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Genetic Analysis of Simian Virus 40 from Brains and Kidneys of Macaque Monkeys

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Simian virus 40 (SV40) was isolated from the brains of three rhesus monkeys and the kidneys of two other rhesus monkeys with simian immunodeficiency virus-induced immunodeficiency. A striking feature of these five cases was the tissue specificity of the SV40 replication. SV40 was also isolated from the kidney of a Taiwanese rock macaque with immunodeficiency probably caused by type D retrovirus infection. Multiple full-length clones were derived from all six fresh SV40 isolates, and two separate regions of their genomes were sequenced: the origin (ori)-enhancer region and the coding region for the carboxy terminus of T antigen (T-ag). None of the 23 clones analyzed had two 72-bp enhancer elements as are present in the commonly used laboratory strain 776 of SV40; 22 of these 23 clones were identical in their ori-enhancer sequences, and these had only a single 72-bp enhancer element. We found no evidence for differences in ori-enhancer sequences associated with tissue-specific SV40 replication. The T-ag coding sequence that was analyzed was identical in all clones from kidney. However, significant variation was observed in the carboxy-terminal region of T-ag in SV40 isolated from brain tissues. This sequence variation was located in a region previously reported to be responsible for SV40 host range in cultured cell lines. Thus, SV40 appears to be an opportunistic pathogen in the setting of simian immunodeficiency virus-induced immunodeficiency, similarly to JC virus in human immunodeficiency virus-infected humans, the enhancer sequence organization generally attributed to SV40 is not representative of natural SV40 isolates, and sequence variation near the carboxy terminus of T-ag may play a role in tissue-specific replication of SV40.

Two related but distinct polyomaviruses are known to infect humans: JC virus (JCV) and BK virus (BKV). These viruses are closely related to each other and to the polyomavirus of macaque monkeys called simian virus 40 (SV40) (12). JCV and BKV differ in the ability to replicate in cells of different tissues, in tissue distribution, and in disease potential (for a review, see reference 40). BKV replicates and persists largely in kidney tissue. JCV replicates well in human glial cells, but its replication is highly restricted in other cell types. Only JCV infects the central nervous system, causing progressive multifocal leukoencephalopathy (PML), a rare demyelinating disorder usually observed in a setting of immunodeficiency.

The macaque monkey polyomavirus SV40 has been a popular model and tool for the study of transcription, gene regulation, DNA replication, and transformation. Essentially all studies have used a particular strain of SV40 called 776, originally isolated by Sweet and Hilleman in 1960 (42). Although polyomavirus infections assumed to be caused by SV40 have been noted in the kidneys and brains of macaque monkeys (15, 18, 19, 23), detailed genetic characterizations have been lacking.

Major determinants of tissue-specific polyomavirus replication have been thought to reside in the transcriptional enhancer elements. The JCV enhancer functions efficiently in glial cells of human and rodent origin but not in cells of other tissues (21). SV40 promoter/enhancer-directed expression of transgenes in transgenic mice targets expression to the brain (4). Tandemly repeated control elements have been found in the enhancer region of a JCV isolate from the brain of a patient with PML, but they were not similarly repeated in JCV from the kidney of the same patient (24). However, determinants of SV40 host range for different cultured cells reside, at least in part, in T antigen (T-ag) (22, 31, 41). Furthermore, the specificity of disease in transgenic mice is determined by both promoter/enhancer elements and T-ag of JCV and SV40 (10).

Recently, Horvath et al. described polyomavirus infections in rhesus monkeys in the setting of simian immunodeficiency virus (SIV)-induced immunodeficiency (19). These infections prompted us to investigate whether some rhesus monkeys may harbor distinct polyomaviruses and to analyze possible determinants of tissue-specific virus replication. All six virus isolates analyzed in this study were clearly SV40; we found no evidence for distinct polyomaviruses analogous to JCV and BKV in humans. All six contained a single 72-bp enhancer, not the two copies found in the laboratory strain 776. Thus, the two 72-bp enhancer elements present in the commonly used laboratory strain of SV40 are not representative of natural isolates and most likely arose via duplication following extensive cell culture passage. SV40 isolated from the brain and kidney showed essentially identical enhancer element sequences, indicating that enhancer sequences are not important determinants of SV40 tissue-specific replication. In contrast, sequences at the carboxy end of T-ag, which determine host range in cultured cells (31, 41), were essentially identical in all kidney samples but showed considerable variation in brain samples. These results suggest that sequence differences at the carboxy end of T-ag may contribute to tissue-specific SV40 replication.

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TABLE 1. Origins of new SV40 isolates

Monkey species	Animal no.	Cause of immunodeficiency	Site of SV40-associated disease	SV40 isolated from:	Year of isolation
M. cyclopis	261-78	Type D retrovirus?	Kidney, lung[a]	Kidney	1982
M. mulatta	246-88	Experimental infection with uncloned $SIV_{MAC-251}$	Kidney	Kidney	1991
	155-88	Experimental infection with cloned $SIV_{MAC-239}$	Kidney[b]	Kidney	1991
	91-78	"Natural" SIV infection	Brain (PML)	Brain	1991
	54-83	Experimental infection with cloned $SIV_{MAC-239}$	Brain (PML)	Brain, kidney	1991
	124-79	Experimental infection with cloned $SIV_{MAC-239}$	Brain (PML)	Brain	1991

[a] SV40 was isolated from various tissues, suggesting viremia (23).
[b] This animal had rare polyomavirus inclusions in the kidney.

