Journal of General Virology (1997), **78**, 1697–1705. Printed in Great Britain

Tissue culture adaptation of natural isolates of simian virus 40: changes occur in viral regulatory region but not in carboxy-terminal domain of large T-antigen

John A. Lednicky and Janet S. Butel

Division of Molecular Virology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030-3498, USA

The regulatory region of natural isolates of simian virus 40 (SV40) is different from that of laboratory-adapted strains of the virus. The latter have a nucleotide sequence duplication within the enhancer region which varies slightly with each strain, whereas the duplication is lacking in fresh isolates of SV40, which contain an 'archetypal' regulatory region. Many isolates also display nucleotide differences in the DNA encoding the carboxy terminus of large tumour antigen (T-ag). To determine whether genetic changes in these two regions of the SV40 genome were detectable during laboratory adaptation and long-term passage, low-passage virus stocks of two laboratory strains which had detailed passage histories spanning more than 25 years (Baylor strain and VA45-54) were analysed using PCR, cloning and sequencing assays. Both laboratory and archetypal regulatory regions were present in low-passage stocks. Following duplication in the regulatory region, no additional changes were detectable. The variable region at the T-ag carboxy terminus did not undergo any change with tissue culture passage and may serve as a useful site for taxonomic classification of different strains of SV40. Cloned genomes containing single or duplicated enhancers derived from both SV40 strains were viable in CV-1 cells. Attempts to induce regulatory region duplications by 14 serial passages of SV40 archetypal strains in monkey cells were not successful. The results are compatible with tissue culture adaptation of SV40, reflecting either selection of a rare variant pre-existing in the original sample or generation of a rare regulatory region duplication in infected cells.

Introduction

We recently described the detection of simian virus 40 (SV40) DNA sequences in human brain tumours that differed from laboratory strains both in the structure of the viral regulatory region and in the nucleotide sequence at the carboxy terminus of the SV40 large tumour antigen (T-ag) gene (Lednicky *et al.*, 1995*a*). Similar differences have been reported for natural isolates of SV40 from monkeys (Illyinskii *et al.*, 1992). Whereas laboratory-adapted strains of SV40, such as strains 776 (Fiers *et al.*, 1978; Reddy *et al.*, 1978), 800 (Ghosh *et al.*, 1982; Hay *et al.*, 1984) and SV40-B2 (Lednicky *et al.*, 1995*b*), contained two 72 bp enhancer elements, natural isolates from both humans (Lednicky *et al.*, 1995*a*) and monkeys [with one exception (Illyinskii *et al.*, 1992)] had only one 72 bp element in the regulatory region. We refer to the arrangement of the viral regulatory region of natural isolates as 'archetypal', which is typified by the absence of 'large' duplications within the enhancer region (Lednicky *et al.*, 1995*a*, *b*); laboratory strains of SV40 contain duplications within this enhancer region. [The standard convention being followed here is that the enhancer elements are between nucleotides 107 and 300 of SV40 reference strain 776, whereas the G/C-rich motifs (nucleotides 40–103 of SV40 strain 776), which also contain repeated sequences, are classified as 'promoter' but not enhancer elements (Salzman *et al.*, 1986).]

Sequence comparison of the entire early region of several isolates of SV40 obtained from monkeys and humans and recovered over a span of 35 years recently confirmed the existence of a short variable domain at the carboxy terminus of T-ag (T-ag-C; encompassing amino acids 622–708) (Stewart *et al.*, 1996). Except for the short variable region, the amino acid sequence of T-ag was absolutely conserved among the virus isolates. The function of the T-ag-C in natural SV40 infections is unclear. Embedded within the T-ag-C of SV40 (amino acid residues 622–708) is a functional domain which encompasses amino acids 682–708, defined as the host-range/adenovirus

Author for correspondence: Janet S. Butel.
Fax +1 713 798 5019. e-mail jbutel@bcm.tmc.edu

