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Expression of Developmentally Defined Retinal Phenotypes in the Histogenesis of Retinoblastoma

Federico Gonzalez-Fernandez,*†‖
M. Beatriz S. Lopes,* **
Jose M. Garcia-Fernandez,¶
Russell G. Foster,‡‖ Willem J. De Grip,#
Sérgio Rosemberg,** Steven A. Newman,†
and Scott R. VandenBerg‖

From the Departments of Pathology (Neuropathology), Opthalmology,† and Biology,‡ Graduate Program in Neurosciences,‖ University of Virginia Health Sciences Center, Charlottesville, Virginia; the Department of Morphology and Cellular Biology,¶ Faculty of Medicine, University of Oviedo, Oviedo, Spain; the Department of Biochemistry,# Center for Eye Research, University of Nijmegen, Nijmegen, The Netherlands; and the Department of Pathology (Neuropathology),** Faculty of Medicine, University of São Paulo, São Paulo, Brazil*

Retinoblastoma, the most common intraocular tumor of childhood, is a malignant neoplasm that arises during retinal development. The embryonal cell target for neoplastic transformation is not yet clearly defined. To better understand the histogenetic potential of this tumor, the expression of photoreceptor and glial cell-associated proteins were examined in 22 primary retinoblastomas. Interphotoreceptor retinol-binding protein (IRBP), cone and rod opsins were selected as the photoreceptor specific proteins due to their different temporal patterns of expression during normal retinal development. Neoplastic Müller cell differentiation, and non-neoplastic reactive astrocytes were identified using cellular retinaldehyde binding-protein (CRALBP), and glial fibrillary acidic protein (GFAP), respectively. Photoreceptor proteins were present in 16 cases and showed different cellular patterns of expression. IRBP and cone opsin were usually abundant. Although rod opsin was clearly identified in eight tumors, its expression was more restricted than either IRBP or cone opsin. This differential pattern of expression, opposite to the normal pattern of photoreceptor gene expression in the adult retina, corresponded to a marked decrease in mRNA for rod opsin. Cone opsin and IRBP colocalized in fleurettes demonstrating that neoplastic human cone cells are capable of IRBP synthesis. Müller cell differentiation was present in 12 of the 16 cases in which photoreceptor proteins were detected. In contrast, GFAP was only present in reactive, stromal astrocytes associated with blood vessels. Our data suggest that the retinoblastoma has the histogenetic potential of the immature neural retinal epithelium which can give rise to both photoreceptor and Müller cell lineages. The differential expression of cone and rod phenotypes in retinoblastoma is consistent with the "default" mechanism of cone cell differentiation. (Am J Pathol 1992, 141: 363–375)

A retinoblastoma arises during retinal development when both alleles of the rb tumor suppression gene are inactivated within a single cell.[1–3] The embryonal cellular target and the period of cytohistogenesis during which neoplastic transformation can culminate in a retinoblastoma is not known. There are three contemporary theories for the histogenesis of retinoblastomas. First, it has been proposed that retinoblastoma arises from a pluripotential neural stem cell that gives rise to both neuroblastic/neurosensory and astroglial cell types.[4–16] A second hypothesis proposes a restricted capacity for neuronal differentiation. This proposal is supported by *in situ*[17–20] as well as *in vitro*[21] studies in which retinoblastoma cells express neuronal specific proteins but not glial skeletal

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Address reprint requests to Dr. Federico Gonzalez-Fernandez, Department of Pathology (Neuropathology), University of Virginia Health Sciences Center, Charlottesville, VA 22908.

proteins. Another theory extends the concept of neuronal differentiation with a further restriction to photosensory cell "lineages."[22–30]

Previous investigations of photosensory proteins in retinoblastomas have not yielded comparable data about the specific cell lineages. Cone-specific proteins have been analyzed only in cultured tumor cells,[28] and in protein extracts of primary tumors.[29] Alternatively, immunohistochemical investigations of retinoblastomas *in situ* have used only IRBP and rod-specific proteins.[30,31] The current investigation is the first to examine the expression of cone opsin and CRAIBP in addition to IRBP and rod opsin to study the histogenetic potential of the retinoblastoma. Northern blot hybridization provided qualitative and quantitative analysis of photoreceptor gene expression in one case.

Material and Methods

Case Material

Enucleation specimens from 13 males and 9 females were examined (Table 1). The age at the time of surgery ranged from 2 months to 4 years. Two cases were surgical specimens removed at the University of Virginia (cases 2 and 22), and 20 cases were collected from the surgical pathology archives of the University of São Paulo, Brazil. Most of the tumors were restricted to the vitreous chamber, but in nine cases, the tumor breached the anterior chamber and invaded the optic nerve. In one case (case 22), unfixed tissue was also available for Northern blot analysis. Multiple tissue sections from all tumors were examined to confirm the diagnosis and characterize the histopathologic features.

Immunohistochemical Techniques

The tumor specimens were fixed in buffered formalin, except case 22, which was fixed in Bouin's fixative. Six-micron sections of paraffin-embedded tissue were deparaffinized in xylene for 10 minutes and rehydrated through graded ethanols. After abolishing endogenous peroxidase activity with hydrogen peroxide (0.5% in methanol for 30 minutes, 22°C), tissue sections were treated with primary antibodies for either 1 hour at 22°C or 15–18 hours at 4°C. Visualization of the primary antibodies was performed by using the avidin-biotin immunoperoxidase technique (Vectastain, Vector Laboratories, Burlingame, CA) with 3-3′-diaminobenzidine-tetrahydrochloride (DAB) as the chromogen. In two cases, the DAB reaction product was intensified with 0.2% osmium tetroxide. Surgical specimens of eyes (for photoreceptors and Müller cell proteins) and cerebral gliosis (for GFAP) were used as positive control tissue. Replacement of the primary antibodies with normal nonimmune rabbit serum served as negative controls.

