Journal of NeuroVirology (1998) 4, 394–406

http://www.jneurovirol.com

Identification of SV40 in brain, kidney and urine of healthy and SIV-infected rhesus monkeys

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Recent reports of simian virus 40 (SV40) sequences in human tumors have prompted investigations into the poorly understood association of this polyomavirus with its primate host, the rhesus monkey (*Macaca mulatta*). In the present study we have used PCR to analyze tissues from 20 monkeys for the presence of SV40. Five of the animals, which were infected with simian immunodeficiency virus (SIV), were found to exhibit SV40-induced lesions and to have SV40 sequences present in their kidney and brain. Lesions associated with SV40 were not observed in 15 SIV$^-$ monkeys, and SV40 DNA was detected in kidney and urine of only one of these animals. Three regions of SV40 DNA were examined in each tissue: the non-coding transcriptional control region (TCR), the sequences encoding the host range domain (HRD) within the carboxy-terminus of T antigen (TAg), and a portion of the VP1 gene. Each region contained nucleotide alterations compared to the SV40 reference strain 776. In all six animals, the TCR had an archetype structure containing a single 72 bp enhancer element. In addition, the TCR amplified from two animals lacked one of three copies of a GC-rich 21 bp repeat which is part of the promoter in strain 776. Multiple clones of unique HRD sequences were derived from different animals, and in some instances from the same animal. No correlation was found between a particular HRD sequence and its presence in a specific tissue type. Nucleotide changes identified within the VP1 gene indicate that this region, as with the closely-related human polyomavirus JCV, may permit the typing of the virus into individual strains. This study is the first to characterize SV40 sequences present in both healthy and SIV-infected animals and supports the suggestion that strain 776 is not the predominant type of SV40 circulating in its natural host.

Keywords: SV40 variants; archetype; strain typing; SIV; JC virus; PML

Introduction

SV40 was first discovered as a contaminant in polio vaccines administered to millions of people between the years 1954 and 1963 (Shah and Nathanson, 1976). Following the report that SV40 was oncogenic in rodents (Eddy *et al*, 1962; Girardi *et al*, 1962), studies were conducted to determine if this accidental human exposure had pathogenic consequences. Epidemiological analyses have failed to rule out the possibility that the incidence of human cancer was influenced by this event (Heinonen *et al*, 1973; Shah and Nathanson, 1976; Geissler, 1990; Strickler *et al*, 1998) and recent PCR-based experiments have forced this question to be reexamined (Carbone *et al*, 1994, 1996, 1997; Lednicky *et al*, 1995a; Martini *et al*, 1996). In addition, SV40 has, on rare occasions, been associated with progressive multifocal leukoencephalopathy (PML), a fatal demyelinating brain disease most often seen in AIDS patients and caused by the closely related human virus, JCV (Frisque and White, 1992; Tornatore *et al*, 1994).

SV40 has been extensively studied in the laboratory because of its potential importance to human disease and its use as a model system to investigate viral gene regulation and transforming mechanisms in mammalian cells. Much of this work has relied upon a limited number of viral strains adapted to grow in cultured cells. Few studies have examined SV40 isolated directly from its natural

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Received 25 February 1998; revised 30 March 1998; accepted 31 March 1998

host, the rhesus monkey, and most of this work has focused upon animals co-infected with simian immunodeficiency virus (Gribble *et al*, 1975; Holmberg *et al*, 1977; Sheffield *et al*, 1980; Horvath *et al*, 1992; Ilyinskii *et al*, 1992; Hurley *et al*, 1997). In these immunocompromised animals, SV40 infection may lead to the development of PML. In addition, SV40 may cause other lesions, including renal tubular necrosis, interstitial pneumonia, and meningoencephalitis (Sheffield *et al*, 1980; Horvath *et al*, 1992; Ilyinskii *et al*, 1992).

The SV40 and JCV genomes are organized similarly and share 69% DNA sequence homology (Frisque *et al*, 1984). Extensive sequence analysis has been conducted on three regions of individual SV40 and JCV genomes (Martin *et al*, 1985; Yogo *et al*, 1990; Ault and Stoner, 1992; Ilyinskii *et al*, 1992; Stoner and Ryschkewitsch, 1995; Agostini *et al*, 1997; Lednicky and Butel, 1997). The first region, called the transcriptional control region (TCR), contains the origin of DNA replication and the promoters and enhancer for transcription. In JCV, the TCR has been found in two forms: archetype and rearranged type. Archetype JCV is found primarily in the kidney and excreted in the urine, and it is thought to represent the form that circulates in the human population (Yogo *et al*, 1990; Markowitz *et al*, 1991). Rearranged types of JCV are variants which appear to arise in an individual by the deletion and duplication of sequences within the archetype TCR (Yogo *et al*, 1990; Markowitz *et al*, 1991; White *et al*, 1992; Ault and Stoner, 1993; Iida *et al*, 1993; Agostini *et al*, 1997). These rearrangements may influence the virus' tissue distribution and replication potential. Rearranged forms have been detected throughout the body, especially in diseased tissues of PML patients (White *et al*, 1992; Newman and Frisque, 1997), and only these variants have been shown to replicate in cell culture (Daniel *et al*, 1996).

Archetype and rearranged forms of SV40 also exist, but unlike JCV, rearranged SV40 variants appear primarily during propagation in cell culture (Ilyinskii *et al*, 1992; Lednicky and Butel, 1997). With one exception, SV40 detected in monkey brain and kidney tissue has been the archetype form. In their analysis of SV40 in five SIV-infected rhesus monkeys, Ilyinskii *et al* (1992) obtained 22 archetype TCR clones containing one copy of a 72 base pair (bp) enhancer sequence. Only a single rearranged variant, having a partial duplication of the 72 bp enhancer and a duplication of two of the three 21 bp promoter elements, was found. Both forms could be propagated in cell culture. Strain 776, one of the most extensively studied SV40 variants, is a rearranged type which contains two copies of the 72 bp enhancer. This rearrangement has not been observed in sequences amplified directly from an animal, although it is possible that a variant with a duplicated enhancer is present as a minor species *in vivo*. Alternatively, duplications like those observed in 776 may be generated during passage in cell culture (Lednicky and Butel, 1997; Lednicky *et al*, 1998).

