Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 8288–8292, November 1986
Genetics

Early regions of JC virus and BK virus induce distinct and tissue-specific tumors in transgenic mice

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Communicated by Chris Anfinsen, August 1, 1986

ABSTRACT **JC virus and BK virus are ubiquitous human viruses that share sequence and structural homology with simian virus 40. To characterize tissue-specific expression of these viruses and to establish model systems for the study of human viral-induced disease, transgenic mice containing early regions of each of the viruses were produced. The viral sequences induced tumors in a distinct and tissue-specific manner that was similar to their tissue tropism in humans. Ten JC virus-containing founder mice were produced, of which 5 survived to maturity. Four of them developed adrenal neuroblastomas, which metastasized to several other tissues. JC virus tumor-antigen RNA was detected at high levels in the tumor tissues and at low levels in other normal tissues of these mice. One of the three BK virus-containing mice was abnormally shaped and died at 2 weeks of age. The other two BK virus-containing mice developed primary hepatocellular carcinomas and renal tumors and died at 8–10 months of age. BK virus tumor-antigen RNA was expressed in tumor tissues of both mice. Since each of the viruses retained the general tissue tropism that it exhibits in humans, these data suggest that transgenic mice harboring human viruses will be useful as animal models for viral-induced diseases.**

The papovaviruses are a group of small, double-stranded DNA tumor viruses that have been isolated from a wide variety of species. Simian virus 40 (SV40) induces subclinical infection in rhesus monkeys and grows lytically in African green monkey cells in culture (1). It oncogenically transforms rodent and human cells in culture and induces tumors in newborn hamsters (1) and sometimes in mice (2, 3). JC virus (JCV) and BK virus (BKV), although structurally related to SV40 (1), have different host ranges, tissue tropisms, and pathology (4). They are ubiquitous human viruses, and approximately 70–80% of the adult population is seropositive for each of the viruses (5, 6). BKV is found predominantly in the kidney, where it induces a subclinical infection (4, 5). In immunosuppressed patients, BKV can be excreted in the urine, although infections usually are asymptomatic (1, 7–9). JCV has been strongly associated with the fatal demyelinating disease progressive multifocal leukoencephalopathy, which occurs in patients whose cellular immunity has been impaired (10). Multiple glial tumors also have been observed in progressive multifocal leukoencephalopathy patients (11–13); however, JCV has not been proven to be the cause of these or any other human tumor. JCV and BKV transform hamster cells in culture, and both viruses induce tumors in newborn hamsters (1). JCV induces tumors in tissues of neural origin, including medulloblastomas, undifferentiated neuroectodermal tumors, glioblastomas, pineocytomas, neuroblastomas, and meningiomas (14–16). BKV causes brain tumors of ventricular surfaces, as well as nonbrain tumors insulinomas and osteosarcomas (17–19). JCV has not been reported to be tumorigenic in mice or to transform mouse cells in culture (1).

The nucleotide sequence and amino acid homology of the tumor (T) antigen and viral capsid protein genes of SV40, BKV, and JCV are between 70 and 80% (20). The T antigens are most conserved in amino acid sequence at the amino-terminal end, and least at the carboxyl terminus. They share antigenic determinants (16, 21–23), suggesting conservation of structure. It has not been determined if the differences in the T antigens play a role in determination of tissue tropism or other properties of the virus. The most divergent region of the viral genomes encompasses the origin of replication and enhancer sequences (20). Although these regions share structural features (direct and inverted repeats as well as palindromes), the nucleotide sequence homology is only about 40%. It has been postulated that differences in the enhancer region are responsible for the differences in host range, tissue tropisms, pathogenicity and oncogenicity of the papovaviruses (24). Further data suggest that viral variants with mutations in the enhancer region are expressed in different tissues (19).

Regions of the SV40 genome have been introduced into transgenic mice, and characteristic choroid plexus papillomas were induced (25, 26). Later studies indicated that the SV40 large T antigen was responsible for the tumor induction and that the SV40 enhancer sequences directed tumors to the choroid plexus (27). In the absence of SV40 enhancer sequences, a different pathology was observed. Under the influence of the mouse metallothionein I promoter, hepatocarcinomas and pancreatic adenocarcinomas were induced. A peripheral neuropathy also was present in those mice, due to a selective demyelination of the axons in peripheral nerve bundles (28). Pancreatic tumors specific to the insulin producing-cells were induced when the SV40 T-antigen gene was fused to the rat insulin I promoter (29). High levels of T antigen were observed in the tumor tissues.

