Multiple Genetic Loci Modify Risk for Retinoblastoma in Transgenic Mice

Anne E. Griep,1 Jeff Krawcek,2 Denis Lee,2 Amy Liem,2 Daniel M. Albert,3 Rey Carabeo2

PURPOSE. Forty percent of cases of retinoblastoma, a childhood malignancy of the retina, are linked to the inheritance of a mutant allele of the retinoblastoma susceptibility gene Rb1. Tumor penetrance varies among carriers in different family pedigrees, indicating that other genetic factors may modify risk for occurrence of retinoblastoma. This study was undertaken to determine whether multiple genetic loci modify the risk for retinoblastoma in mice.

METHODS. A line of aAcry-HPV16E6/E7 transgenic mice expressing the human papillomavirus type 16 E6 and E7 oncogenes (HPV-16 E6 and E7) ectopically in the retina was characterized. E6 and E7 proteins bind to and inactivate the cellular tumor suppressor proteins p53 and Rb, respectively.

RESULTS. Retinoblastomas developed rarely when the aAcry-HPV16E6/E7 transgene was maintained on the FVB background, but tumors arose with high frequency on C57BL/6 × FVB and C3H × FVB F1 hybrid backgrounds. The incidence of retinoblastoma in the LHB-TAG transgenic mice, which express simian virus 40 large tumor antigen (SV40 T-ag), was also influenced by the FVB and C57BL/6 backgrounds. Resistance of the aAcry-HPV16E6/E7 FVB mice to retinoblastoma mapped in part to the retinal degeneration (rd) locus. However, multiple genetic experiments indicate that resistance to retinoblastoma depends on additional loci in FVB mice.


Retinoblastoma is a malignancy of the retina that occurs in young children and is associated with the loss of function of the retinoblastoma susceptibility gene Rb1.1–5 Children who inherit one mutant allele of Rb1 are predisposed to bilateral, multifocal retinoblastoma and secondary tumors in numerous nonocular tissues. In these tumors, loss of function of the remaining wild-type allele is found at the Rb1 locus. Nonhereditary, or sporadic, retinoblastoma is associated with somatic mutations in both alleles of Rb1 and is usually focal. As such, Rb1 is representitive of tumor-suppressor genes, the disruption of which is associated with cancer development.4 Loss of function of both alleles of Rb1 is the cornerstone of the two-hit hypothesis of Knudsen5 for retinoblastoma development in humans. However, the penetrance of retinoblastoma among human pedigrees is incomplete; it is generally estimated to be 80% to 95%, but in some pedigrees it is as low as 20%.6,7 The absence of complete penetrance may relate to differences in the severity of the defect in mutant Rb1 alleles.8 Alternatively, it may indicate that other cellular genes can affect the risk in a person who carries a mutant Rb1 allele for development of retinoblastoma. Through the study of inbred strains of mice, genetic loci that affect the sensitivity or resistance of an animal (that is, those that modify risk) to specific cancers have been identified. For example, the mouse mom locus modifies risk to intestinal tumors in mice that inherit a defect in the mouse homologue to the human adenomatous polyposis coli gene,9 and multiple mouse loci modify risk to liver tumors (for review, see Ref. 10). In this study, we provide experimental evidence that multiple genes modify risk to retinoblastoma in the mouse.

Genes in addition to Rb1 are likely to contribute to the development of retinoblastoma in mice. Mice carrying a germ-line mutation in one Rb1 allele are not predisposed to retinoblastoma14 as are humans; nor are mouse chimeras that harbor Rb1−/− cells in their retinas predisposed to retinoblastoma.12,13 Yet transgenic mice expressing simian virus 40 (SV40) large-tumor antigen (T-ag) gene in the retina show development of retinoblastoma.13–16 SV40 T-ag is a potent oncoprotein that binds to and inactivates the Rb protein.17 In addition to these direct effects on Rb, SV40 T-ag possesses other activities that contribute to its transforming potential in tissue culture18 and tumorigenic phenotype in vivo.19,20 These activities include its abilities to bind and inactivate Rb-like proteins p107 and p130,21,22 to modulate function of the p530 protein,23 and to inactivate the tumor-suppressor protein

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p53. Of these activities, the inactivation of p107 by T-ag may contribute to the induction of retinoblastoma. Mice that are p107-/-/Rb1-/- show retinal dysplasia, a potential precursor to retinoblastoma. In additional, inactivation of p53 may contribute to retinoblastoma formation in mice, because expression in the retina of the E7 oncogene from human papillomavirus (HPV) type 16 causes retinoblastoma only on a p53-/- background.

