performed with Notch1 and Hes1. We found no evidence for *trans*-activation of the *rax* promoter by either Notch1 or Hes1. In fact, both Notch1 and Hes1 suppressed transcription of the *rax* promoter in a dose-dependent manner (data not shown).

Misexpression of a Dominant-Negative *Hes1* Leads to a Reduction of Müller Glial Cells

Strom et al. (1997) created a dominant-negative mutant of the *Hes1* gene, which is able to inactivate the neurite outgrowth induced by wild-type *Hes1* in PC-12 cells. In order to study the effect of a dominant-negative *Hes1* on the composition of clones, a retrovirus encoding the dominant-negative *Hes1* (*D/N-Hes1*) and the human placental alkaline phosphatase reporter gene was created (Figure 7A). Retinas infected by the LIA-*D/N-Hes1* or control LIA virus were harvested at 4 weeks and stained for AP activity to reveal infected clones. About 7% of clones derived from the infection of P0 retinas with the control LIA virus contained Müller glial cells. In contrast,

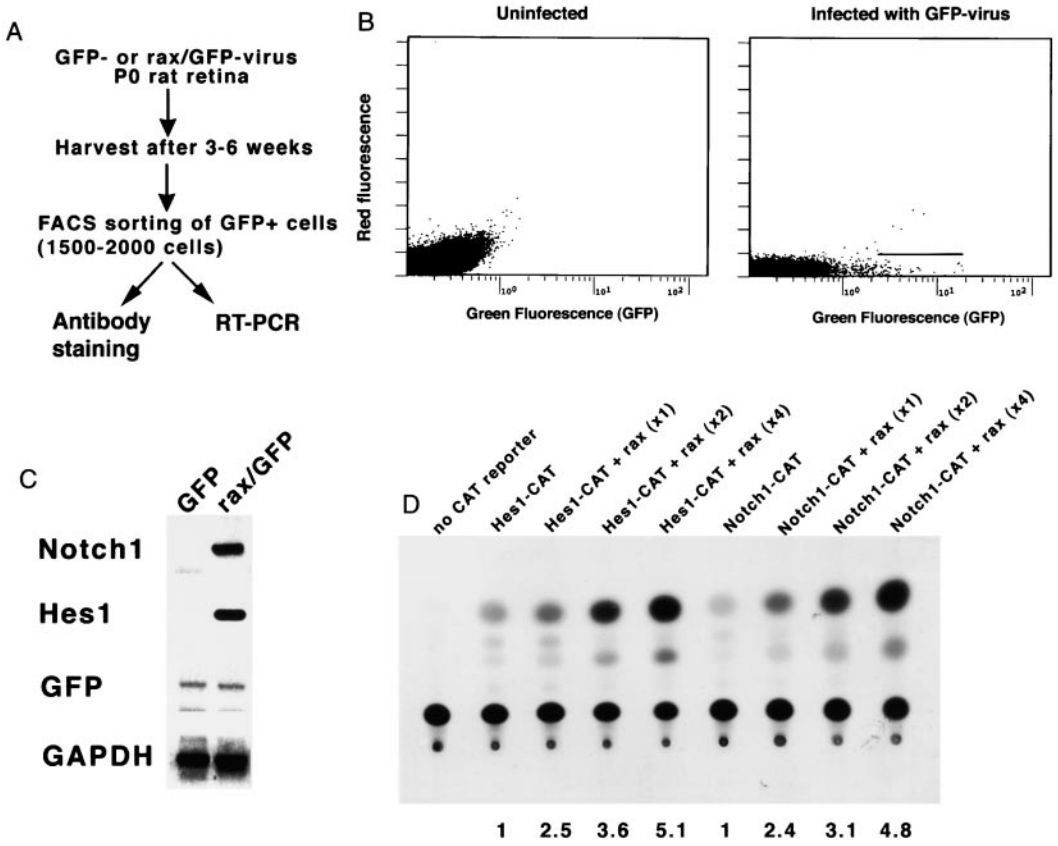

Figure 6. *rax* Induces *Hes1* and *notch1* Transcription

(A) Schema: retinas of P0 rat pups were infected with *rax*-GFP or control GFP retroviruses. After 6 weeks, the retinas were harvested, dissociated into a single-cell suspension and sorted for subsequent analysis by RT-PCR (see below).