The purpose of the work described here was to introduce JC and BK virus early regions into transgenic mice. Comparison of the tissue specificity of expression and pathology induced by the three viruses, coupled with subsequent experiments to introduce recombinant viruses, will demonstrate which regions of the viruses contribute to their host range and pathogenicity.

Ten transgenic mice containing JCV early region genes were produced, of which five survived to maturity. Four of five mice developed adrenal neuroblastomas, which metastasized to the pituitary gland, intestine, stomach, and liver. Three females died at 14–16 weeks of age, and one male died at 10 months. JCV T antigen expression was high in the tumors, and lower, but detectable, in heart, lung, brain, and muscle of the three mice. Three transgenic mice with intact

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Abbreviations: JCV, JC virus; BKV, BK virus; SV40, simian virus 40; T, tumor; kb, kilobase(s).

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BKV early regions also were obtained. One was malformed and died at 2 weeks of age. The two other mice developed liver and kidney tumors and died at 8 and 10 months of age. BKV T-antigen RNA was expressed in tumor tissue of these mice.

METHODS

Transgenic mice were produced as described by Gordon and Ruddle (30). Fertilized eggs were injected with 100–1000 copies of the early region of either BKV (Dun strain) or JCV (MAD-1) (31). Positive mice were identified by Southern blotting of DNA extracted from the distal portion of the tail, as described by Small *et al.* (26). Whole tissue DNA was extracted as described by Gordon *et al.* (32). Whole cell RNA was isolated through a 5.7 M CsCl cushion (33), and 50 μg of RNA was electrophoresed through a formaldehyde/agarose gel. Southern and RNA gel blotting, and radio-labeling of DNA probes was done as described (26). For histological analyses, tissues were fixed in 10% (vol/vol) neutral formalin, embedded in paraffin, sectioned, and stained with hemotoxylin and eosin. For electron microscopy, tissues were fixed in glutaraldehyde, stained with osmium tetroxide, and embedded in epon.

RESULTS

Generation of Mice. *Pvu* II fragments of BKV (Dun) or *Nci* I–*Bal* I fragments of JCV (MAD-1) (31), containing the viral early regions, were injected into the pronuclei of fertilized mouse eggs. Thirteen mice containing JCV sequences (Fig. 1*A*), and 6 containing BKV sequences (Fig. 1*B*) were generated. Several restriction enzyme digests were performed to determine whether the intact regulatory sequences and T-antigen genes were present in the animals (data not shown). A high percentage of these mice contained deleted or rearranged gene fragments, so that in total, 2 mice with intact BKV early regions and 10 mice with intact JCV early regions were obtained.

Five mice, DJC1–DJC5, derived from embryos injected with JCV early region were born dead or died shortly after birth. All of these mice contained intact JC early region genes (Fig. 1*A*). No further analysis was performed on these mice.

Appearance of Tumors in JCV-Containing Mice. Five JC founder mice, three females and two males, survived to maturity. All three female mice, JC62, JC74, and JC86, and one male, JC48, were microopthalmic and developed cataracts at 6–10 weeks of age. The three females succumbed to tumors at 14–16 weeks of age, and JC48 died of tumors at 10 months. Tumors of the adrenal and pituitary glands and large tumor masses on the intestinal wall were present in all four mice. Additional metastatic tumors in the intestinal mesentery and on the stomach wall were detected in JC62 and JC86. In JC62, tumors were also observed in the liver, spleen, and several distinct areas of the brain, including the choroid plexus, cerebellum, and ventricles.

Pathological analyses indicated that the primary tumor in all of the mice was a neuroblastoma originating in the adrenal medulla (Fig. 2). The tumors consisted of small round cells with scant cytoplasm (Fig. 2*A*). They were vascular with extensive areas of necrosis and calcification (Fig. 2*A*). The nuclei of the tumor cells had a diffuse chromatin pattern. Other tumors in the mice were metastases of the adrenal neuroblastoma. An example of a metastatic tumor invading the muscularis layer of the intestine from the outer serosal layer is shown (Fig. 2 *B* and *C*). Electron microscopic analysis of the pituitary gland tumor showed the presence of many neurosecretory granules, indicating that this tumor also was a metastasis of the adrenal neuroblastoma (Fig. 2*D*).

