Expression of Retina-Specific Genes by Mouse Retinoblastoma Cells

Steven L. Bernstein, Geetha Kutty,* Barbara Wiggert,* Daniel M. Albert,† and John M. Nickerson‡*

Purpose. Two cell lines derived from ocular tumors of a transgenic mouse expressing the SV40 large T antigen have been established as models of human retinoblastoma. One line, TM, originated from a metastasis, and the other, TE, originated from the primary tumor. The authors compared these two lines with the normal adult mouse eye by analysis of the expression of five photoreceptor cell-specific proteins: IRBP, opsin, rod- and cone-specific transducins, and S-antigen. The authors sought to determine which of these proteins was expressed qualitatively and to examine semi-quantitatively for changes in the levels of expression in the cell lines.

Method. Western blot analysis was used to detect photoreceptor-specific intracellular or secreted proteins. Total RNA was prepared from cultured cells or from mouse adult whole eye. Specific messenger levels in total RNA were determined either by northern hybridization analysis or by a semi-quantitative polymerase chain reaction (PCR), coupled to complementary DNA (cDNA) substrates prepared from total RNA.

Results. IRBP was present in the retinoblastoma cell lines and secreted into the medium. Neither S-antigen nor opsin were detectable by immunoblotting. IRBP and cone transducin mRNA were present in both cell lines. In contrast, opsin, rod transducin, and S-Antigen mRNAs were not detectable by PCR. β-actin was present in the mRNA populations of whole eye and retinoblastoma. SV40 large T antigen mRNA was present only in retinoblastoma cells.

Conclusions. IRBP and cone transducin expression in mouse retinoblastoma cells is independent of signaling provided directly or indirectly through large T antigen or Rb_{105} regulatory cascades. The pattern of photoreceptor-specific gene expression is similar to that seen in human retinoblastoma cell lines. These murine-derived cell lines may be useful as a tool to study IRBP and cone transducin expression in vitro and to determine early retinoblast expression patterns in the mouse. Invest Ophthalmol Vis Sci. 1994;35:3931–3937.

The sequence of events in visual transduction and retinal development involves a series of proteins, many of which are expressed in the photoreceptor cells.[1,2] The regulation of the genes coding for these proteins is poorly understood, in part because of difficulty in dissecting multiple cell interactions in vivo and because of the inherent limitations of primary retinal culture systems.[3,4] Although transgenic animal models have been used to determine the role of single gene function and dysfunction in retinal development,[5] this technology is complex and may not be appropriate in all instances. Cultured human retinoblastoma cells have been used by a number of investigators to evaluate retinal gene expression in a replicating in vitro system.[6] The most widely employed retinoblastoma cell line, Y-79, derived from a patient with germline mutation of the Rb_{105} gene, expresses photoreceptor-like structures[7,8] and can also express some photoreceptor-specific proteins, such as IRBP, under specific conditions.[6] Retinoblastoma, however, does not spontaneously develop in mice, and it fails to develop even when the Rb_{105} gene is deleted by homologous recombination.[9] Nevertheless, murine retinoblastoma has

*From the *Laboratory of Retinal Cell and Molecular Biology, National Eye Institute, National Institutes of Health, Bethesda, Maryland; the †Department of Ophthalmology and Visual Sciences, University of Wisconsin, Madison, Wisconsin; and the ‡Department of Ophthalmology, Emory University, Atlanta, Georgia.*
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Reprint requests: Dr. Steven L. Bernstein, Laboratory of Retinal Cell and Molecular Biology, National Eye Institute, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD, 20892.

been found to be expressed in a transgenic mouse system[10] derived by inserting a transgene consisting of the protein coding unit of the SV40 large T antigen linked to a luteinizing hormone B-subunit promoter.[11]

Expressed in the cell, T antigen binds to and inactivates Rb_{105}.[12] This inactivation apparently leads to a loss of cell cycle control in a manner similar to spontaneous human retinoblastoma. Retinoblastoma develops in transgenic animals in a fashion identical to that seen in the inherited form of human retinoblastoma.[9] Cell lines derived from these tumors resemble primitive retinoblasts, are homogeneous in their appearance in cell culture, and may express genes active in early retinal development. Two lines were characterized—TE, a retinoblastoma cell line derived from a primary (intraocular) retinoblastoma tumor, and TM, a retinoblastoma cell line derived from a cervical node metastasis. Both cell types have been in culture for 3 years. These cell lines provide an additional system for the study of mouse retinal gene expression and may be useful for further evaluation of the processes involved in retinal development.[13] In this article, we describe our molecular characterization of these cell lines using sequence data corresponding to photoreceptor-specific gene expression.