Sequencing efforts have also been directed towards the portion of the SV40 genome which encodes the carboxy-terminal region of the large T protein (TAg). These sequences include the TAg host range domain (HRD), a region which influences the ability of SV40 to grow in certain cell lines (Manos and Gluzman, 1985; Pipas, 1985; Cole *et al*, 1986). Variation within the SV40 TAg HRD has been noted when comparing sequences obtained from the brain of either single or multiple animals, whereas HRD sequences from the kidneys of different animals have been found to be identical (Ilyinskii *et al*, 1992). The role that these sequences may play in the establishment of an SV40 infection in its host has not been determined.

The third region of the JCV and SV40 genomes to receive recent attention is a portion of the VP1 gene which appears to encode important epitopes of the major capsid protein (Jin *et al*, 1993; Chang *et al*, 1996). Single nucleotide changes within the JCV gene have been used for strain typing and analyzing viral distribution in the human population (Agostini *et al*, 1996a,b; Sugimoto *et al*, 1997). Attempts to use the SV40 VP1 sequence for similar purposes have not been reported.

In the present study we have employed PCR technology to examine the SV40 TCR, TAg HRD, and TAg and VP1 sequences present within one immunocompetent and five SIV-infected rhesus monkeys. Sequence variations were detected in all three of these genomic regions; some of these changes were conserved in different animals and some in different tissues from the same animal.

Results

JCV can be detected in most adults, regardless of their immune status (Frisque and White, 1992; Tornatore *et al*, 1994). The archetype TCR is readily amplified from kidney tissue and urine of individuals with and without PML, and archetype is thought to be the form which circulates in the population (Yogo *et al*, 1990; Flægstad *et al*, 1991; White *et al*, 1992). Rearranged TCR sequences are generally recovered from normal and diseased brain tissue (White *et al*, 1992). Little work has focused upon the JCV TAg HRD sequences present in human isolates, but considerable attention has been given to the VP1 region where sequence variations have permitted identification of different viral strains. A recent SV40 study (Ilyinskii *et al*, 1992) has revealed the presence of the archetype TCR sequences in the brain and kidney of SIV-infected primates, although SV40 DNA amplified from both tissues of a single animal were not sequenced.

Variability in the TAg HRD sequences present in these animals was also noted. Similar studies describing the distribution and identity of SV40 in healthy rhesus monkeys have not been reported. In the present study, we have identified SV40 in a healthy monkey as well as in SIV-infected animals exhibiting SV40-induced lesions. In addition, we have amplified VP1 coding sequences in an attempt to identify different strains of SV40.

SV40 TCR, TAg HRD, and VP1 sequences detected in an immunocompetent rhesus monkey

Tissue samples from 15 healthy rhesus monkeys were examined using two sets of primers that permit amplification of either SV40 TCR or TAg HRD sequences. SV40 DNA was detected in only one animal, L778. TCR and TAg HRD sequences were amplified from the kidney and urine of this animal (Figures 1 and 2), but not from its brain. SV40 DNA could not be recovered from the kidneys of any of the 14 other immunocompetent monkeys, nor from urine and brain specimens obtained from a subset of these animals (six and four animals, respectively).

The TCR obtained from animal L778 represents the archetype sequence, and differs from that of strain 776 by the absence of one of the two copies of the 72 bp enhancer (Figure 1). This archetype strain has been detected previously in SIV-infected rhesus monkeys, human tumors and the ATCC strain Pa-57 (Martin and Li, 1991; Ilyinskii *et al*, 1992; Lednicky *et al*, 1995a, 1997a,b).

As was the case with the TCR clones, TAg coding sequences obtained from L778 kidney were identical to those found in urine (Figure 2, line 9). Unique changes were identified at nucleotide (nt) #2731 and #2668 of the SV40 TAg coding region. An A residue at the former site is either a C or T in other SV40 isolates, and a G residue at the latter site is an A residue in all other strains analyzed to date (Ilyinskii *et al*, 1992; Lednicky *et al*, 1995a, 1997a,b; Stewart *et al*, 1996; Hurley *et al*, 1997; Lednicky and Butel, 1997).

The two VP1 clones obtained from the urine of animal L778 contained three nucleotide alterations relative to the strain 776 sequence (Figure 4, line 4). These same changes were detected in the clones obtained from the five SIV-infected primates examined in this study. The substitution of a C for an A residue at nt #1756 changes amino acid #84 from a glutamic acid to an aspartic acid; the other changes do not alter the VP1 protein sequence.