MATERIALS AND METHODS

SV40-infected animals. Five rhesus monkeys and one Taiwanese rock macaque were diagnosed with SV40 infection on the basis of clinical features and pathological examination (19, 23). A summary of clinical data relevant to those animals is presented in Table 1.

Virus. SV40 was isolated from snap-frozen tissues embedded in OCT compound (Miles Scientific, Kankakee, Ill.) from animals in which SV40 disease was diagnosed (19). Washed and minced kidney or brain tissue from infected animals was cocultivated with Vero cells in complete minimal essential medium. The time from initiation of coculture to preparation of viral DNA ranged from 11 to 24 days, which represented no more than two tissue culture passages. The SV40 strains received from the American Type Culture Collection (ATCC), Rockville, Md., were A2895 (7, 8), PA-57 (20), and EK and DAR (36, 46). Virus samples were received freeze-dried and reconstituted with distilled water. Vero cells were infected with those strains and cultivated as described above. Cells were harvested on day 8 for isolation of viral DNA.

Isolation of SV40 DNA and genomic DNA from SV40-infected cells. SV40-infected cell cultures were used for the isolation of Hirt supernatant DNA (16). One 75-cm^2 flask of virus-producing cells was used for each preparation. Genomic DNA from SV40-infected tissues was isolated both from frozen, OCT-embedded and from formalin-fixed, paraffin-embedded tissue samples. Briefly, frozen tissue sections were washed in phosphate-buffered saline, incubated in extraction buffer (0.01 M Tris-HCl [pH 8.0], 0.1 M EDTA, 20 μg of RNase per ml, 0.5% sodium dodecyl sulfate) for 1 h at 37°C, and treated with proteinase K (3 to 5 h, 50°C) at a final concentration of 100 μg/ml. DNA was then purified with a standard phenol-chloroform extraction, ethanol precipitation procedure. Paraffin-embedded tissue sections were twice extracted with xylene, ethanol, and acetone to remove the paraffin, resuspended in 0.05 M Tris-HCl (pH 8.5) with 0.5% Tween 20, and treated with proteinase K (200 μg/ml) (47). The sample was then incubated for 10 min at 95°C to inactivate the protease, and aliquots were directly used for polymerase chain reaction (PCR) amplification.

Molecular cloning. Viral DNA was cut at the unique *Kpn*I or *Bam*HI site, purified with phenol-chloroform, and ligated into alkaline phosphatase-treated pUC19 by incubation at 15°C overnight. Library Efficiency DH5α Competent Cells (GIBCO BRL, Grand Island, N.Y.) were transformed with the ligation products according to the manufacturer's protocol. Bacterial colonies containing the desired insert were selected by their ability to utilize 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) on ampicillin-containing agar (37). Plasmid DNA was isolated by using either CsCl gradient centrifugation or the Magic Miniprep DNA purification system (Promega, Madison, Wis.). The purified DNA was used for restriction analysis and DNA sequencing.

Blot hybridization. Viral DNAs were digested with restriction enzymes, separated on 0.8% agarose gels, transferred to a nitrocellulose filter, hybridized with SV40 [^{32}P]DNA (2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 68°C), and washed according to standard procedures (37). The GIBCO BRL 1-kb DNA ladder was used as molecular weight markers. Autoradiography was carried out with intensifying screens at −70°C for 1 to 3 days.

DNA sequencing. SV40 clones were sequenced by the primer-directed dideoxy-chain termination method (38) with Sequenase 2.0 (United States Biochemical Corp., Cleveland, Ohio) and a series of primers described below. ^{35}S-labeled reactions were electrophoresed on either 6% polyacrylamide or 5% Long Ranger (AT Biochemicals, Inc., Malvern, Pa.) gels with 8 M urea. Products of PCR amplification (see below) were directly sequenced with the same procedures.

Primers. The primers used for DNA sequencing and PCR amplification were synthesized on a Cyclone DNA synthesizer (Biosearch, Inc., Burlington, Mass.). For the sequencing of SV40, the following primers were used: 5′-CCATCTT TGCAAAGCTTT-3′ (5160 to 5177), 5′-GGCTGACTAATT TTTTTTA-3′ (38 to 20), 5′-CTGACACACATTCCACAG-3′ (255 to 272), 5′-AAATAACCTCTGAAAGAGG-3′ (328 to 310), 5′-GAAAAACCAGAAAGTTAA-3′ (506 to 523), 5′-TT ATTTGTAACCATTATA-3′ (2633 to 2650), and 5′-ACAC CTCCCCCTGAACCT-3′ (2717 to 2700). For the PCR amplifications, the primers used were 5′-GGTACCTCAGTTG CATCCCAGAAG-3′ (GG4915-4936), 5160-5177, 5′-GAGGC GG AAAGAACC-3′ (289 to 275), 5′-GGTACCTTCTGAGG CGGAAAG-3′ (299 to 279), 5′-GGTACCACAACTAGAAT GCAGTGAAA-3′ (GGT2573-2595), 5′-GGTACCTGTGGCT GAGTTTGCTCA-3′ (GGT3069-3049), 5′-GATGCTATTGC TTTATTT-3′ (2621 to 2638), and 5′-GCCAGGAAAATGCT GATA-3′ (2902 to 2885). The SV40 nucleotide numbers (according to the sequence of strain 776) are shown in parentheses. Primers GG4915-4936, GGT2573-2595, and GGT3069-3049 had nucleotides added to the 5′ end of the SV40 sequence in order to create a *Kpn*I site. The M13/pUC (−20)universal and (−24)reverse sequencing primers (both 17-mers) (28) were used for the primary sequencing of the insert regions adjacent to the cloning site.

