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The Polyomavirus Early Region Gene in Transgenic Mice Causes Vascular and Bone Tumors

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Transgenic mice carrying the entire polyomavirus (Py) early region consistently develop both vascular and bone tumors. This tumor spectrum represents a subset of the tumors found in mice infected with Py and an expansion of the vascular tumor spectrum seen in Py middle T antigen (MT) transgenic mice (V. L. Bautch, S. Toda, J. A. Hassell, and D. Hanahan, Cell 51:529–538, 1987). Transgenic mice of three independent lineages develop these pathologies, and mice of individual lineages also develop lymphangiomas and fibrosarcomas. All tumors are of mesenchymal origin, and all tumor tissues express the Py transgene. Some unaffected tissues, including the testes of mice of all lineages, also express the Py transgene. The number of transgene expression sites in mice of a given lineage correlates with the severity and latency of the tumor phenotype in these animals. Analysis of transgene transcripts indicates that RNAs for Py large T antigen (LT), MT, and small T antigen (ST) are present in both tumors and testes of transgenic mice. The ratio of LT RNA to MT and ST RNAs, however, is higher in testes than in tumors and other unaffected tissues, indicating that tissue-specific differences in the relative amounts of the alternatively spliced Py RNAs exist. The finding that some sites of Py transgene expression are susceptible to tumor formation while other expressing tissues such as testes are refractory to tumorigenesis suggests that mechanisms of viral pathogenesis are influenced by a tissue specificity in the effects of the Py early region.

Viral pathogenesis is a complex process involving virus-host cell interactions that can lead to tumor formation (37). One classic model for the study of viral pathogenesis is the interactions of polyomavirus (Py) with its natural host, the mouse (for reviews, see references 13 and 19). Py causes tumors in a range of tissues in newborn and immunocompromised mice. The tumor spectrum of Py includes epithelial tumors such as parotid gland tumors and mammary adenocarcinomas as well as mesenchymal tumors such as osteosarcomas and fibrosarcomas (for a review, see reference 9). Recently, viral strain differences in the tumor spectrum of Py were documented, and the Py strains fit two broad categories: (i) epithelial and mesenchymal tumors and (ii) mesenchymal tumors only (10, 16).

The tumorigenic potential of Py is localized to the early region of the Py genome that encodes three different gene products (6). These proteins are translated from a single primary transcript via alternative splicing (38). Py large T antigen (LT) is a 100-kDa protein required for viral replication, Py middle T antigen (MT) is a 56-kDa protein with transforming activity (24, 39), and Py small T antigen (ST) is a 22-kDa protein that promotes growth in some assays (28). Cell culture experiments have shown that Py LT is also capable of immortalizing primary cells to continuous growth in culture (32) and that Py MT can confer a transformed phenotype to cultured cells. Moreover, these proteins can cooperate with each other and with other oncogenes to fully transform primary cells in culture (31, 33). Thus, Py provides a convenient system for studying both the separate and the concerted actions of viral oncogenes in vivo.

Studies of oncogene action in transgenic mice have shown that both viral and cellular oncogene expression is correlated with tumor formation and that many oncogenes show a cell-type specificity in their action in vivo (for reviews, see references 7, 21, and 22). Surprisingly, a number of papovavirus early regions that show a broad spectrum of expression in cell culture have a restricted expression and tumor profile in transgenic mice. For example, simian virus 40 early region expression is restricted to a few tissue sites and leads to predominantly choroid plexus tumors (5, 29). Likewise, the JC virus and BK virus early regions show restricted expression in transgenic mice, leading to tumors that recapitulate their tropism in humans (36). A Py early region promoter and enhancer linked to a reporter gene showed low-level expression in a number of tissues (25).

To study the effects of the Py early region oncogenes in vivo, we have generated transgenic mice carrying Py early region cDNAs linked to the Py early region regulatory sequences. Transgenic mice carrying the Py MT cDNA showed restricted expression patterns and developed vascular endothelial cell tumors exclusively, transgenic mice carrying the Py LT cDNA developed pituitary tumors with a long latency, and transgenic mice carrying the Py ST cDNA developed no detectable pathology (2, 2a, 3). Here we report that transgenic mice carrying an intact Py early region that is replication defective consistently develop vascular and bone tumors, and with some frequency they also develop lymphangiomas and fibrosarcomas. These tumors are of mesenchymal-cell origin, and they represent an expanded tumor spectrum relative to that of Py cDNA-carrying mice.

MATERIALS AND METHODS

DNA constructions. The Py DNA was of the A1 strain (30), and it has been modified from the wild-type genome as described elsewhere to make Px13 DNA (3). Briefly, the Py genome was linearized at the *Bam*HI site in the late region and inserted into the pML2 vector (26). The features of this construct include a 23-bp deletion and an insertion of a *Xho*I linker at nucleotides 37 to 60 that deletes a Py LT binding site and makes the Py DNA replication defective. A *Cla*I

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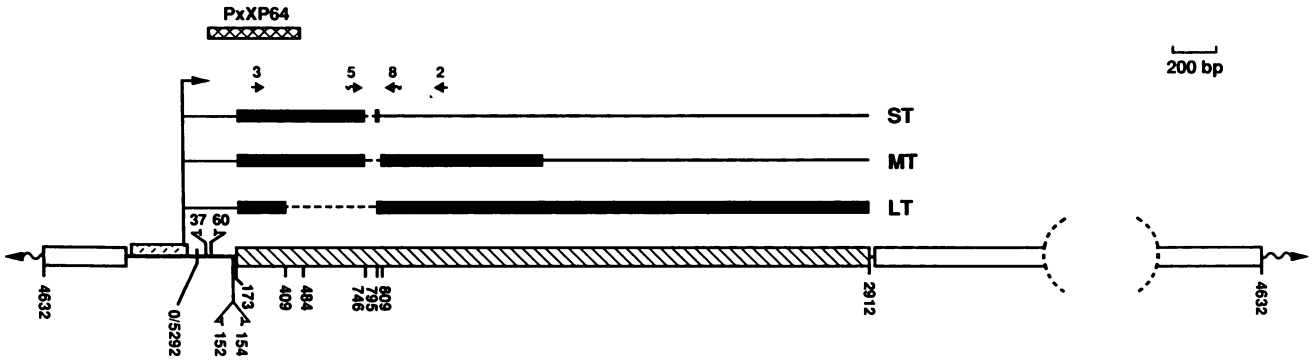

