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JC Virus and Simian Virus 40 Enhancers and Transforming Proteins: Role in Determining Tissue Specificity and Pathogenicity in Transgenic Mice

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When introduced into the germ line of mice, the simian virus 40 (SV40) T antigen under the control of its own transcriptional enhancer and promoter selectively induced tumors in the choroid plexus as well as thymic hyperplasia and kidney pathology. In contrast, the JC virus (JCV) T antigen under the control of its own regulatory sequences induced hypomyelination of the central nervous system and tumors of neural origin. Since SV40 and JCV have extensive sequence homology, except for their transcriptional control regions, these observations suggest but do not prove that, although the diseases induced by the two viruses, are consequences of the transforming gene, they are determined predominantly by the respective viral enhancers and promoters. To test this hypothesis, the regulatory regions of the two viruses were exchanged, and transgenic mice were derived with either chimeric construct. Like wild-type JCV, the construct containing the SV40 T antigen under the control of the JCV regulatory region induced hypomyelination of the central nervous system and neural tumors. Surprisingly, mice with this construct also developed choroid plexus carcinomas. Like the wild-type SV40 transgenic mice, mice with the JCV T antigen under the control of the SV40 enhancer and promoter developed choroid plexus tumors and renal pathology. Unexpectedly, they also had hyperplasia of the thyroid follicular cells. These findings not only provide direct evidence for the specificity of the respective viral regulatory region but also, more importantly, show that the transforming genes play a critical role in determining viral pathogenesis.

The human JC virus (JCV) and simian virus 40 (SV40) are genetically related viruses, with identical genome organizations, that share 70% homology in their early transforming protein T antigen and in their capsid proteins (7). The most divergent region between the two viruses encompasses the origin of DNA replication and the transcriptional enhancer, which are only 40% homologous at the nucleotide level. Despite the extensive sequence homology, the two viruses have completely different host range and tissue specificities.

JCV was isolated from the brains of patients with progressive multifocal leukoencephalopathy, a neurodegenerative disease of which it is considered the etiological agent (16), and SV40 was isolated from the kidneys of rhesus monkeys and induces subclinical infections in those animals (8). However, variants of each virus have also been isolated that showed altered specificities (12, 15). The restricted host range and tissue specificity of JCV are conserved in vitro; the virus propagates only in human glial cells in culture (2, 6). This tissue specificity is regulated at the transcriptional level (6, 10), as demonstrated by the presence of glial cell-specific factors binding to the transcriptional enhancer as well as through *cis*-restricted DNA replication (1, 11, 13). SV40 is transcriptionally active in numerous cell types of human and rodent origin in addition to its natural host, clearly showing a wide host range and tissue tropism in culture (8).

Although in vitro experiments have enabled us to determine features governing the tissue specificities of these viruses, tissue culture cells do not reconstitute the type of cell-cell interactions essential for the development of disease. An appropriate in vivo system is required to determine the basis of viral pathogenesis. Transgenic mice with the SV40 early genes and regulatory sequences have been independently derived in two laboratories (4, 17). Most mice developed choroid plexus papillomas, and a subset of the animals were also found to have kidney pathology and thymic hyperplasia. The JCV early genes and the enhancer/promoter were similarly introduced into the germ line of mice (18, 19). A subset of the transgenic animals displayed a shaking disorder resulting from hypomyelination of the central nervous system (CNS), and the rest of the transgenic mice developed adrenal medullary neuroblastoma that metastasized to several tissues including the pituitary gland and the intestinal mesentery.

