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Malignant melanoma in transgenic mice

(simian virus 40 large tumor antigen/tyrosinase promoter/ocular melanoma/cutaneous melanoma/metastasis)

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ABSTRACT **Ocular and cutaneous melanomas arose in new inbred lines of transgenic mice having an integrated recombinant gene comprised of the tyrosinase promoter, expressed in pigment cells, and the simian virus 40 early-region transforming sequences. The tumors were hypomelanotic and were histopathologically similar to corresponding human melanomas. Eye melanomas often originated at a young age, chiefly from the retinal pigment epithelium, also from the choroid, and rarely from the ciliary body. The eye tumors grew aggressively, were highly invasive, and metastasized to local and distant sites. The earliest formation of these tumors was associated with higher copy numbers of the transgene; mice of different single-copy lines varied greatly in age of onset and frequency of eye tumors. Coat pigmentation was reduced in almost all lines, to various extents. Primary skin melanomas arose later and less frequently than eye melanomas. Hence they were at early stages and of unknown long-range incidence in this investigation, in which autopsies covered the first half-year of life. For both ocular and cutaneous melanomas, the transgenic mice offer numerous possibilities for experimental study of mechanisms underlying formation and spread of melanomas.**

Melanomas have a propensity for metastasis (1) and are increasing in frequency. By the year 2000, 1 in 90 Caucasians in the United States is expected to develop the disease (2). Whereas present five-year survival rates are high for some localized malignant melanoma, survival drops sharply with distant metastases (3).

Spontaneous malignant melanomas in laboratory animals are extremely rare. Although melanomas have been induced by chemical carcinogenesis or ultraviolet irradiation in some rodents (for review, see ref. 4), they arise after a long latency period, tend to be infrequent, and have limited or no metastases in the original host.

We were interested in producing mice with a heritable change, on a uniform genetic background, that would lead consistently to malignant melanomas. We report here the regular occurrence and metastasis of melanomas in inbred-strain transgenic mice with an integrated fusion gene containing the simian virus 40 (SV40) early region under the control of the tyrosinase promoter expressed in pigment cells. This paper deals chiefly with melanomas originating in the eyes and to a lesser extent in the skin, at the age range studied thus far. In the accompanying paper (5), other tumors associated with melanosis occurring in the same animals are described.

MATERIALS AND METHODS

Construction and Preparation of Tyr-SV40E. The SV40 early region, including the coding sequences of the transforming large tumor (T) and small tumor (t) antigens (6) and extending from the *Avr* II (nucleotide 5187) to the *Bam*HI (nucleotide 2533) restriction site, was excised from p6-1ΔL (a gift from James Alwine, University of Pennsylvania). An *Avr* II/*Bgl* II/*Sma* I adaptor was ligated to the *Avr* II site, and the fragment was cleaved with *Bgl* II. Two and one-half kilobases of 5′ flanking sequence of the mouse tyrosinase gene was derived from λgTYR101 (a gift from Siegfried Ruppert; ref. 7) and was used as a promoter. This fragment was bounded by an *Eco*RI site and a *Sau*3A site 65 base pairs downstream of the major transcription start site (7, 8) and 15 base pairs upstream of the initiation codon. The tyrosinase promoter was ligated, in the vector pBS, to the *Bgl* II/*Bam*HI fragment of SV40 early region to generate Tyr-SV40E (Fig. 1). The *Eco*RI/*Bam*HI fragment containing the mouse tyrosinase promoter and the coding regions of SV40 early genes was separated from vector DNA by gel electrophoresis. It was purified for microinjection by using Geneclean (Bio 101, La Jolla, CA) and diluted to a final concentration of 1.5 μg/ml in DNA injection buffer (5 mM Tris/0.1 M EDTA).

Transgenic Mice. Transgenic mice were produced essentially as described by this laboratory (9). Black inbred C57BL/6 (Icr subline) prepuberal females were superovulated and mated to C57BL/6 males. The fertilized eggs were microinjected with ≈1 pl of DNA, corresponding to 300 copies of the transgene. Embryos were transferred, together with uninjected carrier embryos of the albino random-bred ICR strain, to oviducts of ICR pseudopregnant females.