PCR amplification. Hirt supernatant DNA from SV40-infected cells or total DNA from frozen or formalin-fixed animal tissues was used as the template in PCR to specifically amplify either the origin (ori)-enhancer region or the large-T-ag carboxy-terminal coding region. Cloned DNA of SV40 strain 776 (Bethesda Research Laboratories) and the cloned SV40 DNAs of the studied strains were used as

positive controls. DNA of pUC19 (Bethesda Research Laboratories) was used as a negative control. *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) was used for the amplification of desired DNA sequences. The reactions were performed with an automated DNA Thermal Cycler (Perkin-Elmer Cetus) for 30 to 35 cycles (each consisting of a 1-min 94°C denaturation, 1-min 55°C annealing, and 1-min 72°C extension step), followed by a single 10-min extension at 72°C. For nested PCR, two sets of primers (external and internal) were used sequentially. In this case, 1 μl of reaction mixture from the first amplification served as a template for the second. All reagents used for PCR amplification were from Perkin-Elmer Cetus, and the entire reaction was performed according to the manufacturer's recommendations.

Nucleotide and protein sequence analysis. Nucleotide and protein sequences were analyzed and aligned by using MacVector version 3.5 software (International Biotechnologies, Inc., New Haven, Conn.). The sequence of SV40 strain 776 was taken from GenBank (3) (accession number JO2400).

Nucleotide sequence accession numbers. All of the nucleotide sequencing data for the SV40 strains used in this study have been submitted to GenBank under accession numbers M99346 to M99364.

RESULTS

Polyomavirus disease in macaques with AIDS. Polyomavirus disease was diagnosed by histologic examination of formalin-fixed, paraffin-embedded tissues from five rhesus monkeys (*Macaca mulatta* [indicated by the prefix Mm]) and one Taiwanese rock macaque (*Macaca cyclopis* [indicated by the prefix Mc]). The five rhesus monkeys died with SIV-induced AIDS, and the cyclopis macaque died with a natural immunodeficiency syndrome most likely caused by type D retrovirus. Three of these animals, Mm91-78, Mm124-79, and Mm54-83, were adults with PML (19), a fatal demyelinating disease characterized by replication of SV40 in oligodendrocytes of central nervous system white matter (Fig. 1A). Two others, Mm246-88 (19) and Mc261-78 (23), were juvenile animals with severe bilateral tubulointerstitial nephritis caused by polyomavirus replication in renal tubular epithelium (Fig. 1B). The sixth animal, Mm155-88, was a seropositive adult with rare polyomavirus intranuclear inclusions in kidney epithelium. This information is summarized in Table 1. Infection with polyomavirus was confirmed by demonstration of characteristic 40- to 45-nm virions in affected tissues of Mm91-78, Mm54-83, Mm246-88, and Mc261-78 by electron microscopy (Fig. 1C) and by isolation of SV40 from all six animals.

Analysis of the ori-enhancer region in macaque polyomavirus DNA. Polyomavirus was isolated from stored tissue of all six animals, and multiple, full-length, viral DNA clones were derived from infected cell DNA of each sample following digestion with restriction endonuclease *Bam*HI or *Kpn*I. PCR was not used for the isolation of these cloned DNAs. The nucleotide sequences of 22 of 23 clones from these six isolates were absolutely identical in the ori-enhancer region (Fig. 2). This identity in sequence contrasts with differences in T-ag sequences observed in these same clones (see below). All 22 clones with identical sequences in the ori-enhancer region had only a single 72-bp sequence element that is considered to be a core structure of the SV40 enhancer (Fig. 2) (1, 6). The presence of a single 72-bp enhancer element in these clones contrasts with the laboratory strain of SV40, 776, which contains two of these 72-bp motifs (6, 11, 27, 38). These 22 clones were otherwise identical with laboratory strain 776 in the ori-enhancer region (Fig. 2).

