TRILATERAL RETINOBLASTOMA IN TRANSGENIC MICE

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INTRODUCTION

RETINOBLASTOMA, A HERITABLE MALIGNANCY, OCCURS IN THE EYES OF children at a median age of 2 years. It is a malignancy which has been widely studied as a model for genetic predisposition to cancer. The disease occurs in both heritable and nonheritable forms. Thirty to 40% of children with retinoblastoma have a heritable form of the disease.[1] The other 60% to 70% of affected children have spontaneous somatic mutations which will not be transmitted to their own offspring.

Observation of the transmission pattern of retinoblastoma led Knudson et al[2] to formulate a "two hit hypothesis" for tumorigenesis. The heritable form of retinoblastoma arises through transmission of a mutant allele from a carrier parent or through a new mutation which appears in the germline of these children. This mutant allele is carried within every cell. A second somatic mutation at a locus homologous to the mutant locus occurs in the retina, triggering tumorigenesis. The developing retina contains a large number of susceptible cells, and this accounts for high penetrance as well as bilateral expression in the heritable form of the disease. Eighty to 90% of children with heritable germline mutations in the retinoblastoma locus will develop this malignancy. Heritable retinoblastoma is characteristically bilateral and multifocal.

The second form of retinoblastoma is not heritable. This malignancy arises through two unrelated events which occur at homologous loci within a single retinal cell. These double mutation events are unlikely to sporadically occur in more than one retinal cell. The nonheritable form is

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therefore expressed unifocally and unilaterally.[3] Children with this form of retinoblastoma show mutations within tumor cells only. These patients are incapable of transmitting the disease to their own offspring.

Knudson's theory has been substantiated by cytogenetic investigation of deletions specific to retinoblastoma,[4] observation of polymorphisms in linked enzymes,[5,6] and cloning of deoxyribonucleic acid (DNA) loci which correspond to the retinoblastoma gene (Rb-gene).[7-10] Both copies of the Rb-gene are inactivated in retinoblastoma, as well as in some osteosarcomas and soft tissue sarcomas.[8,9,11] Absent or altered messenger ribonucleic acid (mRNA) expression is reported in the majority of sporadic and familial retinoblastomas.[12]

Fifty percent of children who survive bilateral retinoblastoma will develop a second malignancy within 20 years of treatment.[13] Abnormalities in structure of the Rb-gene have recently been noted in human small cell lung cancer,[12] breast cancer,[14] and bladder cancer.[15] These observations suggest that the Rb-gene may have a broad role in restraining tumorigenesis. In the absence of normal gene product a wide variety of malignancies may arise. It is the loss or inactivation of the Rb-gene which predisposes to malignant transformation, earning it the designation tumor-suppressing gene or anti-oncogene.

Evidence for a direct interaction between the Rb-gene as a tumor-suppressing gene and known tumor-promoting genes has recently become available.[16-18] Adenovirus E1A is an oncogene. It immortalizes primary cells,[19-21] transforms cells in conjunction with activated ras or E1B gene, and is tumorigenic.[20,22] It also appears to bind the product of the Rb-gene.[16] Mutational analysis[23,24] of E1A protein has demonstrated that region 2 is required to drive mitosis and to transform cells. Deletions in region 2 diminish or eliminate binding of p105-Rb, the protein product of the Rb-gene.[25] Sequences which are homologous to E1A region 2 have also been identified in papilloma virus (HPV-16),[18,26] c-myc oncogenes, and the CDC 25 mitotic regulator of yeast.[27] HPV-16 DNA is observed in over 50% of cervical carcinoma specimens, suggesting a possible etiologic role for this viral transforming protein in the development of human cancer.[18,26]

Simian virus 40 large T-antigen (SV 40 T-ag) contains a conserved amino acid sequence with homology to the transforming domain of E1A[27] and human papilloma virus 16-E7.[18] This region is required for transforming function of SV 40 T-ag and also for its specific binding to the Rb-gene product.[17] Homologous regions of SV 40 T-ag can functionally substitute for E1A domain 2 in a chimeric molecule which retains transforming function and coprecipitates a 105kd cellular protein.[28] This Rb-binding

oncogene with sequence homology to a variety of known oncogenes was utilized as a transgene in the present model.

Oncogenic proteins may induce malignant transformation by blocking normal cellular proteins which play an important role in the regulation of growth and differentiation. The product of the Rb-gene is known to be a nuclear phosphoprotein which binds to DNA-cellulose columns.[29] The specific function of retinoblastoma protein (p105 Rb) is undefined. It is believed that this protein may have a critical role in maintaining normal cellular growth. Specific binding and functional inactivation of p105 Rb may be one mechanism through which oncogenic proteins induce malignant transformation. Greater knowledge of the function of the Rb-gene product could provide insight into the manner in which normal cell growth is regulated, and the mechanisms by which such regulation is overcome in the course of malignant transformation.

We report the development of heritable ocular tumors with marked resemblance to retinoblastoma in a single strain of transgenic mice. Midline brain tumors in the region of the pineal gland are observed at lower incidence in these animals, consistent with human trilateral retinoblastoma.[30-32] The transgene used to create this model was a chimeric molecule composed of SV 40 T-ag driven by luteinizing hormone (LH) beta-subunit promoter. This construct was intended to direct oncogene expression in gonadotropes of the anterior pituitary. The transgene was microinjected into the pronuclei of fertilized mouse ovocytes. These ova matured and adult mice carrying the gene of interest resulted. In the majority of transgene-bearing murine lines, pituitary adenomas developed as expected. However a single male mouse displayed ocular neoplasms, and these tumors proved to be heritable in greater than 90% of heterozygous transgene-bearing offspring. Murine neoplasms show striking similarity to human retinoblastoma in light, electron microscopic, and immunohistochemical features. Neoplasms are locally invasive with lethality months after birth. The SV 40 T-ag transgene, although present in all tissues, is expressed only in tumor tissue obtained from affected animals.

MATERIALS AND METHODS

The transgene utilized in this model contained the human LH beta-subunit promoter region from -1.09 kb to $+9$ bp relative to the transcription initiation site. This promoter was linked to the SV 40 early region from the Bgl I site to the BamHI site.[33] This restriction fragment lacked the SV 40 early promoter region but contained the protein coding region for T-ag and t-ag, including the translation initiation and transcrip-

tion termination sites. Chimeric gene was purified and injected into fertilized single cell ovocytes.[34] The F_2 generation was produced by matings of transgene-bearing animals with CB6F1/J (C57B1/6J X Balb/J) male and females, obtained from Jackson Laboratory. Transgene-bearing offspring were selected by slot-blot analysis of tail DNA utilizing an SV 40 T-ag specific probe.

Autopsies were performed at 6 weeks to 4 months after birth. Murine tissues were fixed in 5% buffered formalin and 3% phosphate buffered glutaraldehyde-sucrose or quick frozen in liquid nitrogen. Processed tissue was sectioned for light microscopy and transmission electron microscopy or for immunohistochemical analysis.

T-ag mRNA levels were assayed by Northern analysis as previously described.[35] Total RNA was prepared as outlined[36] from the eye and pituitary of a 3-month-old transgenic female and from a panel of tissues from a 2-month-old transgenic male. RNA was also prepared from the eye and pituitary of a control female mouse and from cultured COS cells which functioned as a positive control for T-ag expression.[37]

Southern blot analysis of total genomic DNA was performed using an SV 40 T-ag specific probe.[35] Genomic DNA was subjected to analytical digestion and ligated into cosmid and phage vectors in order to clone the transgene insertion site, and to isolate retinal specific enhancer or promoter sequences within the cloned insertion.[38] Library screening is underway in an effort to isolate SV 40 T-ag containing clones. In situ analysis to murine karyotype is simultaneously in process to confirm the genomic site of transgene integration.