Light and Electron Microscopy. Tissues were fixed in neutral formalin or Omnifix (Zymed Laboratories), embedded in paraffin, and stained with hematoxylin/eosin. Tissues for electron microscopy were fixed in glutaraldehyde, postfixed in osmium tetroxide, and embedded in araldite.

Immunohistochemistry. Sections were immunostained either with the streptavidin-biotin or with the peroxidase-antiperoxidase procedure by using commercial detection systems (Biomeda, Foster City, CA; Dako, Santa Barbara, CA). The primary rabbit antisera used were anti-S-100 (Biomeda) and anti-neuron-specific enolase to detect components characteristic of human melanoma and retinoblastoma, respectively. Monoclonal antibody was used to reveal the human melanoma-specific antigen HMB-45 (Enzo Biochemicals). Normal skin, brain, and cartilage served as internal positive controls; addition versus omission of primary antibody provided negative controls.

RESULTS

Transgenic Lines. Thirteen mice from injected eggs were positive for the integrated Tyr-SV40E transgene. These animals, and the lines to which they gave rise, are serially numbered Tg(Tyr-SV40E)Mi1-13 and will be referred to simply by the line number. The potential founders of lines 1 and 2 were undersize and inviable and are omitted here. Five female and six male founders initiated the remaining 11 lines, listed in Table 1. In all, 45 mice are included in this study: 11

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Abbreviations: RPE, retinal pigment epithelium; SV40, simian virus 40; T antigen, large tumor antigen.

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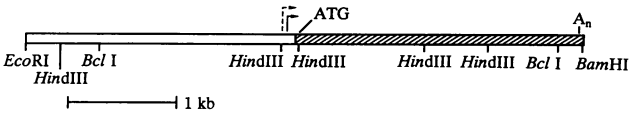

FIG. 1. Map of Tyr-SV40E. The open box represents 5′ flanking sequence of the mouse tyrosinase gene. Major and minor transcription initiation sites (7) are indicated by the solid and dashed arrows, respectively. The SV40 early region, which includes the coding region for the large and small tumor antigen genes, is indicated by the hatched box. kb, Kilobases.

heterozygous founders plus 26 of their heterozygous and 8 of their homozygous descendants. Southern hybridization analysis of tail biopsies revealed multiple separate integrations or late integration of the transgene in lines 3 and 8, with cellular mosaicism confirmed by breeding tests in line 8; tandem multiple-copy single integration in lines 4–7; and single-copy integration in lines 9–13 (data not shown).

Color Change. The coat, characteristically black in the C57BL/6 strain, was lighter than normal in almost all the transgenics and was patterned in some (data not shown). The overall color ranged from very pale grey in line 3 to black in line 13, which corresponded roughly to decreasing numbers of transgene copies (Table 1). However, coat-color differences among single-copy lines imply that the transgene was expressed to different extents in them. Phaeomelanin as well as eumelanin pigments were decreased, as evident in progeny of crosses to congenic agouti black C57BL/6-*A*/*A* animals (data not shown). In some lines, the eyes appeared slightly lighter than those of wild type. Within lines in which homozygotes have thus far been derived, their coats were lighter than heterozygotes of the same line. Hypopigmentation is consistent with the reduced melanization observed in cultured murine skin melanocytes transformed with other oncogenes (10, 11).

Table 1. Ocular preneoplastic lesions (PN), melanoma, and metastases in Tg(Tyr-SV40E) transgenic mice

Line no.	≈ no. of transgene copies	No. of mice	No. with PN only	No. with melanoma*		No. with metastases†
				Early	Advanced	
3	15	1			1	1
4	8	1			1	1
5	6	6		2	4	4
6	4	22‡	4	10	8	4
7	4	1			1	
8	2	2			2	1
9	1	8§		6	2	
10	1	1				
11	1	1				
12	1	1¶				
13	1	1				
Age range, wk			2–4	2–9	8–23	12–19

The data are based on 23 mice completely autopsied, including 5 previously bilaterally enucleated at 8–9 wk; 18 mice with only both eyes studied, from bilateral enucleation at 2–4 wk; the founders of lines 10, 11, and 13, examined only externally; and the line 12 founder, with only unilateral enucleation.