We evaluated IRBP, a protein expressed after birth solely by retinal photoreceptors and pinealocytes. In the mature retina, it is secreted into the interphotoreceptor space, where it transports retinoids between the retinal pigment epithelium and the photoreceptor layer.[14,15] Other retina-specific genes evaluated are mouse opsin, the protein moiety of rhodopsin expressed solely by rod photoreceptors and pinealocytes; the rod- and cone-specific (α) subunits to transducin that bind GTP in the phototransduction cascade; and S-antigen (arrestin), thought to modulate the visual transduction pathway. β-actin was used as an internal control for RNA levels.

We show that, in addition to expressing the mRNA for SV40 large T antigen, the murine retinoblastoma cell lines express IRBP mRNA and protein as well the message for the cone-specific α subunit of transducin and β-actin. Mouse retinoblastoma thus expresses retina-specific genes in a fashion similar to that seen in human retinoblastoma cell lines and may be useful in studies involving IRBP and cone transducin gene regulation and expression and in determining genes involved in early retinal development.

MATERIALS AND METHODS

Polyclonal rabbit–antibovine opsin antibody was a generous gift of Dr. T. Shinohara of the National Eye Institute (NEI). Polyclonal rabbit–antibovine S-antigen antibody was a generous gift of Dr. I. Gery (NEI). For northern blot analysis of IRBP, insert DNA containing a 1.2-kb portion of the first exon of bovine IRBP was generously provided by Dr. D. Borst (NEI). Horse and fetal calf serum, as well as DMEM medium, were obtained from Gibco-BRL (Gaithersburg, MD). RNAzol was purchased from Tel-test (Friendswood, TX); cDNA was prepared from total RNA primed with random deoxynucleotide hexamers from Pharmacia (Rahway, NJ). Oligonucleotides were synthesized on an ABI oligonucleotide synthesizer (Foster City, CA).

All animals were euthanized humanely in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Eyes were enucleated and fast frozen on dry ice before RNA or protein extraction.

TE and TM cells were grown in DMEM with high glucose (4500 mg/ml) supplemented with 15% horse serum and 5% fetal calf serum, along with 10 mM Hepes buffer and penicillin–streptomycin. The cells were grown in suspension, under 8% CO_2, without attachment factors. Cells were harvested, and the conditioned medium was saved for western blot analysis. For Western blot analysis of IRBP, S-antigen and rhodopsin, homogenized cells, and supernatants from both cell lines were electrophoresed on a 10% to 20% gradient-SDS tricine polyacrylamide gels (Novex, San Diego, CA). Western blot analysis of the conditioned cell medium and cell pellets was performed using polyclonal goat–antibovine IRBP antibody (for IRBP), polyclonal rabbit–antibovine opsin, or S-antigen antibody (for opsin and S-antigen). Antibody dilutions were 1:200 (IRBP), 1:400 (S-antigen), and 1:1000 (opsin). The reactions were performed and developed as previously described.[16]

Total RNA was isolated from frozen cell pellets, as described.[17] Total RNA (20 μg) from TE and TM cells, Y-79 cells, human retina and isolated adult mouse whole eye was denatured and run on 1.2% agarose–formaldehyde gels. Levels of total RNA were approximately equal as measured by intensity of ethidium bromide staining of the 18S ribosomal RNA band from each sample. RNA was transferred to nylon membranes. With the exception of opsin, northern hybridization analysis was performed as described.[18] The membranes were prehybridized without formamide in 6 × SSPE, 5 × Denhardts, 10% dextran sulfate, and 0.1% SDS. Prehybridization was performed at 65°C for 8 hours.