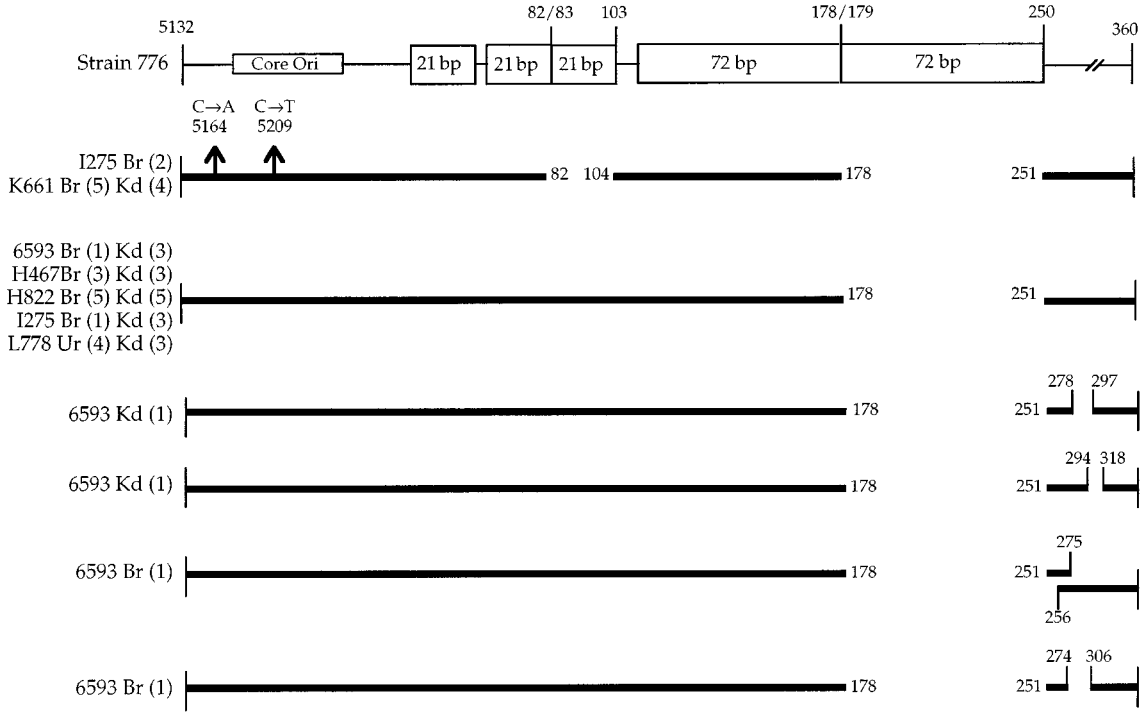

Figure 1 Structures of SV40 TCRs cloned from brain, kidney, and urine of six rhesus monkeys. Clones are named according to the animal (Table 1 for SIV^+ animals; L778 is the SIV^- animal) and tissue (Br=brain, Kd=kidney, Ur=urine) from which they are derived. The number in parentheses indicates the number of clones having the indicated sequence that were obtained from that tissue. Each sequence is represented by a line and is read left to right. A gap in the line indicates a deletion relative to strain 776. The short duplication in 6593 Br is denoted by parallel horizontal lines. Arrows point to the positions of nucleotide changes within a clone relative to strain 776. Nucleotide numbers are based upon the sequence of strain 776 (Buchman *et al*, 1981).

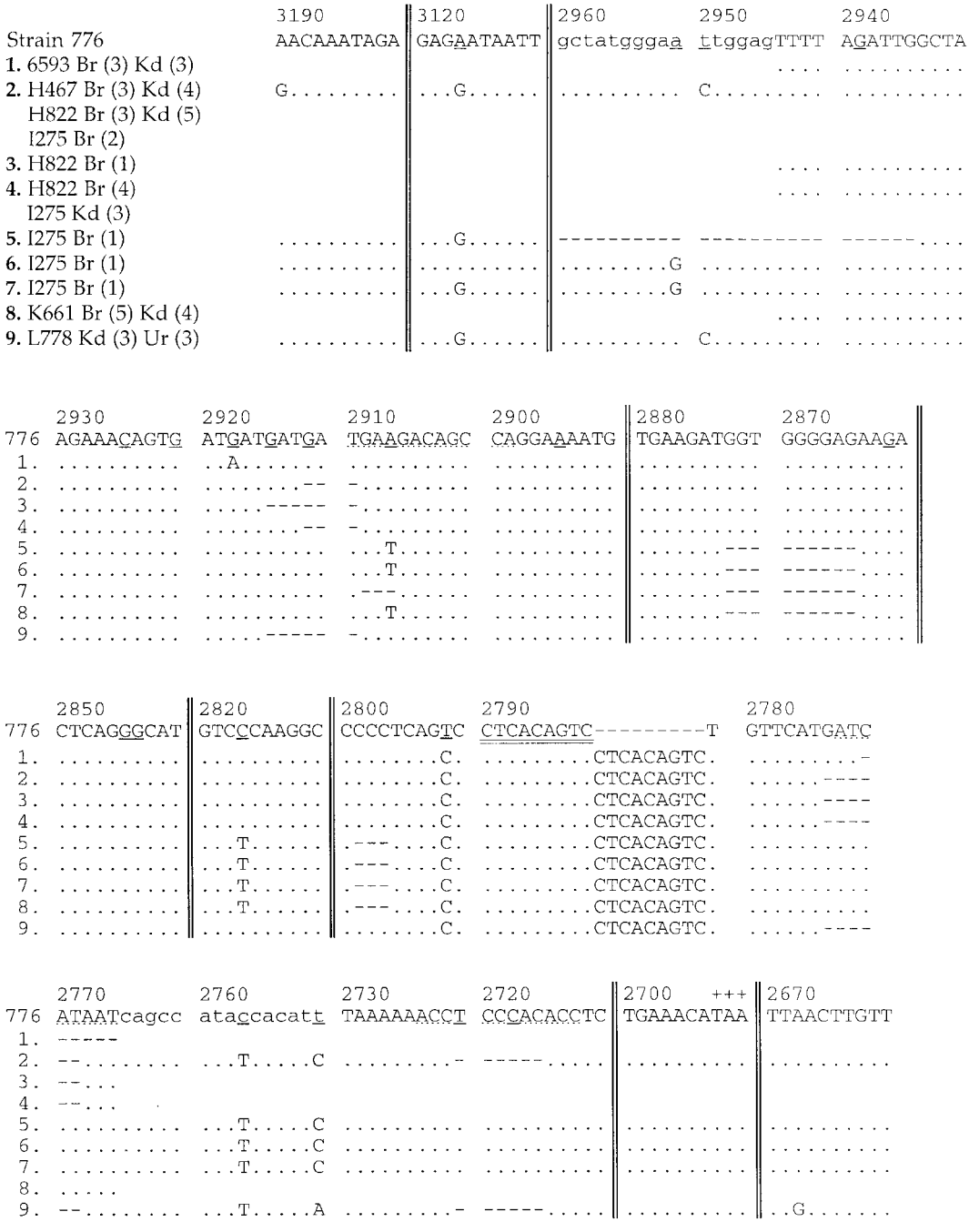

Figure 2 SV40 TAg carboxy-terminal coding sequences amplified from the brain and kidney of five SIV$^+$ monkeys and the kidney and urine of one immunocompetent animal. Underlined nucleotides in the strain 776 sequence indicate previously identified positions which are altered in some strains either by substitution (solid line), deletion (dotted line) or duplication/triplication (double lines) (Ilyinskii *et al*, 1992; Lednicky *et al*, 1995a,1997a,b; Stewart *et al*, 1996; Hurley *et al*, 1997; Lednicky and Butel, 1997). PCR primer sequences (SEP-C1.0, SEP-C2.0) are indicated by lower case letters. Clones obtained from tissues analyzed in this study are shown in lines numbered 1–9 and are named as described in the legend for Figure 1. Dots indicate identity with the strain 776 sequence, dashes indicate deletions. The stop codon for TAg is denoted by three plus signs (+). Regions represented by double vertical lines (nt #3180-3121, 3110-2961, 2890-2881, 2860-2851, 2840-2821, 2810-2801, 2710-2701, 2690-2671) have been omitted from the figure since they are identical in all but one of the clones we have sequenced. This exception is a single clone from I275 Br (line 5) in which nt #3052-2935 have been deleted. It is likely that this deletion represents a lethal mutation in the genome from which it was amplified. Clones shown in lines 2, 6 and 9 may be identical to clones shown in lines 4, 8 and 3, respectively. Alternatively, the latter set of clones may have the same sequence as 776 within the primer binding sites (SEP-C1.0 and SEP-C2.0), and thus they would differ from the first set of clones. The clones in the first set are longer than those in the second set due to the use of alternative primer pairs, but each pair of clones is identical in the regions sequenced.