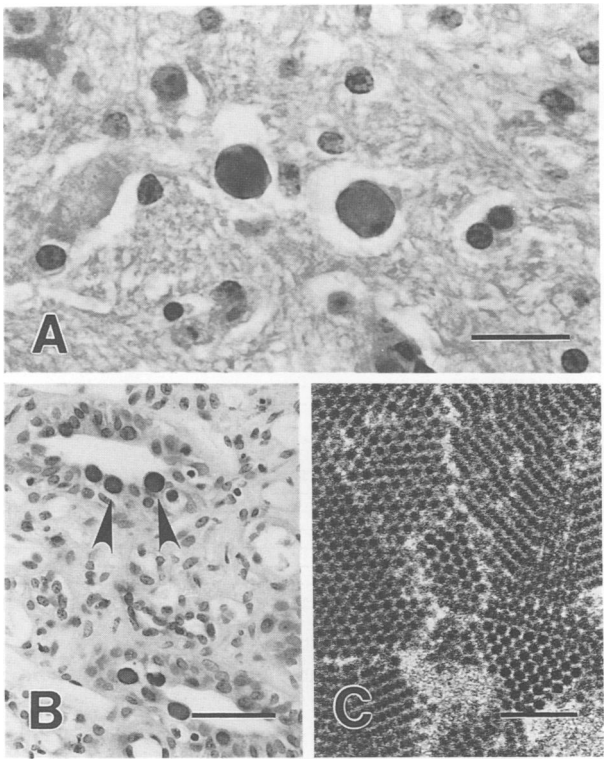

FIG. 1. SV40 replication in rhesus monkey brain and kidney. (A) Basophilic intranuclear inclusion bodies in the nuclei of two oligodendrocytes from Mm91-78 with PML (hematoxylin and eosin; magnification, ×650; bar = 20 μm). (B) Basophilic intranuclear inclusion bodies in the nuclei of renal tubular epithelial cells (arrowheads) from Mm246-88 with tubulointerstitial nephritis (hematoxylin and eosin; magnification, ×215; bar = 50 μm). (C) Characteristic intranuclear paracrystalline array of 40- to 45-nm polyomavirus particles consistent with SV40 in a degenerating renal tubular epithelial cell from Mm246-88 (transmission electron micrograph; magnification, ×50,000; bar = 200 μm).

One of the 23 clones showed an aberrant structure in the ori-enhancer region. In addition to the single 72-bp enhancer sequence and the usual complement of 21-bp repeats, clone 4 from the kidney SV40 isolate of Mm246-88 contained additional copies of the 21-bp repeats and a partial duplication of a region of the 72-bp enhancer element (Fig. 2 and 3).

Four stocks of SV40 were also obtained from ATCC. Our sequence of two of these, from humans with PML, showed significant rearrangements in the region of the 72-bp repeats (Fig. 3), exactly identical to previously published sequences for these same isolates (26). Another strain, A2895, originating from early studies on tumor induction in hamsters (7, 8), also contained an aberrant ori-enhancer structure (Fig. 3). The fourth ATCC strain, PA-57, was also unusual in that it contained mostly smaller, defective viral DNA molecules, which may have resulted from irradiation of the producer cells used for establishing this strain (20). Our sequence of the ori-enhancer region of PA-57 revealed one 72-bp enhancer element identical to that found in the majority of clones in this study.