Rabbit antibodies to the retinal proteins were previously produced by other laboratories: 1) anti-bovine IRBP

Table 1. *Photoreceptor and Glial Cell-associated Protein Immunohistochemistry*

Case (accession no.)	Sex/Age	Cone-opsin		Rod-opsin		IRBP		CRAIBP	GFAP
		Focal	Diffuse	Focal	Diffuse	Focal	Diffuse		
1. (C6644)	M/2y	−	−	−	+	NT	NT	*	NT
2. (S82-6252)	F/15mo	−	−	−	−	−	+ +	*	*
3. (C6645)	F/1y	+	−	−	+	+ +	+ +	*	*
4. (C6646)	M/2mo	+	−	−	−	+ +	−	0	*
5. (C6647)	M/11mo	−	−	−	−	−	−	0	*
6. (C6648)	F/18mo	−	−	−	+	−	−	*	*
7. (C6649)	F/4y	−	−	−	−	−	−	0	*
8. (C6650)	M/2y	−	−	−	−	−	−	0	*
9. (C6651)	M/2y	−	−	−	−	−	−	0	0
10. (C6652)	F/2y	+	+	+	−	−	+	*	*
11. (C6653)	M/3y	+	−	−	−	+ +	−	*	*
12. (C6654)	M/2y	+	+	+	+	+ +	+ +	0	*
13. (C6655)	F/1y	+	+	−	−	−	−	0	*
14. (C6656)	M/3y	−	−	−	−	−	−	0	*
15. (C6657)	M/3y	+	−	−	−	+ +	+ +	*	*
16. (C6658)	F/18mo	−	−	−	−	−	−	0	*
17. (C6659)	M/2y	−	+	−	+	−	−	0	*
18. (C6660)	F/2y	−	−	−	−	+	−	*	*
19. (C6661)	M/9mo	−	−	−	−	+ +	+ +	*	*
20. (C6662)	M/1y	+	−	−	+	+ +	−	*	*
21. (C6663)	M/2y	+	−	+	−	−	+	0	*
22. (S90-2951)	F/4mo	+	+	−	−	+ +	+ +	*	*

Focal = expression in well-defined group of cells or rosette structures; diffuse = random expression. (−) negative; (+) few positive cells; (+ +) many positive cells; (*) present; (0) not present; (NT) not tested.

serum (RB 504, 1:100 or 1:200);[32–35] 2) anti-bovine rod opsin serum (CERN-JS858, 1:2000 or 1:3000);[36–40] 3) anti-chicken cone opsin serum (CERN-874, 1:2000);[41,42] 4) anti-bovine CRALBP IgG (1:400 or 1:1000);[43] 5) anti-bovine GFAP (1:1400, Dako Corporation, Carpinteria, CA). Antiserum RB 504 was prepared against highly purified bovine IRBP[32,33] and its specificity was verified by Western blotting.[32–35] Antiserum against rod opsin (CERN-JS858) was produced in rabbits using purified lipid-free bovine rhodopsin prepared by concanavalin-A-affinity chromatography.[36] The production of rabbit anti-cone antibody (CERN-874) was recently described[41,42] using highly purified chicken cone opsin as the immunogen, and prepared by a modification of previously described methods.[44–46] The specificity of the antiserum (CERN-874) was confirmed by immunoblot analysis of cone (chicken, quail) and rod (chicken, bovine, mouse, rat, hamster) pigments and by immunohistochemistry of retinal tissue (chicken, rat, mouse, monkey). The immune serum cross-reacted with rod pigment only at decreased dilutions (1/<100) using immunoblot analysis. At the higher dilutions (1/>500) used in this study with human retina and elsewhere with mouse retina,[41] this antiserum was selective for cone pigments and intensely labeled the cone outer segments in control tissue. Rabbit anti-CRALBP IgG, supplied by J.C. Saari, was produced from purified bovine protein and shown to react selectively with CRALBP on Western blots from SDS and non-SDS PAGE.[43] Müller cells and retinal pigmented epithelium were selectively immunolabeled with this IgG fraction in rat, bovine, monkey, and human retinas.[43]

Preparation of RNA and Northern Blot Analysis

The enucleation specimen from a 4-month infant with sporadic retinoblastoma (case 22) was immediately transported from the operating room to the laboratory. This exophytic tumor arose from the posterior pole, involved the lamina cribosa, and extended approximately 1 cm into the posterior chamber. Portions of the tumor which were clearly separated from the retina as well as portions of peripheral retina, uninvolved by the neoplasm, were frozen in liquid nitrogen. The remainder of the globe was fixed in Bouin's fixative. Total RNA was prepared by the method of Chomczynski and Sacchi[47] as modified by Gonzalez-Fernandez and Healy.[48] This RNA extraction method was selected because it is suitable for quantitative extractions of RNA from small samples. Total RNA was denatured with glyoxal, subjected to electrophoresis, transferred to Nytran paper (Schleicher and Schuell, Keene, NH) and crosslinked by UV irradiation.[49] Prehybridization and hybridization were carried out at 42°C in 50% formamide, 5x SSPE (0.9 mol/l NaCl; 50 mmol/l phosphate buffer, pH 7.7, 5 mmol/l ethylenediaminetetraacetic acid [EDTA]), 5x Denhardt's solution (0.1% ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin [BSA]), 0.1% sodium dodecyl sulfate (SDS), and 100 μg/ml denatured salmon sperm DNA. The excised cDNA probes were gel purified and labeled with α-^{32}P by the random primer method. Blots were hybridized with 10^6 cpm of radiolabeled probe per ml of the aforementioned buffer. The bovine opsin probe is a full-length cDNA 1.0 kb in length starting at nucleotide number 234.[50] The human IRBP probe corresponds to the full-length human cDNA. The autoradiograms were exposed to XAR film (Kodak, Rochester, NY) overnight at −70°C with an intensifying screen.

Results

Light Microscopy

Sixteen cases had typical rosettes of the Flexner- Wintersteiner and Homer-Wright types that were either focal or diffusely distributed in the tumor (Figures 1C, 2A, 2B, 5A, 5C). A small number of cases had frequent fleurettes (Figure 7B). In three specimens (cases 12, 13, and 21) fields resembling the histopathologic features of retinocytomas[51] with lower cellularity and increased cytologic differentiation were present. Six tumors were primarily composed of poorly differentiated cells with no rosette formation.