0001-4414 © 1997 SGM

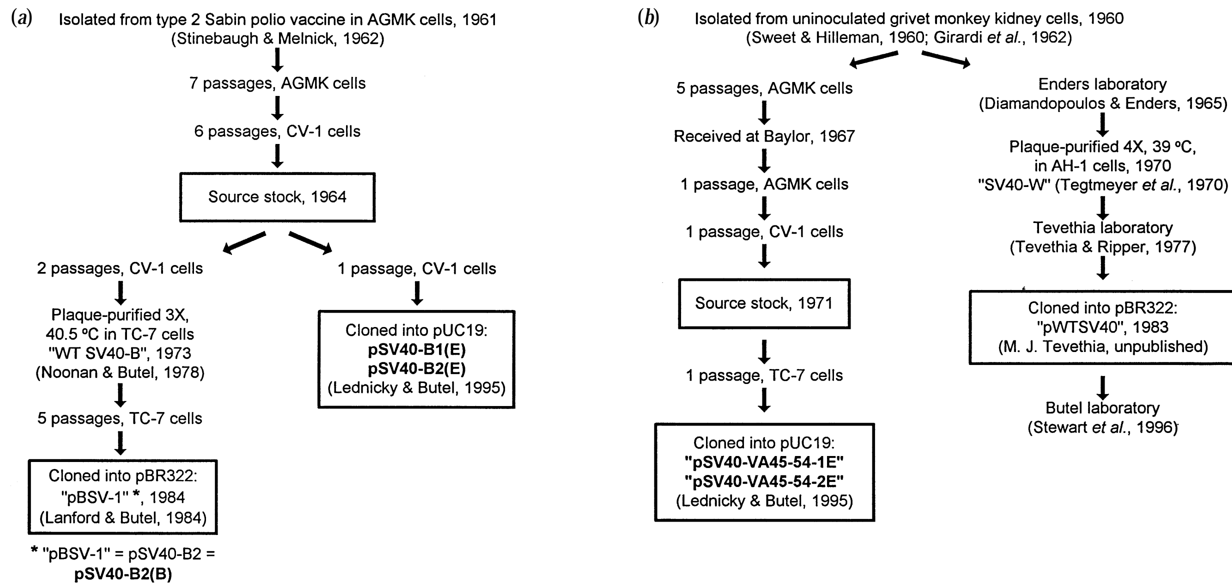


Fig. 1. Passage histories of SV40 laboratory strains SV40-Baylor (*a*) and SV40-VA45-54 (*b*). The two 'source stocks' had been in storage at Baylor College of Medicine for ⩾ 25 years. Numbers of tissue culture passages are indicated if known. Samples enclosed in boxes were compared in this study. Lednicky & Butel (1995) refers to unpublished data.

(Ad) helper function (hr/hf) domain. A carboxy-terminal fragment of T-ag can relieve a block to Ad replication in monkey cells (Rabson *et al.*, 1964; Kelley & Lewis, 1973; Cole *et al.*, 1979) by an unknown mechanism. The hr/hf domain was identified because T-ag carboxy-terminal deletion mutants exhibited different growth properties in monkey cell lines; these mutants grew well in BSC and Vero cells, but not in CV-1 cells (Lewis *et al.*, 1983; Pipas, 1985; Tornow *et al.*, 1985; Cole & Stacy, 1987). Recently, some of these deletion mutants were shown to be defective in virion assembly in CV-1 cells, failing to add VP1 to the 75S assembly intermediates (Spence & Pipas, 1994). These findings are intriguing, as nucleotide differences in the T-ag-C have been detected among viral sequences present in SV40-infected monkey tissues (Illyinskii *et al.*, 1992) and in human tumours (Lednicky *et al.*, 1995*a*). These differences consisted of short polynucleotide insertions and deletions, as well as single nucleotide changes (Illyinskii *et al.*, 1992; Lednicky *et al.*, 1995*a*); many of these changes occurred within the minimal hr/hf domain identified by deletion mapping and just upstream of the separable segments able to supply host range activity (Tornow *et al.*, 1985; reviewed by Spence & Pipas, 1994).

These observations raised the possibility that SV40 might undergo more genetic variation during growth in cultured cells or infected tissues than previously suspected and that there might be mutable hotspots in the viral genome that are prone to change. Knowledge of the genetic stability of SV40 is necessary to permit evaluation of the biological significance of variants found associated with human tumours. We addressed this fundamental question of SV40 variability by doing a molecular archaeological analysis of two well-characterized laboratory strains of the virus. We focused our investigations on two regions of the viral genome, namely the regulatory region and the carboxy-terminal domain of T-ag.

Methods

Viruses. The two laboratory strains of SV40 analysed were the Baylor strain (SV40-B) (Stinebaugh & Melnick, 1962) and VA45-54 (Sweet & Hilleman, 1960; Girardi *et al.*, 1962). We were fortunate to have available relatively low-passage stocks of both SV40-B and SV40-VA45-54 that had been in storage at Baylor College of Medicine for ⩾ 25 years. The detailed passage history of SV40-B is shown (Fig. 1*a*). Following isolation from poliovaccine (Stinebaugh & Melnick, 1962), SV40-B had been passaged seven times in primary African green monkey kidney (AGMK) cells, eight times in CV-1 cells (stable line of AGMK cells), triple plaque-purified at elevated temperature (Noonan & Butel, 1978), passaged five more times in TC-7 cells (a derivative line of CV-1 cells) and finally cloned into pBR322 (Lanford & Butel, 1984).

The passage history of our SV40-VA45-54 stock source is shown (Fig. 1*b*). The virus was isolated originally from an uninoculated grivet monkey kidney cell culture (Sweet & Hilleman, 1960; Girardi *et al.*, 1962). A virus preparation which had been passaged five times in AGMK cells was received at Baylor in 1967, where it was subsequently passaged once each in AGMK cells and CV-1 cells. The history of another lineage of SV40-VA45-54 is also shown.