We used two types of analyses to verify that our cloned

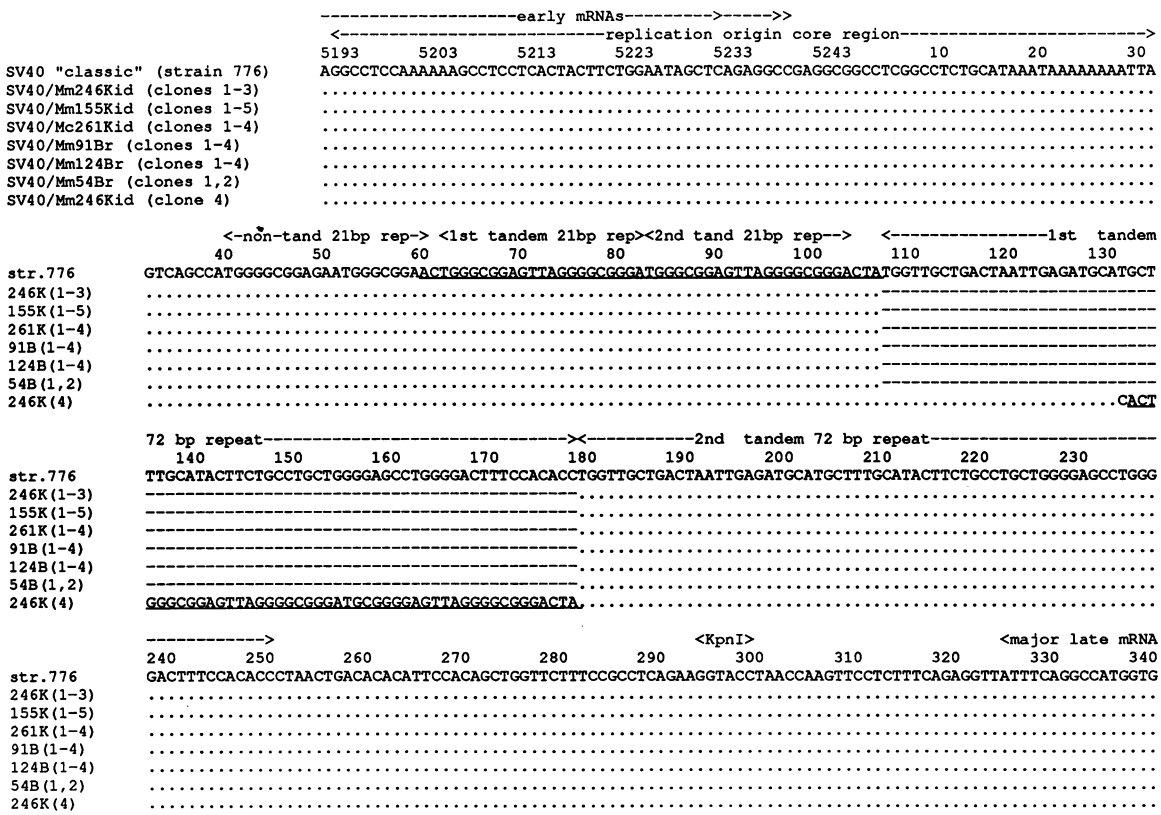

FIG. 2. Alignment of the nucleotide sequences in the regulatory regions of different strains of SV40. The replication origin core and auxiliary regions are shown. The replication origin auxiliary region (three 21-bp repeats and two 72-bp repeats), also required for the transcription of SV40 DNA, is known as the enhancer/promoter region. The transcription start sites are shown for both early and late SV40 mRNAs. The top line shows the sequence of SV40 strain 776 initially determined by Fiers et al. (11, 45) and Reddy et al. (32). Nucleotide numbers are shown above the sequence. The sequences of different clones of SV40 determined in this study are shown relative to the sequence of strain 776. Dots indicate the presence of identical nucleotides; dashes indicate deleted nucleotides.

ori-enhancer DNA sequences were representative of the viral DNA samples from which they were derived: Southern blot hybridization of restriction fragments and PCR amplification of infected cell viral DNA. Digestion of the newly isolated SV40 DNAs present in infected cells with *Kpn*I-*Hin*dIII gave a fragmentation pattern identical to that of strain 776 except for the fragment derived from the ori-enhancer region; the slightly faster migration of the ori-enhancer-containing DNA fragment from the fresh isolates was consistent with the DNA sequences (data not shown). Similarly, DNA spanning the ori-enhancer region was amplified by PCR from the infected cell DNA samples. In each case, the size of the amplified product agreed with the size determined by DNA sequencing of cloned DNA (Fig. 4A). Clone 4 from the kidney of Mm246-88 likely represents a minor component, since a fragment corresponding to the size of its ori-enhancer region was not detected in the amplification of viral DNA from cells infected with SV40 from the Mm246-88 kidney (Fig. 4A).

We succeeded in amplifying SV40 ori-enhancer sequences directly from frozen or paraffin-embedded tissues of two of the animals, Mm124-79 (brain) and Mm246-88 (kidney). In both cases, the size of the amplified DNA fragment exactly corresponded to the size of amplified DNA from infected Vero cells and to cloned DNA of the corresponding SV40, that is, the size containing one 72-bp enhancer element (Fig. 4B).

The results of the ori-enhancer sequence analyses are summarized in Fig. 3.

Analysis of T-antigen sequences in macaque polyomavirus DNA. The nucleotide sequences at the 3′ end of the T-ag reading frame were determined for 22 clones from the six fresh isolates (Fig. 5). The nucleotide sequences were translated into deduced amino acid sequences (Fig. 6). None of the 22 clones had the same sequence as did laboratory strain 776 over the 310 bp that were examined. The 12 clones isolated from the kidney SV40s of the three macaques with kidney disease showed no sequence heterogeneity. These 12 clones were identical in sequence over this region, and this sequence was distinct from that of SV40 776. However, 5 of 10 clones from the SV40 brain isolates showed significant heterogeneity over this stretch (Fig. 5 and 6). Four of four clones from Mm91-78 brain SV40 and one of two clones from Mm54-83 brain SV40 had distinctly variant sequences. The nature of variant SV40 sequences in Mm91-78 brain was different from that in Mm54-83 brain (Fig. 5 and 6). The greatest variation occurred around bp 2764 to 2792 as a result of deletions or duplications in a region of several short direct repeats (Fig. 5).

We used PCR amplification and digestion with *Bsa*BI to examine which sequence predominated in the SV40 DNA from the brain of Mm54-83. The kidneylike SV40 DNA sequence over this region has no *Bsa*BI site, while it is present in the unique sequence in one of two clones from