SV40 TCR sequences detected in SIV-infected rhesus monkeys

Kidney and brain tissues from five SIV-infected rhesus monkeys were tested for the presence of SV40 DNA (Table 1). Three of these animals exhibited SV40-induced lesions in the brain, and two animals had lesions limited to the renal tubules. The brain lesions were distinct from those seen in cases of human and monkey PML (Gribble *et al*, 1975; Holmberg *et al*, 1977; Horvath *et al*, 1992) and were diagnosed as meningoencephalitis, a central nervous system manifestation which affects cerebral gray matter without demyelination.

SV40 TCR sequences were amplified from the kidney and brain of each SIV-infected animal, cloned, and sequenced (Figure 1). All TCRs had an archetype structure, with a single copy of the 72 bp enhancer. The clones obtained from the brain and kidney of animal K661, and two clones from the brain of animal I275, exhibited three differences relative to the TCRs of the other amplified DNAs and of strain 776. These differences included substitutions at nt #5164 and #5209 and a deletion of one of the 21 bp repeats within the promoter region. The two nucleotide polymorphisms have been reported before for SV40 strains isolated from cell culture, monkeys, and humans, although both changes do not always occur in the same variant TCR (Martin and Li, 1991; Stewart *et al*, 1996; Lednicky *et al*, 1997a). Deletion of the 21 bp repeat has not been previously documented.

A second archetype TCR was detected in the brain and kidney tissue of animal I275. These clones contain all three 21 bp repeats and lack the two nucleotide changes to the early side of the promoter-enhancer signals. This TCR sequence was also found in the brain and kidney tissues of animals H467, H822 and 6593 and represents the form found in the healthy monkey L778.

Amplification of the SV40 TCR from the brain and kidney specimens of animal 6593 yielded several related clones containing small duplications or deletions of sequences lying between the enhancer element and the initiation codon for the late protein, LP1. Similar alterations have been observed recently in JCV TCR sequences amplified from a pediatric PML patient (Newman and Frisque, 1997).

SV40 TAg HRD coding sequences detected in SIV-infected rhesus monkeys

H467, K661 and 6593 were the only SIV-infected monkeys examined in which a single SV40 HRD variant was found in both brain and kidney tissue (Figure 2, lines 1, 2 and 8). It should be noted that the sequence of each variant is unique to the individual animal. For example, the variant detected in animal K661 has a 9 bp deletion spanning nt#2873-2865, whereas the SV40 sequence amplified from animals 6593 and H467 is missing 9 bp at nt#2771-2766. The deletion of nt#2873-2865, which was also observed in three clones obtained from the brain of animal I275, had not been noted prior to this study.

PCR primers SEP-C1.0 and SEP-C2.0 were used to amplify SV40 TAg sequences from animals 6593, H822, I275 and K661 (Figure 2, lines 1, 3, 4 and 8). SV40 TAg HRD sequences could not be amplified with these primers from the kidneys of animals H467 and H822 and brain of I275, due to alterations affecting the primer binding sites. This same problem was encountered in the analysis of the kidney and urine of the healthy monkey, L778. Alternate primers were employed, and these

Table 1 SIV-infected rhesus monkeys exhibiting SV40-induced lesions

Animal[a]	*Sex*	*Age (years)*	*Location of SV40 lesions*	*Other diagnoses*
6593	F	15.2	Cerebrum (encephalitis thought to be SV40-induced)	Focal lymphomas in kidney
H467	F	3.3	Renal tubule	Giant Cell disease, anorexia, wasting, Giardiasis, retroviral encephalitis in brain, retroviral pneumonia, Cryptosporidia
H822	M	2.9	Cerebrum, brain stem	Diarrhea, wasting, CNS signs, dilated, nonresponsive pupils, Trichomonas infection, Candidiasis
I275	F	2.3	Renal tubule	Giant Cell disease; minimal retroviral encephalitis in brain, Candidiasis, retroviral pneumonia, retroviral myositis, diarrhea, possible Cryptosporidia
K661	F	2.7	Brain, interstitial tissue of lung, renal tubule, thymus, small intestinal muscularis propria, possibly myenterix plexus	Weakness, anorexia, diarrhea, Cryptosporidia, Candidiasis

[a] Individual rhesus monkeys (*M. mulatta*) are identified by a letter and/or a number

yielded larger amplification products (Figure 2, lines 2, 5–7 and 9). Analysis of this additional sequence identified new sites of variation at nt #3190 (Figure 2, line 2) and #2668 (Figure 2, line 9) of the TAg coding region. In addition, the use of alternate sets of primers revealed the presence of multiple TAg variants within animals H822 and I275, and some of these DNAs were localized to a single tissue (kidney or brain) (Figure 2, line 2 *vs* 3, 4). Alternate primers amplified four SV40 variants (Figure 2, lines 2, 5–7) from the I275 brain specimen. Differences between some of these variants were slight, consisting of single nucleotide substitutions and small deletions (compare lines 6 and 7, Figure 2). Other changes were more extensive, such as the deletion of 119 bp (nt #3052-2935) in one of the clones (Figure 2, line 5) which likely represents a lethal mutation. Similar observations were made upon examination of the tissues from animal H822. Primers SEP-C1.0 and SEP-C2.0 yielded two nearly identical SV40 clones from this animal's brain (Figure 2, lines 3, 4), but none from its kidney. Using the alternate primers, a third clone was derived from the kidney and brain of this monkey (Figure 2, line 2). Partial TAg amino acid sequence based upon the nucleotide sequence data is presented for each clone in Figure 3.

SV40 VP1 coding sequences detected in SIV-infected rhesus monkeys

JC virus has been grouped into four major genotypes on the basis of nucleotide changes within the VP1 coding sequences (Agostini *et al*, 1997). To determine if the corresponding region in the SV40 genome contains potential strain typing sites, DNA was amplified from the kidney tissue of the five SIV-infected primates and the urine of the one healthy animal using VP1-specific primers. A total of 21 clones was examined, and each DNA contained three nucleotide changes (nt #1756, 1939, 1951) relative to strain 776. In addition, a fourth nucleotide change was detected at one of three possible sites (nt #1831, 2044, 2098) in each clone derived from the SIV-infected animals. Only the change at nt #2098 was present in clones derived from more than one animal (Figure 4, line 2). Four distinct VP1 clones were derived from the six animals, and our analysis identified only a single unique VP1 clone in any one animal.