The relatedness between JCV and SV40 constitutes an attractive model system for studying the genetic features of these viruses that confer tissue specificity and pathogenicity. To this end, chimeric viruses in which the control regions from SV40 and JCV were exchanged were constructed (Fig. 1). In each case, great care was taken to conserve the positions of all transcriptional signals. Although JCV tissue specificity is partially controlled by *cis*-restricted DNA replication (13) and SV40 and JCV origins of replication were exchanged in the process of deriving the chimeric constructs, the tissue specificity of the resulting DNAs should not be affected, since they will be integrated into the mouse genome and will not go through viral replication. Transgenic mice were produced as described by Brinster et al. (5). Approximately 200 copies of the JCV enhancer/promoter-SV40 T antigen chimeric gene, designated JC(SV40) and contained within an *Eco*RI-*Bam*HI fragment, were microinjected into fertilized ova at the single-cell stage (Fig. 1A). Mice were screened for the acquisition of novel JCV and

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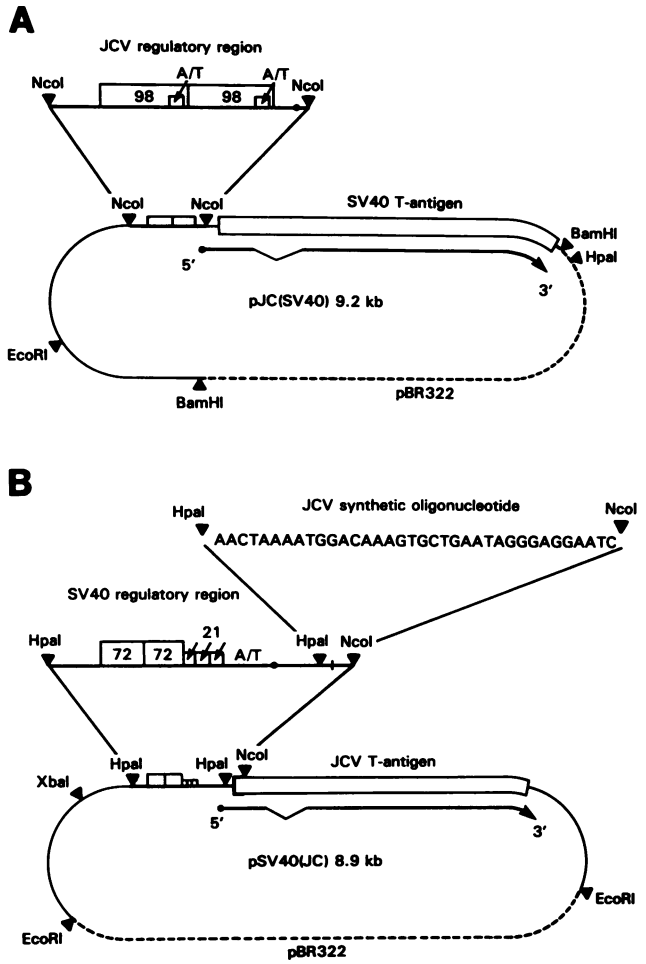

FIG. 1. Features of the chimeric JCV and SV40 hybrid genes. (A) Construct in which the JCV control region directs expression of the SV40 T-antigen gene pJC(SV40). SV40 was cloned into pBR322 at the *Bam*HI site. The SV40 enhancer/promoter was removed by digestion with *Kpn*I and *Sfi*I. An *Nco*I-*Hin*dIII 0.29-kb JCV DNA fragment encompassing its enhancer and promoter and origin of replication was cloned into the SV40 vector after both the vector and the insert were linked with *Nco*I. Indicated in this diagram is the duplicated 98-bp repeat that makes up the JCV enhancer, the AT-rich region, the SV40 T-antigen RNA start-site (●), and its spliced transcript (thick line). The complete transcriptional unit is released by restriction with *Eco*RI and *Bam*HI. (B) Construct in which the SV40 control region directs expression of the JCV T-antigen gene pSV40(JC). The JCV control region was removed from JCV by digestion with *Nco*I and *Hpa*I. In this process, part of the amino terminus of the JCV T antigen was also deleted. A synthetic oligonucleotide with *Hpa*I and *Nco*I sticky ends was inserted to replace the missing amino acid sequence. An *Hin*dIII-*Hpa*I fragment spanning the SV40 regulatory region was then cloned into the JCV vector after conversion of all ends with *Hpa*I linkers. After the cloning step, the region spanning the inserted nucleotide was sequenced by using the dideoxy method (21). The SV40 regulatory region is composed of a duplicated 72-bp repeat, a triplicated 21-bp repeat, and an AT-rich region. The RNA start site for the JCV T-antigen coding sequence is indicated (●), as is the spliced message (thick line). The complete transcriptional unit is obtained by restriction with *Xba*I and *Eco*RI.