Although all four animals were fertile and produced live offspring, the JC sequences were transmitted at a low frequency, suggesting that they were germ-line mosaic. JC62 had eight mice in her first litter, and a second litter of 14-days gestation was present when the mouse was sacrificed. DNA analysis indicated that 3 of the 22 offspring (all in the second litter) were positive for JCV sequences. JC74 produced a single litter containing only three mice. Of those, one, which was born dead and not fully developed, was positive. JC86 produced two litters, with a total of 24 mice, of which only 1 mouse contained intact JCV sequences. JC48 produced 114 offspring, of which 22 (19%) contained intact JCV sequences. JC86 and JC48 had second integration sites, containing only deleted copies of the JCV sequences that were passed to offspring independently. Intact T-antigen genes were not

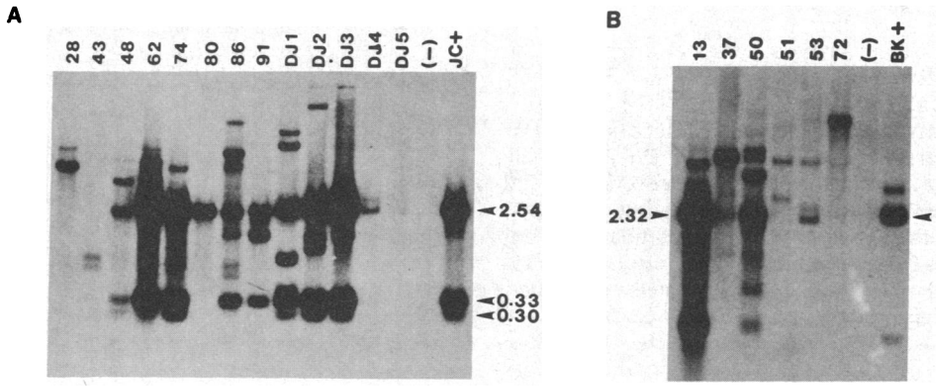

FIG. 1. Blots of DNA from transgenic mice containing JCV and BKV sequences. (*A*) Tail DNA (10 μg) was digested with 20 units of *Pvu* II, electrophoresed in a 1% agarose gel, transferred to nitrocellulose, and hybridized with a JCV early region probe (*Nci* I–*Bal* I fragment). Mice numbered 28, 43, 48, 62, 74, 80, 86, and 91 were born alive and lived to maturity. DJ1–DJ5 were born dead. (−) is DNA from the tail of a negative littermate, JC+ is (−) DNA containing 50 pg of JC early region fragment as a positive control. The expected sizes of fragments are labeled (2.54, 0.33, and 0.30 kilobase pairs). From these data and other restriction analyses (not shown), it was determined that mice JC48, JC62, JC74, JC86, and JC91 contained intact copies of the T antigen and regulatory DNA. All five dead JC mice contained intact sequences. A longer exposure showed that the appropriate bands were present in DJC4 and DJC5. (*B*) DNA was digested with *Mst* II and *Xba* I and treated as in *A*. The probe was the *Pvu* II early region fragment from BKV. Mice 13, 37, 50, 51, 53, and 72 are adult mice. (−) is as *A*. BK+ is (−) DNA containing 30 pg of BKV *Pvu* II fragment. The expected internal *Mst* II–*Xba* I fragment is indicated [2.32 kilobases (kb)]. Mice BK13 and BK50 contained intact sequences based on this digest and a *Pvu* II digest (data not shown). BK37 lacks an undetermined region at the 3′ end of the molecule. Although BK53 appears to contain the 2.32-kb fragment, the mobility is actually slightly faster, and this was confirmed by other digests (data not shown).