Discussion

PCR technology has given JCV investigators a sensitive tool for examining the distribution of viral

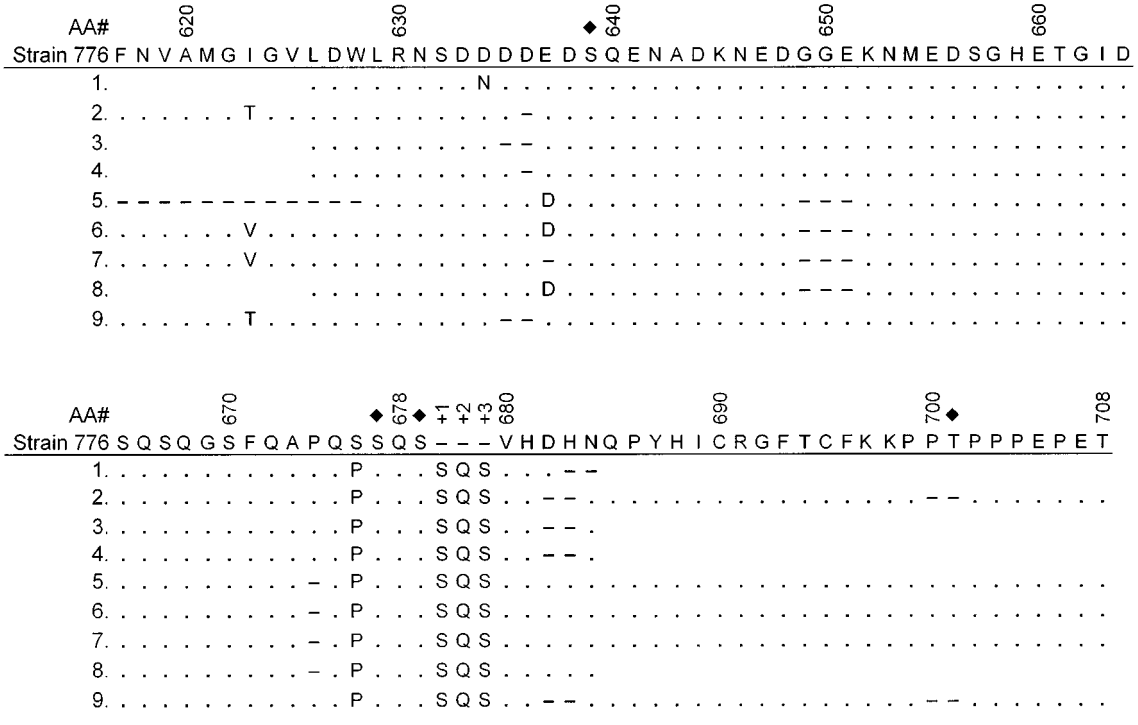

Figure 3 SV40 TAg carboxy-terminal amino acid sequences encoded by DNAs amplified from six rhesus monkeys. Lines 1–9 represent deduced amino acid sequences of cloned DNAs (Figure 2) for the region spanning nt #2970-2694. Dots indicate identity with the strain 776 sequence, dashes indicate deletions. The top two lines represent the amino acid (AA) sequence and numbering for strain 776. The three residues inserted between amino acids #679 and #680 are referred to as +1, +2, +3. Residues experimentally determined to be phosphorylated in the strain 776 TAg are indicated with a diamond (◆) (Fanning, 1992). As in Figure 2, the clones shown in lines 2, 6 and 9 share sequence identity with those shown in lines 4, 8 and 3, respectively, over the shared region amplified by the two different pairs of primers.

	1760	1840	1940	1960	2050	2100
Strain 776	AAAGAACAAC	GATGTGGGAA	AAACCCATTC	TTTTCATTTT	CCCAAAAAAT	CTGTTTTGGA
1. 6593 Kd (4)	.....C....	....	........A.	C.........	...C......	....
2. H467 Kd (3)	.....C....	....	........A.	C.........	....	.......A..
H822 Kd (2)						
I275 Br (3) Kd (3)						
3. K661 Kd (4)	.....C....	A.........	........A.	C.........	....	....
4. L778 Ur (2)	.....C....	....	........A.	C.........	....	....

Figure 4 VP1 coding sequences amplified from six rhesus monkeys. The top two lines show the sequence and numbering of the reference strain 776. Clones shown in lines 1–4 are named according to the system described in Figure 1. Dots indicate sequence identity with that of strain 776. Nucleotides #1597-2122 were amplified and only those 10 bp blocks of sequence containing an alteration are presented; double vertical lines indicate the breaks between these 10 bp blocks. Sequences within the amplified region, but omitted from the figure, match those of strain 776.

DNA in human tissues, for detecting genomic rearrangements which arise during viral replication *in vivo*, and for classifying viral strains which reside in different human populations. Although SV40 has been well characterized in cell culture systems, interactions with its monkey host *in vivo* have not been investigated in detail. It was concluded at a recent N.I.H. workshop entitled 'Simian Virus 40 (SV40): A Possible Human Polyomavirus' (Lewis and Egan, 1997) that this deficiency in our understanding of SV40 biology should be addressed.

Prior to initiating the present study, veterinary pathologists at the Tulane Regional Primate Research Center had conducted postmortem examinations of more than 1000 SIV^+ rhesus monkeys and identified 18 animals with lesions attributable to SV40. Tissues were obtained from five of these animals and all were found to contain SV40 DNA following PCR analysis. SV40-induced lesions have not been observed in any of the Center's SIV^- animals. We did expect to detect SV40 in several of these animals using PCR analysis. Instead, SV40 sequences were amplified from only one of 15 healthy SIV^- monkeys. It is possible that the virus is present in a greater portion of these animals, but at undetectable levels or in tissues which were not tested. JCV, which infects nearly 90% of the human population, may disseminate through the body via lymphocytes (Gallia *et al*, 1997). The possibility that these cells might also serve as a site of SV40 persistence in immunocompetent monkeys is supported by recent PCR analysis of peripheral blood mononuclear cells Lednicky and coworkers (1998). These investigators have also detected anti-SV40 antibodies in six of seven rhesus monkeys from the Tulane Regional Primate Research Center, indicating that SV40 is circulating in this population prior to the animals' exposure to SIV.

