Bilateral Retinal and Brain Tumors in Transgenic Mice Expressing Simian Virus 40 Large T Antigen under Control of the Human Interphotoreceptor Retinoid-binding Protein Promoter

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Abstract. We have previously shown that postnatal expression of the viral oncoprotein SV40 T antigen in rod photoreceptors (transgene MOT1), at a time when retinal cells have withdrawn from the mitotic cycle, leads to photoreceptor cell death (Al-Ubaidi et al., 1992. Proc. Natl. Acad. Sci. USA. 89:1194-1198). To study the effect of the specificity of the promoter, we replaced the mouse opsin promoter in MOT1 by a 1.3-kb promoter fragment of the human IRBP gene which is expressed in both rod and cone photoreceptors during embryonic development. The resulting construct, termed HIT1, was injected into mouse embryos and five transgenic mice lines were established. Mice heterozygous for HIT1 exhibited early bilateral retinal and brain tumors with varying degrees of incidence. Histopathological examination of the brain and eyes of three of the families showed typical primitive neuroectodermal tumors. In some of the bilateral retinal tumors, peculiar rosettes were observed, which were different from the Flexner-Wintersteiner rosettes typically associated with human retinoblastomas. The ocular and cerebral tumors, however, contained Homer-Wright rosettes, and showed varying degrees of immunoreactivity to antibodies against the neuronal specific antigens, synaptophysin and Leu7, but not to antibodies against photoreceptor specific proteins. Taken together, the results indicate that the specificity of the promoter used for T antigen and/or the time of onset of transgene expression determines the fate of photoreceptor cells expressing T antigen.

Expression of viral oncoproteins typically promotes tumorigenesis in various tissues of transgenic mice (18, 29). The oncogene that was most frequently used to induce tumor formation is simian virus 40 (SV40) large tumor antigen (T antigen) (18, 29), which is essential for both viral replication and cell transformation (8, 37). T antigen has been found to interact with several cellular proteins involved in tumor suppression (12, 23), DNA replication (14), and regulation of transcription (15, 38), but its precise role in oncogenesis is not well understood. Accidental expression of SV40 T antigen in the retina of transgenic mice has led to the generation of tumors originating in various retinal cell types (4, 17, 21, 36), except photoreceptors. Brain and retinoblastomalike ocular tumors originating in the inner nuclear layer were induced in one of fifteen transgenic families that harbored a hybrid gene consisting of the human $\beta$-luteinizing hormone gene promoter and the T antigen gene (39). Inheritable human retinoblastomas, typically caused by a defect in the $rb$ gene, arise from retinal embryonic neuroepithelial cells and often express components of the cone, but not the rod phototransduction cascade in tissue culture (5, 20).

We previously showed that T antigen expression driven by the mouse opsin promoter caused an early and rapidly progressing rod photoreceptor degeneration, and at a later stage, the development of brain tumors (2). The rod photoreceptor degeneration, as evidenced by pyknotic nuclei and shrinkage of the outer nuclear layer, began around postnatal day 8 (P8), and was complete by P21. T antigen expression, under control of the opsin promoter, was shown to begin between P1 and P5, and reached maximum levels before onset of the degeneration. In the retina, rod opsin is expressed exclusively in rods, whereas interphotoreceptor retinoid-binding protein (IRBP) is known to be expressed in both rods and cones (31). Both proteins are expressed to a much lesser extent in pinealocytes in the brain (24). Expression of IRBP precedes expression of rhodopsin, and can be detected at embryonic day 17 (E17) (9). Expression of a bacterial reporter gene (chloramphenicol acetyltransferase, CAT) under the control of the human IRBP promoter indicated an even earlier onset of expression (27). To study the effect of onset of expression of T antigen and the cell type specificity of the promoter, the human IRBP promoter fragment was used to direct expression of T antigen to the retina 1.