(B) (Left panel) Live, dissociated cells from an uninfected retina were sorted using green and red fluorescence. Dying cells fluoresce nonspecifically and are both red and green. In the uninfected retina, there is no population of cells that is only green (far right). (Right panel) Dissociated cells from retina infected with the *rax*-GFP virus showing a population of live, GFP expressing cells that fluoresce brightly green (far right of plot).

(C) RT-PCR was performed to examine the expression of *notch1*, *Hes1*, *GFP*, and *GAPDH* in these cells. Although *GFP* and *GAPDH* were detected in both populations, *notch1* and *Hes1* were detected only in FACS-sorted *rax*-GFP virus–infected cells. The size of each band for *notch1*, *Hes1*, *GFP*, and *GAPDH* in RT-PCR is 242 bp, 168 bp, 307 bp, and 450 bp, respectively.

(D) CAT reporter plasmids, pHes1-CAT and pNotch1-CAT, were transfected into NIH3T3 cells with either control vector pME18S (SRα promoter) or pME18S-*rax* plasmid. The amount of pME18S-*rax* plasmid is 5 μg ($\times$1), 10 μg ($\times$2), or 20 μg ($\times$4). The total amount of expression plasmid is adjusted to be 20 μg using pME18S plasmid for each transfection. The relative CAT activity derived from each transfection is indicated below. Loading was normalized by reference to levels of β-galactosidase activity derived form the cotransfected pSVβ plasmid. These numbers below represent the average of three experiments.

we observed a 5-fold decrease in the fraction of clones containing Müller glial cells in retinas infected with the LIA-*D/N-Hes1* virus (Figure 7C). The total number of glia also was reduced. In addition, a moderate decrease in bipolar interneurons was also observed (Figure 7B).

Discussion

In this report, we show that a paired-type homeobox gene, *rax*, is expressed in differentiating Müller glia in the postnatal rodent retina. Misexpression of *rax* in rat retinal progenitor cells using replication-incompetent murine retroviruses gave rise to clones of cells that express the characteristic markers of Müller glial cells. We also found that two other genes that are downregulated in neurons, *notch1* and *Hes1*, continue to be expressed in glial cells early in their development. Misexpression of these molecules in the retina also gave rise to cells expressing glial markers. Misexpression of a dominant-negative *Hes1* led to fewer glial cells. Finally, Rax led to the transcriptional activation of the promoters of the *notch1* and *Hes1*. These lines of evidence suggest that *rax*, *notch1*, and *Hes1* are involved in promoting the choice of glial cell fate in the retina.

Characteristics of Cells Infected with *rax* and *Hes1* Retroviruses

Infection of retinal progenitor cells with a retrovirus expressing *rax* resulted in cells that possessed several features of Müller glia. The virally transduced cells had nuclei in the INL and an apical process at the OLM, which are features of Müller glia. However, there was a difference between the morphology of normal glia and