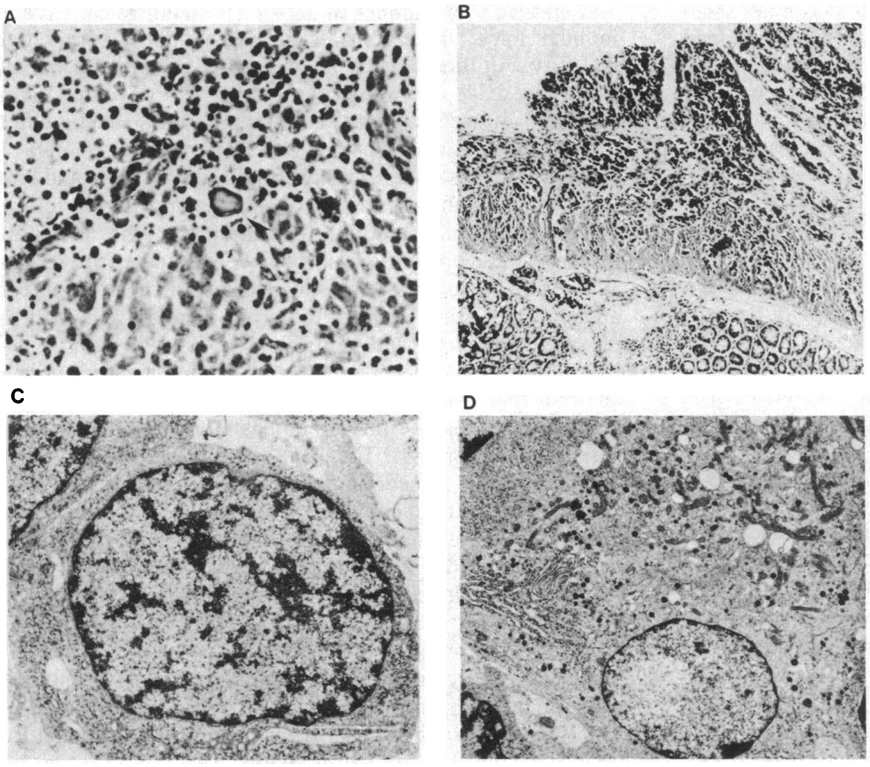

FIG. 2. Pathological analysis of tumors of the adrenal and pituitary glands and the intestinal wall. (*A*) Light micrograph of the adrenal neuroblastoma. There are nests of tumor cells with scant cytoplasm and the nuclei are larger than nuclei of lymphocytes. The nuclei are somewhat irregular in shape and contain a diffuse chromatin pattern. The tumor is rapidly growing, and there are areas of necrosis as shown by the tumor cells containing pyknotic nuclei (small, dark nuclei). In the center (arrowhead) is a calcification, which is characteristic of neuroblastomas. (×130.) (*B*) Light micrograph of the tumor involving the serosal surface of the intestine with invasion into the muscularis by nests of metastatic neuroblastoma. Lower portion demonstrates normal glandular epithelium of the intestine. (×57.) (*C*) Electron micrograph of tumor cell in the metastatic intestinal tumor. Note the scant cytoplasm and large nuclei with diffuse chromatin pattern (×6000.) (*D*) Electron micrograph of the metastatic tumor in the pituitary gland. Electron dense bodies in the cytoplasm are the neurosecretory granules characteristic of a neuroblastoma and tumors of this cell lineage. (×3000.)

present in these mice, and no abnormal phenotype was observed.

The remaining male mouse, JC91, did not develop tumors. JC91 lived for 4.5 months, produced over 100 offspring, and died of causes unrelated to tumor induction. Offspring from all three founders that transmitted intact JCV sequences to live offspring (JC48, -86, and -91) developed a distinctive phenotype, similar to the spontaneously derived jimpy (*jp*) or quaking (*qk*) strains of mice. Tumors have not appeared in the offspring, although none has lived beyond 18 weeks due to a dysmyelination in the central nervous system (34).