1. Abbreviations used in this paper: CAT, chloramphenicol acetyltransferase; GFAP, glial fibrillary acidic protein; IRBP, interphotoreceptor retinoid-binding protein; NF, neurofilament; nt, nucleotides; PDE, phosphodiesterase.
of transgenic mice. In this paper we show that expression of SV40 T antigen under the control of a 1.3-kb human IRBP promoter fragment causes the formation of trilateral tumors involving the eyes and brain. Bilateral retinal tumors appear to arise in the outer nuclear layer of the retina.

Methods

Production of rd/rd, Cat/+ Mice

Mice homozygous for the rd mutation exhibit complete degeneration of the photoreceptor cell layer about the third week of postnatal development (28). FVB/N (rd/rd) mice and transgenic mice homozygous for the IRBP/CAT transgene were mated, and F1 mice, heterozygous for both rd and IRBP/CAT, were then mated to FVB/N to produce mice that are homozygous for the rd mutation and heterozygous for the IRBP/CAT transgene. Mice that were heterozygous for both the IRBP/CAT and rd were used as controls. The presence of the rd allele was detected by the rd specific DdeI polymorphism (30). The IRBP/CAT transgene was detected as described previously (24).

RNAse Protection Assay

Fragments of antisense RNA (259 or 392 nucleotides [nt] for mouse IRBP, 363 nt for CAT, and 300 nt for β-actin) were synthesized in the presence of [α-32P]UTP using T3 or T7 RNA polymerase (Promega Corp., Madison, WI) using corresponding linearized subclones in Bluescript plasmid (25). The synthesis of probes was done with T3 RNA polymerase (Promega Corp., Madison, WI) and with T7 RNA polymerase (Promega Corp., Madison, WI). The probes for CAT and β-actin were radiolabeled and purified by electrophoresis in a 20%–50% polyacrylamide gel. Hybridization was carried out at 42°C overnight in the presence of 50% deionized formamide, 40 mM Pipes pH 6.4, 400 mM NaOAc and 1 mM EDTA. After hybridization the mixture was treated with 10 units of RNAses A and 2,000 U of RNAse T1, and protected fragments were separated by polyacrylamide gel electrophoresis. Autoradiographs were quantitated using a Shimadzu densitometer. The sizes of protected fragments were: 245 or 311 nt for mouse IRBP; 108 nt for mouse opsin; 309 nt for CAT; and 300 nt for β-actin (29). While CAT activity in the rd/rd mice continued to increase during the first few days of retinal development. Mice that were heterozygous for the rd allele and homozygous for the CAT transgene were assayed at different stages of embryonic development by an RNAse protection assay (RPA). The hIRBP promoter directs expression of a bacterial reporter gene, CAT, to the retina and to a much lesser extent to the brain (pinealocytes) of transgenic animals (24). To study the onset of CAT expression in the embryonic retina of a transgenic mouse heterozygous for the hIRBP/CAT transgene, we determined the amount of CAT transcripts at various stages of embryonic development by an RNAse protection assay (RPA). Fig. 1 shows that CAT transcripts and hence transgene expression under the control of the IRBP promoter can be detected as early as E13, at least one week earlier than opsin expression (30).

Cell Specificity

To study the cell specificity of the IRBP promoter, we used a genetic approach in which mice homozygous for the IRBP/CAT transgene were mated with mice homozygous for the rd locus. Mice heterozygous for the rd allele develop normal retinas (32), while mice homozygous for the rd allele suffer a rod photoreceptor degeneration. CAT activity in the retinas of offspring homozygous for the rd allele and heterozygous for IRBP/CAT were assayed at different stages of postnatal development. Mice that were heterozygous for both the rd allele and IRBP/CAT transgene served as controls. As seen in Fig. 1, CAT activity in both rd/rd and rd/+ animals rose during the first few days of retinal development. A sharp increase was seen at P7, a time when the last mitotic activity in the retina of structurally normal rd/+ animals was observed. This de-
delian fashion of inheritance of the transgene and indicated the integration of multiple copies of the transgene at single genomic DNA from the founder mice and their offspring. Human IRBP gene, and the complete coding sequences of the antigen sequences to yield construct HIT1 (Fig. 2). HIT1 contains the transcription start site and upstream promoter of the human IRBP gene and the complete coding sequences to the bacterial CAT gene specifically to photoreceptor cells. These results indicate that the hIRBP promoter directs expression of the viral oncogene. In addition to an early onset of T antigen expression, the IRBP promoter is expected to direct expression of the antigen to both rods and cones. HIT1 was injected into 1-d old FVB/N mouse embryos (35) and five transgenic founder mice were established. Southern blot analysis of genomic DNA from the founder mice and their offspring (data not shown) from a mating to C57BL/6 showed a Mendelian fashion of inheritance of the transgene and indicated the integration of multiple copies of the transgene at single sites (Table I).