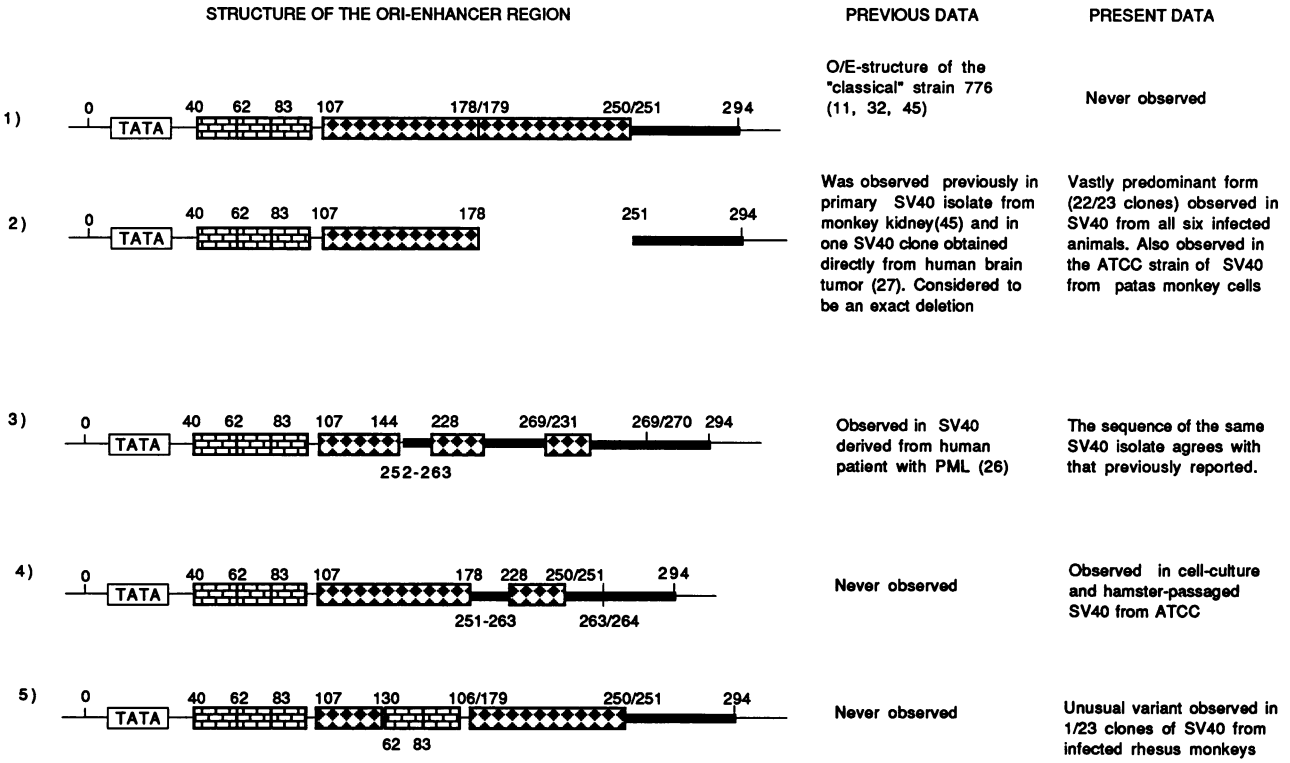

FIG. 3. Schematic structure of ori-enhancer (O/E) region in different strains of SV40. Five types of structures, including the classic structure of strain 776 (line 1), are presented. The data relating to each structure are summarized at the right. The fragment of SV40 sequence from the 0 point to the *Kpn*I site (nt 294) is shown. Strain 776 nucleotide numbering is used. Shown are TATA boxes, 21- to 22-bp repeats (brick boxes), and 72-bp repeats (diamond boxes). The region between nt 251 and 294 is shown as a bold line; other nonenhancer sequences are shown as thin lines.

Mm54-83 brain SV40 (Fig. 5). The vast majority of amplified sequences from this region of Mm54-83 brain SV40 DNA showed the presence of this *Bsa*BI site (Fig. 7A), indicating that the unique variant sequence predominated in this sample. Thus, while kidney-derived SV40 shows an invariant sequence in this region of T-ag, variant sequences predominated in two of three brain-derived SV40s.

To obtain further evidence for the presence of variant sequences in brain-derived SV40, we attempted to isolate SV40 from the kidney of animals with PML from which we had already isolated SV40 from brain. While the kidney of Mm54-83 showed no obvious pathologic lesions, we succeeded in isolating SV40 from this kidney. PCR amplification and *Bsa*BI treatment as described above were used to show that the majority of SV40 DNA derived from Mm54-83 kidney lacked the *Bsa*BI site (Fig. 7B). The product of PCR amplification was also sequenced in the region of interest. Its sequence was indistinguishable from those of other kidney-derived SV40 strains. Thus, SV40 DNA from Mm54-83 kidney was like all other kidney-derived SV40 DNAs in this regard, but it differed from SV40 DNA derived from the brain of the same animal.

The sequences of the two PML SV40s obtained from ATCC were identical to the sequence of A2895 SV40 from ATCC over the T-ag region (Fig. 5 and 6). The sequence of PA-57 closely resembled that of Mm54-83 brain clone 2, with the following changes: A→G (nucleotide [nt] 2818), C→T (nt 2861), and T→C (nt 2895), all silent, and A→T (nt 2907, Asp→Glu) and C→T (nt 2951, Val→Ile).

DISCUSSION

All six polyomavirus isolates described in this study were clearly SV40. No evidence was obtained for the presence of related but distinct polyomaviruses in macaque monkeys, analogous to the situation with JCV and BKV in humans. SV40 appears to be an opportunistic pathogen in the setting of SIV-induced immunodeficiency. In this sense, SV40 is similar to JCV and BKV, which have been observed as opportunistic pathogens in immunodeficient humans (2, 14, 17, 30, 33, 40).