RESULTS

Gross Examination of enucleated eyes shows a spectrum ranging from apparently normal eyes enucleated at 6 weeks to eyes with retinal tumors filling the vitreous cavity enucleated at 3 months. Greater than 90% of animals which carry the transgene develop ocular tumors. The tumors are commonly bilateral and usually multifocal. Smaller tumors are observed within retinal layers (Fig 1); larger tumors are associated with total retinal detachment and optic nerve invasion. Approximately 10% of transgenic animals develop focal midline neoplasms involving the midbrain in the region of the pineal gland. These midbrain tumors are observed in animals that show early intraocular neoplasms which have not yet invaded the optic nerve.

Microscopically intraocular tumors (Figs 2 and 3) are composed predominantly of small cells with relatively large hyperchromatic nuclei and

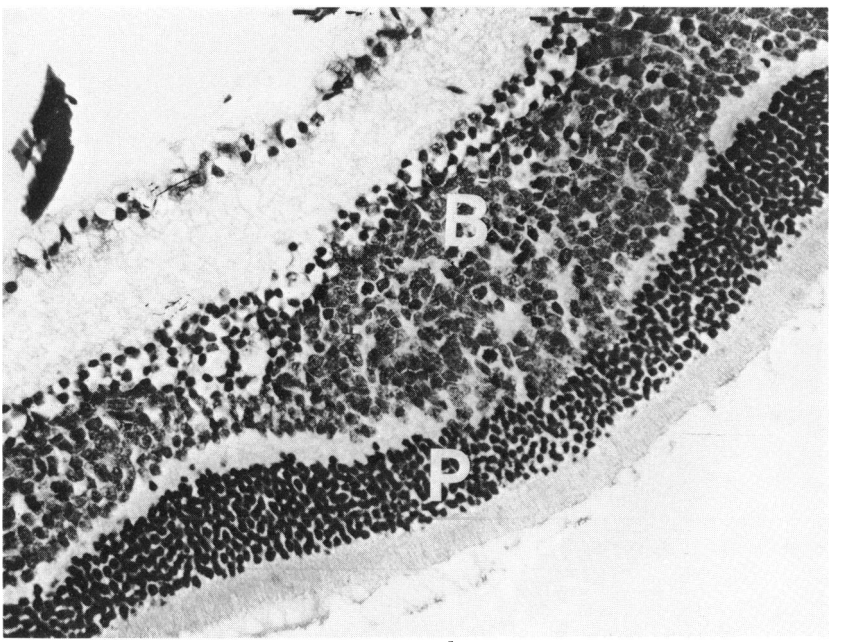

FIGURE 1
Focus of retinoblastoma cells located in the area of the inner nuclear layer of the retina. Ganglion cell layer (B) and photoreceptor cell layer (P) can be observed (H&E, ×700).

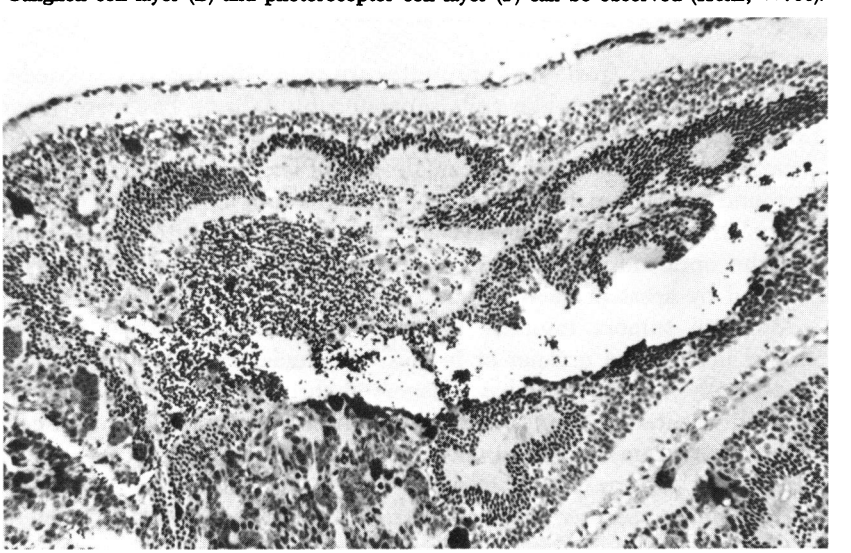

FIGURE 2
Retinal tumor showing cells with photoreceptor characteristics forming large rosettes (H&E, ×500).

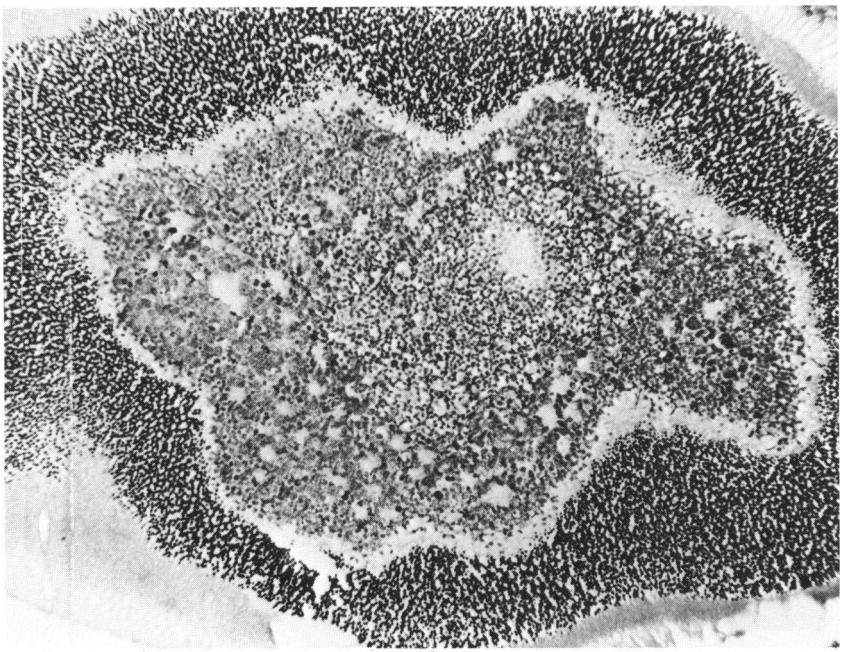

FIGURE 3
Focus of retinoblastoma surrounded by photoreceptor cells. Rosettes can be observed (H&E, × 300).

scanty cytoplasm. Two types of rosettes are seen. The first is composed of rows of photoreceptor-like cells separated by delicate basement membrane from internal photoreceptor-like material. The second rosette structure is characterized by a single row of larger cuboidal cells which surround a fibrous matrix. Tumor cells are adherent to the retinal pigment epithelium and to Bruch's membrane, and are present within the choroid and the optic nerve. Perivascular cuffs of viable tumor cells are surrounded by areas of necrosis and calcification.

Midbrain tumors typically display round undifferentiated cells arranged in a diffuse manner or in clusters. Nuclei are round to oval and occasionally indented and have dispersed chromatin and small nucleoli. More differentiated brain tumors are composed of somewhat smaller cells with hyperchromatic nuclei; these cells are sometimes arranged in a rosette-like pattern (Figs 4 to 6).