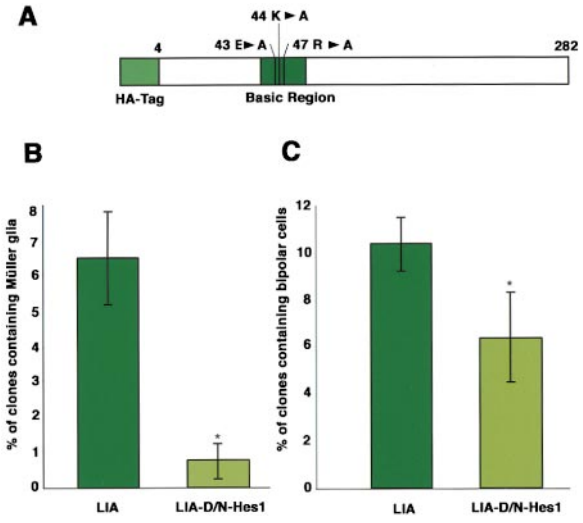

Figure 7. Reduction of Müller Glial Cells by the Dominant-Negative *Hes1* Virus

(A) Structure of the dominant-negative *Hes1* showing mutations at positions 43, 44, and 47. The amino acid preceding the arrow denotes the original, and the changed amino acid follows the arrow.
(B and C) The composition of clones derived from the infection of P0 rat retina with the control virus LIA (dark green bars) and a virus expressing dominant-negative *Hes1*, LIA-*D/N-Hes1* (light green bars).
(B) Reduction of clones infected by LIA-*D/N-Hes1* containing Müller glia (*$p < 0.01$ by two-tailed t test).
(C) Moderate reduction of the percent of clones containing bipolar cells (*$p < 0.05$ by two-tailed t test). All infections were at P0, and harvests were at P21. A total of 496 clones in four retina were counted for LIA-*D/N-Hes1*, and 365 clones in three retina were counted for for LIA.

some *rax*-GFP-infected cells. The endfeet on many infected cells appeared to be in the INL or IPL, as opposed to the inside surface of the fiber layer, as in normal Müller glia. However, these cells were indistinguishable from surrounding Müller glia in terms of the expression of several Müller-specific markers, such as cyclin D3 and CRALBP. *rax* overexpression in the *rax*-GFP-infected cells may have perturbed the morphology of the Müller glia. Alternatively, the virally transduced expression of *rax* on progenitor cells may have been insufficient to specify all the morphological characteristics of Müller glia.

Lineage studies have demonstrated that infection of the rat retina at P0 with a retrovirus carrying only a histochemical marker resulted in a small percentage of clones (3%–7%) containing Müller glia (Turner and Cepko, 1987; Morrow et al., 1999). In an extensive data set containing approximately 1500 clones, clones containing two or more Müller glial cells, and no other cells, were never found (Turner and Cepko, 1987). This observation has suggested that, during perinatal development, Müller glia are not generated by the division of a progenitor exclusively dedicated to the production of Müller glia, or from preexisting glia. In contrast, almost every clone arising from the infection with *rax*, activated *notch1*, or *Hes1* retroviruses contained cells that expressed Müller markers. Two cell clones with both cells showing Müller-like morphology frequently were observed (Figures 3 and 4). Given the fact that lineage analysis does not support the idea of a progenitor dedicated to Müller glia generation, these clones are unlikely to be due to the selective survival of one set of "glioblastic" progenitor cells after retroviral infection. It is also unlikely that the shift toward glial fate observed in *rax*- or *Hes1*-infected progenitor cells was due to a selective toxic effect on neuronal cell types. Since the titers of the control and *rax*-expressing retroviruses were in the same range (10^7 cfu/ml), the effect of specifically killing neuronal cell types would have decreased the apparent rate of infection of *rax*-infected retina by about 10- to 20-fold, which was not observed. It is also unlikely that Müller glia were selectively infected by viruses misexpressing *rax* or *Hes1*. The retroviruses used in this study infect only mitotically active progenitor cells. Previous work has demonstrated that there is no infection of dividing Müller glia in the perinatal period (Turner and Cepko, 1987) and, in fact, that there is very little division of Müller glia during this time.