FIG. 1. Py early region transgenic DNA. The baseline shows replication-defective Py DNA with bp 37 to 60 deleted and replaced by a *Xho*I linker, bp 152 to 154 deleted and replaced with a *Cla*I linker, and a deletion of approximately 500 bp in the late coding sequences. Wavy line, plasmid vector sequences; open box, late region DNA; dotted box, early region enhancer; diagonally striped box, early region DNA; parentheses, deleted sequences. The upper lines show the Py early region transcripts from the transgene. Straight line, RNA; filled boxes, protein-coding sequences; broken line, intron sequences. Immediately above the RNAs are wavy lines with arrows and numbers that denote the locations of PCR primers relative to the map, and above those is a hatched box (PxXP64) that shows the location of the probe sequences used for RNA protection.

linker was inserted at the transcriptional start site (nucleotides 152 to 154), and a deletion of approximately 500 bp in the late region prevents production of late region proteins. PxXP64 was constructed by inserting the 424-bp *Xho*I-*Pst*I restriction fragment of Px13 into SP64 in the antisense direction relative to the SP6 promoter.

Generation and identification of transgenic mice. Px13 DNA was linearized at a *Sal*I site in the plasmid sequences, extracted with phenol and chloroform, ethanol precipitated, and suspended in injection buffer as described elsewhere (3). Microinjection was into F_2 embryos from a mating of B6D2F_1 mice, and all procedures were as described previously (23) with modifications (3). Positive offspring were identified by Southern blot analysis of genomic DNA extracted from tail clips and hybridized to ^{32}P-labeled nick-translated Px13 DNA as described previously (1, 3). Positive mice were mated with B6D2F_1 mice to establish germ line transmission. Subsequent matings of positive transgenic mice were usually male transgenic mice to B6D2F_1 females, and positive offspring were identified by dot blot analysis as described elsewhere (35).

RNA isolation and analysis. Total RNA was isolated from mouse organs by a modification of the hot-phenol method (35) as described before (3). The RNA protection assay was performed by a modification of a previously described method (27). A ^{32}P-labeled antisense RNA probe was synthesized from PxXP64 DNA with SP6 polymerase. The probe was hybridized with 10 or 20 μg of total RNA from mouse tissues, and the mixture was digested with RNases A and T_1. The protected RNA products were analyzed by 8 M urea–5% polyacrylamide gel electrophoresis and autoradiography as described previously (3). The polymerase chain reaction (PCR) was performed by a modification of the method described by Saiki et al. (34). cDNA was synthesized from total RNA as described elsewhere (17) with modifications. A 10-μg volume of total RNA and 1 μg of oligonucleotide Px2 primer were combined in a total volume of 14 μl of H_2O and heated to 75°C. After being cooled to room temperature, a solution was made of 50 mM Tris (pH 8.3 at 42°C), 6 mM $MgCl_2$, 40 mM KCl, 1 mM dithiothreitol, 2.5 mM each deoxynucleoside triphosphate, 1 U of RNAsin per μl, and 0.5 U of avian myeloblastosis virus reverse transcriptase per μl. After incubation at 42°C for 2 h, the reaction mixture was used for the PCR reaction or stored at −20°C. The PCR reaction contained 10 mM Tris (pH 8.4 at room temperature), 50 mM KCl, 1.5 mM $MgCl_2$, each deoxynucleoside triphosphate at 200 μM, each Px oligonucleotide at 0.01 μg/ul, 0.1% Triton X-100, and 0.03 U of *Taq* polymerase (Promega Scientific) per μl in a total volume of 100 μl. A 2-μl volume of the cDNA mixture was added, and the reaction mixture was overlaid with mineral oil. The conditions for PCR with Px oligonucleotides 5 and 8 were 92°C for 10 s and 72°C for 2 min (30 cycles), and the conditions for PCR with Px oligonucleotides 2 and 3 were 93°C for 10 s, 55°C for 2 min, and 72°C for 3 min (30 cycles).

RESULTS

Generation of transgenic mice. To determine the effects of the Py early region gene in transgenic mice, the linearized Py early region DNA illustrated in Fig. 1 was microinjected into mouse embryos. The features of this construct include a deletion of bp 37 to 60 and an insertion of a *Xho*I linker to make the DNA replication defective, a deletion of bp 152 to 154 and an insertion of a *Cla*I linker, and a deletion of approximately 500 bp in the late region to prevent production of late region proteins. Colinear DNA constructs with the same modifications and encoding Py LT or MT cDNAs show expression in transgenic mice (2, 3).

Microinjection of Px13 DNA linearized with *Sal*I into fertilized one-cell mouse embryos produced 44 pups. Eight of these pups (18%) were transgenic by Southern blot analysis of genomic tail DNA extracted from biopsy specimens taken from 2-week-old mice (data not shown). As documented in Table 1, five (62.5%) of the eight transgenic founder mice developed pathology that was fatal either at an early age (e.g., mouse 267 died at 2.5 months) or at a later age (e.g., mouse 268 died at 6 months). The documented pathology in the founder mice was predominantly that of vascular hemangiomas and bone tumors. Two founders (Py-2 and Py-4), however, developed lymphangiomas, tumors of the endothelial cells that line the lymph nodes.

Three of the founder mice, Py-6, Py-7, and Py-8, did not develop any detectable pathology. Both the Py-6 and Py-7 founders were females who died at a relatively early age and were gravid at death, so these mice possibly succumbed to

TABLE 1. Pathology of Py transgenic founder mice

Founder	Mouse no.	Sex[a]	Pathology[b]	Life span (mo)[c]	Transmission	% Transmission
Py-1	164	F	Hemangioma	3.75 (S)	0/13	0
Py-2	256	F	Lymphangiomas, osteosarcomas (skull, spine)	6.0 (S)	0/26	0
Py-3	257	M	Hemangiomas	9.0 (D)	2/113	1.8
Py-4	267	M	Hemangiomas, lymphangiomas, osteosarcoma	2.5 (D)	10/27	37.0
Py-5	268	F	Hemangioma, osteosarcoma	6.0 (S)	24/39	61.5
Py-6	269	F	NAD	2.0 (D)	0/7	0
Py-7	270	F	NAD	1.75 (D)	0/9	0
Py-8	271	F	NAD	>12 (S)	0/41	0

[a] F, female; M, male.
[b] NAD, no abnormalities detected.
[c] D, died; S, sacrificed. All mice sacrificed, except mouse 271 (Py-8), were moribund.
[d] Number of positive progeny/total number tested.

complications of pregnancy. The short life span of these founders may have precluded the development of detectable pathology resulting from the presence of the Py transgene. The Py-8 founder was fertile and lived to an old age, but she did not transmit the transgene to her progeny. Thus, she most likely did not carry the Py transgene in every cell, and this mosaicism may have affected transgene expression and consequent lack of tumor development. In addition, the transgene chromosomal integration site may have prevented transgene expression and the consequent lack of pathology in one or more of this subset of Py founder mice.