Human papillomavirus E6 and E7 oncoproteins belong to the same class of viral oncoproteins as does SV40 T-ag. E6 and E7 are expressed in most HPV-associated cervical cancers and together are necessary and sufficient to transform cells in tissue culture. Similar to T-ag, E6 inactivates p53, although through a different mechanism, by targeting p53 for degradation by a ubiquitin-mediated proteolytic pathway and together are necessary and sufficient to transform cells in tissue culture. Similar to T-ag, E7 binds to and sequesters Rb and Rb-like proteins leading to the deregulation of the E2F transcription factor family. We have shown that expression of HPV-16 E6 and E7 oncoproteins in the ocular lens could induce proliferation and inhibit differentiation in the lens in three independent lines of transgenic mice. These lines of transgenic mice, referred to as aAcry-HPV16E6/E7 mice, were generated and maintained on the FVB inbred mouse genetic background, which is homozygous for a recessive mutation at the retinal degeneration (rd) locus. In the line of aAcry-HPV16E6/E7 mice expressing the highest levels of E6/E7 message, line 19, lens tumors developed in approximately 40% of mice by 1 year of age. Transgene expression in this line of mice was found in multiple ectopic sites including the skin and extralenticular portions of the eye. Expression of E6 and E7 in the skin correlated with the high incidence of skin tumors. However, expression in other tissues such as the extralenticular portions of the eye did not correlate with tumor formation.

In this study we report that, when crossed with other mouse genetic backgrounds such as C57BL/6 and C3H, the line 19 aAcry-HPV16E6/E7 transgenic confers high susceptibility to retinoblastoma formation. Whereas C57BL/6 is wild type at the rd locus, C3H is rd/rd. The retinoblastoma tumors expressed the transgene, appeared to arise from the inner nuclear layer of the retina, and sometimes developed into exophytic tumors. Crosses of line 19 aAcry-HPV16E6/E7 transgenic mice with transgenic mice of the C57BL/6 genetic background in which photoreceptors have been ablated (rdta [rhodopsin promoter driven diphtheria toxin] transgenic mice) also showed development of retinoblastoma. These results suggest that the FVB genetic background in which the transgene originally resides confers protection against retinoblastoma formation associated with expression of E6 and E7. Backcross experiments have shown that the FVB background can also confer resistance to retinoblastoma formation associated with expression of SV40 T-ag. Taken together, these studies indicate that the protective effect of the FVB genetic background is associated not only with the rd/rd allele but also with additional loci.

**METHODS**

**Mouse Strains**

The generation, screening, and characterization of all transgenic mouse lines have been described. Line 19 aAcry-HPV16E6/E7 transgenic mice were generated and maintained on the FVB genetic background. In these transgenic mice, expression of the HPV-16 E6 and E7 genes is directed to the lens using the aA crystallin promoter. The LHβ-TAG transgenic mice express SV40 T-ag ectopically in the retina from the luteinizing hormone promoter. These transgenic mice were originally generated on the C57BL/6 × BALB/c hybrid background and subsequently maintained on the C57BL/6 inbred background. The rdta transgenic mice express an attenuated form of the diphtheria toxin A gene in rod photoreceptors by virtue of the cell type specificity of the rhodopsin promoter that drives its expression. These transgenic mice were generated originally on the FVB background, and subsequently, the transgene was moved onto the C57BL/6 background through backcrossing F1 hybrids. All stock C3H/HeJ and C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME), and FVB mice were purchased from Taconic Farms (Tarrytown, NY). All experiments with animals were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Genotyping**