The phenotype resulting from *rax* misexpression—the shift toward glial cell fate—was incompletely penetrant. Within some of the larger clones, we observed occasional rod photoreceptors, which were morphologically normal and which stained with an anti-rhodopsin antibody. The presence of the few infected photoreceptors found in *rax*-GFP-infected retina again argues against the selective toxicity of *rax* on photoreceptor cells. The partial penetrance of the phenotype may be due to the time of retroviral infection or the level of expression of *rax* in some virally transduced cells. The relationship between the time that a cell can be infected by a retrovirus, which requires that the cell undergo at least one mitotic cycle (Roe et al., 1993), and the time during which the fate of the cell is determined is unknown. The retroviral infection with *rax* and *Hes1* viruses was carried out at P0, a time at which an overwhelming majority of progenitor cells is normally fated to become rod photoreceptors. The retroviruses misexpressing *rax* and *Hes1* may have infected cells in various stages of commitment to their final fate and may have been unable to redirect the fate of the fraction of cells irreversibly committed to the rod fate. Alternatively, *rax* and *Hes1* misexpression may have had to compete with other intrinsic and extrinsic factors, which, at P0, would be expected to drive the cells toward the rod pathway.

Previous studies have reported a potential role for *rax* in the proliferation of retinal progenitor cells (Mathers et al., 1997). Homozygous *rax* mutants do not develop an optic vesicle, suggesting that *rax* plays a role in the designation of tissue as retinal, directly plays a role in retinal proliferation, and/or is required for the survival of early retinal cells. However, using multiple viral constructs, *rax* expression did not alter the size of the clones. At postnatal stages in the rat retina, the rate of proliferation of retinal cells falls dramatically, implying that intrinsic and/or extrinsic signals driving proliferation are limiting. Even if Rax normally drives proliferation at early stages, by P0, when the viral infections were carried out, *rax* expression may not have been sufficient to drive the proliferation of retinal progenitor cells in the absence of other proliferative signals.

Characteristics of *notch1*-Infected Cells

In a previous study, we reported that retinal progenitor cells transduced with the same activated *notch1* gene

used here produced large clusters of cells with processes spanning all the retinal cell layers (Bao and Cepko, 1997). However, the characterization of these cells was limited by the dense signal from the AP histochemical stain within these cells, which prohibited a characterization of gene expression and a more detailed analysis of the morphology. Using the *NIC*-GFP virus, we were able to characterize cells transduced by activated *notch1* further. Cell bodies were in the INL and the ONL, with dense processes extending to the GCL in some cases. Virtually all infected cells stained brightly with antisera to three Müller glial markers and were negative for markers of other cell types, even though some of the infected cells resided in the ONL, where normally only rod photoreceptors reside.

Consistent with our previous study, and in contrast to the results of introducing *rax* or *Hes1*, the introduction of *NIC-notch1* resulted in a significant increase in clone size. The effect on clone size may be due to the fact that we were introducing a constitutively active allele of *notch1*. Progenitor cells that overexpress *rax* can upregulate *notch1* (as discussed below). However, since *notch1* most likely requires its ligand for activation, merely upregulating endogenous *notch1* will not necessarily lead to an increase in signaling by *notch1*. In the postnatal retina, *notch1* ligands, such as Delta 1, are most likely limiting. It is also interesting to note that introduction of *Hes1*, which is thought to be one of the genes upregulated by activated *notch1*, does not result in the same large clones generated by introduction of constitutively activated *notch1*. Activated *notch1* thus may regulate a broader repertoire of genes than *Hes1*, and some subset of these genes appears to be sufficient to drive proliferation of retinal cells.