Expression of HIT1 Causes Trilateral Tumors

All of the transgenic mice families that harbored HIT1 exhibited retinal and brain tumors, albeit at varying incidence levels (Table I). The tumors were consistently trilateral and appear to initiate at embryonic or early postnatal stages. Most ocular and brain tumors were poorly differentiated with a variable degree of anaplasia and bizarre pleomorphic nuclei. The ocular tumors led to total retinal detachment with the tumor cells involving mostly the outer retinal layers (Figs. 3 A and 4 A). The inner retina (inner plexiform and ganglion cell layers) appeared intact and in many sections, the tumor cells merged imperceptibly with the bipolar cell layer (Fig. 4 A). While most of the ocular tumors were poorly differentiated, some tumors were more differentiated and disclosed peculiar rosettes composed of a single layer of cuboidal cells that were centered around a small capillary lined by a single layer of flat endothelial cells (Fig. 3 B).

Figure 1. CAT expression during prenatal and postnatal development of the retina. Prenatal expression of the hIRBP/CAT transgene was monitored by assaying CAT activity (mU/mg protein) (24). Retinal samples were obtained from mice at different stages of postnatal development. Triangles represent the amount of CAT transcripts, as determined quantitatively from an autoradiogram. Triangles represent activity in retinas from mice that are heterozygous for both the rd allele and the CAT transgene, whereas circles represent activity in retinas of animals homozygous for the rd allele and heterozygous for the CAT transgene. Error bars are calculated from three different experiments of the same tissue and at least three different samples.

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Table I. Characterization of Transgenic Mice Families Harboring the HIT1 Fusion Gene

| Family (F2) | Characteristics |
|-------------|-----------------|
| 408         | 413             |
| 414         | 417             |
| 437         |                 |

Phenotype

| None | BT, ET | BT, ET | BT, ET | BT, ET |

Tumor incidence (%)

| 43   | 38     | 55     | 50     | 50     |

Transmittance (%)

| Integration site(s) |
|---------------------|
| 1                   |

| Copies per haplotype |
|----------------------|
| 2                    |

Southern blot analysis (33) performed on tail DNA samples of the founder mice and their offspring from a mating to C57BL/6, was used to obtain the number of copies of the transgene, the number of sites of integration and rate of transmission of the transgene. A total of 145 mice from different families were involved in generating these results. Tumor incidence (%) was calculated by dividing the number of mice that developed tumors after weaning by the total number of weaned mice for each transgenic family. BT, brain tumor; ET, eye tumor.
Scattered erythrocytes were usually observed in the lumens of the capillaries (Fig. 3 B). Characteristic Flexner-Wintersteiner rosettes, typically associated with retinoblastomas (34), could not be identified, but the less specific Homer Wright rosettes (34) were clearly observed in both the retinal and brain tumors (Figs. 3 D, and 4 A). Coronal sections of the brain tumors revealed poorly differentiated neoplasms with the histologic features usually encountered in primitive neuroectodermal tumors of infancy (Fig. 3 C). The cerebral tumors involved mostly the diencephalon, adjacent to the third ventricle, and surrounded the aqueduct and the habenular nuclei without involving the choroid plexus (Fig. 3 C). The tumors also extended to the midbrain and fourth ventricle. The cerebral neoplasms were quite advanced at the time the animals were killed. Many tumor cells showed widespread leptomeningeal infiltration along the Virchow-Robin spaces surrounding the blood vessels as they enter the brain. By electron microscopy, the tumor cells showed large pleomorphic nuclei with formation of nuclear blebs and numerous polyribosomes, scattered mitochondria and dilated cisternae of smooth-surfaced endoplasmic reticulum (Font et al., unpublished results). The exact origin of the tumor was not clearly discernible in the sections examined.