The aAcry-HPV16E6/E7 transgenic mice were screened for the presence of transgene by Southern blot or polymerase chain reaction (PCR) analysis of DNA prepared from tail biopsy specimens, as described previously. The LHβ-TAG transgenic mice were screened for the presence of the transgene using PCR analysis (Jolene J. Windle, personal communication). The rdta transgenic mice were screened for the presence of the transgene by PCR analysis as described. The rd status of F1 hybrid mice backcrossed with the FVB background was performed by PCR analysis. The murine-endogenous retrovirus Xmv is integrated into the first intron of the β-1 subunit of the phosphodiesterase gene, (β-PDE). This retroviral integration event Xmv-28 is not present in the wild-type allele of β-PDE. The following oligonucleotides were generated to amplify the retrovirus-disrupted intron in the rd allele or the intact intron of the wild-type allele of β-PDE in (JFVB line 19H × C57BL/6) × FVB) B6 mice: rd1, 5'-ACCTGCATGT-GAACCCAGTATTC-3'; rd2, 5'-GGGGAACCTGAAACTGAGGT-GGACCCAGTATTC-3'; rd2, 5'-GGGGAACCTGAAACTGAGGT-GGACCCAGTATTC-3'; and rd3, 5'-CTCCTTTCTATTGCCCTGATCCACA-3'. Oligo rd1 is complementary to β-PDE sequences in intron 1 lying upstream of the Xmv-28 integration site, and rd2 is complementary to sequences within Xmv. The combination of oligos rd1 and rd2 selectively amplifies a region in the rd allele, across the junction of the first intron and the integrated retroviral element, generating a 470-bp fragment. Oligo rd3 is complementary to β-PDE sequences in intron 1 downstream of rd1 in the region disrupted by Xmv-28 in the rd allele. The combination of rd1 and rd3 selectively amplifies the intact first intron of the wild-type allele resulting in a 370-bp fragment. The PCR products were resolved on a 2% agarose gel. Animals that were rd/rd at the locus would give rise only to the larger of the two fragments, whereas animals that were rd/+ would contain both fragment sizes in their products. The rdta and rd status was verified phenotypically, when possible, by examining the retina microscopically for the presence or absence of an outer nuclear layer.
Mapping $\alpha$cry-HPV16E6/E7 Transgene Integration Site

Genomic DNA samples were isolated by phenol-chloroform extraction from skin tissues of 49 (FVB|line 19) × C57BL/6/C57BL/6 backcross animals. Each animal was genotyped for simple sequence length polymorphism (SSLP) markers by PCR. Briefly, 100 ng genomic DNA was used as a template in the PCR reaction containing 0.13 mM forward and reverse SSLP primers (Research Genetics, Birmingham, AL), 50 mM deoxyribonucleoside triphosphates, and Taq polymerase in 1X PCR buffer (10 mM Tris-HCl, 1.5 mM MgCl2 and 50 mM KCl [pH 8.3]). Amplification was performed in a GeneAmp PCR System 9600 (Perkin Elmer, Norwalk, CT) in an initial denaturing cycle of 2 minutes at 94°C (1 cycle), 30 seconds at 94°C, 30 seconds at 55°C, and 1 minute at 72°C (50 cycles), 7 minutes at 72°C (1 cycle), and a 4°C soak. PCR products were run on a 7% polyacrylamide gel and visualized under UV illumination. Linkage analysis for the transgene was performed using the MAPMAKER program (Whitehead Institute Genome Center, Cambridge, MA).

Light Microscopy

Eyes were enucleated from mice, fixed in 10% buffered formalin overnight, dehydrated through a graded ethanol series, and embedded in paraffin using an automatic tissue processor (Tissue-Tek, Miles Scientific, Naperville, IL). Sections (5 μm) were cut, mounted on glass slides, stained with hematoxylin and eosin, protected with a coverslip, and examined. The intact skull and brain were fixed overnight in 10% buffered formalin and then decalcified in 5% nitric acid. Brain tissue was processed as described for ocular tissue and also examined by light microscope.

Electron Microscopy

Eyes were fixed for 8 hours in Karnovsky's paraformaldehyde-glutaraldehyde fixative40 prepared in cacodylate buffer immediately before use. Eyes were washed in 0.1 M cacodylate buffer (pH 7.4) on ice for 1.5 hours and then washed in cold ddH2O. After dehydration in a graded series of ethanol, the eyes were infiltrated using a rotator with propylene oxide and Eponate (Ted Pella, Redding, CA), beginning with a 2:1 ratio and gradually increasing the proportion of Eponate over several hours until the eyes were in 100% Eponate. After overnight infiltration with Eponate, each eye was embedded in freshly made Eponate in individual flat-bottomed plastic vials. The sections were cut on a microtome equipped with a diamond knife (Ultracut E; Reichert, Buffalo, NY), stained with uranyl acetate and lead citrate, and examined by electron microscope (H-7000, Hitachi, San Jose, CA) at 75 kV.