Photoreceptor Differentiation

Immunohistochemistry demonstrated that photoreceptor differentiation, defined by IRBP, rod or cone opsin immunoreactivity, was present in 16 cases (Tables 1, 2). Each protein showed a distinctive cellular and cytoplasmic pattern of expression. In six cases, rod and cone opsins were both present. These cases did not show any specific histologic and/or immunohistochemical features, but all had both focal areas of rosettes and more diffuse fields of poorly differentiated cells without a discernible cytoarchitecture. The presence of rosettes or fleurettes was not necessarily associated with the expression of the photoreceptor proteins. In three cases, with rosettes and fleurettes, no immunoreactivity was present in any tumor cell (Table 1, cases 5, 8, and 16). In these cases, however, the neural retina was positive for each of the proteins. Fleurettes were associated only with cone immunoreactivity, whereas regions demonstrating feature of retinocytomas showed both rod and cone phenotypes.

Interphotoreceptor Retinoid-binding Protein

The normal spatial distribution of IRBP in the residual retina of the surgical specimens was disrupted due to de-

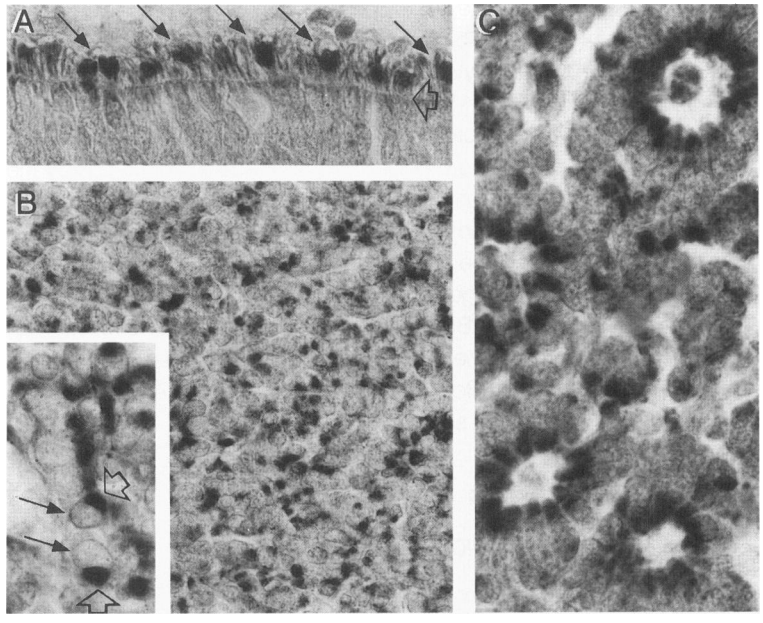

Figure 1. *Immunohistochemical localization of IRBP (case 22).* **A:** *A portion of the retina not involved with neoplasm shows IRBP staining of cone cell inner segments (slanted arrows). Outer segment degeneration is due to retinal detachment secondary to tumor expansion. Open arrow marks the external limiting membrane (×200).* **B:** *Highly cellular field without cytoarchitectural differentiation demonstrates IRBP immunoreactivity in most of the cells (×250). Inset: Higher magnification (×600) of similar field showing cytoplasmic polarization of IRBP (open arrows). Solid arrows indicate nuclei.* **C:** *Flexner-Wintersteiner rosettes with apical IRBP polarization (×400). RB504-ABC immunostaining with 0.2% osmium tetroxide intensification.*

tachment of the neural retina from the pigmented epithelium, degeneration of the photoreceptor outer segments, and expansion of the subretinal space. Figure 1a shows a portion of neural retina uninvolved with the tumor but detached from the pigmented epithelium. IRBP, however, could be identified in the inner segments of surviving cones (Figure 1A, arrows). The specific immunohistochemical distribution of IRBP in the normal control human eyes was similar to that previously described.[33,34,52,76] It was restricted to the region between the external-limiting membrane and the apical surface of the pigmented epithelium, where it was frequently prominent. The matrix surrounding the inner segments stained less intensely than at the level of the outer segments. Prominent labeling at the base of the inner segments was noted. Further evidence for the synthesis of IRBP by cones will be provided later.

IRBP was detected in 12 of the 22 retinoblastoma specimens in which it was present in the majority of the tumor cells. These immunoreactive cells were either diffusely and randomly distributed or formed rosettes. Unlike the opsin proteins, all cells comprising rosettes contained immunoreactivity for IRBP. Prominent cytoplasmic polarization was common not only in rosettes where IRBP was localized at the apical side of the cytoplasm (Figures 1C, 7D), but also in areas without distinct cellular patterns (Figure 1B, inset). IRBP was not found in the bulbous expansions of the fleurettes (Figure 7D), nor it was present in the lumen of these structures or Flexner-Wintersteiner rosettes.

Messenger RNA for IRBP was easily detected by Northern blot analysis of case 22 (Figure 3). The mRNA for IRBP from the tumor had an identical electrophoretic mobility as that from the patient's neural retina which was not involved with tumor, as well as normal adult retina.

Rod and Cone Opsins

Specific immunohistochemical staining for rod opsin was confirmed in normal eyes, where it was most intense in the outer segments of the rod photoreceptors. Unlike IRBP, immunostaining for rod opsin outlined the entire cell body of the rod cells (Figure 2). No crossreactivity of the rod antibody for cone opsin was observed. In Figure 2C, the cone cells, which have nuclei just below the external limiting membrane (elm), are surrounded by the immunopositive rod cells. In the same control specimens, the cone opsin antibody was specific for the cone outer segment (Figure 7A).

Rod opsin immunoreactivity was present in eight cases (Tables 1, 2) but was less abundant than for IRBP. Immunostaining was usually localized to the cellular periphery without cytoplasmic polarization. Rod opsin immunoreactivity was rare in cells forming rosettes (Figure 7G) but was exclusively abundant in areas with retinocytoma-like differentiation in two (cases 12 and 21) of the three cases with this distinctive histopathologic feature. Eleven of the 22 retinoblastomas contained a variable number of tumor cells expressing cone opsin. The immunoreactive cells were distributed in both rosettes (Figure 5B, C) and in areas without specific cytoarchitectural patterns (Figure 5A). The immunostaining was more abundant than rod opsin. The bulbous expansions of the fleurettes frequently had the most intense labeling (Figure 7B, C).