Three of the eight founder mice transmitted the Py transgene to progeny, and the transmission ratios indicate that the Py-3 founder mouse was mosaic for the transgene in his germ line while the other two founder mice were probably heterozygous for the transgene. Two founders with pathology, Py-1 and Py-2, were probably mosaic because they died without transmitting the transgene to progeny. However, all of the founders that transmitted the transgene developed pathology. All three of these founder mice developed vascular hemangiomas, while two of three founders developed bone tumors and one of three founders developed lymphangiomas (Table 1).

Pathology of Py transgenic mouse lineages. The three lineages Py-3, Py-4, and Py-5 were derived from the three founder Py transgenic mice that transmitted the transgene to offspring, and their characteristics are documented in Table 2. In general, the pathology of progeny was similar to the pathology of the respective founder mouse, although some differences were noted. For example, examination of multiple progeny showed the presence of bone tumors in Py-3 mice and the occasional presentation of a fibrosarcoma in Py-3 and Py-5 mice. Transgenic mice of all lineages developed pathology that was eventually fatal to the animal, and there were both similarities and differences among the phenotypes of the three lineages. Mice of all lineages developed vascular and bone tumors, indicating that this tumor profile is specific to the transgene and is not a position effect of the chromosomal integration site. There were differences, however, in the complete tumor spectrum of the lineages. Mice of the Py-4 lineage consistently developed lymphangiomas, and mice of the other lineages never had this type of tumor. Likewise, mice of the Py-3 and Py-5 lineages occasionally developed fibrosarcomas, and mice of the Py-4 lineage never had this tumor. Another striking difference was in the average life span of the three lineages: Py-4 mice had an extremely short average life span of 2.3 months, while Py-3 and Py-5 mice lived much longer, averaging 6.9 and 7.2 months, respectively.

TABLE 2. Pathology of Py transgenic mouse lineages

Lineage	Avg life span (mo)[a]	Pathology
Py-3	6.9	Osteosarcoma, fibrosarcoma (rare), hemangioma (rare)
Py-4	2.3	Osteosarcoma, hemangioma, lymphangioma
Py-5	7.2	Osteosarcoma, fibrosarcoma (rare), hemangioma (rare)

[a] Calculated from 15 to 20 mice that died or were sacrificed when moribund.

The Py-4 transgenic mice presented a consistent set of symptoms and pathological profile at sacrifice or necropsy. They often showed visible signs of lymphangioma formation at 4 to 6 weeks of age, and they quickly developed large cystlike structures in the inguinal and axial areas. At approximately 8 to 12 weeks of age, multiple small blood-filled structures became visible on the ears, tail, and paws. These mice became moribund several days prior to death, although the exact cause of death was not determined. At necropsy, all Py-4 mice showed multiple large cysts filled with a straw-colored liquid at various sites including inguinal, axial, cervical, retroperitoneal, and subcutaneous sites (Fig. 2C). These cysts were sometimes intimately associated with blood-filled cystic structures. Most mice showed evidence of multiple small blood-filled structures on the ears and tail (Fig. 2A), and males had small blood-filled areas on the testes that resembled vascular tumors. In general, the blood-filled tumors were not found at other sites in Py-4 mice. All Py-4 mice also had an obvious thickening of the skull that gave it a white, solid appearance rather than a thin, opalescent appearance (Fig. 2B).

Py-3 and Py-5 transgenic mice showed a less consistent, less dramatic set of symptoms than did Py-4 mice. Mice of these lineages usually developed a single tumor, and the tumors were vascular, bony, or fatty. Mice with vascular tumors usually had a tumor in one or two of several sites that included most organs. The tumors most often were larger than the blood-filled sacs of the Py-4 mice and in both appearance and location resembled the hemangiomas of Py MT mice (3). A subset of mice of both lineages developed hind-limb paralysis at some point. Although tumors were not detected macroscopically in all mice with this symptom, in about 80% of these mice, tumors were found at autopsy, and they were always bony tumors associated with the lower spinal cord (Fig. 2D). Thus, the hind-limb paralysis is probably a result of spinal-cord pinching by the bone tumors. These tumors were also occasionally seen on the rib cage, in