In Situ Hybridization

Eyes for in situ hybridization analysis were enucleated from line 19 $\alpha$cry-HPV16E6/E7 F1 hybrid transgenic mice and fixed in 4% paraformaldehyde overnight at 4°C, transferred to phosphate-buffered saline (PBS), dehydrated through a graded ethanol series, embedded in paraffin, and sectioned at 5-μm thickness. Fixation and paraffin sections were deparaffinized in xylene, rehydrated through a graded series of ethanol, and subjected to in situ hybridization, as described previously.36,41 with the riboprobes described later. Antisense and sense cRNA probes were synthesized in vitro using γ35S-uridine triphosphate and T3, T7, or SP6 polymerase and the following templates: for generation of the $\alpha$ crystallin-specific probes, the clone pGEMcr1 containing a 900-bp fragment of the mouse $\alpha$ crystallin gene was used (obtained from Kathleen Mahon, Baylor University, Houston, TX). For generating the HPV-16 E6/E7-specific probes, the plasmids pAB1.5 and pGEM/E6 containing the E7 and E6 open reading frames of HPV-16, respectively, were used.35 Sections were hybridized with either sense or antisense 35S-labeled riboprobes, washed to remove nonspecifically bound probe, dipped in autoradiographic emulsion (NTB-2; Eastman Kodak, Rochester, NY), and exposed in the dark at 4°C for 2 weeks. After developing, dehydrating, and mounting, sections were examined by dark-field microscopy. Three to four sections from three tumors from three mice were examined.

RESULTS

Effect of C57BL/6 Genetic Background on Tumor Incidence in $\alpha$cry-HPV16E6/E7 Mice

We have reported the generation and phenotypic characterization associated with expression of the HPV-16 E6 and E7 oncogenes in the mouse lens.35 In that study, three independent lines of $\alpha$cry-HPV16E6/E7 mice, lines 4, 18, and 19, were created on the FVB inbred genetic background. In these mice, expression of the viral oncogenes was directed to the lens by the murine $\alpha$A crystallin promoter. Approximately 40% of line 19 transgenic mice, which had the highest level of transgene expression, showed development of lens tumors by 13 months of age. The transgenic mice on the FVB inbred background carry a recessive mutation at the rd locus, which in the homozygous condition leads to a complete loss of rod photoreceptor cells during the first month of postnatal life.42 Because it was possible that the rd/rd-induced defects in the retina of FVB/FVB mice could influence the fate of E6/E7-expressing lens cells, homozygous $\alpha$cry-HPV16E6/E7 mice from line 1935 were crossed with the C57BL/6 inbred strain of mice, which is homozygous for the wild-type rd allele, and the F1 transgenic progeny were characterized. No significant difference in the lens phenotypes was observed in perinatal and young adult $\alpha$cry-HPV16E6/E7 FVB × C57BL/6 F1 mice compared with that of their inbred FVB transgenic parents (data not shown). However, the eye tumor incidence in line 19 $\alpha$cry-HPV16E6/E7 mice increased substantially in these F1 animals (Fig. 1). Less than 10% of line 19 mice on the inbred FVB background had tumors by 10 months. In comparison, by 10 months of age 90% of the F1 line 19 $\alpha$cry-HPV16E6/E7 mice showed tumor development. These tumors began to arise in mice as early as 3 months of age. The high incidence of eye tumor formation seen in the F1, mice was retained when the line 19 $\alpha$cry-HPV16E6/E7 transgene was backcrossed onto the C57BL/6 background to generation N10 (data not shown). A significant proportion of the line 19 $\alpha$cry-HPV16E6/E7 F1 mice had tumors develop in both eyes. Thus, genetic background influences the incidence of tumor formation in $\alpha$cry-HPV16E6/E7 mice.

Development of Retinoblastomas Rather Than Lens Tumors in Line 19 $\alpha$cry-HPV16E6/E7 F1 Transgenic Mice

To determine the cellular origins of the eye tumors in the line 19 $\alpha$cry-HPV16E6/E7 FVB × C57BL/6 F1 mice, microscopic
analyses were performed. Examples of the histology seen in intermediate and advanced line 19 aAcry-HPVl6E6/E7 F1, eye tumors are shown in Figures 2A and 2B, respectively. These histologic features are representative of what was observed in most of the eye tumors in the line 19 F1, aAcry-HPVl6E6/E7 mice. The tumor in Figure 2A appeared to arise in the retina, rather than the lens. A small cataractous lens located within the anterior portion of the eye remained intact, that is, the lens capsule was not broken. The lens is similarly intact in the advanced tumor in Figure 2B. These features suggest that the retina rather than the lens was the source of the tumors. Homer-Wright rosettes, a hallmark of retinoblastoma, were evident in the tumor masses by light and electron microscopic analyses (Figs. 2A, 2B, 3C). Ultrastructural analyses showed that these tumors also possessed basal bodies and trilaminar nuclear membrane (Figs. 3A, 3B, respectively), both characteristics of retinoblastoma in humans. Further histologic analyses indicated that a minority of the retinoblastomas were exophytic, invading the optic nerve (Fig. 2C). Of these, a significant number established metastases within the brain and cervical lymph nodes (Fig. 2C). These metastases also displayed histopathologic characteristics of retinoblastoma including Homer-Wright rosettes (Fig. 2D). The tumors that developed in line 19 aAcry-HPVl6E6/E7 F1 transgenic mice resemble those observed previously in a line of SV40 TAG transgenic mice, LHβ-TAG, that expressed SV40 TAg ectopically in the retina15 and in RBP-TAG transgenic mice expressing SV40 TAg specifically in photoreceptors.16 In each of these transgenic mouse models the retinal tumor bears significant resemblance to human retinoblastoma.