Appearance of Tumors in BKV-Containing Mice. Of the three BKV mice, one (BK13) containing greater than 20 copies of the BKV early region, was abnormally proportioned, grew slowly, and died at 2 weeks. Due to maternal cannibalism, no further analysis was performed on this mouse. The second and third mice, BK37 and BK50, both females, contained about 1–2 and 10 copies of the intact BKV sequences, respectively (Fig. 1*B*). BK37 successfully produced several litters, and several subsequent generations have been produced. Although BK50 mated several times, no offspring were generated. The mouse had a distinctive kyphosis. BK37 and -50 developed enlarged abdomens and became lethargic at 10 and 8 months of age, respectively. The livers and kidneys of the two mice contained many tumor masses, diagnosed as primary hepatocellular carcinomas and primary renal tumors. There was no other detectable pathology. One of two positive female offspring of BK37 that has reached 10 months developed similar tumors.

Expression of T-Antigen RNA in Transgenic Mice. Expression of T-antigen genes was determined in the tumors and normal tissues of two of the JCV mice, JC74 and JC86, and in BK50 (Fig. 3). Low levels of T-antigen RNA were expressed in some histologically normal tissue of the mice and higher levels in the tumor tissue. The JCV mice expressed T-antigen RNA to varying degrees in brain, heart, lung, and muscle, and at a high level in the tumor tissues (Fig. 3 *A* and *B*). JC74 had a high level in the heart, which was hypertrophic, but a low level or no expression in other nontumor tissues (Fig. 3*A*). JC86 expressed various levels of T-antigen RNA in all tissues examined. There was a low level of expression in liver and kidney, slightly higher levels in brain, heart, and lung, and high levels detected in tumor tissues (Fig. 3*B*). Histological examination indicated that there were many small tumors in apparently normal intestinal and stomach tissues in JC86, so that the high levels of RNA observed in these tissues may have been due to contaminating tumor tissue. BK50 expressed a low level of T-antigen RNA in brain, heart, and lung, and a higher level in muscle and the liver tumor (Fig. 3*C*).

To determine whether the increased expression of T-antigen RNA was due to increased transcription or to amplification of the DNA sequences, dot-blot analysis was performed on DNA from the intestinal tumor and normal tissues of mice JC74 and JC86 (Fig. 4*A*). There was a 2- to 3-fold increase in the amount of hybridization to the tumor DNA compared to normal tissue in the JC74 line. Amplification was not detectable in the DNA of JC86. When further analysis was performed on the DNA from JC86, there was a difference in the intensity of the 2.54-kb fragment, which is representative of the insertion site with the intact T-antigen gene (Fig. 4*B*). Analysis of tumor and normal tissue of mouse BK50 indicated that there was no detectable amplification of BKV sequences (data not shown).

DISCUSSION

Transgenic mice containing the early region genes of the human papovaviruses BK and JC developed distinct pathologies. The tissue tropism of the pathology was similar to that which the viruses are thought to have in humans. JC, which is associated with the demyelinating syndrome progressive multifocal leukoencephalopathy and neural tumors in humans (10–13), induced neural tumors in the mice. Additionally, in other mice containing JC, hypomyelination was observed (34). BK, which is thought to replicate in renal epithelial cells and is excreted in the urine of immunosuppressed patients (4, 5, 7), induced renal and liver tumors. This conservation of tissue tropism across species demonstrates that transgenic mice carrying these viruses, and potentially