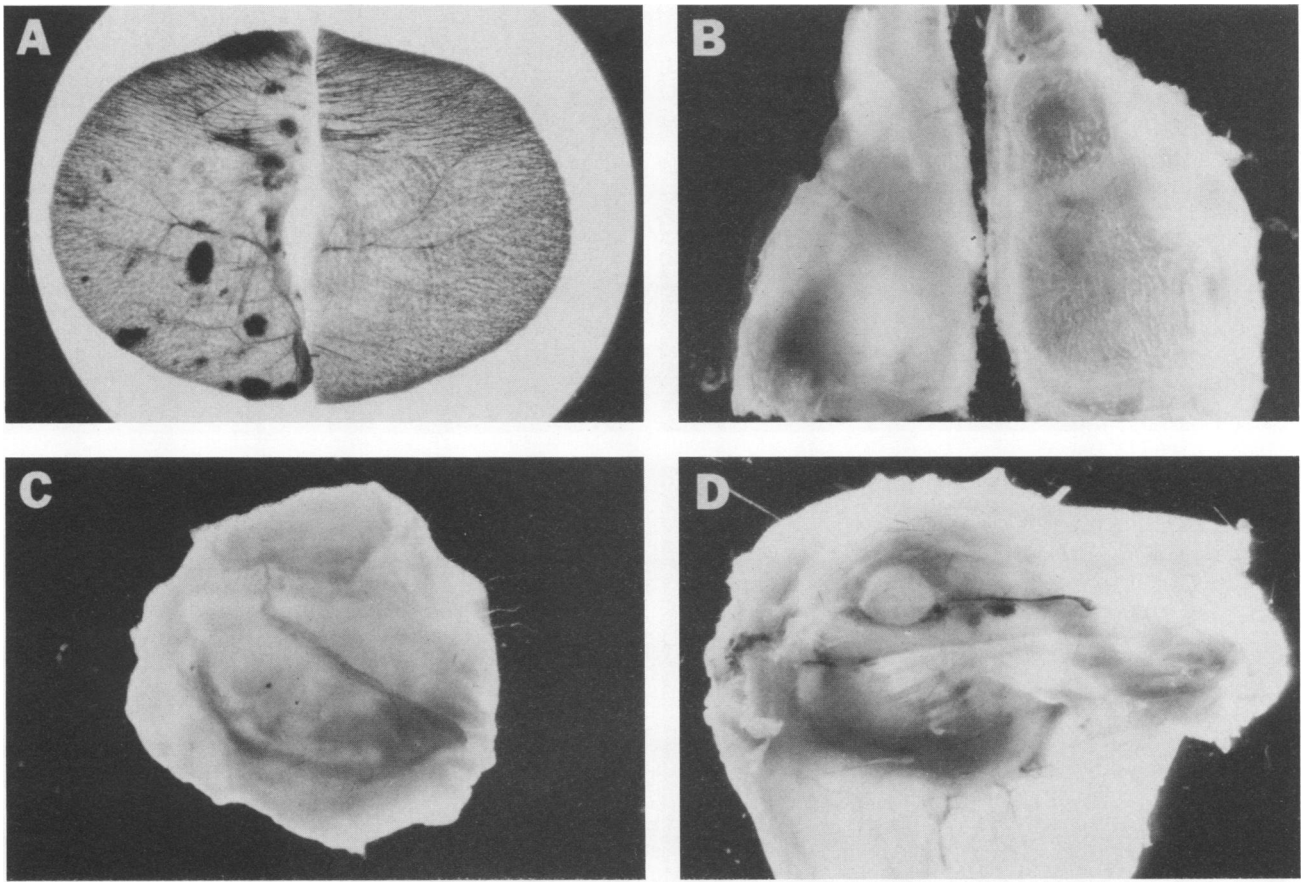

FIG. 2. Macroscopic pathology of Py early region transgenic mice. All photographs were of fresh tissue. (A) Left, ear of an adult Py-4 transgenic mouse; right, ear of an adult nontransgenic mouse. Note the blood-filled sacs visible in the transgenic mouse ear. Magnification, ×5. (B) Left, skull of an adult Py-4 transgenic mouse; right, skull of an adult nontransgenic mouse. Note the white raised areas on the transgenic mouse skull and contrast these with the opalescence of the healthy skull. Magnification, ×3. (C) Cystic structure from the retroperitoneal area of an adult Py-4 transgenic mouse. Note the lack of bloody areas. Magnification, ×3. (D) Section of spine showing a tumor in an adult Py-5 transgenic mouse. The tumor is bony and attaches to the ribs below. Magnification, ×3.

association with joints, and in the skull. On rare occasions, mice of both lineages also developed large solid and fatty protuberant tumors at random locations. Death was most likely due either to hemorrhage or to wasting from paralysis or a large tumor.

The microscopic analysis of tumor tissues is presented in Fig. 3. Histological study of the blood-filled tumors of Py-4 mice showed small blood-filled sacs interspersed among normal tissue structures, as shown in the photomicrograph of a Py-4 ear (Fig. 3A). A higher magnification of this type of tumor showed that the caverns were lined by one or a few layers of thin, elongated cells (Fig. 3B). This histological picture is consistent with the identification of these tumors as vascular endothelial cell tumors, and they resemble the hemangiomas described in Py MT mice (3).

The microscopic examination of a Py-3 bone tumor showed tumor tissue adjacent to normal rib muscle tissue, with no sign of invasion (Fig. 3C). Upon visual examination, the skull thickenings of the Py-4 mice appeared multifocal and seemed to involve more healthy bone tissue than did the bone tumors of the Py-3 and Py-5 mice. At the microscopic level, however, the Py-4 skull had tumors that resembled the Py-3 bone tumor (Fig. 3D). Higher magnification of the tumor tissue revealed individual atypical cells surrounded by extracellular material (inset, Fig. 3D). This histology is characteristic of osteogenic sarcomas, tumors of osteoblast cells that produce bone matrix.

The fluid-filled cysts found in all Py-4 mice consisted of irregular open spaces with some leukocytes and with expansion of both the lining and surrounding fatty tissue (Fig. 3E). The association of some of the cysts with sites of major lymph nodes, and the presence of straw-colored fluid and of leukocytes rather than erythrocytes all contribute to the identification of these structures as lymphangiomas. The large fatty tumors that occasionally were found in Py-3 and Py-5 mice consisted of relatively undifferentiated cells that appeared to be of mesenchymal origin, which is consistent with the identification of these tumors as fibrosarcomas (Fig. 3F).

Expression analysis. To determine the expression pattern of the transgene in the Py transgenic mice, RNA extracted from healthy tissues and from tumors of mice of each of the three lineages was analyzed. The Py early region transgene (Fig. 1) can theoretically produce a primary Py transcript that is alternatively spliced to yield different Py mRNAs encoding different Py early region proteins, as occurs in Py-infected cells (37). To obtain semiquantitative data on the sites of Py transgene expression, we employed an RNA