To assess further the cellular origin of the tumors in line 19 aAcry-HPVl6E6/E7 F1 transgenic mice, eyes with early-stage tumors were examined microscopically. In multiple examined eyes, dysplastic and hyperplastic regions were evident in the retina, whereas the cataractous lens remained intact (Figs. 4A, 4B, 4C). Early lesions arose within the inner nuclear layer of the retina (Fig. 4B), similar to the apparent origin of tumors in the LHβ-TAG transgenic mice15,45 Crystallin-specific immunohistochemical and in situ hybridization analyses indicated that these lesions lacked expression of lens-specific crystallins (data not shown). This further suggests that the tumors arising in line 19 aAcry-HPVl6E6/E7 F1 mice are not lenticular in origin. Characterization of more than 150 tumors arising in line 19 aAcry-HPVl6E6/E7 F1 mice indicate that more than 90% of the eye tumors were retinal in origin. These findings led us to examine additional eye tumors from line 19 aAcry-HPVl6E6/E7 FVB mice. From more than 100 tumors examined, less than 1% of the tumors arising in the inbred FVB background were retinoblastomas. Thus, the genetic makeup of the mouse influences not only the frequency of tumor development but also the type of eye tumor in the line 19 aAcry-HPVl6E6/E7 mice.

**Heightened Expression of the E6 and E7 Genes in the Retinoblastomas in Line 19 aAcry-HPVl6E6/E7 F1 Mice**

Previously, we reported that mRNA specific for the HPV-16 E6 and E7 genes was detected in extraretinal portions of the eye of line 19 aAcry-HPVl6E6/E7 mice35 using the sensitive method of reverse transcription (RT)-PCR analysis. We performed in situ hybridization to assess whether viral transgenes were expressed in the retina and in the retinal tumors. High-level expression of the viral transgenes was detected not only in the lens but also within the retinal tumor mass of this and other aAcry-HPVl6E6/E7 F1 mouse eyes (Fig. 4D). Lower-level expression of the transgene was noted within the microscopic, dysplastic lesions arising within the inner nuclear layer of the retina. Expression in the unaffected regions of the retina was at or below the limit of detection by in situ hybridization, as was the case in the retina of aAcry-HPVl6E6/E7 FVB mice (data not shown). This finding suggests that the basal level of transgene expression was not influenced by the mouse's genetic background. Thus, the level of expression of the viral transgenes seems to be augmented specifically in the retinal tumors of line 19 aAcry-HPVl6E6/E7 F1 mice. Similar increased levels of E6/E7 expression have been noted in squamous cell carcinomas arising in line 19 transgenic mice.36

**Chromosomal Site of Integration of the aAcry-HPVl6E6/E7 Transgene in Line 19 Mice**

Retinoblastoma in humans is associated with mutations at the Rb1 locus. We therefore determined whether the high incidence of retinoblastoma in line 19 aAcry-HPVl6E6/E7 mice was a consequence of the transgene insertion into and disruption of the Rb1 locus in the FVB genome. An insertion of this kind would lead to an Rb1<sup>+</sup> genotype in the FVB × C57BL/6 F1 animals. The chromosomal location of the transgene was mapped in the (FVB × C57BL/6 × C57BL/6 backcross (B1) progeny using SSLP analysis.44 Briefly, linkage of 17 SSLP mark-

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**Figure 1.** Eye tumor incidence as a function of age in aAcry-HPV16E6/E7 transgenic mice on FVB (□) inbred versus with C57BL/6 × FVB F1 hybrid genetic backgrounds (■). Line 19 aAcry-HPV16E6/E7 FVB transgenic mice were crossed with C57BL/6 nontransgenic mice. Transgenic mice maintained on the FVB inbred background and F1 progeny were monitored for the presence of overt eye tumors at the indicated ages and killed when tumors were observed. Mice were genotyped by polymerase chain reaction analysis (see the Materials and Methods section). Indicated are the percentages of mice showing eye tumors at various ages up to 13 months. The number of mice in each group is indicated in parentheses.
Risk Factors for Retinoblastoma in Mice