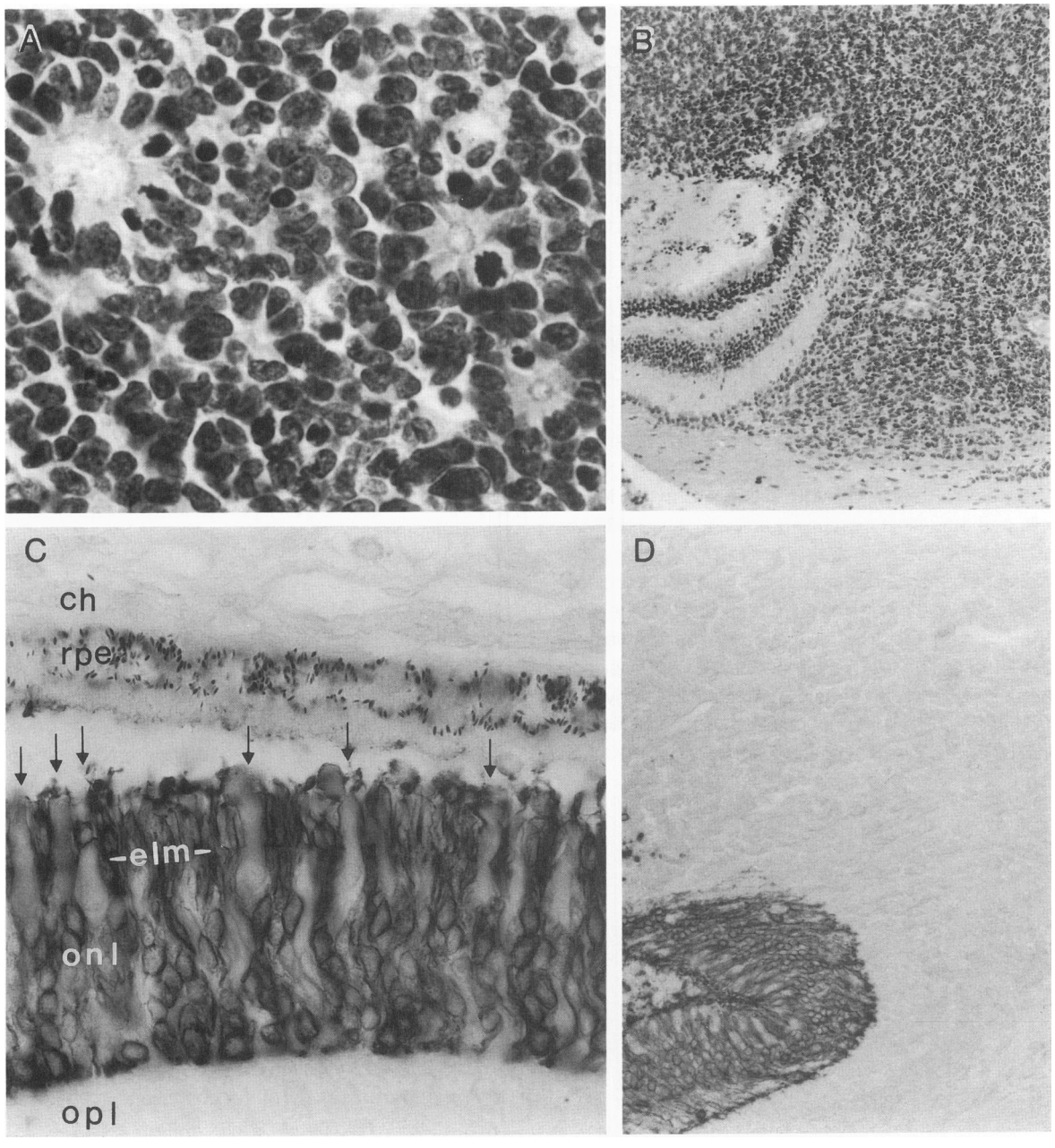

Figure 2. *Immunohistochemical localization of rod opsin (case 22).* **A:** *The tumor contained numerous Flexner-Wintersteiner rosettes (Hematoxylin and eosin, ×400).* **B:** *Portion of neural retina blending in with the tumor (Hematoxylin and eosin, ×150).* **C:** *Portion of neural retina uninvolved with tumor had moderate outer segment degeneration due to detachment of the neural retina from the pigmented epithelium. Rod cell inner segments, residual outer segments, and cell membrane stain intensely with the rod opsin antibody. Immunonegative cone photoreceptors are marked with arrows (CERN-JS858-ABC immunostaining with 0.2% osmium intensification, ×250).* **D:** *The same region as in* **(B)** *is treated with the rod opsin antibody. Although the neural retina is positive, the tumor is strikingly negative for rod opsin (CERN-JS858-ABC immunostaining with 0.2% osmium intensification, ×200).*

The mRNA for rod opsin in case 22 could not be detected by Northern blot electrophoresis (Figure 3, upper panel, lane 2). In contrast, rod opsin transcripts were easily demonstrated in both the patient's retina as well as the adult control retinas. The three forms of the mRNA for rod opsin probably represent use of different termination sequences in the 5′ untranslated region.[52] Quantitative densitometry of RNA dot-blots confirmed that the level of the mRNA for IRBP was significantly higher than that of rod opsin in the tumor (Figure 3). The opposite relationship held in the neural retina where the level of the mRNA for rod opsin was markedly higher than that of IRBP. The trace amounts of mRNA for rod opsin in the tumor may be due to decreased levels of expression of this message, or contamination of included pieces of residual retina. Since high-stringency conditions were used, the small

Table 2. *Photoreceptor and Retinal Glial Differentiation*

Neuroretinal differentiation (16 of 22 cases)					
Photoreceptor differentiation only = 5 cases			Photoreceptor and Müller differentiation = 11 cases		
Opsins only			IRBP only 3 cases	Opsins and IRBP	
C	C&R	R		C	C&R	R
2	1	2		4	4	0

C = cone; C&R = cone & rod; R = rod.

opsin signal in the tumor probably does not represent crosshybridization with cone opsin transcripts.

Glial Differentiation

In the normal retina, CRALBP was found in the Müller cells and the retinal pigmented epithelium. CRALBP immunopositivity was present in the villous processes at the level of the inner-limiting membrane, cell body, and foot process at the inner-limiting membrane (Figure 6A). GFAP immunoreactivity in the normal retina was restricted to astrocytes in the inner nerve fiber layer and often distributed in a perivascular pattern. In reactive retina, intense GFAP immunoreactivity was observed in both inner and outer segments.