TABLE 1. Pathologies of JC(SV40) and SV40(JC) transgenic mice[a]

Gene construct	Mouse	Sex	Age (wk)	Sc	Ag	Mp	Cp	Ky	Ty	Th
JC(SV40)	1-2	Male	3	+	−	−	−	−	−	−
	2-2	Male	(4)	+	−	−	−	−	−	−
	3-1	Male	2	−	+	+	−	−	−	−
	1-11	Female	7	−	+	+	++	−	−	−
	3-6	Male	16	−	+	+	++	−	−	−
SV40(JC)	6-1	Male	(3)	−	−	−	+	−	−	−
	6-5	Male	3	−	−	−	+	−	−	−
	6-6	Male	5	−	−	−	+	+	−	−
	5-6	Female	6	−	−	−	−	+	−	
	1-5	Male	7	−	−	−	+	+	+	−
	5-1	Male	(5)	−	−	−	+	+	+	+
	1-3	Female	(11)	−	−	−	+	+	+	+

[a] Mice were either found dead or sacrificed when in a moribund state. The age at the time of death is shown. When mice were sacrificed, their ages are indicated in parentheses. Various tissues were collected, fixed in formaldehyde, sectioned, and stained with H&E. The tissues analyzed (with disease manifestation) include the following: Sc, spinal cord (hypomyelination); Ag, adrenal gland (adrenal medullary neuroblastoma); Mp, myenteric plexus (myenteric plexus tumor); Cp, choroid plexus (++, carcinoma; +, papilloma); Ty, thyroid (thyroid hyperplasia); Ky, kidney (renal corpuscle hyperplasia and cyst); Th, thymus (thymic hyperplasia).

SV40 DNA sequences by Southern blot analysis of DNA obtained from tail biopsies (20). The T-antigen sequence used contains the open reading frames of both large T and small t antigens. Of 34 mice obtained, 5 had the full-length microinjected *Eco*RI-*Bam*HI fragment integrated in their genomes. The number of integrated copies varied from 1 to 3. Four of the five mice (designated 1-2, 1-11, 2-2, and 3-1) died between the ages of 2 and 7 weeks and bore no offspring. Mouse 3-6 was bred successfully.

Each transgenic mouse was analyzed for pathological changes. Details of individual mice are given in Table 1. Two of the founder mice (1-2 and 2-2) appeared to be runts 1 week after birth. At two weeks, mouse 1-2 developed shaking tremors when moving. This mouse died at 3 weeks, presumably as a result of this phenotype. The other mouse did not shake but had hind leg paralysis. It was sacrificed at 4 weeks of age because of its moribund state. Histological analysis of hematoxylin-eosin (H&E)-stained sections from various tissues did not show any pathological changes. However electron microscopic analysis of sections of the spinal cord from both mice showed clear hypomyelination of the CNS. Compared with those of an age-matched nontransgenic littermate (Fig. 2A), most axons (arrowheads) within the spinal cord of mouse 2-2 did not show a myelin sheath, which is normally seen as an electron-dense structure wrapped around the axon (Fig. 2B). Myelinated axons from the transgenic mice also revealed changes in myelin sheath thickness.

The three remaining JC(SV40) mice did not exhibit any motor dysfunction and did not reveal any pathology of the CNS but died prematurely. On autopsy, mice 1-11 and 3-6 showed greatly enlarged adrenal glands that were half the size of the kidneys. H&E analysis of sections of these adrenal glands showed a complete loss of the adrenal cortex and histological changes characteristic of adrenal medullary neuroblastoma. Mouse 3-1 also showed pathology in the form of adrenal medulla hyperplasia. The lack of a full-blown phenotype most likely is a result of the young age at which this mouse died.