FIGURE 2. Eye tumor histology in αAcry-HPV16E6/E7 line 19 C57BL/6 × FVB F1 hybrid mice. (A, B) Hematoxylin and eosin-stained 5-μm paraffin sections from representative intermediate (A) and advanced (B) eye tumors in the line 19 F1 hybrid mice. Note the small cataractous lens characteristic of line 19 mice and large retinoblastoma with characteristic rosettes. (C) Low magnification of head and neck from a line 19 F1 hybrid mouse shows the large retinoblastoma in the eye (E), the invasion of the tumor into the brain (arrow), and the metastasis to the cervical lymph node (LN). (D) High magnification of a brain tumor from another line 19 F1 hybrid mouse shows the characteristic rosette appearance (arrow). L, lens.

ers to the transgene was analyzed using the Fisher’s exact test. A marker on chromosome 12, D12Mit34, yielded the best linkage significance, with \( P = 4.1 \times 10^{-15} \) (Table 1). SSLP markers that flank the tumor suppressor genes, Rb1 (D14Mit7, \( P = 0.07 \)) and Trp53, (D11Mit19, \( P = 1.0 \); D11Mit14, \( P = 1.0 \)) did not show linkage to the transgene. Based on our genotype data from the backcross, the transgene was positioned 2.1 centimorgans (cM) proximal to D12Mit34, using the MAP-MAKER program (data not shown). The location of line 19 transgene on chromosome 12 discounts the possibility that its position causes the disruption of the Rb1 or Trp53 loci, which are located on chromosomes 14 and 11, respectively. Thus, a direct effect of the transgene’s insertion does not account for the high incidence of retinoblastoma in line 19 F1, αAcry-HPV16E6/E7 mice.

Resistance to Retinoblastoma in an SV40 T Antigen Transgenic Mouse Line on the FVB Background

Alternatively, the high incidence of retinoblastoma in line 19 F1, αAcry-HPV16E6/E7 transgenic mice may reflect an influence of genetic background on transgene expression, a consequence of the location of the transgene integration in the mouse genome. To investigate this possibility, studies were performed on another line of transgenic mice in which retinoblastomas develop. This particular strain of mice carries an LHβ-TAG transgene whose integration site is on chromosome 4. In these mice SV40 large-tumor antigen (SV40 TAG), which is under the transcriptional control of the luteinizing hormone (LHβ) promoter, also is expressed ectopically in the retina. These transgenic mice were originally generated on a C57BL/6 × BALB/c hybrid background and subsequently maintained on the C57BL/6 background in our laboratories. To examine the influence of genetic background on SV40 T-ag-induced retinoblastoma, we crossed the C57BL/6 mice harboring the LHβ-TAG transgene with FVB mice, and the F1 hybrids were then backcrossed for two generations onto the FVB background. Tumor incidence in the LHβ-TAG transgenic B2 generation decreased to approximately 75% of that seen on the inbred C57BL/6 background (Fig. 5). That the FVB genetic background seems to confer resistance to retinoblastoma induced by a second viral oncoprotein, SV40 T-ag, indicates that the genetic influence does not result from specific effects on the HPV transgene in line 19 αAcry-HPV16E6/E7 mice or from the site of transgene integration.

Involvement of the rd Locus in Retinoblastoma Formation

The C57BL/6 and FVB inbred strains are highly polymorphic. Therefore, the difference in susceptibility to retinoblastoma between these mouse strains may be influenced by many genes. We suspected that one allele in particular may influence retinoblastoma incidence—that is, the rd allele that is present on the FVB genetic background but not on the C57BL/6 genetic background. The rd allele present in FVB 42 is a recessive mutant allele of the gene that encodes the β subunit of cGMP...
phosphodiesterase gene (β-PDE). In rd/rd mice (such as inbred FVB), the rod photoreceptors in the outer nuclear layer of the retina degenerate during the first month of life, and subsequently, the cone photoreceptors degenerate as well. We reasoned that the absence of the photoreceptor cells in the retina may directly or indirectly influence the incidence of retinoblastomas. The degeneration of the photoreceptor cells observed in rd/rd mice can be experimentally recapitulated by directing expression of a diphtheria toxin transgene to the rod photoreceptor cells using the rhodopsin promoter. We therefore generated FVB × C57BL/6 F1 animals that were cotransgenic for line 19 αAcry-HPV16E6/E7 and rdta transgenes. In these mice, the percentage of eyes in which retinoblastomas developed was less than half that seen in F1 litter-