Transcriptional Cascade of *rax*, *Hes1*, and *notch1*

rax, *Hes1*, and *notch1* are expressed by retinal progenitor cells and by differentiating Müller glia. In addition, when individually transduced, all three genes are capable of promoting the formation of cells that express markers of Müller glia. Since all three of these genes are presumably transcription factors, these observations raise the possibility that they either directly or indirectly regulate each other. There is evidence that activated *notch1* directly upregulates *Hes1* (Jarriault et al., 1995; Ohtsuka et al., 1999). Here we provide evidence that *rax* leads to upregulation of *Hes1* and *notch1*. Following infection with *rax*-GFP virus, both *notch1* and *Hes1* RNA were detected using an RT/PCR assay, while they were undetectable in cells infected with a control GFP virus when the PCR assay was run with the same number of cycles. We used a novel application of the retroviral technology to detect these RNAs. Previously, we had not been able to recover individual, intact infected cells from retinal tissue. Our analyses of the effects of an introduced gene on retinal cells had been limited to morphological criteria or to expression of a particular antigen that might define a cell type. The detection of *Hes1* and *notch1* RNA in infected cells was made possible by the advent of GFP-expressing retroviruses that can be sorted by FACS, coupled with a very sensitive RT-PCR assay on the sorted cells. In this paper, we directed our analysis toward candidate genes. In the future, it should be possible to use microarray technology to analyze global gene expression changes in infected cells marked by GFP and recovered from infected tissue.

The upregulation of *Hes1* and *notch1* by *rax* may be direct. Reporter constructs with either the *notch1* or *Hes1* regulatory regions showed a 5-fold induction in RNA levels when *rax* was cotransfected. The *Hes1* upstream region encodes two putative sites for a paired-type homeobox gene, such as *rax* (Dr. R. Kageyama, personal communication). The sequence of the 11 kb *notch1* regulatory regions (Lewis et al., 1998) is not yet known, but our data predict that such a site is present in *notch1* as well. Since *rax* is expressed prior to *notch1* or *Hes1* in the retinal anlagen (Tomita et al., 1996; Bao and Cepko, 1997; Furukawa et al., 1997), it is likely that at least the initial period of *rax* transcription is independent of *Hes1* and *notch*, while the subsequent expression of *Hes1* and *notch1* may be dependent upon *rax*. Cotransfection of *notch1* or *Hes1* with a reporter construct encoding 7 kb of the *rax* upstream region did not lead to upregulation of the reporter. Although it is possible that a larger regulatory region of *rax* might reveal regulation by *notch1* or *Hes1*, there is currently no strong rationale for why this should be so. Interestingly, *rax* may lead to upregulation of *Hes1* in two ways, through an increase in levels of *notch1* and through direct activation of *Hes1* transcription. Further studies will be required to define these relationships more clearly.

rax, *Hes1*, and *notch1* Promote the Choice of Glial Cell Fate

Our results demonstrate that the persistent expression of *rax*, *Hes1*, and a constitutively activated allele of *notch1* results in clones of cells that express glial cell markers. Although these three genes are also expressed in retinal progenitor cells, their persistent expression by retroviral transduction did not appear to generate a progenitor cell. Moreover, in addition to this common subset of genes, there are genes whose expression distinguish Müller glia from typical progenitors. Müller glia express cyclin D3 (C. Ma and C. L. Cepko, submitted), while retinal progenitor cells express cyclin D1. Müller glia express CRALBP, a gene not expressed by progenitor cells. Müller glia also express GFAP after injury to the retina (Osborne et al., 1991; Sarthy and Egal, 1995), although GFAP is only weakly expressed by intact retina, thus limiting its use as a marker for Müller glial cells.

Based on these characteristics, it is clear that the virally transduced cells do not represent a typical retinal progenitor cell. However, it is possible that the infected cells represent a hitherto undescribed population of persisting adult progenitor cells whose morphology and gene expression characteristics overlap considerably with those of Müller glia. Alternatively, the virally transduced cells may represent an abnormal cell type, such as a hybrid between progenitors and Müller glia. Even if such a population of late, persistent progenitors in the retina exists, there are currently no markers available that would allow us to distinguish these cells from Müller glia.

It is also unlikely that cells misexpressing *rax* and *Hes1* became Müller glia simply because the progenitor state had persisted in these cells and the infected cells

had acquired the fate of the last-born cell type: Müller glia. Birth-dating studies have shown that Müller glia, rods, and bipolar cells are the last-born cell types (Young, 1985). We found no evidence of the bipolar marker Chx-10 in the cells misexpressing *rax* and *Hes1* and very few cells expressing rhodopsin, a marker of rods. Moreover, when we analyzed the kinetics of CRALBP expression, we found that clones arising from *rax*, *NIC*, and *Hes1* misexpression expressed CRALBP at P5, at the same time as normal, uninfected Müller glia (data not shown).