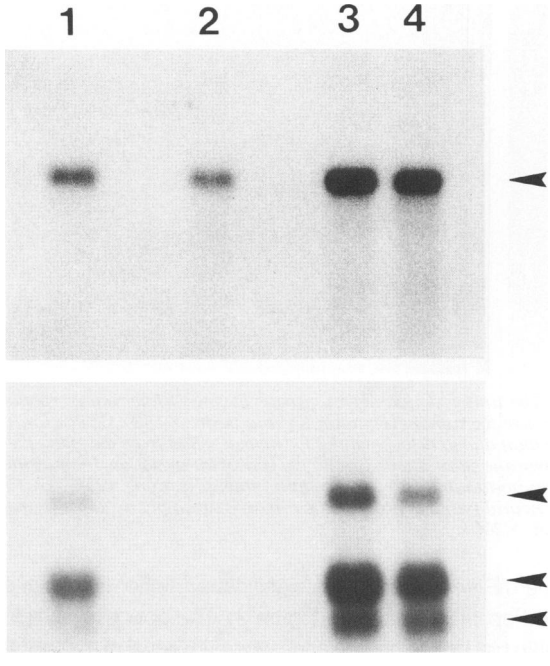

Figure 3. *Northern blot autoradiograms of retinoblastoma and neural retina without tumor. The blot was probed with a ^{32}P-labeled IRBP cDNA (Top), stripped and reprobed with a ^{32}P-labeled opsin cDNA (Bottom). Lanes: 1, uninvolved retina from the same eye containing the tumor (case 22); 2, tumor (case 22); 3 and 4, normal adult human retina (control retina). Arrowheads from top to bottom: 4.3kb, 3.0kb, 2.2kb, 1.9kb.*

CRALBP immunoreactivity was observed in 12 of the retinoblastomas. Immunoreactive cells were usually arranged in small clusters or scattered as isolated cells (Figures 6B, 7E) with elongated cell bodies and short processes reminiscent of Müller cell morphology (Figure 6A, inset). In contrast, no cells with either cytoplasmic pigment or with the characteristic histologic features of retinal pigmented epithelial cells were observed. Immunoreactive cells were also never found in rosettes. The nuclei of these cells were indistinguishable from adjacent tumor cells that did not stain.

GFAP immunoreactivity was abundant in processes associated with reactive astrocytes, which were frequently adjacent to blood vessels (Figure 7F) and residual neural retina. In adjacent serial sections, GFAP and CRALBP immunoreactivity were clearly distributed in different cell populations.

Discussion

The histogenesis of retinoblastoma may be better understood by examining proteins with different temporal and cell type-specific patterns of expression during normal retinal development. The well-defined cell populations constituting this highly organized structure in both developing and mature tissues particularly strengthen this approach. The present study used immunohistochemistry and Northern blot analysis, when unfixed frozen preserved tissue was available. The immunohistochemical assays used polyclonal antisera. These heterogeneous antibodies would have the potential for broad reactivity, and this caveat must be carefully addressed when polyvalent antibodies are applied to define specific histogenetic events. All the polyvalent antibodies in this study were prepared against purified proteins, and the specific protein and selective cellular immunoreactivity were previously verified by Western blot analysis and retinal tissue sections from a variety of vertebrate and mammalian species (Material and Methods). In the case of the homologous opsin proteins,[54,55] the generation of antibodies using opsins from cone- or rod-dominant animals permitted selective recognition as demonstrated by both immunoblot studies on isolated pigments and immunohistochemistry of normal, mixed, and cone- or rod-dominant retinas.[36–42] In the present study normal human retina sections were included as controls to verify the opsin and cellular selectivity of the antisera at dilutions used with the tumor specimens. This study is the first to apply cone opsin and CRALBP antibodies to the analysis of retinoblastomas *in situ*. These antisera were previously advantageous in identifying cone-specific gene expression and Müller cells in normal retina, respectively.[41–43]

The early events of retinal development related to the

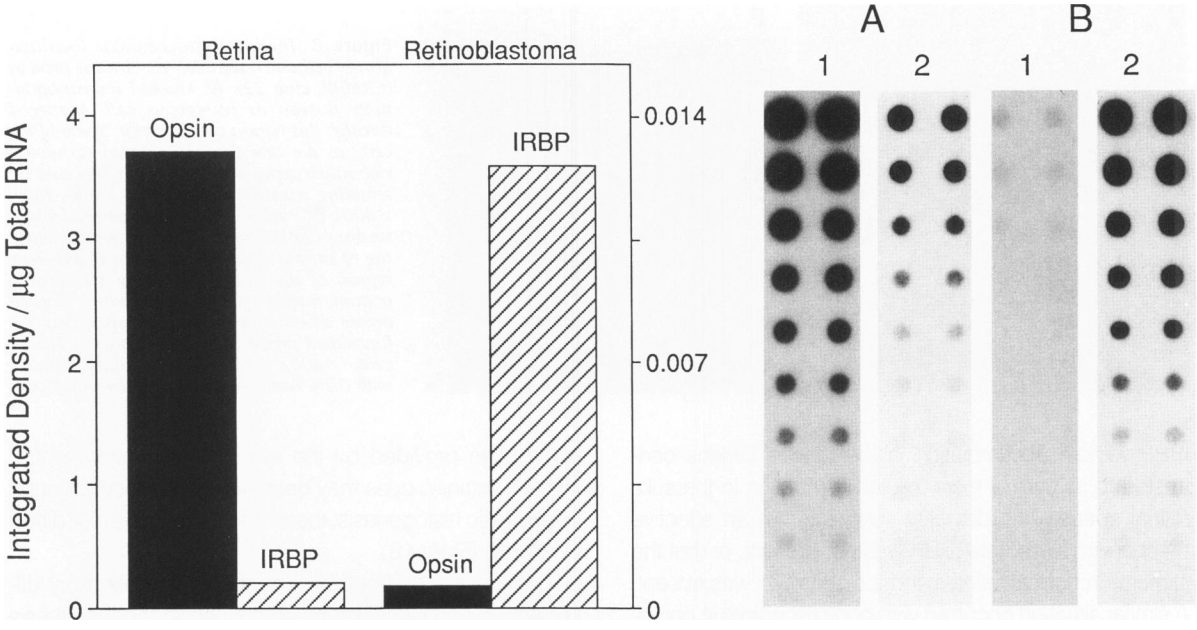

Figure 4. *Quantitative analysis of IRBP and opsin mRNAs.* **Right:** *Dot-blots of total RNA from normal adult retina (A) and tumor (B). Serial dilutions ranged from 5 µg to 0.005 µg total RNA dot. Strips 1A and 1B were probed with ^{32}P-labeled opsin cDNA, and strips 2A and 2B with ^{32}P-labeled IRBP cDNA. Probes and hybridization conditions were the same as in Figure 3. Using only the initial linear portion of the data for calculation of the slope, the ratio of the integrated density for both IRBP and opsin in the normal retina and tumor/µg total RNA were compared* **(left).**