Oligonucleotides. Oligonucleotides RA3, RA4, TA1 and TA2 (Lednicky *et al.*, 1995*a*) were used as PCR primers and DNA sequencing primers. RA3 and RA4 were used to analyse regulatory region sequences between nucleotides 5190 and 300 (numbering based on SV40-776). Primers TA1 and TA2 were used to analyse sequences encoding the carboxy terminus of T-ag (nucleotides 2999–2691; encoding amino acids 607–708).

DNA sequence analysis. Double-stranded PCR-amplified DNA products were sequenced directly from PCR amplification reactions using the Sequenase PCR product sequencing kit (Amersham). Alkali-denatured

plasmids were sequenced [after purification of the plasmids with the QIAGEN plasmid purification kit (QIAGEN)] using the Sequenase version 2.0 DNA sequencing kit (Amersham). The sequence of both DNA strands was determined in every case.

■ **Cloning of viral genomes.** Viral DNA was purified as previously described (Lednicky *et al.*, 1995 *a*), cloned into the *Eco*RI site of pUC19 and transformed into *Escherichia coli* strain XL1-BLUE (Stratagene).

■ **Plaque assays.** SV40 plaque assays were performed in CV-1 and TC-7 cells as previously described (Lednicky & Folk, 1992; Noonan & Butel, 1978).

Results

Genetic analysis of SV40 Baylor strain

We first recovered SV40-B from a low-passage virus source stock frozen in 1964 (Fig. 1 *a*). A virus preparation of SV40-B was prepared by infecting nearly confluent CV-1 cells in a flask (75 cm^2) with 50 μl of source stock virus (of unknown viral titre). A 100 μl aliquot of the source stock was tested by PCR; a mixture of both 'laboratory type' (containing a large repeated segment within the enhancer region) and 'archetypal' regulatory regions was revealed (Fig. 2, lane A). This indicated that 'archetypal' viral genomes were present in a low-passage stock of a standard SV40 strain and that genetic changes characteristic of a laboratory strain were detectable early in the adaptation of a natural virus isolate to tissue culture. PCR analysis of a lysate (Hirt, 1967) prepared from the cells infected with source stock virus revealed the presence of both regulatory types, but with the laboratory type more pronounced than in the earlier passage (Fig. 2, lane B). In contrast, virus stocks of SV40-B2 (the terminal '2' indicates a duplicated viral enhancer region) and SV40-776 that were prepared from cells infected with cloned plasmids lacked archetypal-length regulatory regions, even after 14 passages in SV40-permissive CV-1 cells (Fig. 2, lanes C and D). Thus, revertants (to archetypal-length regulatory regions) were not evident, suggesting that a constant flux between regulatory types did not occur in CV-1 cells.

We next determined whether changes had occurred at the variable T-ag-C. Virus stocks of SV40-B had been passaged many times prior to the cloning of pSV40-B2(B) in 1983 (the terminal letter 'B' denotes use of the *Bam*HI cloning site) (Lanford & Butel, 1984), including triple plaque purification at elevated temperature (Noonan & Butel, 1978; Fig. 1 *a*). It was expected that changes at the T-ag-C of SV40-B2 would be evident, comparing cloned pSV40-B2(B) with source stock virus, if this region was a mutation hotspot during growth in tissue culture.

We performed a direct PCR sequence analysis on the lysate of cells infected with the source stock virus, expecting ambiguities of the DNA sequence if the uncloned source stock contained a mixture of viruses with changes at the T-ag-C (similar to the mixture of regulatory region types described above). However, no ambiguities were detected (Fig. 3 *a*). For more detailed analysis, virus DNA was purified from the same

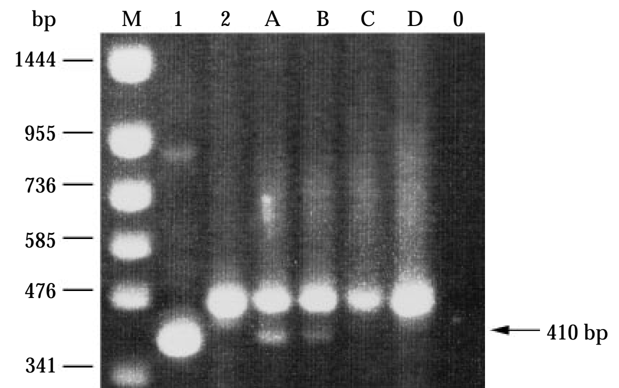


Fig. 2. SV40 regulatory region PCR analysis. Aliquots of SV40 Baylor strain virus preparations (Fig. 1 *a*) were digested with proteinase K and then extracted with phenol. DNA was ethanol-precipitated from the treated digests and subjected to PCR analysis using primers RA3 and RA4 (Lednicky *et al.*, 1995 *a*). Positive controls were plasmids pSV1X72 (lane 1) and pSV2X72 (lane 2); pSV1X72 has an archetypal regulatory region, whereas pSV2X72 is SV40 laboratory strain 776, which has a duplication within the enhancer region (Lednicky *et al.*, 1995 *b*). Lanes: A, low-passage SV40-B source stock; B, virus propagated once from the SV40-B source stock; C and D, virus prepared from plasmids pSV40-B2 and pSV2X72, respectively, and serially passaged 14 times in CV-1 cells; M, molecular mass markers; 0, negative control PCR reaction. The approximately 410 bp PCR product generated from archetypal regulatory region genomes is identified by the arrow to the right of the figure.