Electron microscopic studies of intraocular tumors confirm light microscopic findings of generally uniform small cells with large nuclei and scanty cytoplasm (Fig 7). Rosettes composed of photoreceptor cells with delicate internal limiting membranes and inner segment material are seen

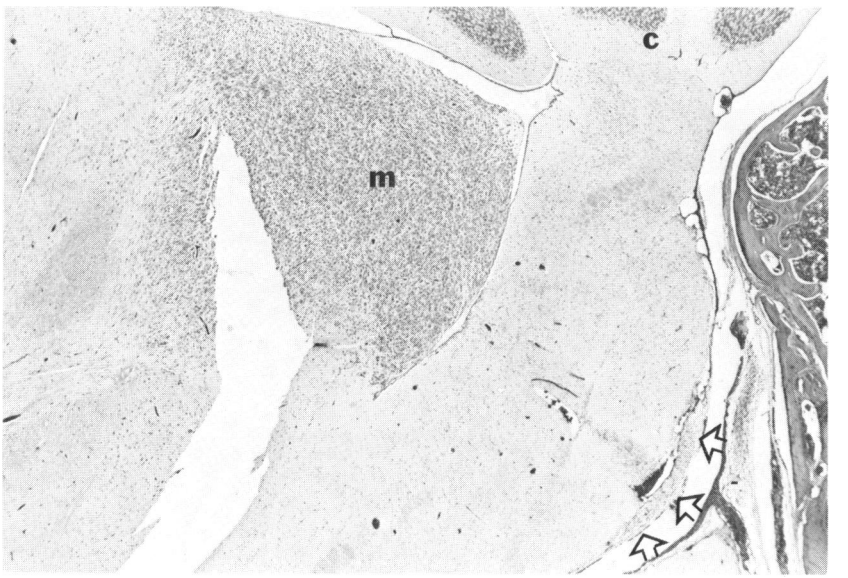

FIGURE 4
Neuroblastic brain tumor localized to the midbrain (M), anterior to the cerebellum (C) in the region of the pineal gland (*arrow*). Dysplastic cells stream between the pineal gland (*arrows*) and the midbrain neoplasm (H&E, ×300).

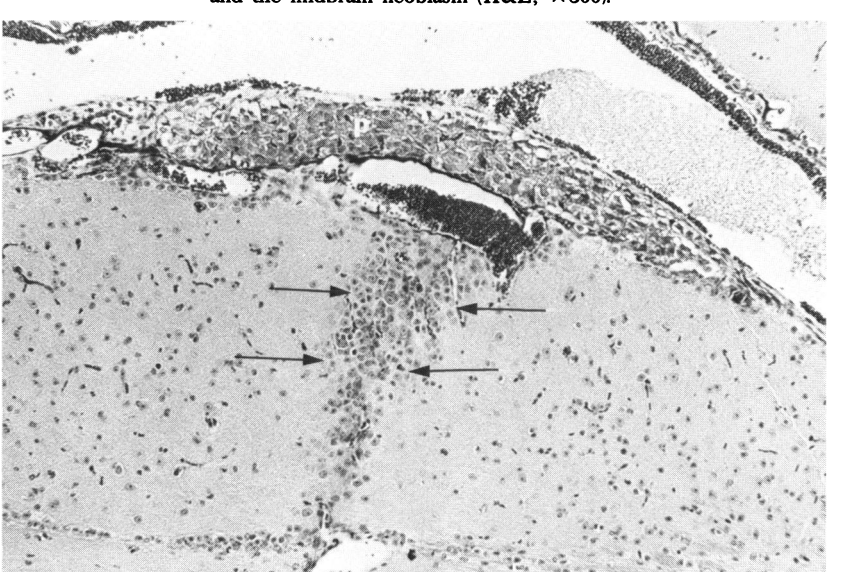

FIGURE 5
Higher power view of pineal gland (P) with adjacent dysplastic cells (*arrows*) (H&E, ×750).

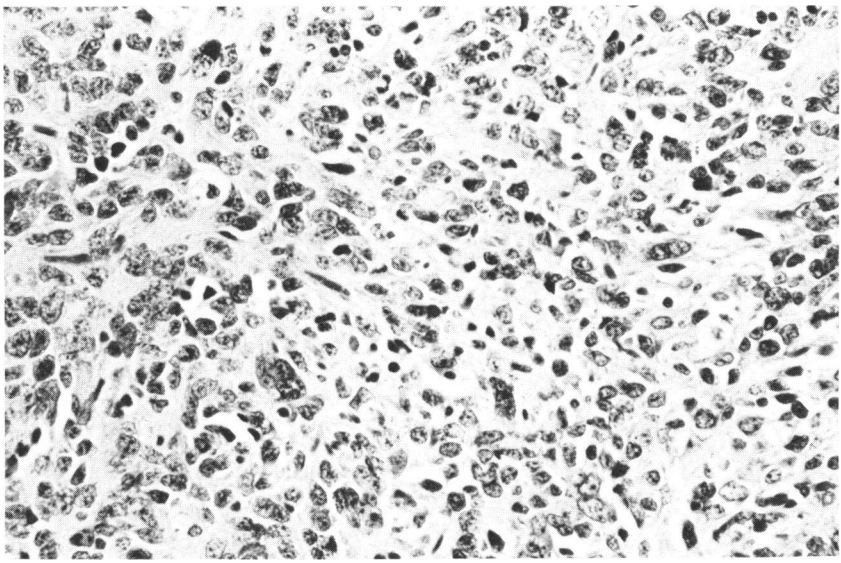

FIGURE 6
Less differentiated midbrain tumor; cells show large nuclei with prominent nucleoli, anisonucleosis, anisocytosis, and cell groupings without rosette formation (H&E, ×800).

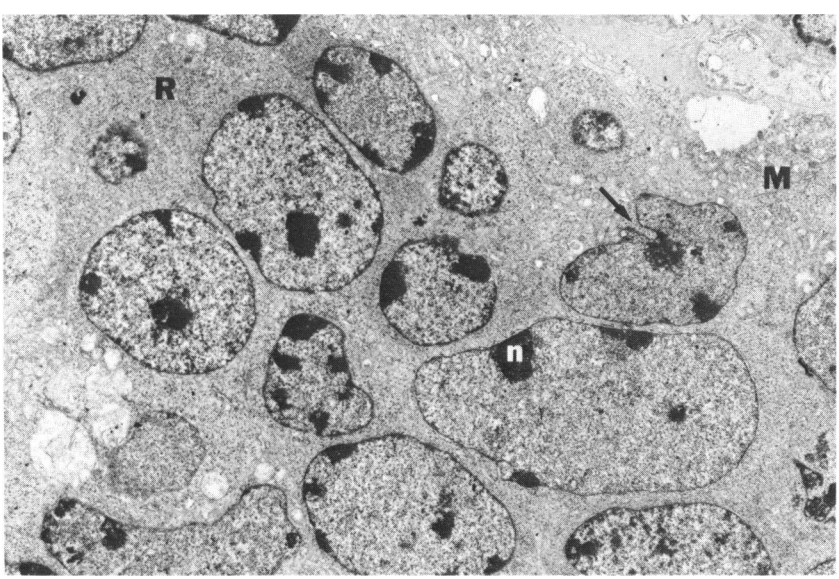

FIGURE 7
Undifferentiated tumor cells arising from the retina, having small round nuclei (N), one or more nucleoli (NI), and dispersed chromatin (C). Note invaginations of the nuclear envelope (*arrow*). The cytoplasm contains mitochondria (M) and ribosomes (R) (×3080).

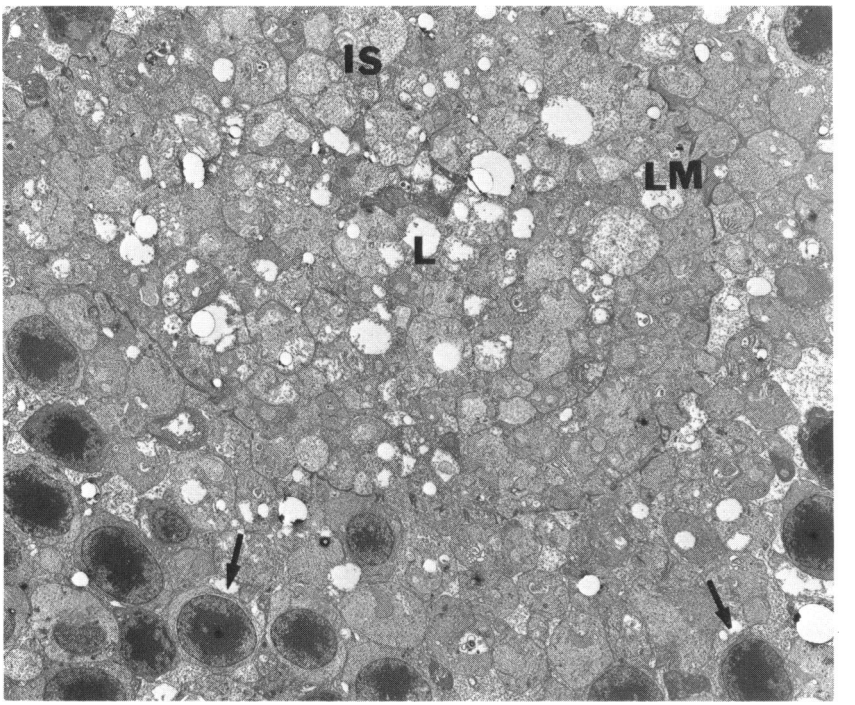

FIGURE 8
Photoreceptor cells from retinal tumor (*arrows*) in the inner portion of a rosette. Limiting membrane (LM) and inner segment material (IS) are present within the lumen (L) (× 1560).