The major form of the ori-enhancer region of all six SV40 isolates described in this study was distinctly different from that of laboratory strain 776 in that it had a single 72-bp enhancer element. We did not observe a single clone of the 23 analyzed with two 72-bp elements as are present in strain 776. A single 72-bp enhancer element in SV40 was previously observed twice, in SV40 present in a human brain tumor (27) and in one of the original SV40 sequence determinations (45); on both occasions, the difference with strain 776 was described as a deletion. Our data now strongly suggest that the enhancer sequences present in SV40 laboratory strain 776 are not representative of natural SV40 isolates. It seems likely that a duplication arose in strain 776 upon extensive cell culture passage, but we were unable to obtain early passage stocks of strain 776 to confirm this possibility. Remarkably, this situation seems to parallel duplication events in the JCV and BKV enhancers. Early reports indicated that these viruses had repetitive enhancer

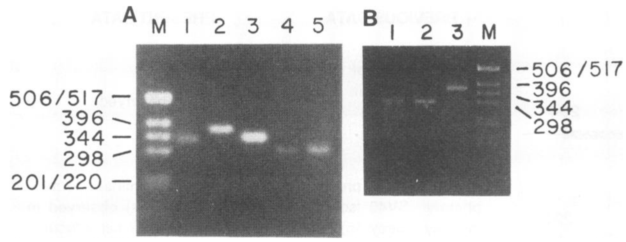

FIG. 4. (A) PCR amplification of the ori-enhancer regions in different strains of SV40. SV40 DNAs contained in Hirt supernatant of cells infected with ATCC strains A2895 (lane 1) and PML-1 (lane 3), newly isolated strains KID/246 (lane 4) and BR/91 (lane 5), and cloned DNA of SV40 strain 776 (lane 2) were used as templates. Primers 5160-5177 and 289-275 were used for amplification. Sizes of the amplified fragments are 336 bp (A2895), 372 bp (776), 341 bp (PML-1), and 300 bp (KID/246 and BR/91). (B) PCR amplification of the ori-enhancer regions from SV40-infected tissues. Total DNA isolated from paraffin-embedded SV40-infected tissues was amplified with two sets of primers (GG4915-4936 and 299-279 [external]; 5160-5177 and 289-275 [internal]) (nested PCR). Lanes: 1 and 2, nested PCR amplification products from the brain of Mm124-79 and from the kidney of Mm246-88, respectively (size of amplified fragments is 300 bp); 3, PCR amplification of SV40 strain 776 cloned DNA with primers 5160-5177 and 289-275 (size of amplified fragment is 372 bp). Lanes M, size marker (positions indicated in nucleotides).

elements (12, 24, 34), but more recent data indicate that fresh isolates contain a single such element (25, 35, 44) which begins to change during cultivation in vitro (35).

A striking feature of the opportunistic SV40 infections described in this report is the tissue-specific nature of active virus replication observed upon pathologic examination. Three of the cases were limited to lesions in the brain, and three were largely confined to kidney. The materials and reagents generated in this study have allowed preliminary investigation of the genetic determinants of SV40 tissue-specific replication. Sequence differences in the ori-enhancer region are apparently not responsible for tissue-specific SV40 replication, since these sequences were found to be virtually identical in both brain- and kidney-derived SV40. However, considerable sequence variation was observed at the carboxy end of T-ag that might be responsible, at least in part, for the SV40 tissue-specific replication. All fresh isolates from kidney had the same sequence in this region, but two of the three isolates from brain contained primarily variant sequences. Much of the variation appeared to arise from deletions or duplications in regions of short direct repeats. Additional support for the notion that these variant sequences contribute to SV40's ability to replicate in the brain comes from studies in cell culture. Deletion mutations in this region were previously found to influence the host cell range in which SV40 would replicate in cell culture (22, 31, 41). More experiments are needed, however, to demonstrate whether the observed sequence variations indeed influence SV40's ability to replicate in the brain.

The carboxy end of T-ag is also highly variable in an evolutionary sense, since this region shows considerable divergence in JCV and BKV (12). Furthermore, sequence variation between different isolates of JCV and of BKV is confined to the same region (24, 43).

How might sequence changes at the carboxy end of T-ag influence SV40's capacity to replicate in the brain? We of course can only speculate at this time, but one of the myriad functions of SV40 T-ag is to orchestrate the directional

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3000                                                                                    2912
   GTTTAGTTTGTCAGTGTATCAAAAAATGAAGTTTAATGTGGCTATGGGAATTGGAGTTTTAGATTGGCTAAGAAACAGTGATGATGATGA
A) .................................................C........................T.....A........
B) .................................................C..............................A........
C) .................................................C..............................A........
D) .......................G.................................................................
E) .................................................C..........A............................

2911<BbsI>                                                                                 2821
   TGAAGACAGCCAGGAAAATGCTGATAAAAATGAAGATGGTGGGGAGAAGAACATGGAAGACTCAGGGCATGAAACAGGCATTGATTCACA
A) ...........................................................................................
B) ...........................................................................................
C) ...........................................................................................
D) ...............G...............................A...........................................
E) ------------...............................................................................