Probes for murine IRBP, opsin, S-antigen, the α-subunit of cone transducin, and β-actin were generated by random priming with ^{32}P-dCTP. Hybridization was performed at 65°C for 12 to 16 hours. After hybridization, the membranes were washed twice for 30 minutes with 2 × SSC, then washed twice with 0.2 × SSC at 65°C for 30 minutes. Kodak X-AR film (Eastman Kodak, Rochester, NY) was exposed to the radioactive membranes at −70°C for varying lengths of time (1 to 14 days), using a Dupont (Wilmington, DE) lightning plus intensifying screen.

For reverse-transcribed PCR (RT-PCR) analysis of mRNA levels, first-strand cDNA was synthesized from total RNA isolated from cells and mouse adult whole eye, as well as from human adult retina and Y-79 (suspension culture) cells using random hexanucleotide primers. PCR reactions were then performed using the exon–exon priming method.[19] For mouse IRBP, the sequences were derived from exon 1, 5′ TCCACAGCCCAGGACATAGTGG (22mer), and from exon 2, 5′GAATCTCAAGTAGCCAATGT (20mer), generating a 369-bp product (Nickerson, unpublished data, 1990). Human IRBP primers were from exon 1, 5′ ATGGCCACCAAACTGAGCGGT (21mer), and from exon 2, 5′ CTTCAGGGGAAGGGATCTGT (20mer), generating a 348-bp product. Mouse opsin primers were from exon 1, 5′ TCGAGCAGCCGCAGTACTACCTG (23mer), and from exon 2, 5′AGCACAGGCCAACGCCATGATCCA (24mer), yielding a 434-bp product. Mouse rod transducin (α-subunit) primers were from exon 1, 5′ CTGAGAAGGATGCCCGCACTG (21 mer), and from exon 3, 5′CTCACCTGCCGGGCTGAATCT (21mer), yielding a 269-bp product. Mouse cone transducin (α-subunit) primers were from the cDNA sequence at nt 205, 5′ TAGAGTTCAAGTCTGTCATCT (21mer), and downstream (nt 522 to 501), 5′ CTGCTCGTTAGGGAGGTAGTT (21mer), yielding a 317-bp product. Primer pairs used for PCR of transducin subtypes were unique for the specific subtype, as evaluated by the FASTA homology computer program available through the GCG computer program package (Genetics Computer Group, Madison, WI). S-antigen primers were from exon 7,5′ AGAGATCCTGAGGAGGACAAGATC, and from exon 8, 5′ CAGTTTCTAGGCATCTAGGCTAAG, yielding a 730-bp product. SV40 primers were from exon 1, 5′ AATATTCCTCTGATGAGAAAG, and from exon 2, 5′ TACTAAACACAGCATGACTCA, yielding a 436-bp product. Genbank accession numbers were M36695 (mouse opsin), M24086 (mouse S-antigen), M25506 and M5508 (mouse rod transducin), L10666 (mouse cone transducin), and J05253 (human IRBP). Mouse β-actin primers from exon 1 and exon 2 were purchased from Clontech (Palo Alto, CA) and yielded a 562-bp product. SV40 large T antigen exon primers were from published sequence data.[20]

PCR was initiated with a 1.5-minute incubation at 94°C, followed by 30 rounds of denaturing at 94°C for 1 minute. Annealing temperatures were 55°C for mouse and human IRBP and opsin, 51°C for rod transducin, 57°C for S-antigen, and 52°C for β-actin, cone transducin, and SV40 large T antigen, all for 1 minute. Extension was at 72°C for 2 minutes. After thermal cycling, the reactions were incubated for 10 minutes at 72°C and stored at 4°C. Samples were extracted with $CHCl_3$ and run on polyacrylamide gels in TAE buffer.

FIGURE 1. Western immunoblot analysis of proteins secreted from TE and TM cells and from adult mouse whole eye. Protein extract from adult mouse whole eye and conditioned medium from TE and TM cells were analyzed for IRBP as described in Materials and Methods. An IRBP-specific protein standard and molecular weight standards were run simultaneously. TE = A retinoblastoma cell line derived from a primary (intraocular) retinoblastoma tumor; TM = a retinoblastoma cell line derived from a cervical node metastasis.