(Fig 8). Less differentiated rosettes, characterized by cuboidal tumor cells arranged in a radial pattern around a tangle of fibrils, are also abundant (Fig 9).

Additional ultrastructural features of intraocular tumors include cilia with a characteristic 9 + 0 configuration; nuclear triple membrane structures with a central dense layer of either granular or fibrillar chromatin bounded on both sides by membrane; cytoplasmic microtubules; and dense-core secretory granules (Figs 10 to 13). Midbrain tumors are generally less differentiated than intraocular tumors, however these ultrastructural features are also observed within some midbrain tumor cells. In addition midbrain tumors show numerous membrane-bound mitochondria with resemblance to inner segment structures of normal retina. The cytoplasm of midbrain tumors contains stacks of lamellae which are similar to outer segment lamellae within normal retina, but may also represent golgi apparatus (Figs 14 to 17).

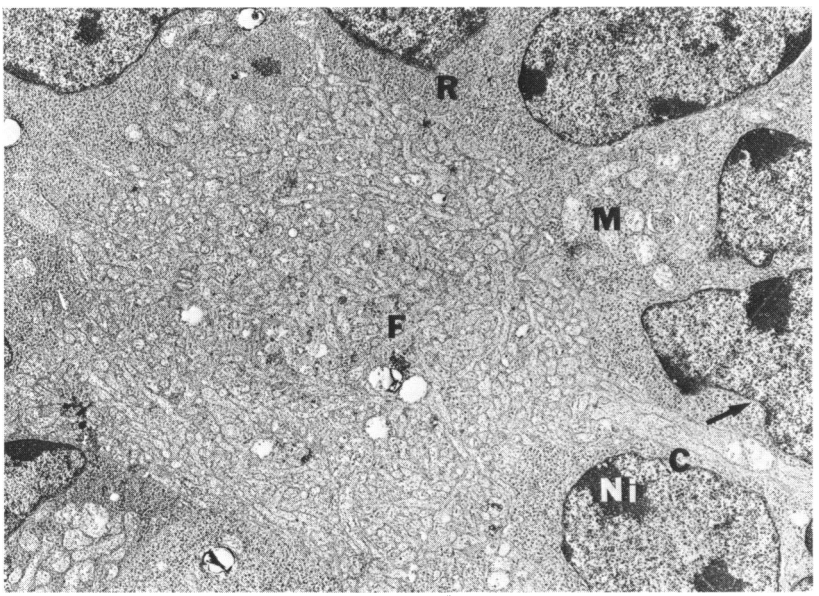

FIGURE 9
Area of cuboidal retinal tumor cells forming a rosette pattern around a fibrous matrix (F). The nuclei contain nucleoli (Ni), marginal chromatin (C), and have invaginated nuclear membranes (*arrow*). In the cytoplasm there are mitochondria and groups of ribosomes (R) (×6055).

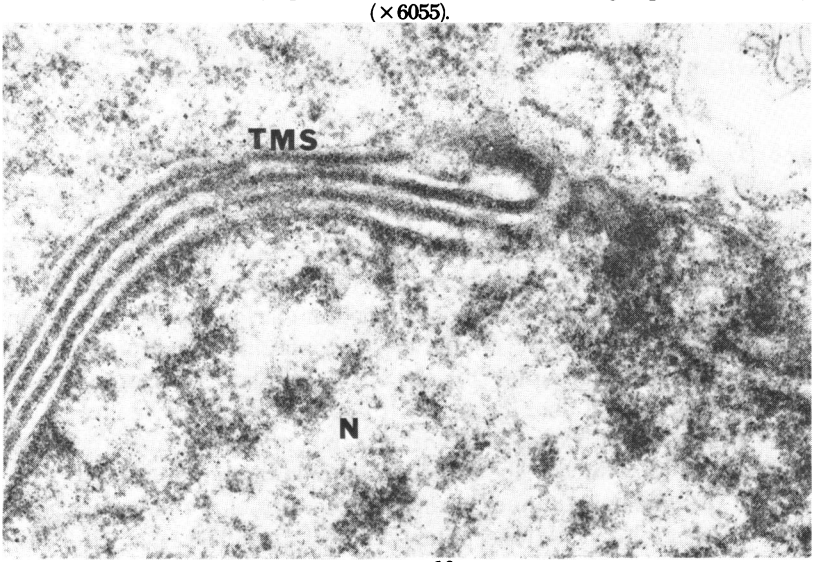

FIGURE 10
Portion of tumor cell having triple membrane structure (TMS) with dense chromatin material involving the nuclear envelope. A portion of the nucleus (N) is also present (×71,000).

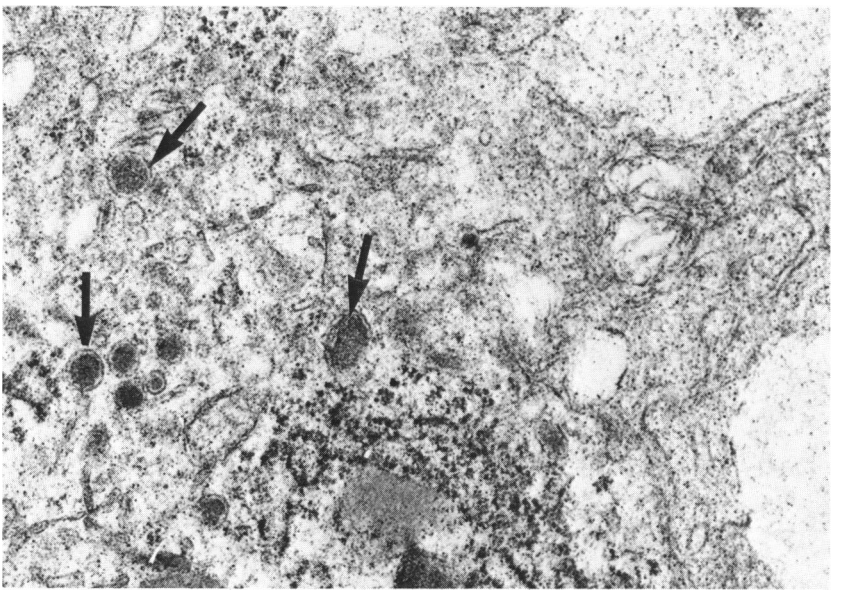

FIGURE 11
Neurosecretory granules (*arrows*) found in tumor cells, having a diameter of approximately 900 to 100A (×41,000).

Immunohistochemical analysis reveals intraocular and midbrain tumors to be positive for neuron specific enolase (NSE) and negative for S-100 protein and vimentin. Glial fibrillary acidic protein (GFAP) is identified within the stroma of the tumor but not within the tumor itself.

Molecular biologic studies: The progeny of matings between transgene-bearing animals and CB6F1/J breeders carry the SV 40 T-ag transgene in 50% of cases. This heterozygous transmission pattern was confirmed through ten generations by dot-blot analysis of purified tail DNA utilizing an SV 40 T-ag specific probe (Fig 18). The transgene acts dominantly in determination of phenotype; greater than 90% of dot-blot positive animals develop ocular neoplasms with diagnostic features of retinoblastoma. Preliminary results suggest that 10% of these transgene-bearing animals develop midbrain tumors.