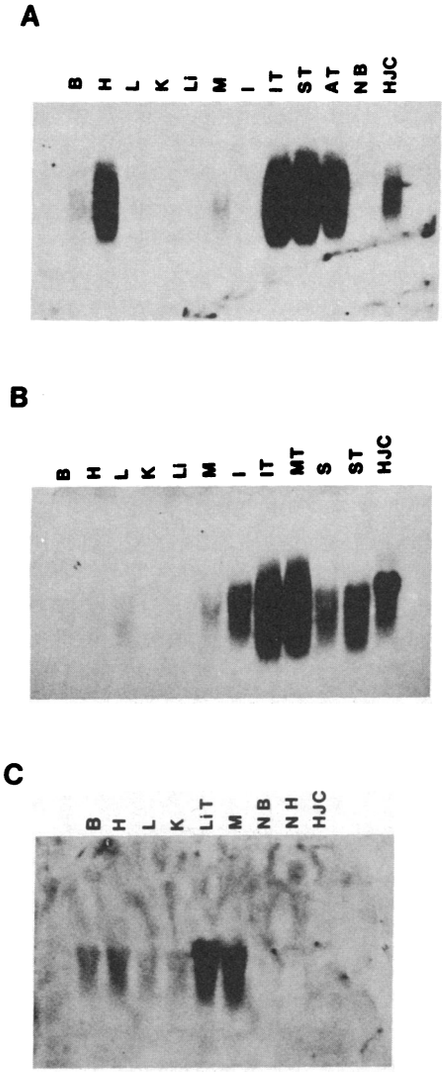

FIG. 3. Analysis of RNA from normal and tumor tissues of mice JC74 (*A*), JC86 (*B*), and BK50 (*C*). HJC is RNA isolated from a JCV-transformed hamster cell line (kindly provided by E. Majors). Lanes: B, brain; H, heart; L, lung; K, kidney; Li, liver; M, muscle; I, intestine; IT, intestinal tumor; LiT, liver tumor; S, stomach; ST, stomach tumor; AT, adrenal gland tumor; MT, tumor located in the intestinal mesentery; NB and NH, brain and heart from normal, virus-negative littermates. *A* and *B* were probed with a JCV early region probe; *C* was probed with a BKV early region probe.

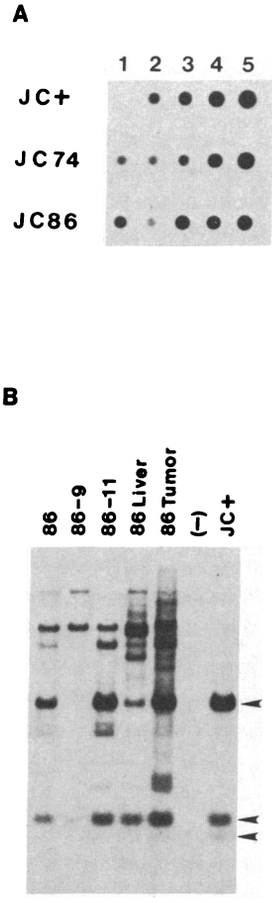

FIG. 4. DNA analysis of normal and tumor tissues from JCV transgenic mice. DNA dot-blot analysis was performed as described (39). (*A*) Dot-blot analysis of DNA from JC74 and JC86. DNA (10 μg) is present in each sample. JC+: 10 μg of negative DNA containing 0 pg (column 1), 10 pg (column 2), 20 pg (column 3), 50 pg (column 4), and 100 pg (column 5) of JCV early region DNA. JC74: JC74 (column 1), JC74-1 (column 2), JC74 liver (column 3), JC74 intestine (column 4), and JC74 intestinal tumor (column 5) are compared. JC86: JC86 (column 1), JC86-9 (containing partial JCV sequences) (column 2), JC86-11 (containing intact JCV sequences) (column 3), JC86 liver (column 4), and JC86 intestinal tumor (column 5) are compared. (*B*) Southern analysis of DNA from JC86 line. DNA was digested with *Pvu* II. Expected bands of 2.54, 0.33, and 0.30 kb are present in JC+ [50 pg of JCV early region DNA in 10 μg of negative DNA (−)]. The bands at 2.54 and 0.33 kb are indicative of the intact T-antigen sequences and are increased in intensity in the tumor tissue.

other human viruses, may be excellent models for the study of human viral-induced disease. Furthermore, the distinctive phenotypes induced by SV40 (25–28), JC, and BK suggest that further experiments in which hybrid molecules are introduced will demonstrate which areas of the viruses (e.g., the enhancer elements, promoter sequences, and/or T-antigen coding regions) are responsible for their tissue tropism and pathology.

The frequency of generating live mice containing intact JCV or BKV early region sequences (6%) was considerably lower in these experiments than in other experiments in our laboratory (20–30%). The high correlation between mice born dead and presence of intact sequences (five out of five mice) suggests that JCV might have a toxic effect on the developing embryo. If the site of integration or copy number of the viral sequences in those mice permitted expression during early development, the T antigen may have been sufficient to prevent normal development. It has been shown that polyomavirus, a mouse papovavirus, has a detrimental effect on embryos when the mother is infected during the preimplantation stages of development (36).