cell lysate by CsCl–ethidium bromide density gradient centrifugation, cut at the *Eco*RI site and cloned into *Eco*RI-linearized pUC-19. One hundred recombinant plasmids of the expected size range that contained an *Sfi*I restriction site (present in SV40-B but lacking in pUC-19) were chosen for analysis. We designated laboratory-type clones as pSV40-B2(E) clones and archetypal-type clones as pSV40-B1(E) clones (the terminal letter 'E' denotes use of the *Eco*RI cloning site) (Fig. 1 *a*). Using a combination of restriction enzyme and PCR analysis, we determined that the apparent regulatory region length of 92 of the 100 clones was like that of SV40-B2, whereas eight appeared to have an archetypal-length regulatory region.

DNA sequence analysis of four randomly chosen clones of pSV40-B1(E) and pSV40-B2(E) revealed that the T-ag-C terminus of each was exactly like that previously reported for pSV40-B2(B) (Fig. 3 *b*; Lednicky *et al.*, 1995 *a*). The sequence of the variable domain of T-ag for the Baylor strain is shown and compared to that of SV40-776. Exactly the same sequence was found for virus clones pSV40-B2(B), pSV40-B1(E) and pSV40-B2(E) (Fig. 1 *a*). As the sequence determinations were made on virus samples having different culture histories, this established that the T-ag-C region had not undergone any sequence drift. Furthermore, the regulatory region of pSV40-B2(E) clones contained precisely the same duplicated sequence previously detected in pSV40-B2(B) (Lednicky *et al.*, 1995 *b*). This indicated that once the duplication of the enhancer region occurred, it remained stable thereafter, despite differing virus culture histories. However, this duplication was lacking in pSV40-

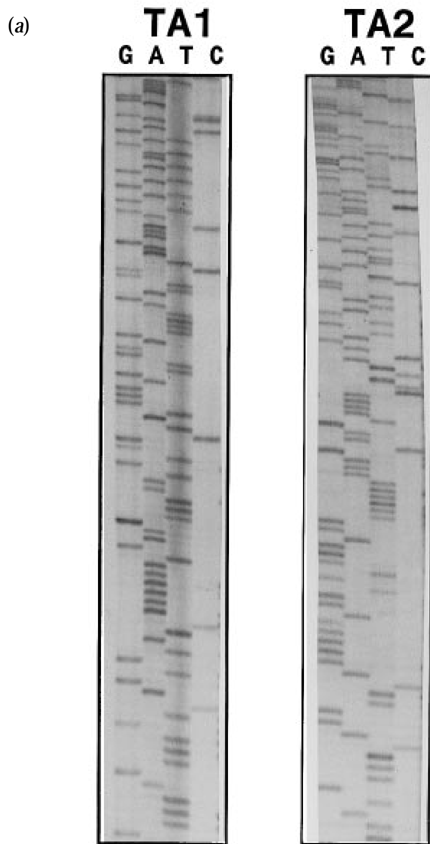
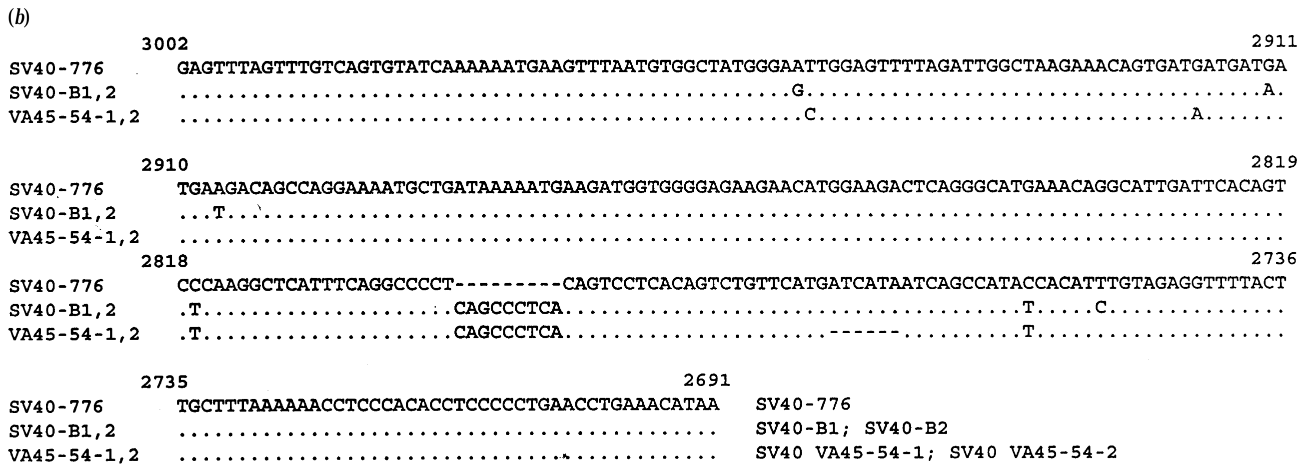