Northern blot analysis of total RNA prepared from a variety of transgenic tissues shows specific expression of SV 40 T-ag within ocular tissues. Southern blot analysis of total genomic DNA utilizing an SV 40 T-ag specific probe reveals a relatively simple restriction pattern in the region of transgene integration (Fig 19).

FIGURE 12
Tumor cell cytoplasm containing microtubules with a diameter of about 200 to 250A (*arrows*) (× 131,000).

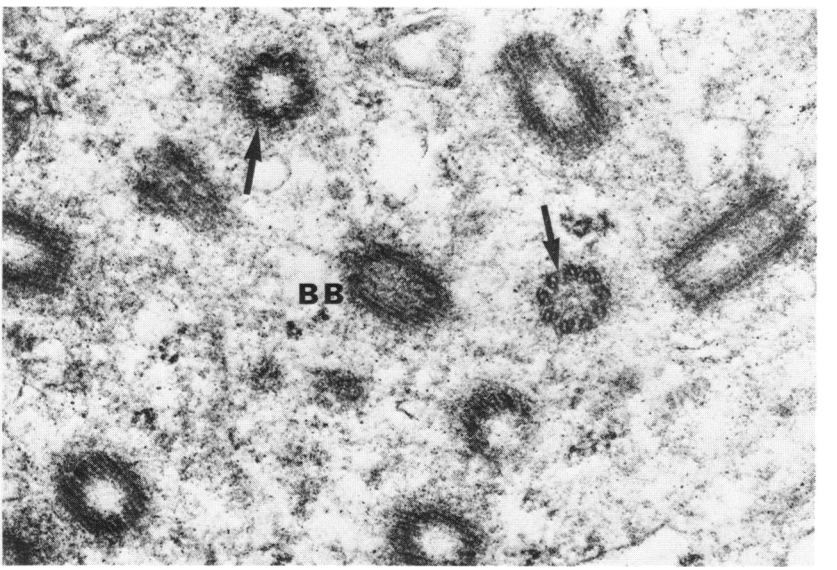

FIGURE 13
Tumor cell cytoplasm showing transverse section of cilia with a 9 + 0 pattern (*arrows*), and basal bodies (BB) (×41,000).

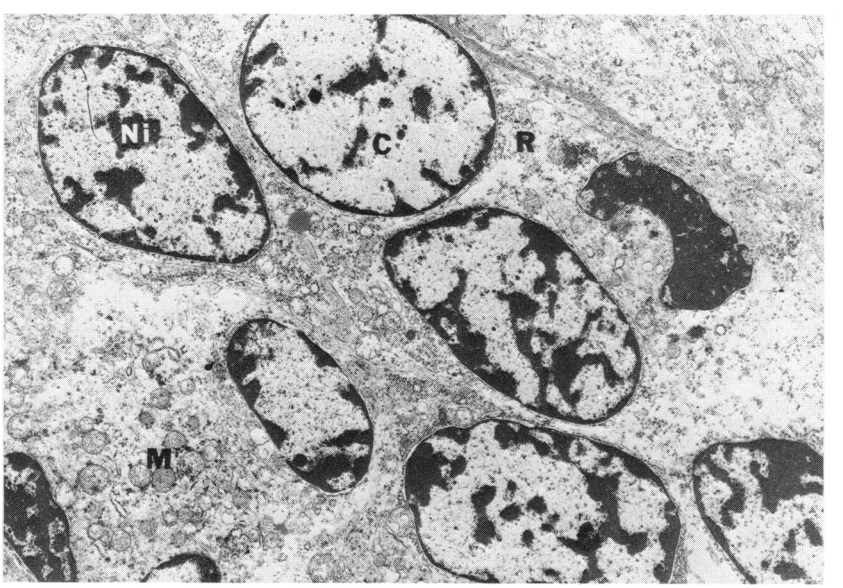

FIGURE 14
Low magnification of a cluster of undifferentiated tumor cells in midbrain with small round nuclei (N) and dispersed chromatin (C). Note numerous mitochondria (M) in the cytoplasm (×2600).

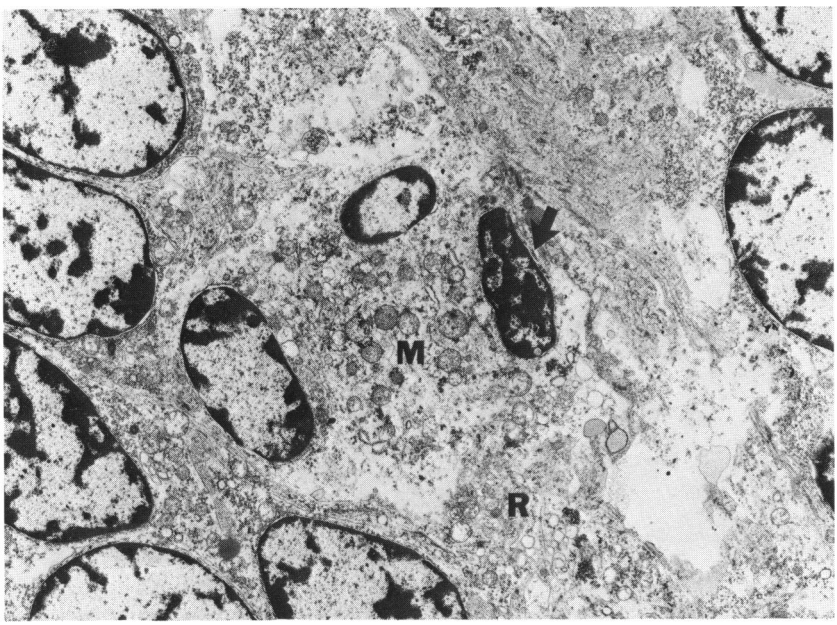

FIGURE 15
Higher magnification of the undifferentiated tumor cells in midbrain showing numerous mitochondria (M), ribosomes (R) and pyknotic cell debris (*arrow*) (×5700).

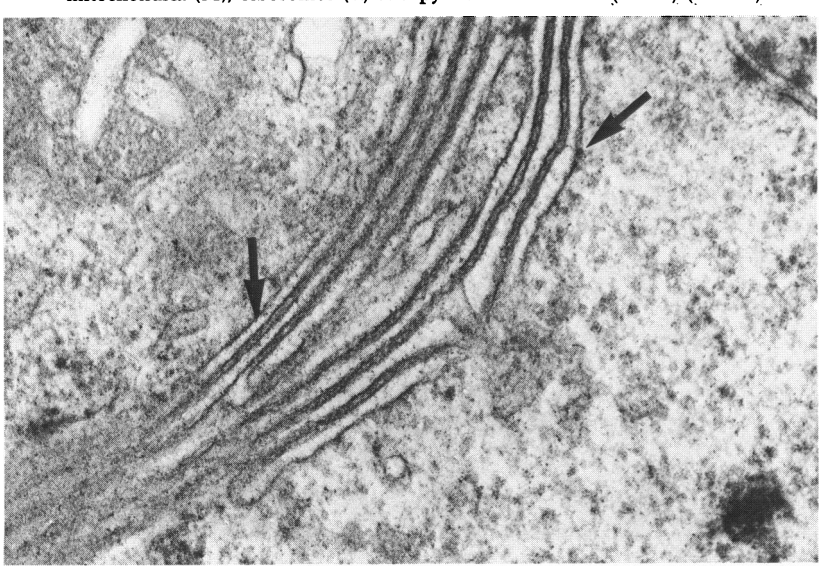

FIGURE 16
Stacks of lamellated membrane structures (*arrows*) found in the midbrain tumor cell cytoplasm (×29,400).