*Of the 18 cases of early melanoma, 8 were unilateral and 10 were bilateral; of the 19 cases of advanced melanoma, all were bilateral. Individual mice often had more than one eye tumor.

†All metastases occurred in animals with advanced melanoma.

‡Includes four homozygotes. Their eyes were removed at 4 wk; two had preneoplastic lesions only, and two had early melanoma.

§Includes four homozygotes. Their eyes were removed at 4 wk; all had early melanoma.

¶One eye only, removed in a preliminary study of this founder at 35 wk, had a small preneoplastic lesion and hypomelanotic retinal pigment epithelium (RPE).

Ocular Melanoma. Externally, the eyes became markedly enlarged, starting at 4 wk of age in some founders with multiple transgene copies and at 11 wk in the first single-copy case (in line 9) to show the defect; enlargement was followed by corneal ulceration, hemorrhage, and partial destruction of the orbit. However, surgical removal of the eyes of progeny at younger ages disclosed that ocular melanoma had in fact developed much earlier. To date, of the 41 mice in lines 3–9 who have had both eyes histologically examined, all had multiple preneoplastic lesions and/or frank melanomas (Table 1). Among the 18 cases (in lines 6 and 9) enucleated at 2–4 wk, 14 mice already had early melanoma. Bilateral occurrence of tumor became universal as the disease progressed. Despite aggressive and rapid growth, two or three independent tumors were sometimes seen in one eye.

A majority of the melanomas appeared to originate in the RPE, three were distinctly in the choroid, some were in the RPE–choroid interface and difficult to attribute to one or both layers, and one was in the ciliary body. The earliest observed lesions, in 2-wk animals, were multiple preneoplastic foci of RPE (Fig. 2*a*). The RPE was locally hypomelanotic or amelanotic and acquired two or more layers of large polyhedral cells with prominent irregular nuclei. In contrast, the normal RPE had smaller cuboidal cells with relatively small nuclei and many melanin granules. Hyperplastic areas often contained atypical nuclei suggestive of an *in situ* neoplasm. These lesions were always present in eyes examined at 4 wk and were often accompanied by hypercellularity of the underlying choroid layer. Lesions became situated at the RPE–choroid interface (Fig. 2 *b* and *c*), usually in the posterior hemisphere of the eye, where they expanded into larger masses of neoplastic tissue. In three cases, there were independent early tumors that arose deeply in the choroid. In one of these, choroid and RPE appeared to contribute to two independent tumors. The tumors occupied the entire eyeball in 4–8 wk, infiltrating the orbital tissues and the optic nerve. Early growths became S-100- and HMB-45-positive after the tumor had reached at least 0.5–1 mm in size (Fig. 2*d*).

Histologically, the tumors were pleomorphic with epithelioid, spindle, small-cell, glanduliform, or, more rarely, myxoid patterns, as in human ocular and cutaneous melanomas (12–14). Most were mixtures with areas of specific types sometimes seeming to originate from separate tumors that had become apposed. The most frequent type (or predominant area) of tumor was epithelioid (Fig. 2*d*), consisting of large polyhedral cells similar to the human epithelioid ocular melanoma (12, 15). Of 20 epithelioid tumors, all contained a large proportion of S-100-positive cells. Next in frequency was the spindle-cell type. Glanduliform differentiation occurred in a few tumors. Small-cell tumors were similar to the glanduliform variant, but without lumina. The myxoid type (in 3 tumors) included a mucinous matrix. All the tumors were negative for neuron-specific enolase. HMB-45 immunostain was detectable in a majority of tested tumors (19 out of 27). Melanin granules were present in cells of most epithelioid tumors and occasionally in the spindle or small-cell variants, even in the ones without obvious pigmentation.

Metastases of Ocular Melanomas. Of the 18 mice with advanced eye tumors, all were locally invasive (Fig. 2*e*), and 11—killed at 12 wk or older—had distant metastases (Fig. 2 *f* and *g*). The sites included occurrences in 2–4 animals in lymph nodes (regional or distal), lung, bone, muscle, brain; and single occurrences in salivary gland, thymus, and subcutaneous tissue. Animals with tumor in the brain often developed uncontrollable circling and/or lateral-leaning behavior.