Extensive rearrangements within the viral TCR were not observed in any of the animals examined here. Archetype SV40 was amplified from all six animals, including diseased and normal brain and kidney tissue. The TCR sequences amplified from five of the monkeys matched those of strain 776 except for the absence of a second copy of the 72 bp enhancer. In two of these five animals, I275 and 6593, additional TCR variants were detected. The only SIV^+ animal which lacked the common TCR sequence in both kidney and brain was K661. The modified archetype form amplified from the kidney and brain of K661, and the brain of I275, lacked one of the three copies of the 21 bp promoter element. This is the first time naturally occurring SV40 isolates have been found lacking this sequence. Mutants constructed *in vitro* which lack one copy of the 21 bp element remain viable in cell culture (Lednicky *et al*, 1995b).

The presence of archetype SV40 in all of the infected monkey tissues indicates that rearrangement of its TCR is not required to cause disease in the animal. This is in apparent contrast to what is seen with JCV. Two forms of JCV, archetype and rearranged, are found in human tissues, with the former present in kidney and urine, and the latter predominating in diseased brain (Yogo *et al*, 1990; Markowitz *et al*, 1991; Newman and Frisque, 1997). It has been suggested that this rearrangement process generates JCV variants with enhanced replication and pathogenic potential *in vivo* (Loeber and Dörries, 1988; Yogo *et al*, 1991; White *et al*, 1992). Similar differences between the two viruses are observed when their replication activities are compared *in vitro*. Only the rearranged form of JCV will replicate in cultured cells (Daniel *et al*, 1996), whereas archetype SV40, like the rearranged 776 variant, can be propagated in a variety of cells in culture (Lednicky *et al*, 1995b; Lednicky and Butel, 1997).

SV40 TAg sequences were amplified from kidney and brain of the 5 SIV-infected animals and from the kidney and urine of the immunocompetent animal. Unlike amplified TCR sequences which exhibited little variation between tissues or between individual animals, TAg sequence variability was readily detected. However, no correlation could be made between a specific TAg sequence alteration and the tissue from which it was retrieved, and therefore we

did not conclude that changes in the TAg HRD sequences altered the tissue specificity of SV40 *in vivo*. As more examples of sequence variation become available, it is possible that some of the single nucleotide changes observed in the TAg HRD will prove useful in identifying different strains of SV40.

Sequences of all of the SV40 TAg clones (total of 49) derived in this study are altered at two positions relative to the strain 776 sequence. The first change involves a single nucleotide substitution (T to C) at position #2792. This particular nucleotide difference has been documented in nearly every SV40 isolate obtained from humans, primates, and cultured cells (Ilyinskii *et al*, 1992; Lednicky *et al*, 1995a, 1997b; Stewart *et al*, 1996; Hurley *et al*, 1997; Lednicky and Butel, 1997). The second change involves nucleotides at position #2790-2782. There are several ways to align the sequences between position #2800-2781 due to short polynucleotide repeats within this region. By shifting the boundaries of deletions and insertions in the sequence of clones published earlier, these repeats become apparent and some DNAs, once thought to differ from each other in this region, are now found to be identical (Figure 5). Realignment of these DNA sequences, as shown in Figure 5, reveals some of these identities and clearly illustrates the presence of one, two or three copies of nt #2790-2782.

The TAg of SV40 strain 776 has been extensively characterized by a large number of laboratories. It is possible that TAgs encoded by some of the DNAs amplified in this study would exhibit functional differences with the 776 protein due to sequence alterations. For example, phosphorylation of multiple threonine and serine residues in TAg has been identified and some of these modifications are known to influence TAg function (Scheidtmann *et al*, 1984; Mohr *et al*, 1987; Prives, 1990; Chen *et al*, 1991; Scheidtmann *et al*, 1991; Cegielska *et al*, 1994). The threonine at position 701 is phosphorylated in the 776 TAg, and this residue is absent in several of the clones sequenced in this study (Figure 3, lines 2 and 9). In addition, new sites of phosphorylation might occur in the TAgs encoded by the clones derived here. A 9 bp insertion in these DNAs would specify three additional amino acids, including two serines, between residues 679 and 680 (Figure 3; +1, +3). These serines might be phosphorylated since they are part of the same consensus sequence recognized by the kinase(s) which modifies serines 677 and 679 of the strain 776 TAg (Hathaway and Traugh, 1982; Scheidtmann *et al*, 1984; Chen *et al*, 1991).

VP1 coding sequences amplified from the six animals examined in this study differed at a limited number of sites from those of strain 776 (Figure 4). Within the same animal, multiple clones of this sequence were identical, even though variations had readily been detected in the TCR and TAg HRD sequences. We had anticipated that two VP1 sequences might be amplified from animal I275, since both the common and modified archetype TCRs had been found in this animal, suggesting a dual infection. Furthermore, we predicted that one of the VP1 clones would match that obtained from animal K661. This latter monkey was the only other animal from which the modified archetype TCR had been obtained. However, only a single VP1 sequence was amplified from the kidney and brain of monkey I275 and it differed from that found in animal K661.

Additional SV40 VP1 sequences will have to be amplified from a greater number of animals before specific sites of variation can be identified for developing a strain typing scheme such as the one being used for JCV (Agostini *et al*, 1996b). The monkeys housed at the Tulane Regional Primate Research Center have been obtained from several sources and one might predict that they have been exposed to different strains of SV40. It is possible that some of the VP1 sequence variability already noted here reflects the existence of multiple strains within the animals at this facility.

The sequence data presented in this study lends further support to the suggestion that SV40 strain 776 is not representative of the virus found in rhesus monkeys. The form of SV40, whether it be archetype and/or rearranged type, to which millions of people were accidentally exposed during the early days of the poliovirus and adenovirus vaccination programs, remains unknown.