RESULTS

Western Blot Analysis: IRBP Expression in Mouse Retinoblastoma Cell Lines and Adult Mouse Whole Eye

Western blot analysis showed that IRBP was expressed and secreted into the culture medium in detectable levels in both cell lines. The IRBP concentration was at lower levels than that seen in mouse whole eye, using a polyclonal antibody probe (Fig. 1). There was no staining for S-antigen or opsin in the cell extracts of either of the two murine cell lines. In contrast, strong positive signals were seen from protein extracts prepared from murine whole eye and known protein standards (data not shown).

RT-PCR based detection of specific messengers β-actin, IRBP, rod and cone transducin, mouse opsin, and S-antigen mRNA levels were measured under conditions that yielded single major product bands at the expected sizes of 369-bp (mouse IRBP), 269-bp (rod transducin), 317-bp (cone transducin), 434-bp (opsin), 730-bp (S-antigen), and 562-bp (mouse β-actin) when amplified from mouse retina cDNA derived from mouse adult whole eye total RNA. Primers for SV40 large T antigen mRNA yielded a major product band at the expected size of 436-bp only from cDNA prepared from mouse retinoblastoma cell lines. PCR results are seen in Figure 2. PCR-based amplification of cDNA reverse transcribed from total RNA purified from TM or TE cells gave a readily detectable signal for IRBP mRNA, but it was lower than that expressed in mouse adult whole eye using equivalent amounts of total RNA as starting material for the cDNA reaction. A similar result was seen for cone transducin. Neither S-antigen, rod transducin, nor mouse opsin were detected using this assay technique (rod transducin data not shown). In contrast, cDNA prepared from mouse whole eye with S-antigen and opsin primers yielded strong signals of the predicted size cDNA fragments for both these mRNAs (Fig. 2). Thus, PCR product levels for these photoreceptor-specific mes-