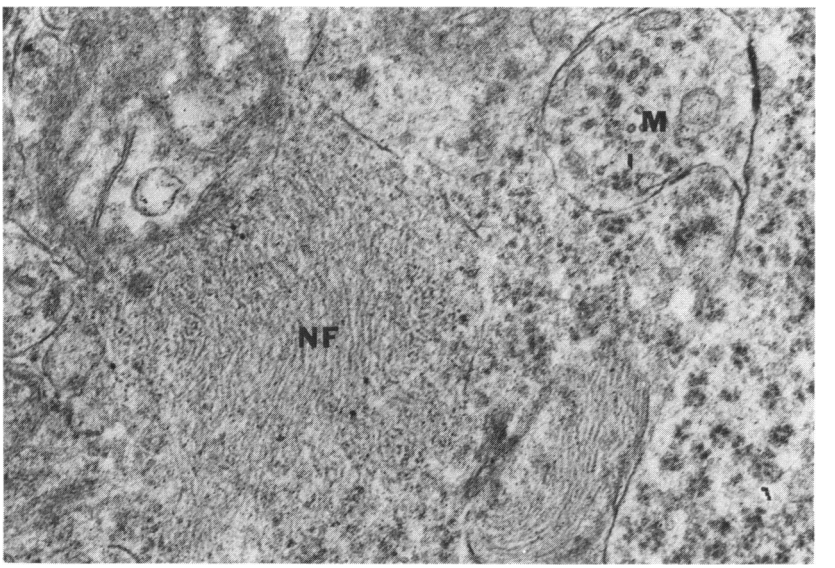

FIGURE 17
Tumor cells from midbrain revealing neurofilaments (NF) and transverse section of microtubules (M) (×56,000).

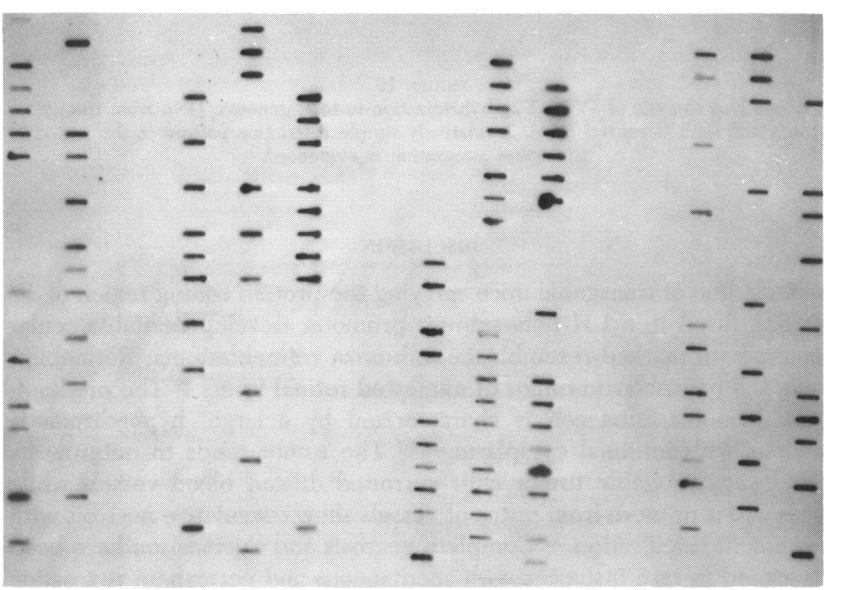

FIGURE 18
Slot-blot analysis of SV 40 T-ag hybridization to purified tail DNA from matings between transgene-bearing animals and CB6F1/J breeders. Approximately 50% of offspring carry the transgene.

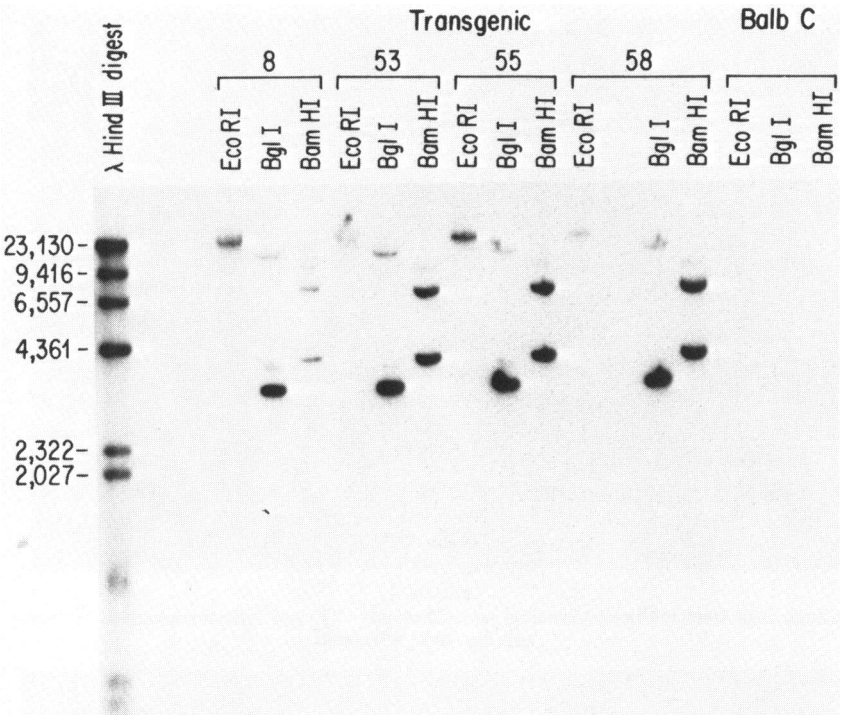

FIGURE 19
Southern blot analysis of SV 40 T-ag hybridization to total genomic DNA from transgenic animals and Balb C control mice. A relatively simple restriction pattern in the region of transgene integration is evidenced.

DISCUSSION

A single line of transgenic mice carrying the protein coding region of SV 40 T-ag fused to a LH beta-subunit promoter develop heritable ocular tumors with marked resemblance to human retinoblastoma. Retinoblastoma is a neuroblastic tumor of nucleated retinal layers.[39] The predominant retinoblastoma cell is characterized by a large, hyperchromatic nucleus with minimal cytoplasm.[39,40] The tumor tends to outgrow its blood supply; viable tumor cells surround dilated blood vessels while areas 110 μ or more from nutrient vessels show coagulative necrosis with prominent calcification.[39] Complete necrosis and calcification have been associated in rare instances with spontaneous and permanent regression of this malignancy.[41] Ocular tumors in transgenic mice display these characteristic histologic features of human retinoblastoma.

Rosette formation is also characteristic of retinoblastoma. No other neural tumor contains such highly differentiated rosettes in such large numbers.[39] Cuboidal cells attached at apical ends by terminal bars surround a central lumen. This Flexner-Wintersteiner rosette is a diagnostic feature of retinoblastoma. The Homer Wright rosette is characteristic of retinoblastoma, but has been described in other neural tumors such as neuroblastoma, sympathicoblastoma, and cerebellar medulloblastoma. It is composed of a single row of cuboidal cells surrounding a tangle of fibrous cytoplasmic processes.[39,40] Both Flexner-Wintersteiner and Homer Wright rosettes are observed in every transgenic intraocular tumor examined. More differentiated midbrain neoplasms also display rosette formation.

Electron microscopic studies of transgenic retinoblastomas confirm characteristic ultrastructural features including cilia with 9 + 0 pattern (the shafts lack central processes), cytoplasmic microtubules, and lamellated membranes.[42] As in human retinoblastoma, Müller cells which normally separate the inner segments of photoreceptor cells are absent. Tumor cells differentiating along a photoreceptor line are joined directly by terminal bars.[42]

Immunohistochemical stains are also consistent with human retinoblastoma. While undifferentiated human retinoblastoma cells demonstrate both NSE and GFAP, differentiated cells display either glial or neuronal features.[43] These transgenic tumors, staining positively for NSE and negatively for GFAP, display a neuronal pattern of differentiation. Immunohistochemical features of transgenic retinoblastoma are identical to a widely studied human retinoblastoma cell line, Y-79.

The mode of extension of intraocular retinoblastoma is characteristically by local invasion.[41] Endophytic tumors grow from the inner retinal layers into the vitreous space. Exophytic tumors grow toward the choroid producing retinal detachment. Invasion of the optic nerve with extension to the brain is observed. Transgenic tumors demonstrate both endophytic and exophytic growth, with replacement of retina and vitreous, and with optic nerve invasion.