The fact that live mice were also obtained with intact early region sequences suggests a different pattern or level of expression in these mice. Of five mice containing JCV sequences that survived to maturity, four developed tumors. The frequency of transmission of the intact JCV sequences suggested that all four mice were mosaic, i.e., they didn't contain the JCV sequences in every cell. This hypothesis was supported by comparisons of the intensity of JC-specific bands on Southern blots, in which positive offspring had more copies per cell than the founders (data not shown). We believe that the mosaicism accounts for the different phenotypes exhibited by the founders and offspring. The founders, which probably did not contain JCV early regions in all oligodendrocytes, made sufficient myelin to allow them to live long enough to develop tumors. The offspring, which contained JCV DNA in all oligodendrocytes, died of dysmyelination before tumors had time to appear. JC91, who was not mosaic, and his offspring died at 4–5 months due to dysmyelination. Thus in this case too, the founder and offspring died of dysmyelination prior to the appearance of tumors.

In humans, JCV antibodies are present in 70–80% of sera tested (5, 6). These data indicate that a large fraction of the population has been exposed to JCV, without any apparent clinical symptoms. Although papovavirus particles usually are not detected in healthy individuals (1), they can be detected in kidney and are excreted in urine of immunosuppressed individuals (7, 8, 35, 37). Additionally, during the last trimester of pregnancy, JCV and BKV have been detected in the urine (38), perhaps due to decreased immunocompetency. In the degenerative demyelination disease, progressive multifocal leukoencephalopathy, JC viral particles can be isolated from brain lesions (10). This disease is usually

observed only in patients that have been immunocompromised by illness or by therapy.

Tumor induction in JCV-containing mice occurred earliest in females. Since an immunocompromised state is induced by pregnancy (38), and these mice were pregnant for 3–6 weeks, it is tempting to speculate that cells expressing T antigen were not eliminated as actively. The reduction in immunosurveillance may have allowed a rapid proliferation of neoplastic cells in susceptible tissues. This hypothesis is supported by the findings that decreased immune function seems to make mice more susceptible to SV40-induced tumors (2, 3). Although the current numbers are only suggestive, we have developed lines of mice containing JC and BK early region genes that will allow us to pursue this hypothesis, by following the tumor induction in females after pregnancy and in artificially immunosuppressed mice.

T-antigen RNA was detected in both normal and tumor tissues of the JCV transgenic mice. Low levels of T-antigen RNA were detected in brain, heart, and lung. In JC74, there was a high level of expression in the heart. There was little to no detectable expression in the liver or kidney of the mice. The presence of T-antigen RNA in normal tissue was surprising and suggests that moderate levels of T antigen may be tolerated by some cell types. The low level of expression seen in whole tissue may have been due to high expression in a subset of the cells, which, therefore, did not affect the functioning of the tissue as a whole. In tumors, uniformly there were high levels of T-antigen RNA. This could be explained by a higher level of T antigen per cell or by increased levels in a subset of cells.

Of the three BK-containing mice, one was runted, developed abnormally, and died at 2 weeks. It is possible that the T-antigen gene was expressed shortly after birth in specific tissues and adversely affected its growth and development. The second mouse, BK50, lived for 8 months and was kyphotic and infertile. T-antigen RNA was expressed in normal and tumor tissue. The level was low in brain, heart, and lung, and higher in muscle. The third mouse, BK37, lived for 10 months and was fertile. Several offspring of BK37 also have developed tumors.

Introduction of SV40 sequences into transgenic mice resulted in induction of choroid plexus papillomas (25, 26). Injection of the early regions of BK and JC viruses into transgenic mice caused the development of different and distinctive pathologies. Both viruses caused tumors consistent with the tissue type commonly affected; BKV affected epithelial type tissues, and JCV affected neural tissues. An interesting and logical extension to these studies is to make hybrid viral sequences containing enhancer/promoter regions from one virus and T-antigen genes from another and to inject those molecules into transgenic mice. These experiments will allow further dissection of the viral sequences to determine which part of the viral genome is responsible for the distinctive tissue tropisms and pathologies.

We thank Charles Bieberich for many helpful discussions and assistance, Tim Triche for help with the pathological analysis, Eugene Majors for providing the HJC cell line, and Yvette Johnson for animal care. This work was supported in part by an award from the Eukaryotic Genetics Program of the National Science Foundation (G.S.). G.S. is a Leukemia Society of America Scholar.

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