	2793	2779
1. Strain 776	GTC<u>CTCACAGTC</u>----------TGT	
2. Present Study	G**C**CCTCACAGTCCTCACAGTC---------TGT	
3. SV40-B2	G**C**CCTCACAGTCCTCACAGTC---------TGT	
4. Gang. 13	G**C**CCTCACAGTCCTCACAGTC---------TGT	
5. Ost. 2	G**C**CCTCACAG**C**CCTCACAGTCCTCACAGTCTGT	
6. Ost. 9	G**C**CCTCACAGTCCTCACAGTCCTCACAGTCTGT	

Figure 5 Alignment of SV40 sequences obtained from monkeys and humans showing duplication or triplication of a nine nucleotide sequence within the TAg coding region. Line 1 shows nt #2793-2779 of SV40 strain 776. The motif which is repeated in some DNAs is underlined. Sequences from monkeys in the present study are diagrammed in line 2. Sequences of SV40 Baylor strain (SV40-B2), ganglioneuroma 13 (Gang. 13), and osteosarcomas (Ost.) 2 and 9 have been publsihed previously (Lednicky *et al*, 1995a, 1997b). Nucleotides that differ from the strain 776 sequence are shown in bold.

Materials and methods

Rhesus monkey tissue and urine samples

The tissues analyzed in this study were collected at the Tulane Regional Primate Research Center. Included in this investigation were brain, kidney, and urine specimens from 15 immunocompetent

rhesus monkeys (*Macaca mulatta*), and brain and kidney samples from five SIV-infected animals (Table 1). All specimens were received frozen and stored at −80°C in individual vials; specimens from immunocompetent and SIV-infected animals were collected, stored and handled separately.

Prior to PCR amplification, tissue fragments measuring approximately 8 mm^3 were digested in 100 μl tissue digestion buffer (White *et al*, 1992). Each sample was vortexed, incubated at 56°C for 90 min, and then heated to 95°C for 10 min to inactivate the proteinase K in the buffer. The digested samples were centrifuged and the supernatant was subjected to PCR analysis.

Crude urine was dispensed into 1 ml aliquots for testing. Urine samples were processed as described by Agostini *et al* (1996a). The urine was spun at 16000 g for 10 min and the pellets washed with PBS before suspending in 50 μl of tissue digestion buffer (Agostini *et al*, 1996b). The digestion and subsequent analysis of the urine-derived pellets were conducted as described above, except that the proteinase K inactivation step was conducted at 100°C.

PCR amplification

Three sets of PCR primers capable of amplifying the SV40 TCR (nt #5132-360), the TAg HRD coding region (nt #2944-2766), or a portion of the VP1 gene (nt #1597-2122) were used to detect SV40 sequences in the monkey specimens. Oligonucleotides used as primers for PCR or for subsequent sequencing are listed in Table 2.

To minimize the potential for contamination, several precautions were taken. These included setting up reactions in areas separate from that used to prepare tissues and employing single use aliquots of reagents, positive displacement pipettors (Gilson, Villiers-le-Bel France), and multiple negative controls. Before preparing the tissues for PCR analysis,

Table 2 Nucleotide sequence of PCR and sequencing primers

Primer	*Sequence 5′→3′*	*Nucleotide positions*	*Primer use*[a]
SEP-C1.0	CCTCTACAAATGTGGTATGGCTG	2743–2765[b]	P/S
SEP-C2.0	GTGGCTATGGGAATTGGAG	2963–2945[b]	P/S
SEP-C3.0	GACTCAGGGCATGAAACAGGC	2852–2832[b]	S
SEP-C4.0	CCATGAATGAGTACAGTGTGCC	3238–3217[b]	P/N/S
SEP-C5.0	GAGCTGCAGGGTGTGTTAGC	1985–2004[b]	P
SEP-C6.0	GTAGAGGAGGTTAGGG	2489–2504[b]	S
SEP-C7.0	CCTGGAACGCAGTG	3150–3137[b]	S
SEP-C8.0	CCTCAGTCCTCACAGTCT	2798–2781[b]	S
SRP-1.0	GCTGCAAAGATTCCTCTCTG	5127–5146[b]	P
SRP-2.0	GACCTACGAACCTTAACGGAG	384–364[b]	P
SRP-3.0[C]	CTGAGGCCGAAAGAACC	291–275[b]	S
SRP-4.0	GCCTCCTCACTACTTC	5204–5219[b]	S
SRP-5.0	GACCTAGAAGGTCCATTAGCTGC	5109–5131[b]	P/N
SRP-6.0	CTACGAACCTTAACGGAGGCC	381–361[b]	P/N
SRP-7.0	CCTCAGAAGGTACCTAACC	286–304[b]	S
SRP-8.0	CTTCATCTCCTCCTTTATCAGG	5016–5037[b]	N
SRP-10.0	CAGACACAGTAGCAATTAGGTC	610–589[b]	N
SV923E	ACAGCCATTCCTGGTTGTTG	923–904[b]	N
SV1232L	TTAGGCCTACAATGGTGAGA	1232–1251[b]	N
SV1576L	GCCAAAGCTCGTCATAAAAGG	1576–1596[b]	P/N
SV2142E	GGATCAGGAACCCAGCACTC	2142–2123[b]	P/N
SV2386L	GTCTGTGAAAAACCCCTACCC	2386–2406[b]	P/N
SV2369E	TTTTAAAATATCTGGGAAGTC	2369–2349[b]	N
SV2721E	TCCCACACCTCCCCCTGAACC	2721–2701[b]	S
SV3126L	TAACAAAAACTCACTGCGTTCC	3126–3147[b]	S
SV3628E	TGTTGCCCAAAATGGATTCAG	3628–3608[b]	P/N
SV4852L	TGCACACTCAGGCCATTGTT	4852–4871[b]	N
SVBam1	GGGGATCCAGACATGATAAGATACA	2531–2555[b]	N
Rhesus-1	GGTAACTGTCAAGTCACCAAAC	4900–4879[d]	P
Rhesus-2	GCCTTCCTCCTTATTGCCTAC	4665–4765[d]	P
M13 Rev	CAGGAAACAGCTATGACC	205–222[e]	S
T7 Promoter	TAATACGACTCACTATAGGG	365–346[e]	S
PCRII#1	GATCCACTAGTAACG	253–267[e]	S
PCRII#2	GTGATGGATATCTGC	304–290[e]	S

[a] P=PCR, N=Nested PCR, S=Sequencing.
[b] Numbering system for SV40 776 is from Buchman *et al* (1981).
[c] The double underlined nucleotide (nt# 284) is a G in strain 776.
[d] Numbering system for the rhesus internal control primers is from Golos *et al* (1991).
[e] Numbering system for vector pCR2.1 is from the Original TA Cloning Kit Manual (Invitrogen)

working surfaces were treated with 10% bleach and UV irradiation. To further minimize the potential for cross-contamination of samples, disposable forceps, scalpels, and tubes were used whenever feasible.