Bilateral and multifocal tumors are associated with heritable retinoblastoma in children. This transgenic model parallels human germline mutation in that tumors are expressed in both eyes, with multiple discrete foci of tumor development.

The appearance at lower incidence of midbrain tumors involving the region of the pineal gland suggests further correspondence with heritable human retinoblastoma. The appearance of these neoplasms is consistent with independent primary tumors as opposed to metastatic lesions.[30] In

the majority of cases these tumors arise in conjunction with well-differentiated intraocular neoplasms which do not invade the optic nerve. Brain tumors are characteristically observed as unifocal discrete masses involving the midbrain in the region of the pineal gland. Diffuse spread along meninges is not observed. At autopsy, metastatic lesions are absent elsewhere in the body. These midbrain neoplasms may correspond to midline brain tumors observed in children with trilateral retinoblastoma.

Why should SV 40 T-ag fused to a LH beta-subunit promoter produce intraocular tumors with marked resemblance to human retinoblastoma? Carcinogenesis is believed to be a multistep process. Perhaps one step toward malignant transformation involves the expression of SV 40 T-ag within mouse retinal cells. The transforming region of T-ag specifically binds the protein product of the Rb-gene.[17] This binding may lead to inactivation of the Rb-protein or at least to reduction in dosage of free Rb-protein. Abnormalities in Rb-gene expression have been associated with malignant transformation in an increasingly wide variety of tumors.[8,11,12,14,15] However, loss of p105 Rb has been most frequently associated with development of retinoblastoma, and it is possible that the retina has a specific susceptibility to malignant transformation when p105 Rb is inactivated, or when free p105 Rb dosage is reduced.

This is only a partial explanation for tumorigenesis in our model. Although the same chimeric molecule was introduced into numerous pronuclei, only a single F_0 mouse developed retinoblastoma. Other F_0 mice developed the intended pituitary adenomas. It is likely that some random integration event in a single F_0 mouse placed the SV 40 T-ag under other, unexpected regulatory controls.

Half of all transgenes injected into fertilized mouse pronuclei are expressed; those which are unexpressed may have been incorporated into translationally silent areas of genomic DNA.[44] Transgenes which are expressed have become randomly integrated into areas of translationally active DNA. Some of these transgenes are expressed in a manner which reflects the regulatory controls of the promoter and enhancer regions included in the chimeric molecule. Nonviral tissue-specific regulatory units in the Elastase I,[45] Insulin II,[46] and alpha-crystallin[47,48] genes successfully direct SV 40 T-ag expression and subsequent tumorigenesis in a tissue specific manner. In these cases only several hundred base pairs of 5′ upstream sequences are required to direct expression[44] and such regulatory units can be included in the chimeric molecule. Other genes require more complicated regulation of expression; human globin gene expression relies on both 3′ and 5′ regulatory sequences.[49,50]

Despite intended tissue specificity, many investigators have found unexpected patterns of expression for their transgenes.[51,52] SV 40 early

region metallothionein (MT) fusions result in choroid plexus tumors with associated thymic and renal pathology.[51] When MT gene sequences are deleted, transgenic mice show no change in tissue specific tumorigenesis. Investigators conclude that the 72 base pair repeat region of the SV 40 enhancer has its own tissue specificity for choroid plexus, thymus, and kidney. When this viral enhancer is deleted and the MT gene is replaced, transgenic mice show liver and pancreatic tumors with peripheral neuropathy. Palmiter and co-workers[51] speculate that in the absence of an otherwise dominant SV 40 viral enhancer, the MT enhancer directs SV 40 expression in a tissue specific manner.

Microinjected transgenes integrate into the genome randomly. When several lines of transgenic mice show an identical pattern of tissue-specific expression, then investigators must conclude that regulatory signals within the transgene direct this tissue specific expression. Such regulatory signals may be viral enhancers like the SV 40 72 bp repeat or they may be other cell-type specific regulatory units like MT or beta-subunit of LH. However, if a single transgenic animal shows a unique pattern of tissue specific expression, then regulation of this expression probably lies outside the microinjected chimeric molecule. Chromosome positional effects have been reported.[53,54] It is possible that a genomic enhancer or promoter region specific for the retina became dominant over regularly units intrinsic to the microinjected chimeric molecule in our model. Northern blot analysis confirms the likelihood of such an event; the SV 40 T-ag transgene is specifically expressed within ocular tissues. Identification and cloning of this regulatory region could eventually provide an avenue for directing other genes of interest to be expressed specifically within retinal cells.

Studies are currently underway to investigate SV 40 T-ag and p105-Rb co-expression in the retina and brain of transgenic animals over time. Elucidation of integration events which may have participated in tumor formation will be achieved by in situ to karyotype, and by retrieval of transgenic flanking sequences. Cloning of retinal specific regulatory sequences is currently in progress. Since the eye is not an essential organ for viability, animals may be studied after enucleation for development of second malignancies. The development of these second malignancies and the molecular biologic correlates of this process will be available for study. The ontogeny of retinoblastoma, and its correlation with SV 40 T-ag oncogene and Rb anti-oncogene expression will be explored. No appropriate model for this malignant and heritable disease of childhood has previously been developed. Transgenic mice will be useful for study of chemotherapeutic agents as well as for testing of interventions which

target responsible mutations at the level of DNA expression.

Children with retinoblastoma develop this disease through homozygous mutations in the Rb-locus. With the Rb-gene deleted or mutated, functional Rb-protein cannot be generated and retinal cells undergo malignant transformation. In this murine model of retinoblastoma the retinoblastoma locus is intact, but an oncogenic protein which binds Rb-protein is specifically expressed within retinal cells. Rb-protein is functionally inactivated through specific binding with an oncogenic protein. The endpoint is the same; Rb-protein is not available to direct normal cellular growth and differentiation, and retinoblastoma results.

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DISCUSSION

Dr Lorenz E. Zimmerman. What an epochal paper we have just heard! It is fitting that it was presented at this historic meeting of the American Ophthalmological Society, and it is also most appropriate that it followed the Frederick Verhoeff Lecture. As most of you know, Doctor Verhoeff and the American Ophthalmological Society made history back in 1926 when his term, retinoblastoma, was officially adopted by the AOS, and subsequently by the American Registry of Pathology as the best of many names that had been used for this retinal tumor, believed to originate from undifferentiated neuroblastic cells of the retina. Reflecting back even further to 1864, when the AOS held its first meeting, retinoblastoma was then an invariably fatal cancer, as it continued to be almost to the turn of the century. Ophthalmologists should be extremely proud of what has been accomplished since then. Today, in the United States and in many other socio-economically privileged countries, we are more concerned with preservation of vision and genetic counselling than in saving life in our management of retinoblastoma.

During recent years we have learned that this tumor occurs in three clinical forms. One is relatively rare but very important. It is observed in some children who have a variety of abnormalities associated with a deletion of the long arm of chromosome 13. This form was crucial to the discovery of the locus for the genetic abnormality associated with retinoblastoma, but in over 95% of patients with retinoblastoma there is no obvious chromosomal abnormality.

In about a third of patients who have no detectable chromosomal abnormality, there is nevertheless a genetic defect affecting all of the body's cells. In the remainder of patients (about two-thirds all retinoblastoma patients), there is neither a chromosomal deletion nor a genetic abnormality. Yet despite these fundamental differences between the three groups, the tumor—retinoblastoma—is indistinguishable whether viewed by the ophthalmologist, by the pathologist using light microscopy, or by the electron microscopist. This always seemed enigmatic to me.

It led me some years ago to postulate that while the tumors are indistinguishable, what is different in those patients who have either a chromosomal deletion or a genetic abnormality is their susceptibility to whatever it is in the environment that actually causes retinoblastomas to develop. Knowing that retinoblastomas are worldwide in occurrence, affecting all populations and all races, and realizing that they are often apparent at birth or very soon after birth, I postulate that the oncogenic agent could very well be a lowly pathogenic virus transmitted from the mother to her baby, either in utero as with the rubella virus, or acquired during the neonatal period, or later in childhood (*Am J Ophthalmol* 1970; 69:947-964).