emergence of specific matrix proteins and the temporal differentiation of photosensory cell populations suggest that these are suitable for the study of neoplastic differentiation in retinoblastomas. This tumor arises from retinoblasts derived from the immature retinal epithelium. During normal development, the outer layer of the optic cup becomes the retinal-pigmented epithelium, which is separated from the inner layer by the interphotoreceptor matrix. This extracellular matrix mediates interactions between the neural retina and the pigmented epithelium.[56–58] The major soluble component of the matrix is IRBP, a 145-kd glycolipoprotein. The function of IRBP is unknown; however, it may have a role in the transport of vitamin A through the subretinal space.[59–62] During retinal development, IRBP has an earlier pattern of expression than rod opsin, suggesting that independent mechanisms exist for the control of their genes.[63–66] The cells comprising the inner layer of the optic cup, as the immature neural retinal epithelium, maintain the potential to differentiate into photoreceptors, neurons, or retinal glia, even as daughter cells of the final mitosis.[67–69] Cone and rod photoreceptor cells arise in a temporally organized manner through a combination of extrinsic cues acting on genotypically competent progenitors.[70,71]

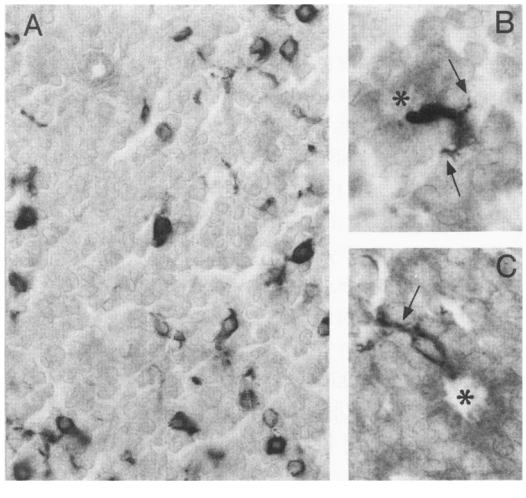

Figure 5. *Immunohistochemical localization of cone opsin (case 22).* **A:** *Strongly cone opsin-positive cells were scattered throughout solid areas of the tumor.* **B, C:** *Frequently, these cone-positive cells contained short processes polarized toward one end of the cell (arrows). Cone-positive cells were occasionally present within the rosettes. The asterisk marks the lumen of a rosette. A rounded apical protuberance extends into the lumen of the rosette marked by an asterisk, while the opposite end of the cell ends in two branching processes (solid arrows). CERN-874-ABC immunostaining with 0.2% osmium tetroxide intensification (×200).*

This study of 22 retinoblastomas *in situ* has demonstrated a potential for divergent retinal differentiation with the presence of photosensory (IRBP and opsins) and Müller cell (CRAIBP) phenotypes. In the tumors, IRBP immunoreactivity was the most ubiquitous photoreceptor-associated protein. It was present even when the opsins could not be detected. Of the photoreceptor-associated proteins studied, IRBP was unique for its polarized cytoplasmic distribution, even in the absence of morphologic differentiation. Intracellular localization of

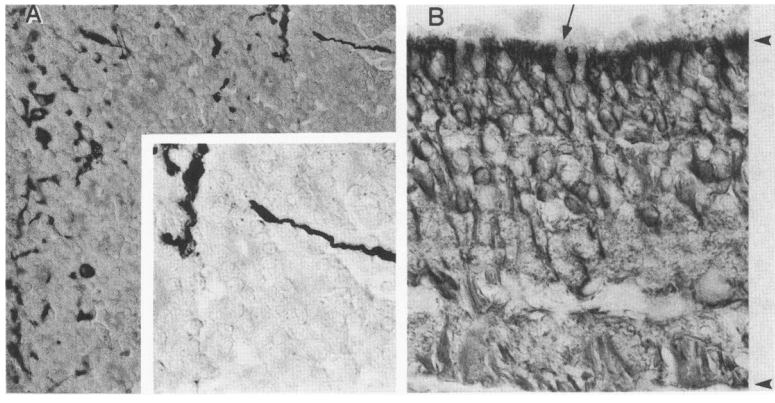

Figure 6. *Immunohistochemical localization of cellular retinaldehyde-binding protein (CRAlBP; case 22).* **A:** *Marked immunopositivity is seen in numerous cells scattered through this region of the tumor. Some of the cells, as the one shown in the inset, have a ribbonlike appearance with short processes resembling normal Müller cells (×200, inset, ×400).* **B:** *Portion of detached retina demonstrating CRAlBP immunohistochemical staining of normal Müller cells. Arrowheads: Top, region of the external limiting membrane; bottom, internal limiting membrane. Slanted arrow shows a photoreceptor separating the fimbriated processes of two adjacent Müller cells (×400). CRAlBP-ABC immunostaining with 0.2% osmium tetroxide intensification.*

IRBP without accumulation in the rosette lumens contrasted to its normal extracellular distribution in the subretinal space. This disparity suggests that an effective mechanism to secrete IRBP was not present, or that the stimulus in normal retina to induce secretion was absent. A similar situation occurred with photoreceptors in chronically detached retina (Figure 1A), and in photoreceptors from mice with retinal dystrophy (rd) that were maintained in culture.[72] Another explanation for the absence of IRBP in the extracellular space is that IRBP is secreted, but either degraded or not sufficiently concentrated extracellularly to be identified with the methods used. However, retinoblastoma cell lines appear to secrete IRBP in culture.[73,74]

Normally, IRBP is synthesized uniquely by the photoreceptors and secreted into the subretinal space. Although it is clear that the rod cells synthesize IRBP,[52,73,75–80] some[80,81] but not all laboratories[77–79] have been able to document IRBP synthesis by the cone cells. In the present study, we have obtained further evidence that human cone cells are capable of IRBP synthesis. First, cytoplasmic accumulation of IRBP was observed in the inner segments of cones in regions of detached retina. More dramatic evidence was obtained in tumors that demonstrated fleurettes. In these differentiated structures, cone but not rod immunoreactivity was demonstrated at the tips of the cytoplasmic protrusions while IRBP was present in the cytoplasm closer to the nucleus.