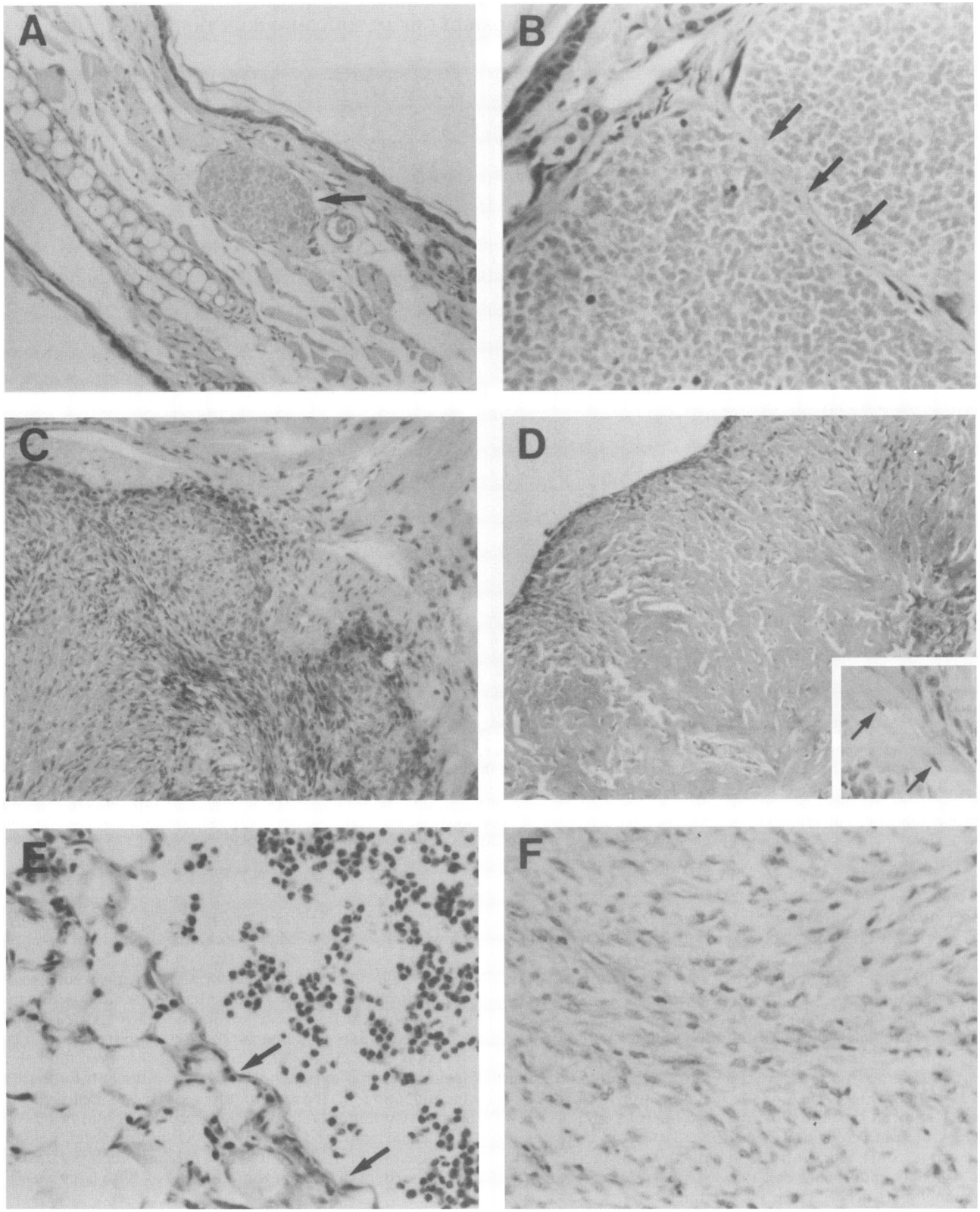

FIG. 3. Microscopic pathology of Py early region transgenic mice. All tissues were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. (A) Cross-section of an ear of a Py-4 transgenic mouse, showing a blood-filled sac surrounded by healthy ear tissue. Magnification, ×184. (B) Higher magnification of a different area of the Py-4 ear shown in panel A. Note the blood-filled sacs surrounded by a few layers of elongated endotheliumlike cells marked by arrows. Magnification, ×368. (C) Rib tumor and surrounding tissue from an adult Py-3 transgenic mouse. The tumor was bony and attached to the rib. Note the lack of invasion into the surrounding muscle tissue. Magnification, ×184. (D) Skull tumor of an adult Py-4 transgenic mouse. Note the deposits between the cells. Magnification, ×184. Inset, higher magnification of bone tumor showing individual cells (marked by arrows) surrounded by deposited material. Magnification, ×368. (E) Cystic structure from an adult Py-4 transgenic mouse. Note the irregular spaces containing some heavily stained cells and lined by elongated cells (marked by arrows). Magnification, ×368. (F) Solid tumor from an adult Py-3 transgenic mouse. Note the undifferentiated swirling cells. Magnification, ×368.

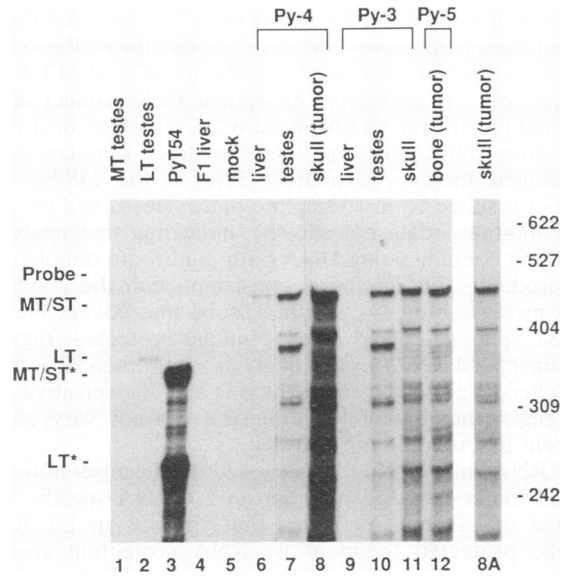

FIG. 4. RNA protection analysis of transgenic mouse tissues. Total RNAs (20 μg except in lane 8, which contained 10 μg) were isolated from mouse tissues and used in an RNA protection assay. The products were electrophoresed in 8 M urea–5% polyacrylamide gels and subjected to autoradiography at −80°C with an intensifying screen. Lane 1, MT-1A (Py MT transgenic) testes; lane 2, LT-1 (Py LT transgenic) testes; lane 3, Py T54 control cells transformed with Py; lane 4, B6D2F1 (nontransgenic) liver; lane 5, mock reaction; lane 6, Py-4 liver; lane 7, Py-4 testes; lane 8, Py-4 skull (with tumor) (loading, 25% of that in other lanes); lane 9, Py-3 liver; lane 10, Py-3 testes; lane 11, Py-3 skull (with abnormal area); lane 12, Py-5 bone (with tumor); lane 8A, same as lane 8 (Py-4 skull with tumor). Lane 8A was exposed for 18 h, and lanes 1 to 12 were exposed for 6 days. Left margin shows fragments corresponding to protection by transcripts: probe, ^{32}P-labeled antisense RNA corresponding to a *Xho*I (nucleotide 60 linker)-to-*Pst*I (nucleotide 484) restriction fragment; MT/ST, transgenic MT-ST transcript; LT, transgenic LT transcript; MT/ST*, viral MT-ST transcript; LT*, viral LT transcript. Transgenic RNA protects a larger fragment because the RNA initiates upstream of the major viral transcriptional start site (3). Right margin shows size markers (in base pairs) (pBR322 digested with *Hpa*II).

protection assay. Our probe in this assay allowed for the identification of a unique protected fragment for Py LT RNA but did not distinguish among MT, ST, and unspliced RNAs. We therefore used PCR analysis to unambiguously identify each of the species of Py RNA.