Now, as a result of remarkable progress made by the concerted research efforts of teams of clinical ophthalmologists, geneticists, and basic scientists using the most modern pioneering techniques of genetic exploration, a wealth of new information is being acquired, but we still don't know what are the specific causes of the genetic alterations that lead to the transformation of embryonic retinal cells into neoplastic cells.

A major advance was the discovery that while the familial form of retinoblastoma appears clinically to be the result of a dominant gene, the tumors are actually the consequence of a homozygous loss of the normal gene function at the retinoblastoma locus. In the past, when genetically determined retinoblastoma was thought to be the consequence of one's having acquired an abnormal dominant gene, the term "retinoblastoma gene" was used to reflect the concept of a dominant oncogenic gene. Today, the term "retinoblastoma gene" is used differently—it signifies the normal genetic material at the retinoblastoma locus. This normal gene behaves as a dominant that protects the cell, preventing it from undergoing neoplastic change (ie, an anti-oncogene). If only one of the alleles at the retinoblastoma locus becomes abnormal, losing its protective function, it is recessive to the remaining normal dominant allele that is still protective, preventing the cell from undergoing malignant change. When both alleles suffer a loss of normal function, the cell becomes cancerous.

As the authors have indicated, there are now several reports describing the inactivation of normal function of the retinoblastoma gene protein by various

oncogenic viruses, including an adenovirus, a papilloma virus, and simian virus 40. Their remarkably complete experimental model of genetic retinoblastoma in transgenic mice was a serendipitous complication of research aimed at the development of functionally active pituitary adenomas. Why their genetic engineering, which was successful in accomplishing the initial objective, should have also produced in one, and only one mouse, such a nice model of genetic retinoblastoma is a mystery that these investigators will be exploring further. Their great good fortune derived from this freakish accident was that the affected mouse became a sexually active male that initiated this strain of murine retinoblastoma, a phenomenon analogous to the new genetic mutations that develop spontaneously in humans, and which are responsible for the genetic form of sporadic retinoblastoma.

Heretofore, basic research has been retarded by the nonavailability of naturally occurring retinoblastoma in any animal species other than man. While many attempts have been made, using various cancerogenic chemicals and oncogenic viruses, to create a strain of retinoblastoma in animals, this goal has never before been achieved.

Let me emphasize the fact that I am convinced the authors have, indeed, produced what I never before believed possible—an almost perfect model of genetic retinoblastoma. They have characterized their tumor very thoroughly, and they have allowed me to study sections of their retinal and midbrain tumors. Using clinical and pathologic observations of the natural history of the tumor, how it grows in the eye, how it spreads, how it kills the animals, these investigators found it is just like human retinoblastomas, with very few differences. When judged by histologic, ultrastructural, and immunohistochemical criteria, their experimental tumor appears to be identical to human retinoblastoma. The frequency of ectopic midline intracranial foci is greater than the estimated frequency of trilateral retinoblastoma in man, and these investigators have not described the fully differentiated benign variant of retinoblastoma, the retinocytoma in their murine model.

What remains for them to demonstrate as their research continues is whether, after successful treatment of the affected mice, second cancers will develop at other anatomical sites and with what frequency. Also, if they treat their experimental tumors with radiation rather than by enucleation, will there be an exceptionally high incidence of osteosarcomas, epithelial cancers, melanomas, and other neoplasms in the irradiated tissues as has plagued the child who has received radiation therapy?

Finally, let me congratulate the original investigators whose interests were focused on the pituitary—not the eyes—how easy it would have been for them to have destroyed that one male mouse that fouled up their experiments by developing bilateral retinoblastomas! While this unanticipated result of their original experiments proved to be a scientific bonanza, this experience also serves to warn us to expect the unexpected when one begins tinkering with genes.

Thank you for the opportunity of discussing this outstanding paper, which has launched a new era in the already remarkably productive research on the pathogenesis of retinoblastoma.

DR IRENE H. MAUMENEE. Ladies and gentlemen. The pathogenic mechanism underlying the variable severity of the retinoblastoma gene, even in a single family, has to be explained. I would like to propose a hypothesis for the development of retinoma and possibly an extension to spontaneous regression. It cannot be tested in the transgenic mouse model because the transgene is present in multiple copies, but it rather needs every clinician's cooperation for its corroboration.

It is now widely accepted that the retinoblastoma (rb-) gene can only become manifest if not opposed by the wild type normal (rb+) gene. In a previously published family (Cavenee et al, *Science* 1985; 288:501-503; case 2) the father had an enucleation in Teheran at age 2 years, presumably because of retinoblastoma. In his fellow eye he has a retinoma. In his son, bilateral retinoblastoma was diagnosed at age 4 months and the boy has since lost both eyes to the disease.

On DNA analysis of tissue taken from a retinoblastoma after enucleation in the child, homozygosity of the region containing the retinoblastoma gene was present compared to peripheral blood DNA of the father and child which contained the retinoblastoma gene in a single copy only.

Homo- or hemizygosity of the rb-gene develops at metaphase through either of four distinct abnormal mitotic divisions all of which have been found in human retinoblastoma. I would like to postulate that tumors with less aggressive behavior arise through aberrant mitotic dysjunction leading to clonal trisomy 13, as a correlary to monosomy combining a double retinoblastoma gene complement and wild type normal gene (rb- rb− rb+).

The reduction in severity would arise through the mitigating effect of the normal wild type gene product as compared to homozygosity of the rb-gene. Trisomies are unstable and one can easily postulate conversion to the diploid state, be it rb- rb− with unmitigated tumor growth or rb- rb+ with lacking further growth or even regression of the tumor.

This hypothesis can be easily tested by DNA analysis of such tumors, and I would like to submit a plea that eyes harboring a retinoma be submitted for DNA analysis and not be placed into formalin per primam.

DR JOHN R. HECKENLIVELY. I want to congratulate Doctor Albert and co-workers for a fabulous paper! We have been looking for a tissue model for retinoblastoma for quite a while, so it is very exciting that a transgenic mouse has been developed. There might be a question among the membership as to the value of mouse models. We are currently using mouse models for other ocular diseases, such as retinal degenerations to examine mechanisms in retinitis pigmentosa and other retinal dystrophies. The reason that we believe this is a valid approach, is in comparing the human gene map with the mouse map, we are discovering a large portion of the genes are held in common. They are not necessarily on the same chromosome, for example, mouse chromosome number 1 might actually correlate with human chromosome 3 and 7. But there are large regions of the human chromosome which map in segments on the mouse chromosome, and there actually is a 70% homology between the two. The retinoblastoma "off" gene also

seems to be playing a role in other types of human tumors, such as neuroblastoma and breast carcinoma. This type of work is opening up a great deal of knowledge. Interestingly, it has been ophthalmology's work with retinoblastoma that has focused other branches of medicine to look at retinoblastoma as a major model for carcinoma.

DR DEVRON H. CHAR. I would like to add my congratulations. I have a single query about Doctor Maumenee's intriguing hypothesis. I am slightly skeptical in one regard. In vitro study of the retinoblastoma gene product shows it to have an incredibly potent control over tumor cell proliferation. Preliminary data from a number of groups suggests that even a very small amount terminates cell growth and is often cytotoxic. While it is conceivable that the mechanism Doctor Maumenee mentions for retinocytoma development is correct, I would be surprised if there were sufficient genetic material for a normal copy, but insufficient not to totally abrogate retinoblastoma or retinocytoma growth. Certainly her idea of testing the hypothesis makes good sense. Also the intriguing ability to take archival paraffin tissue and analyze DNA is an interesting one worth pursuing.

DR DANIEL M. ALBERT. I would like to thank Doctor Zimmerman for his very thoughtful and generous discussion of my paper. Doctor Maumenee's hypothesis is certainly an interesting one. I also appreciate Doctor Char and Doctor Heckenlively's remarks.