Although the retinal and brain tumors were highly undifferentiated, the neoplastic cells reacted with antibodies to neuron-specific enolase, S antigen and Leu7. S-antigen (arrestin) is not a photoreceptor specific protein and has been identified in the non-neoplastic state in pinealocytes, lens fiber, and epithelial cells, cerebellum and cerebral cortex (7), and also in several primitive neuroectodermal tumors of man (21). The tumor cells were not reactive with either antibodies against neurofilament and GFAP, or with photoreceptor specific antibodies (see legend of Table II), but were reactive with antibodies to T antigen (Fig. 4 B).

**Discussion**

Expression of SV40 T antigen in the retina of transgenic mice driven by the mouse opsin promoter leads to a rapidly progressing retinal degeneration associated with sustained DNA synthesis and mitotic cycles in the cells of the outer nuclear layer (2). To investigate whether the onset of expression of the oncogene or the cell specificity of the promoter is responsible for the resulting phenotype, another retina specific promoter was used to direct expression of T antigen to the photoreceptor cells of transgenic mice. The human
Table II. Immunohistochemical Characterization of Retinal and Brain Tumors

| Antibodies against | Mouse (F0)  |
|-------------------|-------------|
|                   | NSE Synapo. | Leu 7 | T antigen | S antigen |
| 666 OD (417)      | + +         | -     | +         | NP        |
| 666 OS (417)      | + +         | +     | f         | NP        |
| 666 Br (417)      | + +         | + f   | +         | NP        |
| 1257 OD (417)     | -           | +     | NP        | NP NP     |
| 1257 Br (417)     | NP +        | +     | NP        |
| 1258 OS (417)     | NP +         | + f   | +         | + f       |
| 1258 Br (417)     | NP + + f     | + f   | + f       | + f       |
| 726 OD (437)      | -           | + f   | NP        |
| 726 OS (437)      | -           | +     | NP        |
| 726 Br (437)      | +           | -     | + +       | + +       |
| 916 Br (437)      | +           | +     | -         | + +       |

Immunoreactivity of tumors to different antibodies were assessed by staining sections with the avidin-biotin immunoperoxidase method and using hematoxylin as counter stain. The presence of brown precipitant was used to indicate immunoreactivity. All mice were adult when killed, but the developmental stage of the tumor differed depending on the individual mouse. The results shown were from an average of 15 sections from each antibody. Column mouse F., founder mouse nomenclature (Br, brain; OS, left eye; OD, right eye. Numbers in parentheses refer to individual mouse families). NSE, neuron-specific enolase; Synapto.; synaptophysin; Leu 7, human natural killer cells specific antigen. f, focal (few foci only); NP, not performed; +, weak; ++, moderate; + + +, strong; -, negative. Immunohistochemical stains for glial fibrillary acidic protein (GFAP), neurofilament (NF), rhodopsin, and rod cGMP phosphodiesterase (PDE) were negative (not shown).