RNA protection analysis with a PxXP64 antisense probe revealed multiple sites of Py transgene expression in the mice (Fig. 4 and Table 3). The major transcription initiation site in the transgene is 5′ of major viral transcription start sites (compare controls in Fig. 4, lanes 1 to 3 [3]). Thus, the presence of a protected band of 424 bases results from the presence of MT, ST, and/or unspliced RNAs, while a protected band of 349 bases results from the presence of LT RNA. All tumors and some unaffected tissues, including the testes of all lineages, expressed the Py transgene (Fig. 4 and Table 3). Although the testes of Py-4 mice had multiple small vascular hemangiomas that may have been solely responsible for transgene expression in this organ, it is likely that there was substantial contribution from unaffected testicular tissue, because the pattern of protected fragments (see below) resembled that of unaffected testes and not that of tumor tissue.

TABLE 3. Summary of expression analysis of Py transgenic mice

Source	Py RNA assayed for[a]	Results of analysis of[b]: Py-4 PRO	Py-4 PCR	Py-3 PRO	Py-3 PCR	Py-5 PRO	Py-5 PCR
Tumor[c]	M + S	+	+	+	+	+	+
	L	−	+	−	+	−	+
Testes	M + S	+	+	+	+	+	+
	L	+	+	+	+	+	+
Liver	M + S	+	+	−	+	−	−
	L	−	+/−	−	−	−	−
Spleen	M + S	+	+	−	+	−	−
	L	−	−	−	−	−	−
Kidney	M + S	+	+	−	+	−	+
	L	−	−	−	−	−	−
Brain	M + S	−	+	−	+	−	−
	L	−	+/−	−	+/−	−	−

[a] Total RNA was assayed for either Py MT and ST RNAs (M + S) or for Py LT RNA (L) in separate experiments.

[b] PRO, RNA protection assay; PCR, PCR analysis. All tissues but Py-3 kidney were analyzed with at least two different tissue samples. +, positive signal in all tissue samples; −, negative signal in all tissue samples; +/−, one positive and one negative signal from tissues.

[c] Tumor represents skull osteosarcoma and lymphangioma for Py-4, hemangioma and fibrosarcoma for Py-3, and osteosarcoma for Py-5.

Mice of the Py-4 lineage (Fig. 4, lanes 6 to 8 and 8A, and Table 3) had the greatest number of unaffected sites that expressed the transgene, including the liver. In contrast, RNAs from mice of the Py-3 lineage (Fig. 4, lanes 9 to 11, and Table 3) did not show protected fragments indicating liver expression of the transgene (Fig. 4, lane 9). However, RNAs from Py-3 testes, an unaffected tissue, and an abnormal Py-3 skull that probably carried a tumor were positive for the protected fragments (Fig. 4, lanes 10 and 11). Mice of the Py-5 lineage showed an expression pattern similar to that of the Py-3 mice, with transgene expression restricted to tumors and testes (Fig. 4, lane 12, and Table 3).

It was possible to determine the relative levels of Py transgene RNA expression in a comparative manner. The amount of total RNA, the amount of reaction mixture loaded on the gel (see the legend to Fig. 4), and the relative intensity of the signals showed that the level of expression in unaffected testes of all lineages was approximately equivalent, whereas the level of transgene RNA expression in tumor tissue was substantially higher. For example, RNA from a skull tumor of a Py-4 mouse (Fig. 4, lanes 8 and 8A, short exposure) was used, so that lane 8 represents 15% of the RNA in lane 7, yet the signal appears 5- to 10-fold more intense. Thus, the overall level of transgene expression relative to total RNA is higher in tumors than in unaffected tissues.

The pattern of transgene expression differed among the different sites of expression. A protected fragment corresponding to Py LT RNA protection was clearly visible in reaction mixtures of testis RNAs of mice of all lineages (Fig. 4, lanes 7 and 10, and Table 3), and this protected fragment was not detected in reaction mixtures of tumor RNAs or RNAs from other unaffected sites. The presence of background does not exclude the possibility that low amounts of LT RNA are present in nontesticular RNAs, and subsequent PCR analysis (see below) revealed LT-specific products in a subset of these RNAs. However, RNA protection analysis of 8 different testis RNAs and 15 tumors and positive tissues of various lineages showed that the pattern of an LT-