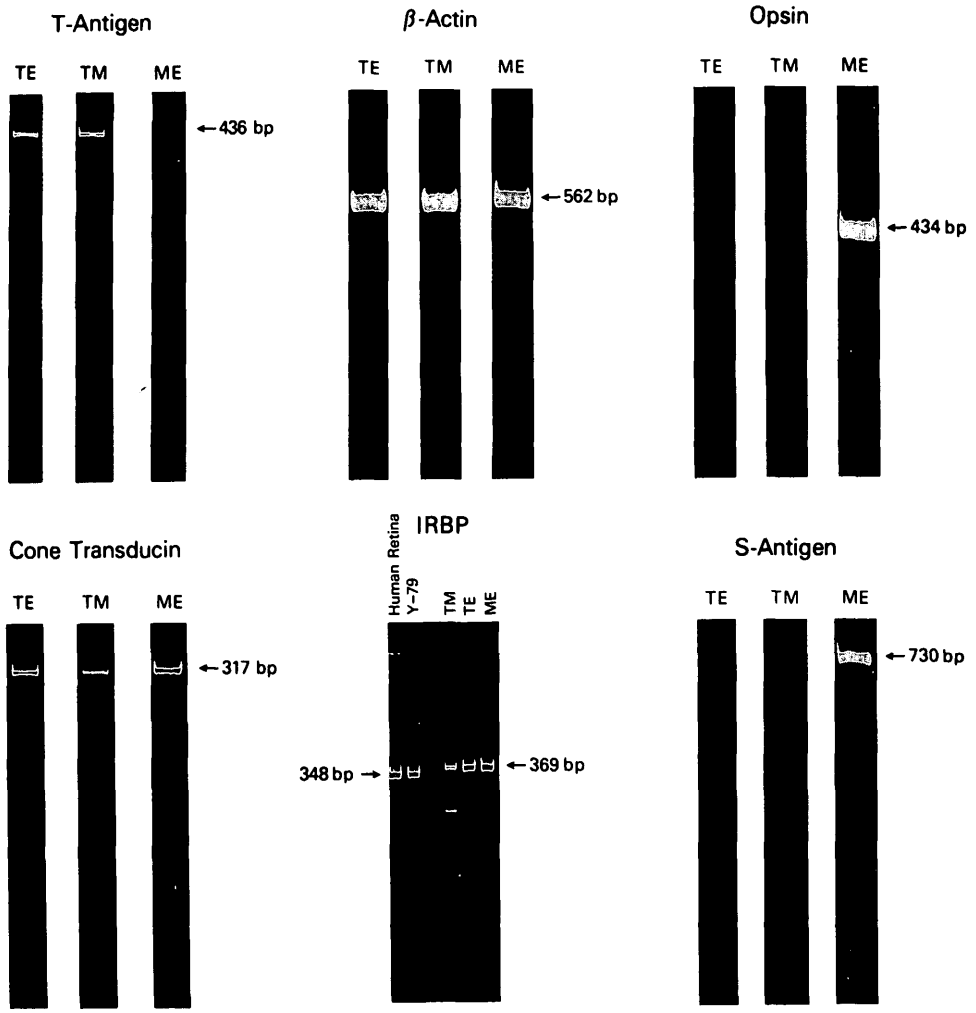

FIGURE 2. Reverse-transcribed PCR (RT-PCR) generated fragments from mRNA-specific sequence primers. Exon-specific oligonucleotide primers for SV40 large T antigen (T antigen), mouse β-actin (β-actin), mouse opsin (opsin), mouse α-subunit for cone transducin (cone transducin), mouse IRBP (IRBP), and mouse S-antigen (S-antigen) were used in PCR reactions employing cDNA generated from total RNA from either TE or TM cell lines, or adult mouse whole eye (ME). Exon-specific primers for human IRBP were used for cDNA generated from total RNA from human retina and Y-79 cells and yielded a DNA fragment with a predicted length smaller than that from mouse IRBP. RT-PCR products were analyzed as described in Materials and Methods.

sages were present at either lower levels or were unexpressed in the mouse retinoblastoma cell lines, compared to mouse whole eye total RNA, which expressed strong signals for all six nonviral messages. The signal for β-actin was strong in mouse retina as well as in both cell lines (Fig. 2). cDNA prepared from human-derived Y-79 and human retina total RNA yielded a strong signal of the predicted fragment size (348 bp) for human IRBP mRNA (Fig. 2).

Northern Blot Analysis of IRBP, Cone Transducin, Opsin, S-Antigen, and β-Actin

Northern blot analysis using a 1.2-kb, ^{32}P-labeled bovine IRBP probe hybridized against total RNA from mouse retina, TM, and TE mouse retinoblastoma cell lines is seen in Figure 3. A band, estimated at 4.1 kb, is detectable in all lanes. The signal is strongest for mouse eye, with positive but progressively weaker signals from TE and TM cells, respectively. The probe was also hybridized against total RNA from human retina and Y-79 cells grown in culture. A strong signal is detected at approximately 4.2 kb in human retina, with a weaker signal for Y-79 cells (Fig. 3).

Probing with a 317-bp, ^{32}P-labeled probe for the α-subunit of mouse cone transducin gave a band at the expected size of 1.8 kb, when hybridized to total RNA from mouse retina, TE cells, and TM cells (cone transducin; Fig. 4). A 1.8-kb, ^{32}P-labeled bovine opsin