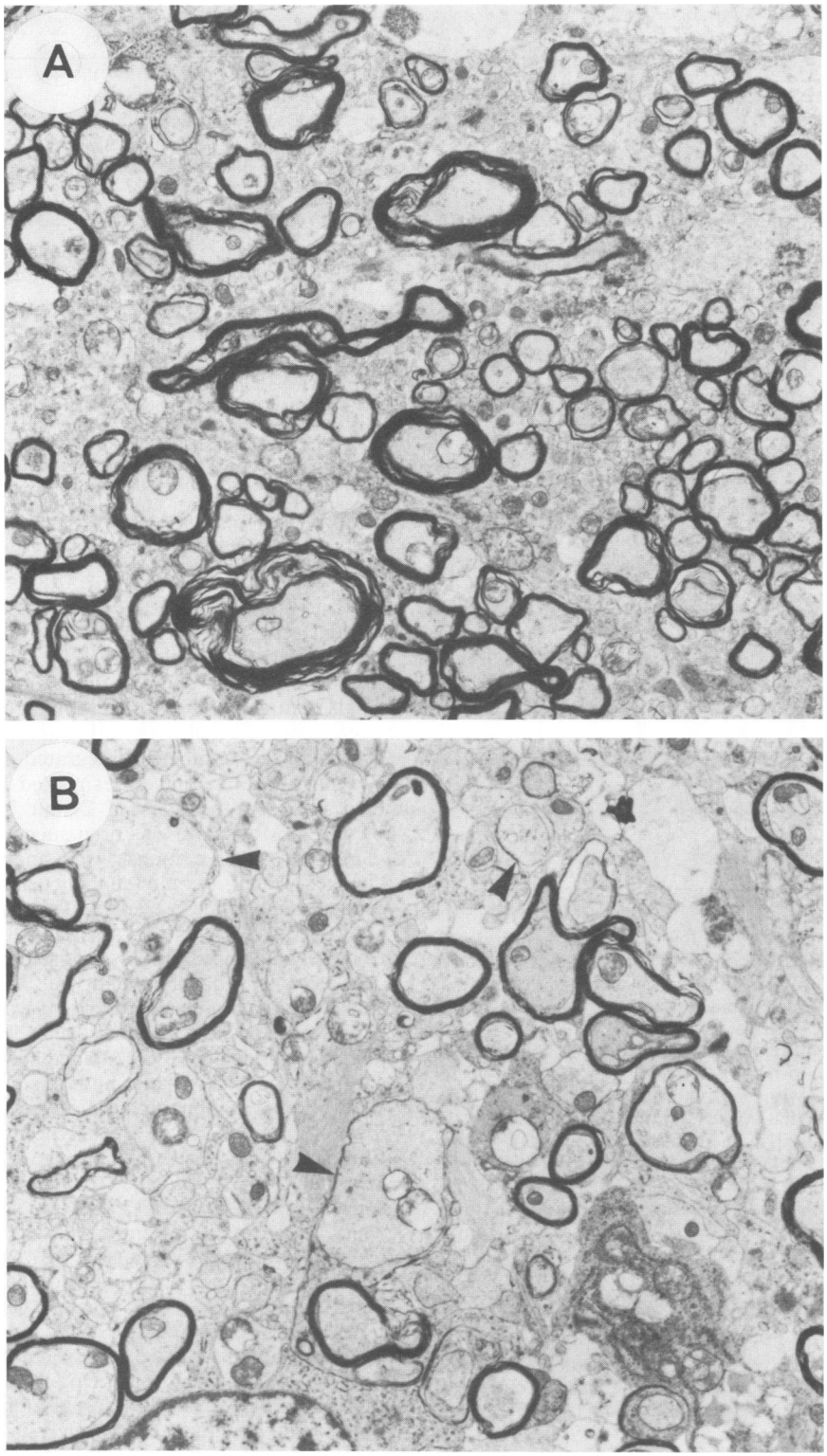

FIG. 2. Electron microscopic analysis of the JC(SV40) mice. Tissues were fixed with glutaraldehyde, embedded in Epon, sectioned, and stained with uranyl acetate. (A) Cross section of axons in the spinal cord of a normal mouse. (B) Cross section of the spinal cord from JC(SV40) mouse 2-2 with axons lacking myelination (arrowhead). Magnification, ×7,000.