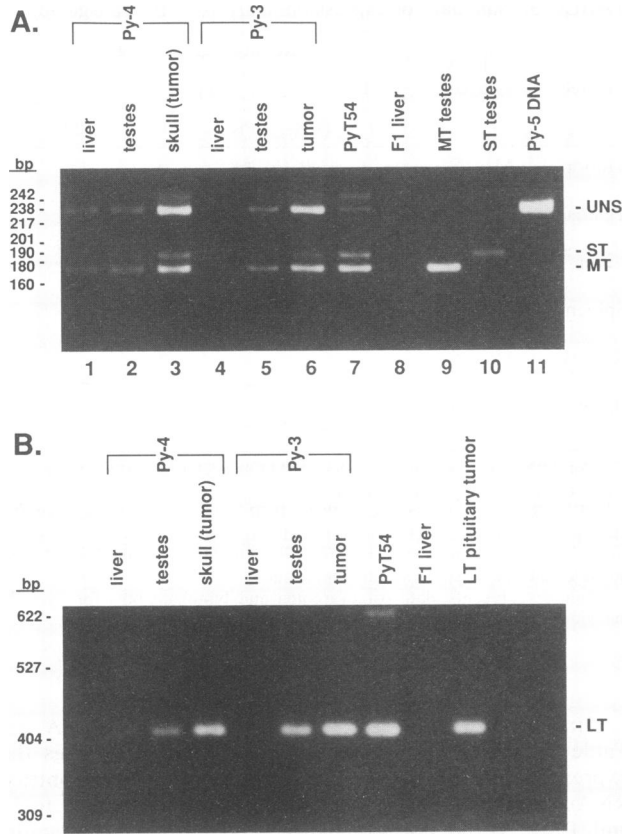

FIG. 5. PCR expression analysis of transgenic mouse tissues. Total RNAs from mouse tissues were reverse transcribed using Px oligonucleotide 2 as a primer (see Fig. 1 for location). The cDNAs were subjected to PCR analysis, electrophoresed through an 8% polyacrylamide gel, and stained with ethidium bromide. (A) PCR analysis using Px oligonucleotides 5 and 8 as primers. Lane 1, Py-4 liver; lane 2, Py-4 testes; lane 3, Py-4 skull (with tumor); lane 4, Py-3 liver; lane 5, Py-3 testes; lane 6, Py-3 tumor (fibrosarcoma); lane 7, Py T54 control cells transformed with Py; lane 8, B6D2F1 (nontransgenic) liver; lane 9, MT-1A testes; lane 10, ST-1 (Py ST transgenic) testes; lane 11, Py-5 tail DNA used without reverse transcription. Left margin shows size markers (in base pairs) (pBR322 digested with *Hpa*II). Right margin shows positions of expected PCR products: UNS, unspliced RNA. (B) PCR analysis using Px oligonucleotides 2 and 3 as primers. Lanes 1 to 8, as described for panel A; lane 9, LT-1 pituitary tumor. Left margin shows size markers (in base pairs) (pBR322 digested with *Hpa*II). Right margin shows positions of expected PCR products.

protected fragment only in testis RNA is consistent (data not shown). The relative ratio of the different Py RNAs thus seems to be regulated differently in testes than in tumors and other tissue sites of expression.

To determine the exact pattern of expression of each of the Py RNAs, we employed a sensitive PCR assay to cDNAs synthesized from RNAs of various tumors and tissues. One set of primers (oligonucleotides Px5 and Px8; see Fig. 1 for locations) gives PCR products unique to templates for unspliced, MT, and ST RNAs, and a second set of primers (oligonucleotides Px2 and Px3; see Fig. 1 for locations) allowed for detection of a PCR product specific to LT RNA.

The results of the PCR are shown in Fig. 5. Lanes 1 to 5 of the PCR analysis are the same RNA samples as were analyzed by RNA protection in lanes 6 to 10 of Fig. 4. Analysis of Py MT and ST RNAs (Fig. 5A) revealed that all sites of transgene expression were positive for PCR products of 189 and 175 bp that were specific to ST and MT RNA, respectively. An additional prominent PCR product of 238 bp could result from an unspliced RNA template, or it could result from DNA contamination of the RNA samples. It was determined that approximately 50% of the 238-bp PCR product resulted from a template that is destroyed by alkaline conditions (data not shown), indicating that unspliced RNA and contaminating DNA both contribute templates to this product. This finding is consistent with the results of RNA protection analysis with a probe that covers the MT and ST splice sites and gives a unique protected fragment with unspliced RNA. In this analysis, more than 90% of the "unspliced" protected fragment was alkaline sensitive, and the relative amount of this fragment did not vary among different RNAs (data not shown).

LT RNA analysis (Fig. 5B) showed that a subset of tissues and all tumors was positive for an LT RNA-specific PCR product of 414 bp. All testes that showed an LT RNA-specific protected fragment by RNA protection analysis produced the LT-specific PCR product (Fig. 5B, lanes 2 and 5, and Table 3). The tumors and Py-4 liver, however, did not have detectable LT-specific protected fragments by RNA protection, yet they also produced an LT-specific PCR product. This discrepancy probably results from the high sensitivity of the PCR reaction and the lack of background in this assay. The PCR reaction was not quantitated, so absolute amounts of LT RNA were not determined, but it is likely that Py transgenic-mouse tumors contain very low but detectable levels of LT RNA.

The expression analysis using both assays was extended to other tissues, and the results are documented in Table 3. Several results were confirmed and extended by this complete analysis. First, as noted above, all tumors and testes from mice of all lineages expressed the Py transgene. Second, other unaffected organs were sites of transgene expression in a lineage-dependent manner. For example, Py-4 mice showed transgene expression in the liver, spleen, kidney, and brain, and Py-5 mice showed expression only in the kidney. Third, some sites of expression, such as brain tissues of Py-4 and Py-3 mice, were negative by RNA protection analysis but positive by the more sensitive PCR analysis. Finally, expression of the LT RNA was not detectable in some sites where expression of ST and MT RNAs was documented, such as spleens and kidneys of Py-4 mice. It thus appears that both lineage-specific differences in expression patterns and tissue-specific differences in the relative amounts of the different Py mRNAs contribute to a complex expression pattern of the Py early region transgene.

DISCUSSION

The analysis of these transgenic mice shows that the Py early region is tumorigenic in transgenic mice. Five members (62.5%) of a group of eight founder mice had detectable pathology that was fatal to the animals in all cases. Three lineages of Py transgenic mice were established in which the Py early region transgene was passed through the germ line to progeny. All mice that carried the transgene developed fatal tumors. Mice of all lineages developed vascular and bone tumors, indicating that this tissue specificity for Py transgene action is a result of the *cis*-acting sequences associated with the transgene.

The tumors induced by the Py transgene are found exclusively in tissues of mesenchymal origin. In addition to the

vascular and bone tumors, the lymphangiomas and fibrosarcomas that were scored are also mesenchymal tumors. No evidence of epithelial tumors was seen, and the common parotid tumor induced by viral infection was not scored in any Py transgenic mice. The reason for this restricted tumor spectrum in the transgenic mice is not known, and it may reflect either the tumor spectrum of the Py strain from which the transgene is derived or the specific properties of the transgene construct.