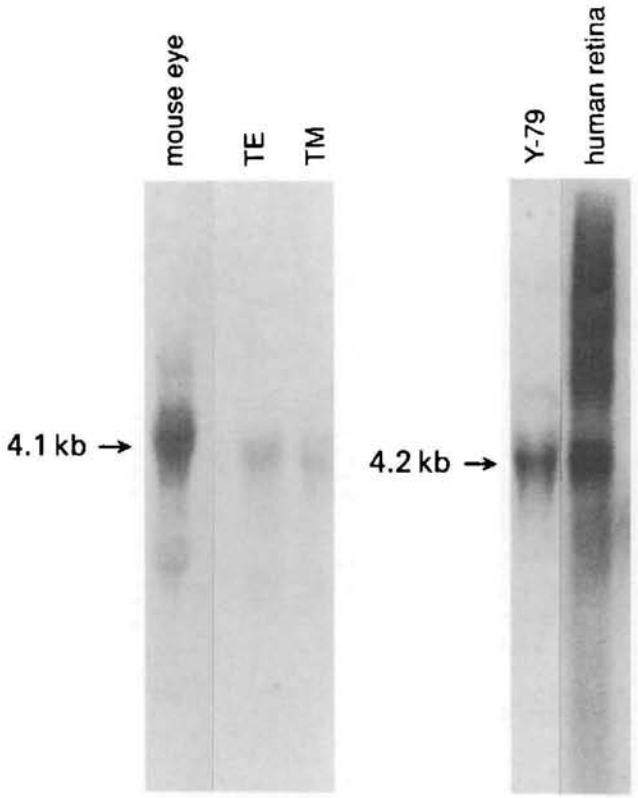

FIGURE 3. Northern hybridization analysis of IRBP expression in total RNA from mouse eye, human retina, TE and TM cells, and Y-79 cells. Twenty micrograms of total RNA was run in each lane. ^{32}P-labeled probes for mouse or human IRBP mRNA were hybridized and analyzed as described in Materials and Methods. Exposure was at −70°C. Exposure times were 3 days for human retina and Y-79 cells and 2 days for mouse eye, TE cells, and TM cells.

probe revealed bands at 5.1, 4.3, 3.2, 2.15, and 1.6 kb when hybridized against total RNA from mouse retina (opsin; Fig. 4). These results agree closely with published data.[21] Surprisingly, hybridization of total RNA from TE and TM cell lines with the opsin probe showed only two bands: an intense band at approximately 5.1 kb and a fainter band at approximately 1.9 to 2.0 kb (Fig. 4). The results of northern hybridization of total RNA from mouse whole eye, TE cells, and TM cells, with the ^{32}P-labeled probe for S-antigen mRNA, are shown (S-antigen; Fig. 4). Only total RNA from mouse yielded the expected band at 1.9 kb; RNA from TE and TM cells was negative for this message. Probing with a PCR-derived, ^{32}P-labeled fragment for mouse β-actin yielded an intense band at approximately 1.9 kb when hybridized to total RNA from mouse retina, TE cells, and TM cells (β-actin; Fig. 4).

DISCUSSION

Primers derived from known sequence data for mouse β-actin, S-antigen, IRBP, the α-subunit for mouse cone transducin, and opsin yielded single bands at the predicted sizes when RT-PCR was performed using cDNA from mouse adult whole eye as the substrate. Primers for SV40 large T antigen mRNA yielded a major band at the predicted size only from the mouse retinoblastoma cell lines. These results imply that the product(s) of RT-PCR were identical to the expected messenger sequence, although this was not verified by sequence analysis. The mouse retinoblastoma cell lines used in this study expressed the message for IRBP and the α-subunit of cone transducin. β-actin was also expressed.

The level of IRBP expression is similar to that seen in the Y-79 (human) retinoblastoma cell line, whereas β-actin message was expressed in high abundance in all tissues, as measured by RT-PCR, or when normalized for variation in the amount of total RNA employed for northern hybridization. S-antigen protein was absent in the cell pellets of both TE and TM cell lines as measured by polyclonal antiserum and western blot analysis. The mRNA for this protein was not detectable by northern blot analysis, although a faint band was seen by RT-PCR analysis. Similarly, there was no signal seen using α-subunit specific primers for mouse rod transducin. These results suggest that the level of these two mRNAs, if present, are far less than that seen in mouse whole eye. Results of RT-PCR analysis of murine retinoblastoma RNA are in reasonable agreement with the northern blot analysis data of the mature messages. This indicates that RT-PCR of retinal RNA, using exon-specific primers, can be used as a rapid and sensitive alternative method of specific messenger RNA detection. Because of the intrinsic ability of RT-PCR to amplify small amounts of starting material, however, slight differences in the starting samples may result in inaccurate mRNA concentration estimates. In the absence of PCR-based internal sequence standards,[22] sequence-specific mRNA quantitation may be best estimated directly by northern blot analysis.

Although northern hybridization suggested the presence of at least two transcripts with significant homology to opsin (Fig. 4b), opsin messenger sequence was not detected among cDNA generated from mouse retinoblastoma cell mRNA by RT-PCR analysis. The transcripts seen in our northern blot analysis results may represent unique sequence with significant homology to opsin, but with a distinct function, such as mouse red–green cone opsin. Hybridization of a homologous opsin probe to mouse retina total RNA yielded the multiple-band autoradiographic pattern reported by other investigators.[21] This has been proved to derive from the variable length of the 3′ untranslated region of opsin mRNA species.[23] The photoreceptor-specific pattern of messenger expression in these mouse retinoblastoma cultures is similar to that seen in some early passage human retinoblas-