Cutaneous Melanoma. In four mice (two heterozygotes of line 5, one homozygote of line 6, and one heterozygote of line 8) autopsied at 12–18 wk, a small melanoma was found in the superficial dermis at the junction with the hair follicle epi-

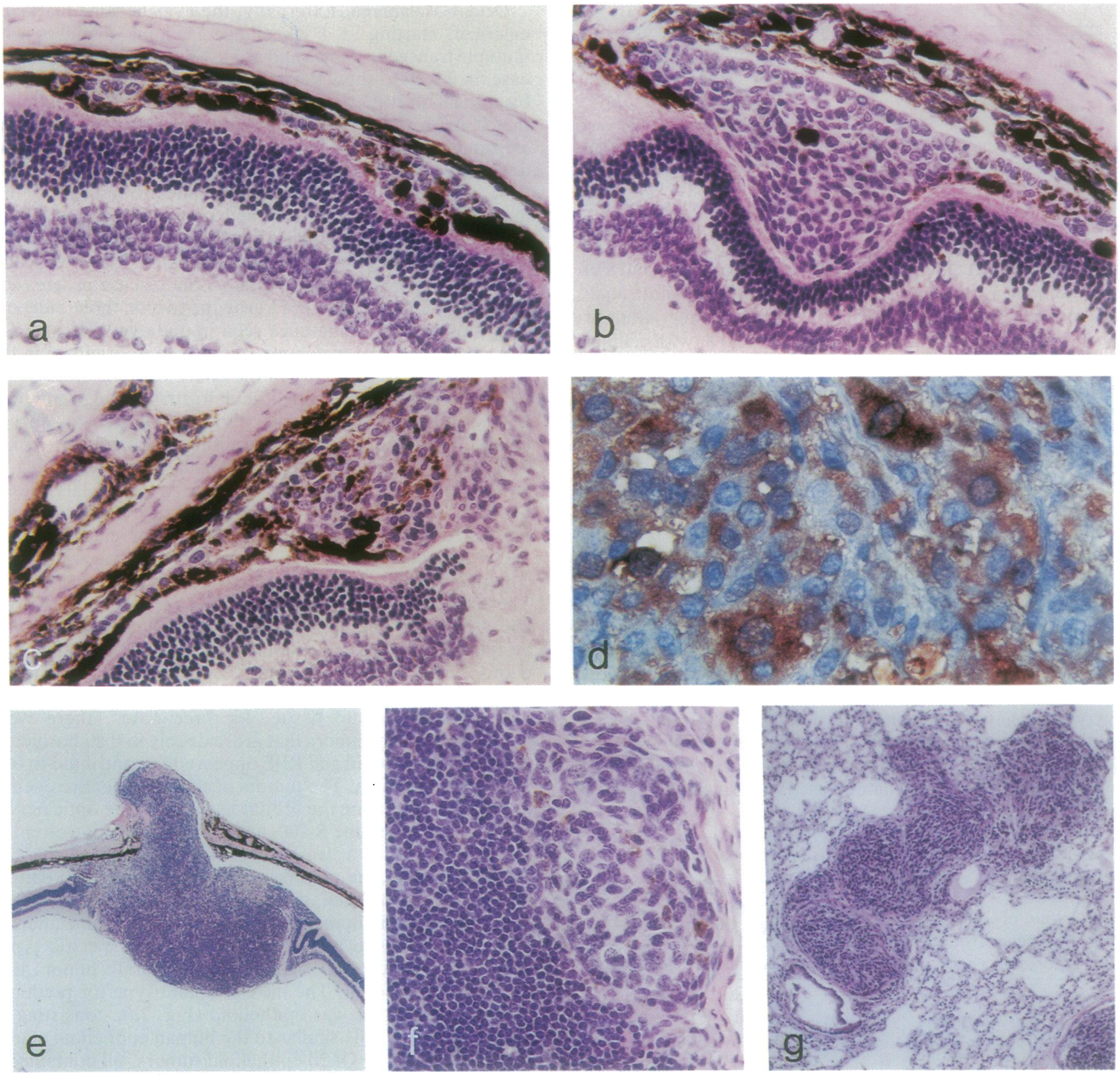

FIG. 2. Stages of ocular lesions, and examples of invasion and metastases of ocular melanomas, in the transgenic mice. (*a*) Early RPE lesions, characterized by hyperplasia and hypopigmentation. (*b*) Early melanoma in the RPE–choroid interface, with the tumor cells contiguous with the RPE. Note the hypercellularity of the pigmented choroid (above the tumor). (*c*) Small, partly pigmented melanoma (upper right) near the optic nerve; and remnants of the RPE, neighboring the tumor. (*d*) Epithelioid melanoma with many S-100-positive cells visualized with hematoxylin and S-100 immunohistochemistry. (*e*) Tumor growing into the posterior eye chamber and invading the optic nerve. (*f*) Early lymph node metastatic nodule with some melanin-containing cells. (*g*) Spindle-cell melanoma metastasis in the lung. Tumor cells are leaving blood vessels and invading the lung parenchyma. (*a–c*, and *f*, ×160; *d*, ×250; *e*, ×50; *g*, ×80.)

thelium. The tumor of one was in the skin of the lumbar back region; in the other three, the tumor was closely associated with a hair or vibrissa follicle on the snout (Fig. 3). All consisted of atypical epithelioid hypomelanotic cells and were S-100- and HMB-45-positive. They clearly differed from the one apparent metastasis of ocular melanoma situated deeply in subcutaneous tissue. Although metastasis from ocular tumors in the same animals cannot be ruled out, the differences support the conclusion that these were four early primary cutaneous melanomas.

DISCUSSION