There are similarities and differences between previous work on *notch* and *Hes1* and the results presented here. Similar to the rat retina, in the adult *Xenopus* retina, the *notch* homolog, *Xotch*, continues to be expressed in a small population of cells located in the INL that express Müller glial markers, but not markers of any neuronal cell type (Dorsky et al., 1995). Introduction of activated *Xotch* by lipofection into *Xenopus* retinal progenitor cells resulted in a blockade of neuronal differentiation (Dorsky et al., 1995). The transduced cells appeared to be single neuroepithelial cells. Cell division was not promoted, nor was formation of Müller glia. When the same allele of activated *notch* used in our study was introduced into the murine cell line, P19, glial differentiation was supported, and neuronal differentiation was blocked, once again implicating *notch* in the glial pathway (Nye et al., 1994). In a previous study of the mouse retina, *Hes1* misexpression led to clones whose cells were distributed throughout the retina and that were morphologically dissimilar to any cell type, including neuroepithelial cells and Müller glia (Tomita et al., 1996). The differences among these studies may be due to different constructs and/or the fact that different species, and perhaps different target cells within each species, were transduced. Alternatively, subtle differences in the level of expression of these genes lead to different outcomes.

A simple model that can be proposed is one in which the persistent expression of these genes leads to the promotion of the glial fate. All of the data reported here are consistent with this model, but they also raise questions concerning additional roles of these genes in retinal development. For example, we found that introduction of a dominant-negative allele of *Hes1* led to a decrease not only in Müller glia, but also a modest decrease in bipolar cells. Misexpression of the *D/N-Hes1* may have had several effects on the retina. *D/N-Hes1* may have interacted with one or several other members of the *Hes1* family of genes in the retina and thus affected the genesis of Müller glia and bipolar cells. But while the lack of specificity is an important caveat for any analysis of misexpression of dominant-negative genes, our results could also be explained by a specific and partial loss of *Hes1*. A role for *Hes1* in bipolar development is supported by the results of Tomita et al. (1996) in which they found no bipolar cells in cultures from *Hes1* knockout retinas. Relevant to a possible role of these genes in bipolar development are also our findings on the role of *neuroD* in retinal development. Since *neuroD* is a positive acting bHLH gene, its activity might act in opposition to *Hes1*. Introduction of *neuroD1* using a retrovirus vector led to an increase in both amacrine neurons and rod photoreceptors, accompanied by a complete loss of Müller glia and a modest reduction in bipolar cells. In the *neuroD1* knockout mouse, both Müller glia and bipolar cells were present at higher levels.

Taken together, the data discussed above suggest that a balance of positive and negative bHLH genes regulates the production of Müller glia and bipolar cells. *rax* and *notch1* may control this balance. It is also possible that *notch1* controls the differentiation of all retinal cell types. We have proposed that retinal progenitor cells are heterogeneous and change states of competence to make different cell fates over time (Cepko et al., 1996). In addition, at one point in time, we believe that more than one progenitor type is present. Introduction of *rax*, *Hes1*, and *notch1* may promote one progenitor cell state relative to others and/or may block production of certain cell types by particular progenitor cell types. For example, infected P0 cells may be blocked from producing rod and amacrine cells, which are normally made at an earlier time and thus by an earlier progenitor cell type, than Müller glia and bipolar cells. The infected cells may progress to a progenitor cell state that is among the last, one in which rods, Müller glia, and bipolar cells are made. Among the cell types normally made by late progenitor cells, only Müller glia normally maintain expression of *rax*, *Hes1*, and *notch1*. While in this progenitor state, it appears that Müller glia are induced by these genes. Since bipolar cells and rods do not maintain expression of these genes, formation of rod and bipolar cells may not be favored by their overexpression, and in fact, rods may be blocked by them. Rod induction may have a dependence upon a positive bHLH gene, such as *neuroD*. Thus, in a *neuroD* knockout, more progenitor cells may be available to enter the latest stage of progenitor cells, when bipolar cells and Müller glia are produced, perhaps explaining why there are more bipolar cells and Müller glia in a *neuroD* knockout retina.