Fig. 3. Carboxy-terminal T-ag gene sequence analysis. (*a*) Direct PCR sequence analysis on cell lysate of culture infected with low-passage SV40-B stock. Primers TA1 and TA2 (Lednicky *et al.*, 1995 *a*) were used to amplify the carboxy-terminal sequence of T-ag, and sequencing was performed as described (Lednicky *et al.*, 1995*a*). The labels TA1 and TA2 above the panels refer to the sequencing primer used for each sequence reaction and serve to orient the figure. In the panel topped with the letters TA1, the sequence corresponding to nucleotides 3000–2892 of SV40-776 is shown. For the panel headed TA2, the sequence shown corresponds to nucleotides 2691–2806 of SV40-776. No ambiguities in DNA sequence are apparent. This indicated that the uncloned low-passage SV40-B stock did not contain a detectable mixture of viruses having changes in the variable domain at the carboxy terminus of the T-ag gene. (*b*) Carboxy-terminal T-ag sequences of cloned viruses derived from low-passage source stocks of strains SV40-B and SV40-VA45-54. With each virus strain, the T-ag sequence was the same for variants having archetypal (type 1) or duplicated (type 2) regulatory regions (from this study) and identical to the sequence determined earlier using virus with different passage histories (Lednicky *et al.*, 1995*a*; Stewart *et al.*, 1996). The T-ag-C sequence is also shown for prototype SV40 strain 776.



B1(E) clones. The structures of the two regulatory regions (with and without the duplication) are summarized in Fig. 4 and compared to that of SV40 reference strain 776.

Apart from a difference in length of the regulatory region, the cloned viral genomes appeared to be of similar size by restriction enzyme analysis. To determine whether viable genomes had been cloned, several pSV40-B1(E) and pSV40-B2(E) clones were linearized with *Eco*RI and lipofected into CV-1 cells as previously described (Lednicky & Folk, 1992). All clones tested formed SV40-type cytopathic effects, characterized by cell vacuolation prior to cell death. The pSV40-B2(E) clones formed larger plaques and yielded higher virus titres than the pSV40-B1(E) clones (data not shown). These results were consistent with the relative replication abilities of SV40-776 and SV1X72, a derivative of SV40-776 in which one of the 72 bp enhancer elements has been deleted (Lednicky *et al.*, 1995 *b*).

Genetic analysis of SV40 strain VA45-54

Similar patterns of genetic change during tissue culture adaptation were displayed by SV40 strain VA45-54. Regu-

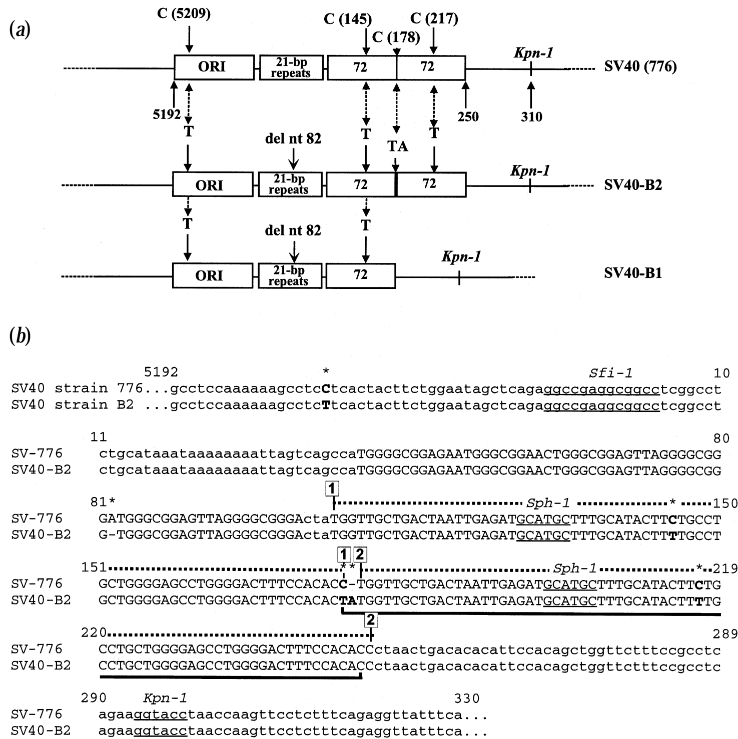


Fig. 4. Structure of regulatory region of Baylor strain of SV40. (*a*) Schematic representation of the regulatory regions of SV40-776, SV40-B1 and SV40-B2. The viral origin of DNA replication is identified by the acronym 'ORI', and boxed number 72 refers to the 72 bp sequence within the enhancer region that is often reiterated in tissue-culture-passaged strains of SV40 (Lednicky *et al.*, 1995*b*). All comparisons are relative to the DNA sequence of standard reference strain SV40-776. (*b*) DNA sequence of the regulatory region of SV40-776, SV40-B1 and SV40-B2. The 21 bp repeat region is identified by uppercase letters between nucleotides 40 and 103. Enhancer repeats are identified by a dotted overline that is bordered by boxed numbers '1' or '2'. The enhancer DNA sequence lacking in SV40-B1 is underlined with a heavy line. Single nucleotide changes between SV40-776 and Baylor strain are identified with bold letters. Asterisks above the letters identify sites where nucleotide differences were observed.