Melanomas in Tg(Tyr-SV40E) transgenic mice occurred in conjunction with SV40 T-antigen expression (Fig. 4). The eye and skin tumors are histopathologically similar to corresponding human melanomas, with a preponderance of epithelioid and spindle cells, frequent S-100 and HMB-45 reactivity (16), and premelanosomes or melanosomes (Fig. 5). Thus, the mice provide an opportunity to examine experimentally the ontogeny and behavior of melanomas.

Ocular melanomas in these animals arose chiefly in the RPE, less often in the choroid, and only once in the ciliary body. In humans, ocular melanomas are reportedly mainly choroidal (1), but RPE melanomas are apparently difficult to diagnose and are said to be "frequently confused with malignant melanomas of the uvea" (17). Abundance of early-stage material in the transgenic mice (but not in humans) has enabled many instances of preneoplastic lesions to be seen in the RPE and progression toward frank tumors to be documented (Fig. 2 *a–c*). The transformability of the RPE has

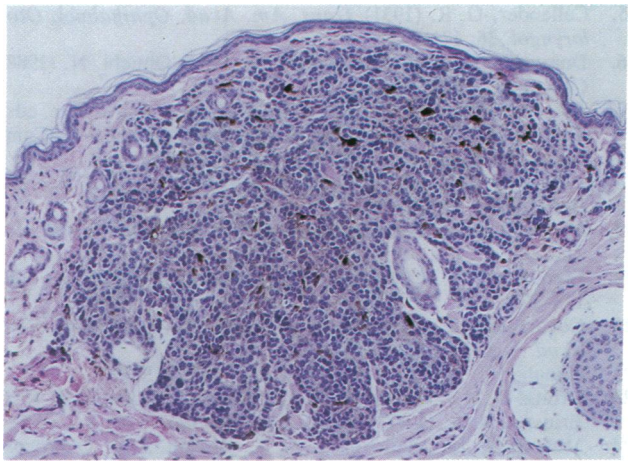

FIG. 3. Early skin melanoma on the snout. The tumor cells, including some that are pigmented, form a nodule in the superficial (papillary) dermis. (×110.)

been directly demonstrated in the hamster by establishment of tumor-producing cell lines from RPE isolated in culture and infected with the SV40 virion (18).

The high visible incidence of preneoplastic lesions in the transgenic mouse RPE raises the question of the capacity for ostensibly normal RPE cells in the same animals to form these lesions and for all such lesions to become tumors. Grafting of appropriate small tissue samples from transgenic eyes to immunoincompetent hosts or sites could provide the answers and serve as a useful model of the mechanisms underlying tumorigenesis.