In conclusion, *rax*, *Hes1*, and *notch1* appear to be in a transcriptional cascade that promotes the formation of Müller glia. They may also regulate the formation of bipolar cells. Further studies in which progenitor cell states are defined molecularly will aid in our understanding of potential additional effects.

Experimental Procedures

In Situ Hybridization and Immunohistochemistry

The full-length *rax* and *Hes1* cDNAs were used as probes for in situ hybridization as described (Sasai et al., 1992; Furukawa et al., 1997). Texas red–HNPP labeling and FISH was performed according to the manufacturer's protocol (Roche).

For immunohistochemistry, slides were blocked for 2 hr in PBS with 0.1% Triton X-100 (Sigma), called PBST, to which 3% donkey serum (DS) was added (PBST-DS), and washed three times in PBST. Primary antibody was diluted in PBST-DS and incubated for 1 hr. The primary antibodies, species, dilution, and source used are as follows: Rho4D2, mouse monoclonal, 1:400 (Molday, 1989); Chx10, rabbit polyclonal, 1:500, (Liu et al., 1994); VC1.1, mouse monoclonal, 1:400 (Barnstable et al., 1985); CRALBP, rabbit polyclonal, 1:2000 (Bunt-Milam and Saari, 1983); and mouse monoclonal, 1:2000 (gift of Jack Saari); cyclin D3, rabbit polyclonal, 1:100 (Santa Cruz Biotech, cat# sc-182). After washing three times in PBS with 0.1% Tween, the slides were incubated for 1 hr in PBST-DS and 1:200 dilution of appropriate dye-conjugated secondary antibodies (Jackson Labs). Slides were washed three times in PBS with 0.1% Tween

before mounting. All washing and reactions were carried out at room temperature.

Transactivation Assay

For the expression in NIH3T3 cells, the entire *rax* cDNA was subcloned into pME18S driven by the SR promoter. *Hes1* promoter (2 kb) –CAT reporter construct was a gift from Dr. R. Kageyama (Takebayashi et al., 1994). The *notch1* promoter–CAT reporter construct was made by inserting an XhoI-NaeI 11 kb *notch1* promoter fragment (Lewis et al., 1998) into the HindIII-BamHI site of pBLCAT3 with synthetic linkers (Luckow and Schütz, 1987). NIH3T3 cells on 10 cm dishes were transfected with 0, 5, 10, 15, or 20 μg of the *rax* expression vector, plus 7.5 μg CAT reporter vector and 2.5 μg of the *lacZ* expression vector (pSVβ, Clontech). After 2 days, cells were harvested, and CAT activity was measured according to Sambrook et al. (1989). These experiments were carried out three times. CAT activities were measured using a phosphoimager, and the values were averaged.

Virus Construct

A 1.2 kb XbaI mouse *rax* cDNA fragment containing the entire *rax* coding region was blunted and subcloned into the blunted BamHI site of the pGFP virus vector (pBABE-MN-IRES-E-GFP; gift of Dr. Gary Nolan, Stanford University) to create p*rax*-GFP. An EcoRI-AhaIII 1.1 kb rat *Hes1* cDNA fragment containing the entire *Hes1* coding region was blunted and subcloned in the blunted BamHI site of the pBABE-MN-IRES-EGFP virus vector (p*Hes1*-GFP). A 2.6 kb cDNA fragment encoding the mouse *notch1* cytoplasmic domain initiating at amino acid 1753 (Bao and Cepko, 1997) was subcloned into the StuI site of the pBABE-MN-IRES-EGFP vector to create the viral construct *NIC*-GFP.