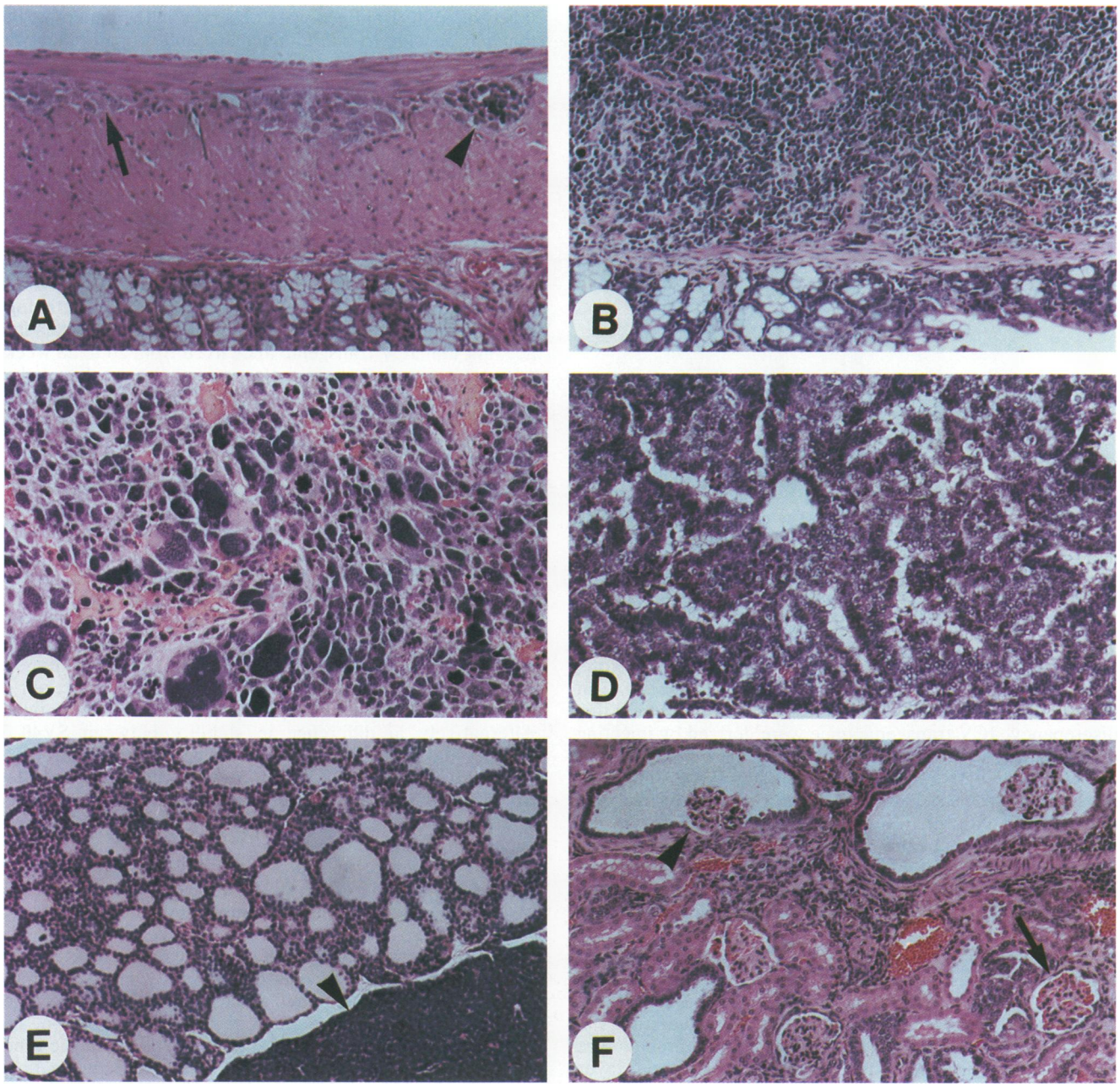

FIG. 3. Microscopic examination of different tissues from JC(SV40) and SV40(JC) transgenic mice. Paraffin-embedded sections were stained with H&E and examined microscopically. (A) Intestine from JC(SV40) mouse 3-6 (magnification, ×40). (B) Intestine from JC(SV40) mouse 3-6 (magnification, ×40). (C) Choroid plexus from JC(SV40) mouse 3-6 (magnification, ×400). (D) Choroid plexus from SV40(JC) mouse 1-3 (magnification ×400). (E) Thyroid gland from SV40(JC) mouse 1-3 (magnification, ×100). (F) Kidney from SV40(JC) mouse 1-3 (magnification, ×100).

Large intestinal masses located in the ileum just above the cecum were also visible in two of the JC(SV40) mice (1-11 and 3-6). These masses were tumors that appeared to originate from the intestinal serosa. A typical histological analysis of early lesions seen in these mice is shown in Fig. 3A. The section shows a portion of normal myenteric plexus (arrow), a neural structure that innervates the two muscle layers by which it is surrounded. The nuclei of the neural cell bodies that make up the ganglion are round and lightly stained by H&E. As shown in Fig. 3A (arrowhead), early lesions are clearly originating from the myenteric plexus, where the neural cell bodies have become hyperchromatic and are highly proliferative. Given time, these lesions, which are aggressive in nature, develop and displace the muscle layers, giving rise to the large tumors seen at autopsy (Fig. 3B). These tumors, which are neural in origin, were not