**Figure 3.** Ultrastructural analysis of retinoblastoma in line 19 αAcry-HPV16E6/E7 C57BL/6 × FVB F1 hybrid mice. Electron micrographs show characteristic ultrastructural features of retinoblastoma in an eye tumor from a line 19 F1 hybrid mouse. (A) Basal body located in center of field; (B) trilaminar nuclear membrane on nucleus in upper left; and (C) Homer-Wright rosette. Magnification: (A) ×19,500; (B) ×31,200; (C) ×3,200.
FIGURE 4. Microscopic and in situ hybridization analyses of early retinoblastomas in line 19 aAcry-HPV16E6/E7 C57BL/6 × FVB F1 hybrid mice. (A, B, C) Hematoxylin and eosin-stained paraffin section from a representative early retinoblastoma in a line 19 aAcry-HPV16E6/E7 F1 hybrid mouse. (A, C) Low and high magnification shows tumor in peripheral retina and (B) high magnification of central retina. Note the dysplastic foci (arrows) in the inner nuclear layer in (B). (D) In situ hybridization analysis for E6/E7 transgene expression. A sequential slide was hybridized to an 35S-labeled antisense E6 and E7 probe. The peripheral retina, corresponding to (C), is shown. Hybridization was also observed in the central retina corresponding to the region shown in (B). No hybridization was observed in the retina when sense E6/E7 or antisense αA crystallin probes were used (data not shown). INL, inner nuclear layer; ONL, outer nuclear layer; L, lens; R, retina.

mates transgenic only for the line 19 aAcry-HPV16E6/E7 transgene (Fig. 6). This result indicates that the degeneration of photoreceptor cells in line 19 aAcry-HPV16E6/E7 FVB mice that resulted from the rd/rd genotype probably contributed in part to the low incidence of retinoblastomas.

Involvement of FVB Alleles in Addition to rd in Resistance to Retinoblastoma in Line 19 aAcry-HPV16E6/E7 Transgenic Mice

Retinoblastomas developed in line 19 aAcry-HPV16E6/E7, rdta cotransgenic FVB × C57BL/6 F1 animals (Fig. 6) at a frequency (35% had at least one retinoblastoma by 7.5 months or age) far above that seen in FVB inbred mice carrying only the line 19 aAcry-HPV16E6/E7 transgene (<1% had retinoblastomas by 10 months). Thus, there must be recessive alleles in the FVB mice that, in addition to the rd allele, confer resistance to retinoblastoma in the FVB background. To test this hypothesis, we scored the incidence of retinoblastomas in F1 hybrid mice generated by crossing the aAcry-HPV16E6/E7 mice (inbred FVB background) to the C3H inbred mouse strain (Fig. 7). C3H and FVB inbred strains are also highly polymorphic, but both strains carry the rd/rd genotype. The F1 animals, all of which retain the rd/rd genotype, displayed a moderately high incidence of retinoblastoma (30%). This result supports the premise that FVB mice possess recessive alleles in addition to rd that confer resistance to retinoblastoma.

DISCUSSION

In this study we report that retinoblastomas arose in mice transgenic for the HPV type 16 E6 and E7 oncogenes and that the animal's genetic background strongly influenced the penetrance of this tumorigenic phenotype. Our data suggest that recessive alleles at multiple loci in the FVB inbred mouse contributed to the resistance of these mice to development of retinoblastomas and that these loci also influenced the induction of retinoblastoma in mice transgenic for SV40 Tag.

Role of Viral Oncogenes in Retinoblastoma

The induction of retinoblastoma in the aAcry-HPV16E6/E7 mice specifically occurred in the line that ectopically ex-

TABLE 1. Significance of Linkage to the Line 19 aAcry-HPV16E6/E7 Transgene of Different Mouse Markers on Chromosome 12

<table>
<thead>
<tr>
<th>Markers</th>
<th>Position (cM)</th>
<th>Linkage Significance (P)</th>
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<tbody>
<tr>
<td>D12Mh146</td>
<td>17.0</td>
<td>2.3 × 10^-6</td>
</tr>
<tr>
<td>D12Mh12</td>
<td>19.0</td>
<td>1.3 × 10^-6</td>
</tr>
<tr>
<td>D12Mh34</td>
<td>29.0</td>
<td>4.1 × 10^-13</td>
</tr>
<tr>
<td>D12Mh45</td>
<td>38.0</td>
<td>1.4 × 10^-9</td>
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(cM, centimorgan.)
pressed its transgenes in the retina (Fig. 2), implying that E6 and E7 in the retinal tissue is necessary for development of retinoblastoma at ages up to 5 months for LHB-TAG C57BL/6 mice and 7 months for LHB-TAG B2 mice. The number of mice in each group is indicated in parentheses.

pressed its transgenes by microscopic analysis. Indicated are the percentages of mice in each group showing retinoblastomas at ages up to 5 months for LHB-TAG C57BL/6 mice and 7 months for LHB-TAG B2 mice. The number of mice in each group is indicated in parentheses.