Reaction mixes (50 μl) were composed of assay buffer (FisherBiotech; 50 mM KCl, 10 mM Tris-HCl [pH 8.3]), 1.5 mM $MgCl_2$ (FisherBiotech), 2.5 U of Taq DNA polymerase (FisherBiotech), 200 μM each of dATP, dCTP, dGTP and dTTP (Pharmacia Biotech, Uppsala, Sweden) and 0.5 μM of each oligonucleotide primer. To enhance the sensitivity of primer pair SRP-8.0 and SRP-10.0, the concentration of SRP-8.0 was increased to 2.5 μM. Between 1 and 5 μl of a tissue digest was used in each analysis. In most experiments, reactions were overlaid with 40 μl mineral oil (Nujol Mineral Oil, Perkin Elmer). In some experiments, mineral oil and $MgCl_2$ were replaced with HotWax Mg^{2+} (Invitrogen, San Diego, CA) to increase sensitivity and reduce non-specific priming. Reactions were amplified through 40 cycles in either a Perkin-Elmer Cetus DNA thermal cycler or an MJ Research PTC-200 Peltier thermal cycler as previously described (White *et al*, 1992). We have not attempted to use fewer cycles to determine if SV40 would still be detectable in our samples. An initial preheating step of 2 min at 94°C was added to the cycling conditions when the HotWax Mg^{2+} beads were used. To monitor the sensitivity of the reaction conditions, control tubes were spiked with 10^1 to 10^6 input molecules of cloned SV40 DNA (pSV40, Haggerty *et al*, 1989). Amplification of 10^1 or 10^2 molecules of input DNA could be detected routinely using 40 cycles.

Three sets of primer pairs were originally employed to amplify SV40 sequences from brain, kidney, and urine. Primer pair SRP-C1.0 and SRP-C2.0 was used to amplify the SV40 TCR from the kidney of animal K661. Because these primers did not yield consistent results when the DNA copy number was low, they were replaced with primer pair SRP-5.0 and SRP-6.0 for amplification of SV40 from all other samples. Primer pair SEP-C1.0 and SEP-C2.0 was used to amplify the TAg HRD region. The sites to which these primers bind were found to be altered in some of the SV40 variants detected in this study. In these cases, alternate primer pairs were employed: SEP-C4.0 and SEP-C5.0, SV2386L and SEP-C4.0, or SV2386L and SV3628E. Primer pair SV1576L and SV2142E was used to amplify a portion of the VP1 region.

In some instances, it was necessary to use nested PCR and hot start to amplify SV40 DNA to a level that permitted cloning. In a nested reaction using SV2386L and SV3628E as the first primer pair and SVBam1 and SEP-C4.0 as the second pair, SV40 TAg HRD sequences were amplified from the brain of animal I275, the kidney and brain of animal H467, and the kidney and urine of animal L778. To amplify TCR sequences from H467 brain, primers SRP-8.0 and SRP-10.0 were nested by primers SRP-5.0 and SRP-6.0. This combination of primers failed to amplify SV40 TCR sequences from L778 kidney and urine, so SV4852L and SV923E were used in conjunction with SRP-5.0 and SRP-6.0 instead. To amplify the targeted VP1 region from the brain of animal I275, primers SV1232L and SV2369E were nested by SV1576L and SV2142E. In each nested reaction the outer primer set was used to amplify target DNA through 30 cycles and 1 μl of the resulting product was amplified through 25 cycles with the inner primer pair.

Rhesus monkey internal control primers (Rhesus-1 and Rhesus-2), which amplify a portion of the glycoprotein alpha subunit gene, were used in multiplex reactions with SV40-specific primers to confirm that DNA in the digested samples could be amplified (Golos *et al*, 1991).

PCR products were visualized on gels containing 0.7% molecular biology certified agarose (Eastman Kodak, Rochester, NY), 2% NuSieve GTG agarose (FMC Bioproducts, Rockland, ME), 1 × TBE buffer and 0.5 μg/ml ethidium bromide.

DNA cloning and isolation

PCR products were cloned into TA cloning vector pCRII or pCR2.1 according to the protocol supplied with the original TA cloning kit (Invitrogen). Recombinant DNA was prepared from ampicillin-resistant transformants (*E. coli*, INVaF′ One Shot, Invitrogen) using the Wizard Plus Minipreps kit (Promega, Madison, WI). Each DNA was digested with BstXI, electrophoresed on a 7% polyacrylamide gel, and stained with ethidium bromide to identify those clones containing an SV40 DNA insert. DNA amplified from the brain of animal I275 using primer pair SEP-C4.0 and SEP-C5.0 was difficult to clone directly from the PCR reaction mix. Gel purification of this product, followed by the use of a micropure-0.22 separator (Amicon) and microcon a 30 microconcentrator (Amicon, Beverly, MA) to recover the desired fragment, solved this cloning difficulty.

Sequence analysis

Clones containing SV40 DNA fragments of expected size were either sequenced manually by the method of Sanger *et al* (1977), or automatically using an ABI 377 Prism Sequencer (Perkin-Elmer, Oak Brook, IL) at the Nucleic Acids Facility of the Pennsylvania State University Biotechnology Institute. Sequencing reactions employed primers listed in Table 2. DNAs in manual sequencing reactions were radioactively labeled using sequencing grade Klenow enzyme (1 U/μl) and 20 μCi α-^{32}P-dATP (New England Nuclear, Boston, MA). Products were electrophoresed on denaturing 7% or 8% polyacrylamide gels for 4 or 2 h, respectively. Gels were exposed to BioMax film (Eastman Kodak) for 24–36 h.

Acknowledgements

We are grateful to our colleagues in the RJF laboratory for critical reading of the manuscript. We also thank Drs Lednicky and Butel and their colleagues at the Baylor College of Medicine for helpful advice and for graciously sharing a preprint of their work (Lednicky *et al*, 1998) during the preparation of this manuscript. While comparing our results, it was revealed that tissues from some of the same animals had been analyzed by both our laboratories. This work was supported by Public Health Service Grant CA-44970 from the National Cancer Institute (to RJF), by a Sigma Xi Grants-in-Aid of Research (to JSN), and by grant RR-00164 from the National Center for Research Resources (to GBB).

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