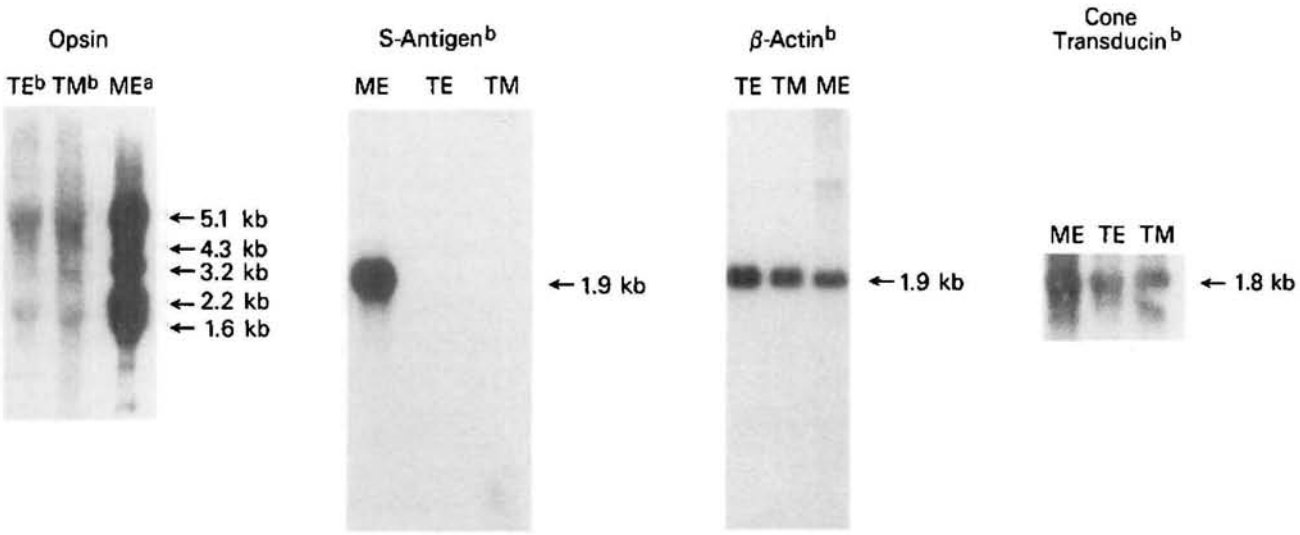

FIGURE 4. Northern hybridization analysis of total RNA from mouse eye (ME), TE cells, and TM cells. Twenty micrograms of total RNA was run in each lane. Probes for mouse opsin, cone transducin, S-antigen, and β-actin were hybridized and analyzed as described in Materials and Methods. Superscript letters indicate exposure times at −70°C: [a]2 days; [b]5 days.

toma cell cultures. These human cultures were shown to express mRNA, which hybridized at high stringency to labeled probes derived from bovine cone transducin and either red- or green-cone opsin.[24]

It is interesting that IRBP, a retina- and pineal-specific gene, is expressed with the level of mRNA similar to that seen in the Y-79 (human) retinoblastoma cell line (Fig. 2). Although IRBP is expressed most abundantly by terminally differentiated (4 days after birth or older) rod and cone photoreceptors, it is also expressed prenatally in both rat and mouse[25,26] and in the cow.[27] This early expression of IRBP, as well as its expression in retinoblastoma cell lines, suggests that, apart from its later role as a carrier of retinoids across the interphotoreceptor matrix, IRBP may play an important role in early retinoblast function and in the developing fetal visual system.

In spite of the difference in genetic origin, mouse and human retinoblastoma are apparently committed, before terminal differentiation, within the cone cell lineage. Photoreceptor-specific gene expression in mouse retinoblastoma cells is thus similar to the expression seen in nonviral-derived human retinoblastoma cell lines and independent of signals controlled by SV40 large T antigen. Retinoblastoma cell lines derived from murine transgenic retinoblastoma tumors may be useful in further evaluating the control of such expression and the role of IRBP and photoreceptor-specific genes in the developing retina.

Key Words

murine retinoblastoma, interphotoreceptor retinoid binding protein (IRBP), reverse-transcription based polymerase chain reaction (RT-PCR), messenger RNA, retina-specific genes

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