2820                                                           <-BsaBI-->                 2740
   GTCCCAAGGCTCATTTCAGGCCCCTCAGTCCTCACAGT---------CTGTTCATGATCATAATCAGCCATACCACATTTGTAGAGGTTT
A) ...T........................C.........CCTCACAGT.........------.........T.................
B) ............................C.........CCTCACAGT.........------.........T.....C............
C) ............................C.........---------.........------.........T.................
D) ............................C.........CCTCACAGT.........................T.....C............
E) ............................C.........CCTCACAGT.........------.........T.....C............

2739                                            2691
   TACTTGCTTTAAAAAACCTCCCACACCTCCCCCTGAACCTGAAACATAA   Strain 776
A) .................................................   KID/246,155,261(cl.1-4), BR/54(cl.1)
B) .................................................   BRAIN/124(clones 1-4)
C) .................................................   BRAIN/91(clones 1-4)
D) .................................................   BRAIN/54(clone 2)
E) .................A..------.......................   ATCC-SV40(cl.1-4), PML/SV40(cl.1-4)
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FIG. 5. Alignment of T-ag carboxy-terminal coding nucleotide sequences of different SV40 strains. Top line, strain 776 reference sequence. Nucleotide numbers are shown above the sequence; A, sequence of isolates KID/246, KID/155, KID/261, and BR/54 (clone [cl] 1); B, sequence of isolate BR/124; C, sequence of isolate BR/91; D, sequence of isolate BR/54 (clone 2); E, sequence of ATCC strains of SV40 and of those obtained from humans with PML. Numbers of clones sequenced for each strain are shown in parentheses. Direct repeats possibly related to either insertions or deletions are underlined. Bold letters signify nonsynonymous nucleotide substitutions. The sites of *Bsa*BI and *Bbs*I cleavage are also shown. Dots indicate identity in sequence, and dashes indicate deletions.

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                               623             *                         o o  o 675
        a/acids 605-622        IGVLDWLRNSDDDDEDSQENADKNEDGGEKNMEDSGHETGIDSQSQGSFQAPQ
A/B)        -- " --            T..........N.........................................
C)          -- " --            T..........N.........................................
D)          -- " --            .....................................................
E)          -- " --            T...N.........----...................................

      * *                      *      708
     SSQS---VHDHNQPYHICRGFTCFKKPPTPPPEPET  Strain 776
A/B) P...SQS...--.......................  KID/(246,155,261), BR/(124,54(1))
C)   P...---..--........................  BR/91
D)   P...SQS............................  BR/54(2)
E)   P...SQS....................--......  ATCC-SV40, PML-SV40
```

FIG. 6. Alignment of deduced protein sequences of T-ag carboxy termini in different strains of SV40. The carboxy-terminal region representing amino acids 605 to 708 is shown. Top line, reference amino acid sequence of strain 776. The ATCC strain of SV40 is A2895; PML-derived SV40s were also obtained from ATCC. The identified phosphorylation sites are shown by asterisks; those found so far only in T-ag overexpressed in insect cells are shown by circles (5, 9, 39). Dots indicate identity in sequence, and dashes indicate deletions.

switch from early to late transcription during the course of lytic infection. T-ag likely needs to interact intimately with host cell transcription factors in this process (13), and the complement of transcription factors is known to vary in a cell-type- and tissue-specific fashion (29). It is interesting in this regard that the deletion and insertion variations observed in this study are likely to affect known sites of T-ag phosphorylation (5, 9, 39) (Fig. 6). Recently, mutations in these sites were shown to affect T-ag binding to the ori-enhancer region and to diminish SV40 transformation capacities (39).

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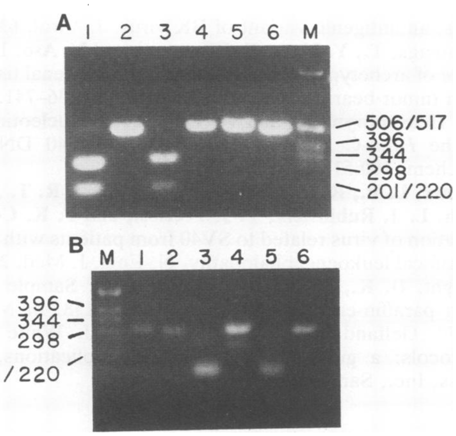

FIG. 7. PCR amplification of T-ag carboxy-terminal regions in different SV40 strains and subsequent digestion of the products with *Bsa*BI. After amplification, *Bsa*BI was added to the reaction mixture for 2 h (37°C). (A) SV40 DNA contained in Hirt supernatants of cells infected with strains BR/54 (lanes 3 and 4) and BR/91 (lanes 5 and 6) and cloned strain 776 DNA (lanes 1 and 2) were used as templates. Primers GGT2573-2595 and GGT3069-3049 were used for amplification. Lanes 1, 3, and 5, treated with *Bsa*BI; lanes 2, 4, and 6, not treated. (B) SV40 DNA contained in Hirt supernatants of cells infected with strains from kidney (lanes 1 and 2) and brain (lanes 3 and 4) of Mm54-83 and cloned strain 776 DNA (lanes 5 and 6) were used as templates. Primers 2621-2638 and 2902-2885 were used for amplification. Lanes 1, 3, and 5, treated with *Bsa*BI; lanes 2, 4, and 6, not treated. Lanes M, sizes standards (positions indicated in nucleotides.)

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