To produce virus, the plasmids were transfected in to a Phoenix cell line (gift of Dr. Gary Nolan); supernatant was collected and concentrated (Cepko, 1997).

FACS Sorting and RT-PCR

Retina of rats at P0 were infected with control GFP and *rax*-GFP viruses as described (Cepko, 1997). After 4 weeks, four retinas resulting from infection with each virus were harvested, washed three times in HBSS with 10 mM HEPES (pH = 7.4) (HH solution), cut into small pieces, and incubated for 45 min in HH solution containing 5 mg/ml Collagenase (Type IV, Sigma) at 37°C. Retinal pieces were washed two times in HH solution and digested for 45 min with 100 U papain (Worthington) and RNAse-free DNAse (Worthington). After papain digestion, retinas were washed three times in HH and gently triturated through a Pasteur pipette to generate a single-cell suspension. These cells were then washed and resuspended in HH at a concentration of 5×10^6/ml. Typically, 90% of cells were found to be alive by the Trypan-Blue staining method.

After trituration, the resulting material was filtered through a 40 μm filter and FACS sorted. Previously, we had shown that the suspension material could be divided into three categories based on red and green fluorescence. Cell debris and dying cells were dimly red and green (i.e., nonspecific fluorescence). Live uninfected cells were neither red or green. GFP virus–infected cells were brightly green (10- to 100-fold brighter than background) without red fluorescence. In each of three trials, we recovered 2000 control GFP and *rax*-GFP-infected cells from four retinas.

Total RNA from the GFP-infected sorted cells was extracted using Trisol solution (GIBCO) and RNAse-free DNAse (Roche). cDNA was synthesized, and RT-PCR was conducted. RT-PCR primers for detection of rat GAPDH were 5′-AACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′ (Clontech), and of EGFP gene were 5′-GGACGGCGACGTAACGGCCACAAG-3′ and 5′-CACCAGGGTGTCGCCCTCGAACTTC-3′, which amplified 307 bp product. Product sizes were 450 bp and 307 bp for GAPDH and EGFP genes, respectively. PCR conditions were 94°C, 1 min, followed by 94°C, 30 s; 60°C, 50 s; 72°C, 50 s; 40 cycles. The rat *notch1* and *Hes1* gene products were amplified by nested RT-PCR. The first set of primer pairs for rat *notch1* transcripts were 5′-CTGCAGTGACAACATTGATGACTGTGCC-3′ and 5′-ACACTGACACTCGAAAGAGCCCAGTGTG-3′. The second set of primers was 5′-AGTGCCGCCTGTTTTCAGGGTGCCACCTG-3′ and 5′-TTGGCACCTAGAGCGCACTCATCCACG-3′, which amplified 242 bp product. The first set of primer pairs for rat *Hes1* transcripts was 5′-ACCGGACAAACCAAAGACAGCCTCTG-3′ and 5′-CTGCAGGTTCCGGAGGTGCTTCACTG-3′. The second set of primers was 5′-GACAGCCTCTGAGCACAGAAAGTCATC-3′ and 5′-CACTGTCATTTCCAGAATGTCTGCCTTC-3′, which amplified 169 bp product. PCR condition for either first or second round PCR were the same as described above.

Acknowledgments

We thank S. Fields-Berry, M. Samson, Juanita Campos, and J. Zitz for their technical assistance, F. J. de Sauvage for the gift of the *notch1* regulatory region, G. Nolan for providing the GFP retroviral plasmid, M. Caudyt for the dominant-negative *Hes1* allele, and R. Kageyama for providing the rat *Hes1* cDNA and the *Hes1* promoter-CAT plasmid. This work was supported by the Howard Hughes Medical Institute, the Harvard–MIT Health Sciences and Technology Program (S. M.), a grant from the National Eye Institute (EYO 8064), and a fellowship from National Institutes of Health to Z.-Z. B. (EY06726-01).

Received December 15, 1999; revised April 26, 2000.

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