Human eye melanomas have been found to metastasize chiefly to the liver, lung, bone, kidney, and brain (1). Metastases in the transgenic mice were found in lung, bone, brain (and other organs) but not in liver or kidney at the ages observed. Whether the mouse tumors are capable of further spread is unknown, as early fatalities due to brain invasion were common.

Not surprisingly, eye melanomas occur at much younger ages in the mice (Table 1) than in people (1) due to expression of the mouse tyrosinase gene in midgestation, when pigment first appears in the eye. Hence the T antigen would be produced when the cells are actively proliferating and providing a large candidate population for neoplastic conversion (19). Human sporadic eye melanoma occurs when there is a much smaller proliferating population, for example, in the RPE (20).

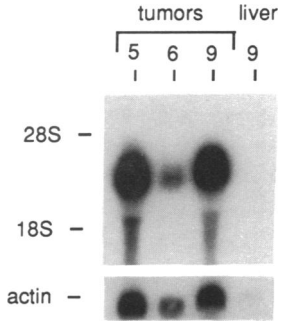

FIG. 4. Northern blot analysis demonstrating expression of SV40 T-antigen message in ocular melanomas. Total RNA samples from tumors of transgenic lines 5, 6, and 9 and from the liver of line 9 were resolved by formaldehyde gel electrophoresis, transferred to nylon, and hybridized with an SV40 T-antigen-specific probe (*Upper*). Relative levels of RNA loaded are indicated by their hybridization to an actin-specific probe (*Lower*).

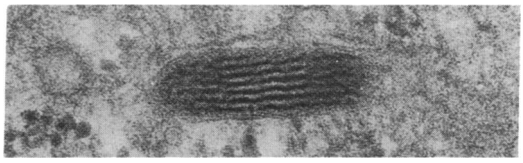

FIG. 5. Electron micrograph of an ocular melanoma cell with an early-stage melanosome. (×31,000.)

The founders of lines 10–13 do not yet have external evidence of eye tumors at 48 wk of age, although a preneoplastic RPE lesion was found at 35 wk in line 12 upon unilateral enucleation. These animals all have a single transgene copy. However, their coats are progressively darker than in the single-copy line 9, which has had advanced eye melanoma. The coat-color differences presumably reflect decreasing levels of expression of the transgene, possibly depending on the host chromosomal site or on rearrangements (21) at the site of integration.

Cutaneous melanomas in our mice are much less frequent and occur later than do ocular melanomas; the reverse is true in humans. The disparity may be due partly to earlier activation of the mouse transgene in pigment cells of the eye (prenatally) than in the skin (postnatally) and partly to much greater exposure of human than of mouse skin to ultraviolet irradiation. The effects of administered ultraviolet light on the transgenic animals will therefore be of interest. Early enucleation should also enable long-term observations of cutaneous melanoma and possible metastases.

The injection, into fertilized mouse eggs, of DNA containing a transforming sequence such as the SV40 T-antigen gene, driven by a tissue-specific promoter, has been shown to be an effective strategy to generate specific kinds of tumors (22, 23). There have been two reports describing eye tumors in transgenic mice: lens tumors consistently resulted from expression of SV40 T antigen under the control of the α-crystallin promoter (24), and retinoblastoma (with characteristic neuron-specific enolase activity) arose unexpectedly in one mouse with the SV40 T-antigen gene under the control of the luteinizing hormone β-subunit promoter (25). No metastases were described in either case although local invasion occurred. There have been no reports of eye or skin melanoma in transgenic animals.

Our mice enable experimental studies of the etiology, progression, metastasis, and treatment of malignant melanoma to be carried out in animals with different numbers of copies of an oncogenic transgene. They are also a source of relevant tissues and reagents.

Note Added in Proof. After this paper was submitted, ocular lesions were found in transgenic mice of two lines concerning which there had been little or no previous information. A homozygote of line 12 had a tumor in one eye, detected at 22 wk and diagnosed, after autopsy at 25 wk, as an advanced glanduliform melanoma; the other eye contained preneoplastic RPE lesions. One eye was surgically removed from a homozygote of line 13 at 31 wk and found to have preneoplastic RPE lesions.

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