Opsins were used as photoreceptor cell type-specific proteins because antibodies to IRBP cannot distinguish rods from cones. Although 16 of the 22 cases demonstrated photoreceptor differentiation, there was a significant disparity between the abundance of specific cone and rod cells as determined by opsin immunoreactivity. Recent studies of the normal development of the retina offer an explanation for the predominance of cone over rod expression. These studies[70] suggest that cone differentiation occurs through a default mechanism,[71] whereas commitment to the rod phenotype requires specific cues provided by the retinal microenvironment.[82] These extrinsic cues may be absent or ineffective during neoplastic histogenesis, thereby favoring cone-cell differentiation (Figure 8).

Previous morphologic evidence for neurosensory differentiation in retinoblastomas has led to the hypothesis that this tumor arises from the retinal photoreceptors.[22–27] More recently, cone but not rod specific mRNA expression was demonstrated in retinoblastoma cells maintained in culture.[28] However, cones may have a selective survival advantage over rods under these conditions.[28] Furthermore, biochemical evidence of cone but not rod cGMP phosphodiesterase was demonstrated in extracts of primary tumors.[29] Alternatively, two different laboratories studying primary enucleation specimens demonstrated rod opsin by immunohistochemistry.[30,31] These apparently contradictory findings may be explained by the different experimental approaches used. Since Hurwitz et al[29] did not perform immunohistochemical analysis of their specimens, the possibility exists that focal rod specific expression may be occurring in their cases. Rod opsin expression was present in 50% of the cases in this study, although significantly less abundant and more focal than cone opsin expression.

Although neuronal differentiation in retinoblastoma has been thoroughly documented,[17–21] unequivocal documentation of neoplastic gliogenesis has been technically problematic and the occurrence of neoplastic astroglial differentiation has been disputed. This may be due, in part, to the restricted gliogenesis that can occur in the normal retina. Müller glial cells, in contrast to extrinsic astrocytes migrating from the optic nerve, are the only glia originating from retinal progenitor cells.[83–86] Therefore, the astroglial cell population in retinoblastomas are probably reactive and infiltrate from the vascular stroma of the residual retina. This was confirmed by immunohistochemical localization of GFAP and S-100 in primary tumors. The presence of these proteins was also interpreted as evidence for gliogenesis in retinoblastomas *in situ*[4,7–12,16,87–89] although this concept has remained