described as such in earlier wild-type JCV transgenic studies (18), where intestinal tumors were observed in four of five JCV transgenic mice but were classified as metastases from the adrenal medullary neuroblastoma. The discrepancy in

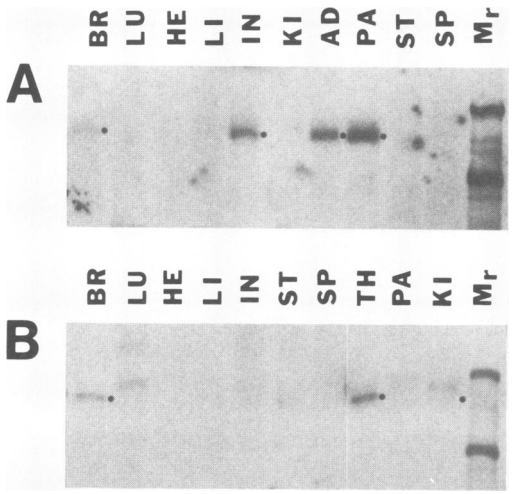

FIG. 4. Tissue distribution of SV40 T antigen and JCV T antigen in transgenic mice. (A) Expression of SV40 T-antigen protein in JC(SV40) mouse 3-6. The 88-kDa SV40 T antigen is indicated (●). (B) Expression of JCV T antigen in SV40(JC) mouse 1-3. The 83-kDa JC T antigen is indicated (●). The tissues analyzed include the following: BR, brain; LU, lung; HE, heart; LI, liver; IN, intestine; KI, kidney; AD, adrenal gland; PA, pancreas; ST, stomach; SP, spleen; TH, thymus. Protein markers of 92.5 and 69 kDa are shown in lane M_r.

these results could be attributed either to the presence of the SV40 T or t antigens in the JC(SV40) transgenic mice or to an error in diagnosis of the wild-type JCV mice. To address this discrepancy, JCV transgenic mice were derived by injecting an *Ava*II DNA fragment encompassing the early region of JCV together with its enhancer/promoter. Five transgenic mice were obtained. These mice had a longer lifespan than did the JC(SV40) mice but exhibited myenteric plexus tumors that were identical to those seen in the JC(SV40) mice. In addition, it is unlikely that these tumors were a result of metastases from the adrenal medulla neuroblastoma since one mouse displayed myenteric plexus lesions but no adrenal lesions.