FIGURE 5. Retinoblastoma incidence in LHB-TAG C57BL/6 inbred (Δ) or FVB B2 transgenic (▲) mice. LHB-TAG transgenic mice maintained on the C57BL/6 background were crossed to FVB mice, and the F1 hybrids then were backcrossed to FVB mice for two generations. Mice were genotyped by polymerase chain reaction analysis (see the Materials and Methods section). Transgenic mice on the C57BL/6 and B2 backgrounds were monitored for the presence of overt eye tumors at the indicated ages and killed when tumors were observed. Tumors were confirmed to be retinoblastomas by microscopic analysis. Indicated are the percentages of mice in each group showing retinoblastomas at ages up to 5 months for LHB-TAG C57BL/6 mice and 7 months for LHB-TAG B2 mice. The number of mice in each group is indicated in parentheses.

FIGURE 6. Retinoblastoma incidence in line 19 αAcry-HPV16E6/E7, rtda C57BL/6 × FVB F1 transgenic mice. Homozygous line 19 αAcry-HPV16E6/E7 FVB transgenic mice were crossed with heterozygous rtda C57BL/6 transgenic mice, and F1 hybrid progeny were monitored for the presence of overt eye tumors at the indicated ages. Mice were genotyped for the presence of the αAcry-HPV16E6/E7 and rtda transgenes by polymerase chain reaction analysis (see the Materials and Methods section). (■) Mice with rtda transgene; (○) mice without rtda transgene. Tumors were confirmed to be retinoblastomas by microscopic analysis. Indicated are the percentages of mice in each group showing retinoblastomas at ages up to 12 months. The number of eyes in each group is indicated in parentheses.
useful for identifying strain-specific modifiers of tumor induction in a given tissue and may provide insight into the variable penetrance of tumor development in humans.

Role of the Retinal Degeneration Phenotype in Modifying Risk to Retinoblastoma

We think that the phenotype resulting from the rd allele in FVB mice contributes in part to their resistance to retinoblastoma. This is based primarily on our observations that ablation of the photoreceptor cell layer by the directed expression of a diphtheria toxin transgene caused a twofold decrease in the incidence of retinoblastoma in line 19 aAcry-HPV16E6/E7 FVB × C57BL/6 F1 transgenic mice. How the physical ablation of the photoreceptor cell layer in rd/rd mutant and rdta transgenic mice contributed to the reduced incidence of retinoblastoma remains unclear. The retinoblastomas that arose in the HPV-16 transgenic mice clearly possessed histopathologic and ultrastructural characteristics of photoreceptor cells. The early dysplastic lesions that arose in the retinas of these mice and are the presumed precursors of the retinoblastomas were located within the inner nuclear layer of the retina, where bipolar, amacrine, and horizontal cells reside (Fig. 4). Similarly, the retinoblastomas that develop in the LHβ-TAG mice arise within the inner nuclear layer. However, photoreceptor cells normally reside in the outer nuclear layer. One possible explanation is that retinoblastoma in these animals results from metaplasia, that is, the conversion of a differentialed cell type present in the inner nuclear layer to a tumor cell displaying characteristics of photoreceptor cells. An alternative explanation is that retinoblastoma arises from an undifferentiated precursor cell, that is, a retinoblast, that is retained within the inner nuclear layer. We know from cell fate experiments that many cell types arise from a common progenitor cell. Therefore, differentiation of a retinoblast residing in the inner nuclear layer could result in a tumor displaying characteristics of a photoreceptor cell.

Role of Alleles in Addition to rd in Modifying Risk to Retinoblastoma

We observed a high incidence of retinoblastoma in line 19 aAcry-HPV16E6/E7 FVB × C3H F1 mice that are rd/rd. Thus, recessive alleles on the FVB genetic background other than rd must contribute to resistance to retinoblastoma. We have begun backcross experiments to determine whether homozygosity at other loci, in conjunction with homozygosity at rd, correlates with resistance to retinoblastoma in the aAcry-HPV16E6/E7 (FVB × C57BL/6) × FVB B1 mice. Preliminary results indicate the existence of at least two alleles; homozygosity at either one decreases risk of development of retinoblastoma in rd/rd mice (data not shown). Confirmation of these preliminary results and more refined mapping of these loci are under way.

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References