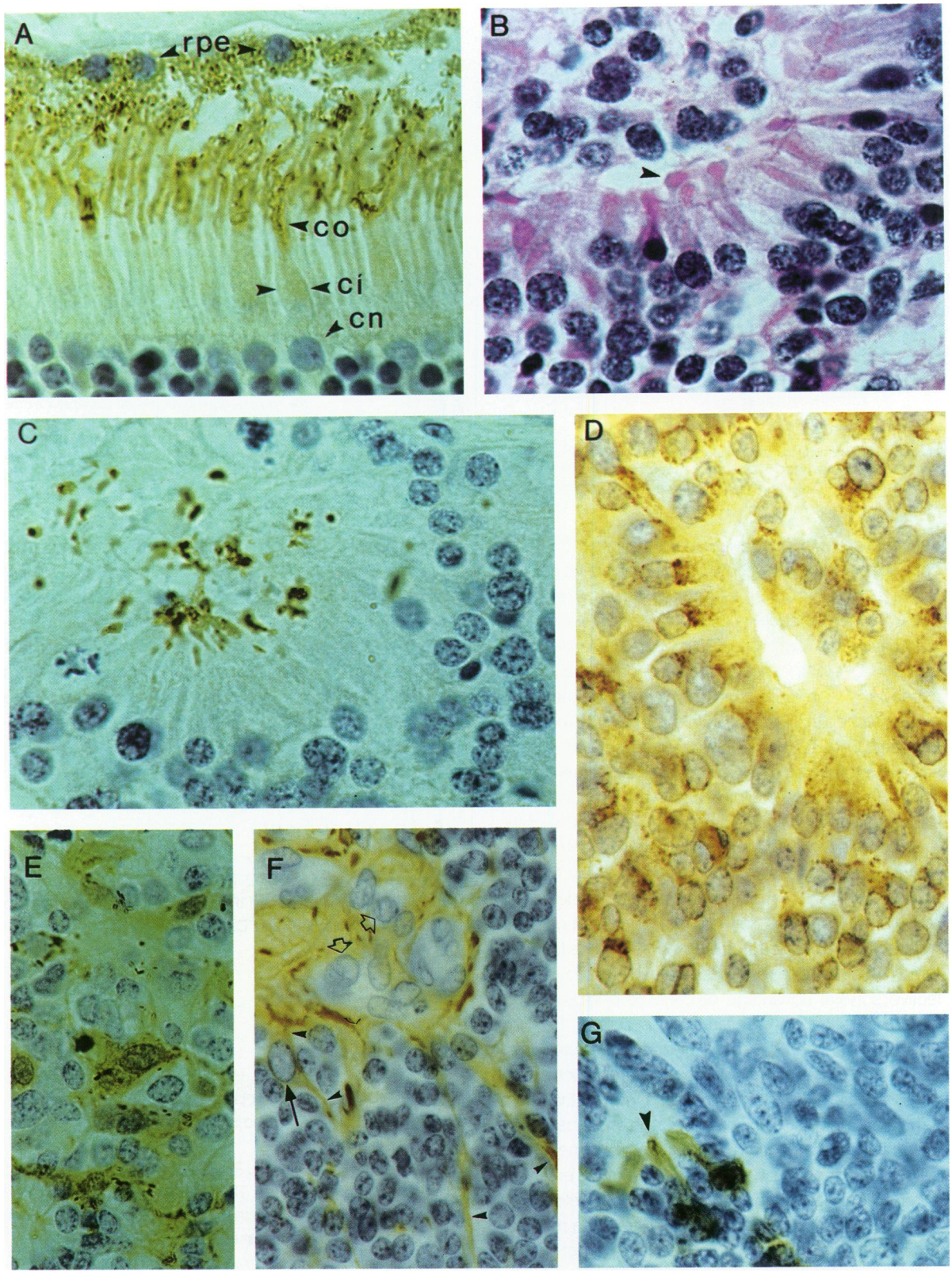

Figure 7. *Immunohistochemical localization of cone and rod opsins, IRBP, CRALBP, and GFAP in retinoblastoma (Case 3)* **A**: *Localization of cone opsin in normal human retina. This control specimen was enucleated for a uveal melanoma [(cn), cone cell nucleus; (ci) cone cell inner segment; (co), cone cell outer segment] (CERN-874-ABC immunostaining with hematoxylin counterstain, ×1,000).* **B**: *Retinoblastoma fleurette. Arrow indicates cytoplasmic process extending into the fleurette lumen (Hematoxylin and eosin, ×1,000).* **C**: *Same field as* **(B)** *from an adjacent section treated with the cone opsin antibody. Immunopositivity was restricted to the tips of the cytoplasmic processes which enter the fleurette lumen (CERN-874-ABC immunostaining with hematoxylin counterstain, ×1,000).* **D**: *Similar field from a section adjacent to* **(A)** *and* **(B)** *is treated with IRBP antibody (RB 504-ABC immunostaining with hematoxylin counterstain, ×1,000).* **E**: *CRALBP was identified in scattered cells within the tumor (CRALBP-ABC immunostaining with hematoxylin counterstain, ×1,000).* **F**: *GFAP was usually restricted to perivascular reactive astrocytes (solid arrow) which extend fine processes (arrowhead) into the nearby tumor. Open arrows indicate endothelial cells (GFAP-ABC immunostaining with hematoxylin counterstain, ×1,000).* **G**: *In our series, this was the only example of opsin positive cells participating in rosettes (arrowhead) (CERN-JS858-ABC immunostaining with hematoxylin counterstain, ×1,000).*

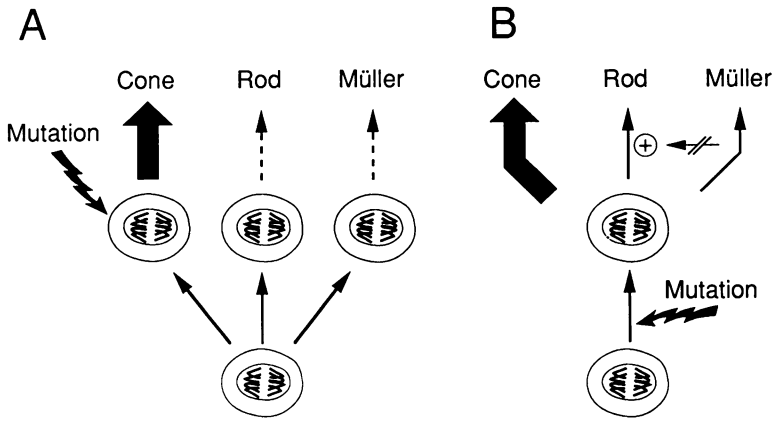

Figure 8. *Two models of the cytohistogenesis of retinoblastoma. In the normal retina immature retinoblasts can give rise, even as daughter cells from the final mitotic division, to both photoreceptors, neurons, and Müller glial cells. The cone phenotype develops by default, while the rods require specific extrinsic cues(⊕).* **A:** *In the first model, the mutational event occurs in a hypothetical sublineage committed to the cones. Rod photoreceptors and Müller cells would not arise in the tumors because their lineage would be bypassed.* **B:** *In the second model, the target of the mutation is the multipotential neural retinoblast. The cone phenotype would be favored in the differentiating neoplastic cells because the normal extrinsic signal(s) to induce the rod phenotype would be disrupted in the tumor (as indicated by the broken arrow). Therefore the cone phenotype would predominate by default with rare expression of the rod cell phenotype. The transformed neural retinoblasts also have the capacity for neoplastic Müller cell differentiation. Our data, taken together with the observations of others, is more consistent with the second model than with the first (see text). In both models, the differentiation of other retinal neurons is omitted for clarity.*

controversial with conflicting interpretations.[89,90] Müller cells appear to contain GFAP only in reactive retina.[92–95] Since extrinsic retinal astrocytes strongly express GFAP,[96] immunoreactivity of this intermediate filament is not suitable for identifying Müller cell differentiation. In contrast, CRALBP is not expressed normally by retinal astrocytes[97] and is uniquely synthesized by the retinal pigmented epithelium and Müller cells.[43,98] Therefore, we used CRALBP as a Müller cell-associated protein, since no morphologic evidence for retinal pigmented epithelial differentiation could be observed. Retinoblastoma cells expressing CRALBP were identified in 50% of the cases and in 69% of tumors demonstrating photoreceptor cell gene expression. The only other study that has examined CRALBP in retinoblastoma did not detect the mRNA for CRALBP by Northern blot analysis of polyadenylated RNA extracted from retinoblastoma cells in culture.[70] This negative result may be explained by the selective survival advantage of conelike photoreceptors over other retinal cell types in tissue culture.[82,99]

The present demonstration of divergent differentiation in retinoblastomas *in situ* with the presence of both retinal photosensory and Müller glial cells suggests that these tumors are derived from immature cells of the neural retinal epithelium. The differential expression of neoplastic photoreceptor cell types is consistent with the emerging concept that normal retinal cytohistogenesis relies more on positional extrinsic signals derived from the microenvironment rather than on the early commitment of specific cell lineages.[100,101] The observation that a common progenitor for neurons and Müller glia persists even late in the normal development of *Xenopus* and rat retinas complements this hypothesis.[67–69]

Previous studies using human retinoblastoma lines in culture have provided conflicting data concerning divergent differentiation. Retinoblastoma cells grown in monolayers demonstrated extensive divergent differentiation, suggesting that retinoblastomas arise from the most primitive retinoblastic epithelium.[102] In contrast, matrix/organotypic culture could not demonstrate neoplastic astrocytic or retinal pigment epithelial differentiation.[21] Although our study does support the concept of divergent differentiation in retinoblastomas, this appears to be more restricted, in the primary intraocular neoplasms, to the cytogenetic potential of the immature neural retinal epithelium.

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