The two oldest JC(SV40) mice (1-11 and 3-6) also showed erythematous masses in their brains. Similar lesions were observed in the transgenic offspring of mouse 3-6. In these young mice, the early lesions clearly originated from the choroid plexus. As the lesions progressed, the architecture of the choroid plexus, normally seen as a single cell layer arranged in a papillomatous array, was replaced by grossly enlarged cells with hyperchromatic nuclei and total loss of cell morphology (Fig. 3C). Large areas of necrosis and calcification were also seen. Based on the aggressive nature of this tumor and the cellular changes observed, this tumor is classified as choroid plexus carcinoma. All the above phenotypes were correlated with expression of the transgene at the protein level (Fig. 4A). SV40 T antigen was detected by Western immunoblot analysis. A 2.5-mg sample of protein extract was immunoprecipitated by using a hamster antitumor serum against SV40 T antigen (9). The immunoprecipitate was fractionated on a 10% polyacrylamide–sodium dodecyl sulfate gel and transferred to a nitrocellulose membrane. The blot was then probed with the same hamster antitumor serum followed by incubation with ^{125}I-labeled protein A. Expression of SV40 T-antigen is observed in the brain, intestine, and adrenal gland, the same tissues that showed pathologic changes (Fig. 4A). In addition, high levels of SV40 T antigen were observed in the pancreas, but this was specific to one founder line and no pathology was observed.

Our findings provide strong evidence that the JCV enhancer/promoter has a significant role in determining the tissue specificity of this neurotropic virus, since the major phenotypes caused by the JCV control region and SV40 T antigen are identical to pathologies observed in the wild-type JCV transgenic mice. However, it is surprising that all JC(SV40) transgenic mice, with the exception of animals with CNS involvement, developed choroid plexus carcinomas. The choroid plexus is an epithelial tissue and has not been shown to be a target of the JCV enhancer driving JCV T antigen in five transgenic mice. It is unlikely that the lack of pathology reflects insufficient time for tumors to arise if one takes into account that JCV transgenic mice lived from 20 to 28 weeks whereas aggressive choroid plexus tumors were detected as early as 7 weeks after birth in the JC(SV40) mice. Previous in vitro experiments with hybrid papovaviruses similar to the ones in this study demonstrated that the transforming potential of SV40 T antigen is stronger than that of JCV T antigen (3). Since transformation is a very sensitive assay requiring that an event occur in only one cell for scoring, the combination of a strong transforming protein such as the SV40 T antigen and adequate expression of this protein in a cell in which it has a vulnerable subcellular target will lead to transformation. It is therefore reasonable to suppose that leakage of expression of SV40 T antigen in the choroid plexus, when under the control of the JCV enhancer/promoter, leads to the carcinomas observed. This phenotype is not seen in JCV transgenic mice, most likely as a result of the weak transforming capacity of the JCV T antigen. Alternatively, a difference in stability between SV40 T antigen and JCV T antigen could be the cause of these results. It is unlikely that the observed phenotype resulted from selected mutations within the JCV control region, since a similar phenotype was observed in all of the mice from each founder line.

Transgenic mice in which the SV40 enhancer/promoter controls expression of the JCV T antigen were also derived by microinjection of single-cell embryos with an *Xba*I-*Eco*RI fragment from the pSV40(JC) construct (Fig. 1B). The presence of complete copies of the transgene was assayed by genomic DNA digestion with the same enzymes. Seven mice were obtained that contained at least one full-length copy of the transgene (Table 1). Four of the mice (6-5, 6-6, 5-6, and 1-5) died between the ages of 3 and 7 weeks, and the other three mice (6-1, 5-1, and 1-3) were sacrificed between 3 and 11 weeks. Because of the early death of the founder mice, no transgenic lines were established.

A cranial bulge was observed in the four mice that survived longest (5-6, 1-5, 5-1, and 1-3). H&E staining of brain sections revealed papillomatous tumors in each case (Fig. 3D). These tumors, originating from the choroid plexus, contain cells that had lost their original cuboidal shape, had enlarged, and had hyperchromatic nuclei. The three mice without a cranial bulge (6-1, 6-5, and 6-6) also developed similar histological lesions. The appearance of the tumors was always papillomatous, and the tumors never formed tightly packed masses even in a mouse with advanced lesions, such as mouse 1-3. Although the choroid plexus tumors in these SV40(JC) mice is reminiscent of the phenotype in wild-type SV40 transgenic mice, the SV40(JC) mice died at an earlier age (3 to 7 weeks) than did the SV40 mice, which were reported to succumb between 14 and 17 weeks of age (22).