IRBP-promoter has previously been used to direct expression of the bacterial CAT gene to the retina and pineal gland of transgenic mice (24). Expression of the IRBP/CAT transgene, irrespective of the site of integration, starts at embryonic day 13 (Fig. 1), a time when developing retinal cells have just begun the initial differentiation into rod and cone photoreceptor cells (40). Utilizing a hybrid gene composed of the human IRBP promoter and the bacterial β-galactosidase gene, Yokoyama et al. (40) detected expression in both rod and cone photoreceptor cells similar to the pattern of expression of the endogenous IRBP gene (31). We chose to test the tissue specificity of the human IRBP promoter by following the CAT activity in the retina of mice homozygous for the rd mutation. The rod photoreceptor cells of these mice rapidly degenerate starting at P10, while nuclei of the cone photoreceptor cells survive for at least 6 mo (28). We allowed matings of IRBP/CAT transgenic mice to rd/rd mice and selected for mice that are homozygous for the rd allele and carry the IRBP/CAT transgene. Our results show that expression of the CAT gene was directed to the photoreceptor cell layer in the retinas of the transgenic animals.

Because hIRBP/CAT transgene expression was shown to start as early as El3, this promoter was chosen to direct T antigen expression to the retina of transgenic mice. We found that expression of T antigen driven by the hIRBP promoter led to the formation of tumors in the brain and retina of mice. Tumors occurred at varying degrees of severity in different transgenic families, and this was most likely due to the effect of site of integration on the levels of expression of the transgene. Animals exhibiting retinal tumors consistently developed brain tumors, unlike the mouse model for retinoblastoma reported earlier (39) where only 27% of the transgenic mice produced brain tumors whereas 100% of transgenic animals produced retinal tumors. This variation in the levels of tumor occurrence has been attributed to the effect of the site of integration of the transgene on the levels, cell type, or onset of expression (39). We also observed tumors in all founder mice that were homozygous for the rd mutation, suggesting that either tumors arose from surviving cone cells (22) or from photoreceptor cells before the onset of degeneration. We favor the latter possibility due to the early onset of expression of the transgene driven by the human IRBP promoter.

Normal retinal photoreceptor cells show varying degrees of reactivity to neuronal specific antibodies. Antibodies directed against neuron specific enolase, S antigen and Leu7 show moderate to intense labeling of photoreceptor cells (11). However, photoreceptor cells show little or no reactivity with antibodies against neurofilaments, synaptophysin and GFAP. Therefore, it is not surprising that neuron specific enolase, S antigen and Leu7 were detected in the tumors. It

Figure 4. (A) Neuronal specific enolase staining of a retinal tumor (120X) for a 90-d old mouse (F0 437). Tumor, which is in close proximity to the lens (asterisk) shows the readily identifiable intact inner retinal layers (arrowhead). Also shown is the Homer-Wright rosette formation (arrow). (B) Immunodetection of T antigen in the retinal tumor of a mouse (family 417) killed at age 60 d (305X). Tumor cells in active mitosis (arrows) display positive staining for T antigen.

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was also expected that the tumors would show absence of reactivity with antibodies against GFAP and neurofilament because the tumors arose from the retinal outer layer. However, while normal photoreceptor cells did not react with anti-synaptophysin antibodies, the tumor cells reacted moderately. This result combined with the negative staining of tumor cells with anti-opsin and anti-phosphodiesterase antibodies (not shown) suggest that either the tumors lost expression of some antigens and gained expression of others, due to the transformation by T antigen, or the early onset of expression of T antigen in the stem cells before the final differentiation into rod and cone photoreceptors. Immunoreactivity to T antigen was restricted to mitotically active cells (Fig. 4 B), which may be caused by the accumulation of T antigen before the mitotic division.

The tumors observed in transgenic animals expressing HIT1 were of highly undifferentiated nature and therefore were designated primitive neuroectodermal tumors. The development of retinal tumors in animals carrying HIT1, in contrast to the earlier observed retinal degeneration in animals carrying MOT1 (2) may have been due to expression of HIT1 in both the rod and cone photoreceptor cells. Therefore, while rod photoreceptor cells degenerated, differentiating cone photoreceptor cells may have reacted differently to the expression of an oncogene resulting in the formation of tumors. This may explain the previous detection of cone-specific and the absence of rod photoreceptor-specific antigens in retinoblastomas (5, 20). Alternatively, the earlier onset of expression of T antigen driven by the human IRBP promoter may be responsible for the formation of tumors.

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