The Py transgene was derived from the A1 strain of Py, and the tumor spectrum of this strain has not been characterized. The A1 genome closely resembles that of the A2 strain of Py in the noncoding region (3, 30), but the entire genome has not been sequenced. The tumor spectrum of the Py transgene is remarkably similar to the tumor spectra of two strains of Py, A3 and RA, that produce only mesenchymal tumors; it is not similar to the spectra of two other Py strains, A2 and PTA, that produce both mesenchymal and epithelial tumors (4, 10, 16). The noncoding regions of the different Py genomes were found to influence the frequency of tumor formation, but the primary determinant of tissue tropism for viral tumorigenesis was localized to a single amino acid polymorphism in the VP1 capsid protein encoded by the late region (14).

The late region of the Py transgene carries a large deletion that includes this polymorphic codon, so this primary determinant of viral tumor tropism is presumably not expressed in Py transgenic mice. Therefore, the mesenchymal specificity of both the Py transgene and the RA and A3 Py strains may result from the absence of a dominant determinant in the late region that results in epithelial tumors. This hypothesis is supported by coinfection experiments showing that the expanded tumor spectrum is dominant over the restricted mesenchymal-only tumor spectrum (10). It is notable, however, that although the Py transgene shares with the RA and A3 strains a mesenchymal-tumor spectrum, it does not share a weakly oncogenic phenotype. Rather, the Py transgene is highly oncogenic, inducing tumors with 100% penetrance and with fatality as early as 11 to 12 weeks of age.

The mechanism responsible for the different tropisms of the Py strains is not elucidated but seems to involve interactions with cell surface receptors, because both viral replication patterns in mouse organs and cell surface properties correlate with the different tumor spectra (12, 15). However, the Py transgene does not require cell entry yet shows a mesenchymal cell specificity in its action. This result suggests that virus entry, while necessary, is not sufficient to confer epithelial tropism. There appears to be an additional intracellular requirement for epithelial tropism that is lacking in the Py transgene. Further evidence for this requirement is that the Py virus tumor spectrum in *nu/nu* nude mice is a subset of the persistently infected tissues (11).

Although the Py transgene has a restricted tumor spectrum relative to that of Py infection by some strains, the finding of tumors in multiple tissues of transgenic mice is an expanded tumor spectrum relative to that of Py MT transgenic mice, which develop only vascular hemangiomas (3), and Py LT mice, which develop only pituitary tumors (2, 2a). The Py transgenic mice do not live long enough to develop the overt pituitary tumors of the Py LT mice, and the lack of vascular and bone pathology in the Py LT mice suggests that the Py MT oncogene is required for tumorigenesis at these sites.

An explanation of the lack of bone pathology in the Py MT mice is less obvious. The Py transgene and the Py MT transgene are colinear except for the presence of 63 bp of MT intron sequences that are missing in the Py MT cDNA. Possible reasons for the difference in tumor spectrums are (i) that the presence of the Py LT and/or ST gene products is required for tumorigenesis in bone and (ii) that the presence of the MT intron sequences leads to expression in bone or to increased levels of expression in general. Evidence to support the latter hypothesis is that when triply transgenic mice carrying all three Py transgene cDNAs were bred from preexisting transgenic mice, these triply transgenic mice did not develop detectable bone pathology (41). However, we have not detected gross differences in expression levels between the Py early region transgene and the Py cDNA transgenes in either tumors or unaffected tissue, and the expression of the Py transgene was not detected in unperturbed skull tissue of Py-3 and Py-5 mice (41). Therefore, if differences in site or expression levels are responsible for the expanded tumor spectrum of the Py transgene, the alterations are subtle enough to have escaped detection thus far.

The Py early region transgene was expressed in the tumors that developed as well as in some unaffected organs. The major site of expression was the testis, and all testes of males of all lineages expressed the Py transgene. Other nontumor sites of expression, such as the liver, spleen, kidney, and brain, varied with lineage. Thus, mice of the Py-4 lineage showed the broadest spectrum of tissues expressing the transgene, and they also developed the most severe pathology and died at a very young age. It is likely, therefore, that the Py-4 transgene integrated in a chromosomal site that promotes widespread expression early in life, whereas the Py-3 and Py-5 integrations may have occurred in a chromosomal context that is more restrictive of expression. These differences in expression of the same transgene among different lineages have been documented, and they sometimes reflect temporal patterns of tumorigenesis (20, 40).

The Py early region transgene can produce three different mRNAs by alternative splicing, and these RNAs encode the three early region proteins Py LT, MT, and ST (37). Differences in the relative amounts of the three RNAs were found in transgenic mouse tissues, and specifically, the relative amount of Py LT RNA was higher in testes than in all other sites of expression. This finding suggests that regulation is at the level of alternative splicing, although it is formally possible that differential RNA stability leads to higher relative levels of Py LT RNA in testes. Splicing of Py LT RNA, which has a unique 5′ donor site and a 3′ acceptor site common to Py ST, thus appears to be favored in testes relative to other sites of expression. In contrast, the coexpression of Py MT and ST at all expression sites is consistent with the reported cooperativity between the two different 3′ acceptor sites in vitro (18). Other alternatively spliced genes such as that for calcitonin-CGRP show a tissue-regulated splicing pattern that is reconstituted in transgenic mice (8).

Regardless of how differential expression is effected, the low levels of Py LT RNA (detectable only by PCR) that are present in tumor RNAs suggests that high levels of Py LT RNA are not required for tumorigenesis in Py transgenic mice. In contrast, the presence of all three Py early region mRNAs in the testes suggests that coexpression of all early region gene products is not sufficient for tumorigenesis in the testes and that other events or conditions are required for Py-mediated tumorigenesis. This hypothesis is supported by the finding that the testes of Py-4 mice express the early region proteins as well as the RNAs (18a).

Analysis of Py early region transgenic mice has documented that Py-mediated tumorigenesis in mesenchymal tissues does not require the viral life cycle and virus repli-

cation. Moreover, these mesenchymal sites of tumor formation in transgenic mice do not require high levels of Py LT RNA for tumor formation. Yet other sites of transgene expression elaborate the full complement of early region proteins and do not form tumors, suggesting that other events are required for testicular tumorigenesis. Taken together, these results show that the mechanisms of viral pathogenesis are influenced by tissue specificity in the effects of the Py early region gene products. Further analysis of tissue-specific differences in tumor susceptibility of Py transgenic mice may further elucidate the mechanisms of tumorigenesis.

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