latory region PCR analysis of an aliquot of our source stock (prepared in 1971) (Fig. 1*b*) showed a mixture of archetypal and laboratory-type genomes, with the archetypal genome predominating (data not shown). CV-1 cells were then infected with an aliquot of the SV40-VA45-54 source stock, and viral DNA was isolated and cloned into pUC-19 at the *Eco*RI cloning site (Fig. 1*b*). Eighty clones were tested; 74 had archetypal regulatory regions [pSV40-VA45-54-1(E) clones] and six had a duplication within the enhancer [pSV40-VA45-54-2(E) clones]. We do not have an explanation for the different relative amounts of archetypal and duplicated genomes present in the low-passage stock of VA45-54, compared to that of Baylor strain. The variable amounts may reflect the fact that our source stock of VA45-54 had been passaged fewer times in culture (7 times vs 13 times for SV40-B), that there may have been differences between the original materials from which the strains were recovered, or that there were unknown differences among the ways in which the early passages of the different virus stocks were made. The regulatory region sequences of the SV40-VA45-54 clones, compared to that of SV40-776, are shown in Fig. 5.

The T-ag-C of each type of clone was identical to each other (Fig. 3*b*) and with the sequence determined previously for an independent lineage of SV40-VA45-54 (Fig. 1*b*; Stewart *et al.*, 1996), indicating that the T-ag-C sequence of strain SV40-VA45-54 had not changed over many years. It is noteworthy, however, that the sequence of the T-ag-C variable region differed among the three SV40 isolates (strains 776, SV40-B and VA45-54; Fig. 3*b*), confirming our previous observations (Stewart *et al.*, 1996) that the carboxy terminus of T-ag contains a variable domain. Four randomly chosen clones of each genome type of pSV40-VA45-54 were linearized with *Eco*RI and transfected into CV-1 cells. Each clone tested was viable and, as observed with the SV40-B clones, type 2 viruses formed larger plaques and yielded higher virus titres than the type 1 clones.

Attempts to induce SV40 regulatory region changes by passage in permissive cells

We next tested whether we could induce duplications within the enhancer region of archetypal SV40 by passage in permissive cells, in an attempt to determine if this change characteristic of SV40 laboratory strains occurs readily during passage in tissue culture. Archetypal SV40 strains SV40-B1, SV40-VA45-54-1 and SV1X72 (Lednicky *et al.*, 1995 *b*) were each serially passaged 14 times in CV-1 cells. Each passage was made by inoculating 2×10^6 CV-1 cells at an m.o.i. of approximately 0·01 p.f.u./cell and incubating the culture until cells lysed completely (10–15 days). PCR analysis of the viral lysates at each passage revealed no changes in the length of the regulatory region. Identical results were obtained after 14 consecutive passages of the three archetypal strains in TC-7 cells, another line of green monkey kidney cells. No duplicated regulatory regions were detected with any of the SV40 lineages. This may indicate that duplications in the SV40 enhancer region are generated very rarely during virus replication and that many more virus generations than those

(*a*)