The two oldest SV40(JC) mice (5-1 and 1-3) also had greatly enlarged thymuses, which, upon analysis by H&E staining of sections, revealed pathology that was similar to the thymic hyperplasia seen in wild-type SV40 mice (4, 17). Microscopic analysis of the thyroid from 3 mice (1-5, 5-1, and 1-3) revealed extensive proliferation of the epithelial cells lining the thyroid follicles (Fig. 3E). These epithelial cells, which are normally cuboidal in shape and present as a single layer of cells surrounding the follicles, have round hyperchromatic nuclei and are arranged in multiple cell layers. In some cases, replacement of the colloid follicle with tumor was also observed. This phenotype was not reported in previous studies of SV40 pathogenesis in transgenic mice (4, 17). Our results suggest that, when taken from its original context, the JCV T-antigen has subcellular targets in cells other than those of neural origin.

With the exception of those of mice 6-1 and 6-5, the kidneys of the SV40(JC) mice appeared pale and granular. Histological analysis of the involved kidneys revealed numerous cysts in the renal cortex. Involvement was bilateral, with sparring of the medulla. A high magnification of an area in the involved cortex shows several abnormalities of the renal corpuscle (Fig. 3F). The epithelial cells that form the Bowman's capsule are hyperplastic, and their nuclei are well rounded and hyperchromatic. Cysts seen in the renal cortex represent enlarged Bowman's space. These pathologic changes were not observed in mice 6-1 and 6-5, probably because of their early death. Although kidney pathology was observed in the wild-type SV40 transgenic mice, the nature of the lesions was different in that the reported cysts were of tubular origin and that glomerular involvement was in the form of glomerulosclerosis (14). The absence of glomerulosclerosis in the SV40(JC) mice is most likely the result of early death, since this condition developed only at 14 to 16 weeks in the SV40 mice (14). The renal corpuscle pathology suggests that JCV T and t antigens again play a role. It is not clear, however, whether the discrepancy in observations results from a qualitative difference between the two T and t antigens, from the difference in expression level between the two transgenes, or a combination of both. Expression of the JCV T antigen was observed in tissues with pathological changes, including the brain, thymus, and kidney (Fig. 4B). The protein of higher molecular weight seen in the lung and kidney is a cross-reacting protein detected by our SV40 antitumor serum and does not represent the JCV T antigen. Because of the small size of the thyroid, it was not possible to document pathology and expression of the transgene.

The occurrence in JC(SV40) and JCV transgenic mice of identical neural pathologies such as hypomyelination of the CNS, adrenal medulla neuroblastoma, and myenteric plexus tumors and the fact that both SV40(JC) and SV40 transgenic mice developed choroid plexus papilloma, thymic hyperplasia, and kidney pathology clearly demonstrate the primordial role of the transcriptional enhancer and promoter elements from each virus in determining the tissue specificity of viral pathogenesis. However, the appearance of phenotypes specific to the newly introduced transforming genes, in the form of choroid plexus carcinomas for SV40 T antigen under the control of the JCV enhancer/promoter and thyroid hyperplasia for JCV T antigen under the control of the SV40 regulatory sequences, suggest the involvement of the transforming proteins.

These results also suggest a close interplay between the regulatory sequences and the transforming protein in specifying viral tissue tropism. JCV and SV40 are two closely related viruses with totally different tissue tropisms. This dichotomy results from the evolution, in the case of JCV, of a tissue-specific transcriptional enhancer and a weak transforming protein, which will keep in check any leakage of expression to non-neural tissues. On the other hand, a virus such as SV40, which does not display any tissue specificity, will have every advantage in having a promiscuous transcriptional enhancer and a strong transforming protein to maximize transforming events in every cell in which it has a subcellular target.

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