ORI | 21-bp repeats | 72 | 72 — Kpn-1 — SV40 (776); 5192, 250, 310

ORI | 21-bp repeats | 72 | 72 — Kpn-1 — SV40-VA45-54-2

ORI | 21-bp repeats | 72 — Kpn-1 — SV40-VA45-54-1

(*b*)

```
                5192                                                Sfi-1         10
SV40 strain 776...gcctccaaaaaagcctcctcactacttctggaatagctcagaggccgaggcggcctcggcct
SV40 VA45-54-2....gcctccaaaaaagcctcctcactacttctggaatagctcagaggccgaggcggcctcggcct

          11                                                                   80
SV-776     ctgcataaataaaaaaaattagtcagccaTGGGGCGGAGAATGGGCGGAACTGGGCGGAGTTAGGGGCGG
SV-VA-2    ctgcataaataaaaaaaattagtcagccaTGGGGCGGAGAATGGGCGGAACTGGGCGGAGTTAGGGGCGG

                                  [1]
          81                       |.............................. Sph-1 ...................150
SV-776     GATGGGCGGAGTTAGGGGCGGGActaTGGTTGCTGACTAATTGAGATGCATGCTTTGCATACTTCTGCCT
SV-VA-2    GATGGGCGGAGTTAGGGGCGGGActaTGGTTGCTGACTAATTGAGATGCATGCTTTGCATACTTCTGCCT

                                    [1]                  [2]
         151........................|                    |.......................199
SV-776     GCTGGGGAGCCTGGGGACTTTCCACACC---------------------TGGTTGCTGACTAATTGAGAT
SV-VA-2    GCTGGGGAGCCTGGGGACTTTCCACACCCTAACTGACACACATTCCACATGGTTGCTGACTAATTGAGAT

                                                              [2]
         200Sph-1.............................................|                269
SV-776     GCATGCTTTGCATACTTCTGCCTGCTGGGGAGCCTGGGGACTTTCCACACCctaactgacacacattcca
SV-VA-2    GCATGCTTTGCATACTTCTGCCTGCTGGGGAGCCTGGGGACTTTCCACACCctaactgacacacattcca

         270                       Kpn-1                            330
SV-776     cagctggttctttccgcctcagaaggtacctaaccaagttcctctttcagaggttatttca...
SV-VA-2    cagctggttctttccgcctcagaaggtacctaaccaagttcctctttcagaggttatttca...
```

Fig. 5. Structure of regulatory region of VA45-54 strain of SV40. (*a*) Schematic representation of the regulatory regions of SV40-776, SV40-VA45-54-1 and SV40-VA45-54-2. The symbols used are as described in the legend to Fig. 4 (*a*). The DNA sequence corresponding to nucleotides 251–271 of SV40-776 is represented by filled boxes. (*b*) DNA sequence of the regulatory regions of SV40-776, SV40-VA45-54-1 and SV40-VA45-54-2. The DNA sequence is presented as described in the legend to Fig. 4 (*b*). The DNA sequence corresponding to nucleotides 251–271 of SV40-776 is represented by filled boxes.

involved during 14 tissue culture passages are required to detect them.

Discussion

We have demonstrated here that early passages of two standard laboratory strains of SV40 contained mixtures of viruses with archetypal or duplicated regulatory regions. This established that in both cases the laboratory-adapted version of the virus was derived from original material that contained genomes with the archetypal regulatory region that we believe to be typical of natural isolates of SV40. Comparison of the regulatory region structures of the low-passage virus stocks with those of laboratory-adapted virus with different passage histories revealed that, once virus with a duplicated enhancer region was selected in culture, the structure was genetically stable thereafter. This study did not determine whether tissue culture adaptation selected for a rare variant present in the original inoculum material or reflected a rare duplication event that occurred in the cultured cells. We also observed that the variable domain at the carboxy terminus of T-ag did not undergo any genetic change during tissue culture adaptation and passage.

The functional basis for the duplication of the 72 bp element in laboratory strains of SV40 is unknown, as is the question of whether a particular regulatory region sequence with a duplication of the 72 bp element at the enhancer is favoured over others and under what conditions. We have noted that both SV40-B2 and SV40-VA45-54-2 form larger plaques and produce higher virus yields in CV-1 and TC-7 cells than does SV40-776. This effect might be due to the differences in regulatory region structure, but it might also be influenced by the variation in T-ag-C sequences. It should be noted that SV40-776 was formerly referred to as the 'small-plaque' strain of SV40 (Takemoto *et al*., 1966), so there may be some intrinsic difference in SV40-776 replication, as plaque size usually correlates with SV40 replication efficiency in CV-1 cells (Lednicky & Folk, 1992).

It is established from previous studies with BK and JC viruses that papovavirus regulatory regions may change during passage in tissue culture (Hara *et al*., 1986; Watanabe & Yoshiike, 1986; Loeber & Dörries, 1988; Rubinstein *et al*., 1991; and references cited in Lednicky *et al*., 1995*b*). Differences are also detected among the DNA sequences encoding the carboxy termini of their respective T-ags (Frisque *et al*., 1984; Tavis *et al*., 1989). Thus, it appears these phenomena may be characteristic of polyomaviruses in general.

We were not able to mimic the change in the structure of the SV40 regulatory region by 14 serial passages of three archetypal versions of SV40 in two different stable monkey kidney cell lines that are permissive for SV40 growth. Perhaps more than 14 passages are necessary to detect a rare event leading to a duplication in the viral enhancer. Alternatively, changes within the enhancer region may arise more frequently when nonclonal cells (such as primary AGMK cells), which are likely to contain a mixture of permissive, semipermissive and

non permissive cell types, are used to isolate and propagate the virus (Figs 1, 4 and 5). In support of that notion, SVPML-1 and -2, SV40 isolates from human cases of progressive multifocal leukoencephalopathy recovered by passage in AGMK cells (Weiner *et al*., 1972; Martin, 1989), contained a mixture of viral genomes that were designated α and β types (Martin, 1989). Viruses of the α type had a 39 bp duplication within the enhancer region (that was lacking in β types) and predominated among clones prepared from tissue-culture-passaged virus (Martin, 1989). However, it is equally possible that such changes reflect selection of rare variants present in host-derived material. Perhaps archetypes were represented in our samples because the viruses were isolated from kidneys derived from apparently healthy animals. Existing tissue culture models provide a precedent for this notion. Genetic changes that occur in SV40 in response to passage in semipermissive and permissive human cells (leading to the accumulation of defective viral genomes) have been documented (O'Neill & Carroll, 1981 and references cited therein). The defective genomes contained deletions at sites other than the regulatory region, although some apparently had duplications within the regulatory region.

The regulatory region sequence of SV40-VA45-54-2 determined in these experiments was exactly like the sequence determined for an independently cloned isolate of SV40-VA45-54-2 (M. J. Tevethia, personal communication). This sequence was also exactly like that reported for SV40 strain 800 (SV40-800) (Ghosh *et al*., 1982; Hay *et al*., 1984). SV40 strain Rh911 (SV40-Rh911), originally isolated from rhesus monkey kidney cells (Girardi, 1965), was renamed SV40-800 (Mertz & Berg, 1974). We noted that the non-archetypal regulatory region sequence reported for SV40-800 was different from the archetypal regulatory region reported for SV40-Rh911 by Van Heuverswyn & Fiers (1979). These results could be explained if the original SV40-Rh911 stock contained a mixture of archetypal and non-archetypal genomes, similar to our findings of mixed archetypal and non-archetypal populations in our early stocks of SV40-B and SV40-VA45-54. However, it was unexpected that the SV40-Rh911 sequence coincided exactly with that of SV40-VA45-54, especially as the two viruses were reportedly recovered from different monkey species and because the virus strains that we have analysed in detail (SV40-776, SV40-B2 and SV40-VA45-54-2) have slightly different regulatory regions. We were able to reconcile these apparent inconsistencies by analysing a low-passage stock of SV40-Rh911 (obtained at Baylor in 1970 and held in storage) and determining that at least five distinct types of virus were present. The regulatory region and T-ag-C sequences of two components corresponded exactly with the sequences of SV40-VA45-54 and SV40-VA45-54-2, whereas the rest had a mixture of archetypal and duplicated enhancers with an identical T-ag-C that was different from that of VA45-54 (results to be presented in detail elsewhere). Both SV40-VA45-54 and SV40-Rh911 were isolated by the same laboratory, and it appears that early stocks of SV40-Rh911 contained a mixture of viruses and that different variants predominate in various passage lineages. In any case, as SV40-VA45-54 was handled differently in several laboratories, and yet the regulatory region remained identical, this suggests that when a growth-promoting duplication within the SV40 enhancer is selected, the structure is stable thereafter in permissive cells.

Our observations that changes at the enhancer did not occur when wild-type isolates of SV40 were passaged in CV-1 cells were unlike the results obtained when an SV40 genome with a mutated archetypal-length SV40 regulatory region was passaged in CV-1 cells, the latter resulting in rapid acquisitions of regulatory region duplications (Herr & Gluzman, 1985). In a related set of experiments, we have determined that a similar phenomenon occurred when SV40 genomes with changes at the 21 bp repeat region were serially passaged in CV-1 cells. One construct, SVGC3,5-N, in which one synthetic SP1 site replaces the 21 bp repeat region (Lednicky & Folk, 1992), gave rise to and was overgrown by a faster growing evolutionary mutant SVGC3,5-N*dup*1 (in which the single Sp1 site of SVGC3,5-N was duplicated) after seven passages in CV-1 cells. The DNA sequence of SVGC3,5-N*dup*1 verified that it was a newly generated mutant. (A detailed report of these findings will be presented elsewhere.) Thus, it appears that, whereas the natural sequence is relatively stable, artificially induced changes of the natural regulatory region sequences somehow promote the rapid emergence of mutated forms in CV-1 cells.

An accumulating body of literature substantiates the hypothesis that archetypal SV40 regulatory region structures predominate in virus strains circulating in nature (Lednicky *et al*., 1995*a*, *b*). These observations include the sequence analysis of SV40-B1, SV40-VA45-54-1 (this report), SVMEN (Krieg & Scherer, 1984; Martin & Li, 1991), several fresh simian isolates of SV40 (Illyinskii *et al*., 1992), SV40 A2895 (Illyinskii *et al*., 1992), SV40 sequences in human tumours (Lednicky *et al*., 1995*a*), the SV40 genome contained within the SV40–adenovirus 7 hybrid virus, PARA(cT) (Lanford *et al*., 1986) and two SV40-wt830 clones that had been considered 'deletion mutants' (Van Heuverswyn & Fiers, 1979). In this regard, SV40 is like papovaviruses JC, BK and mouse polyomavirus, in which archetypal genomes predominate in persistent infections (Markowitz *et al*., 1991; Rochford *et al*., 1992; Tominaga *et al*., 1992). Our data seem to support the notion that archetypal papovavirus genomes are favoured for persistent infections, whereas rapidly growing papovavirus strains (with changes in the regulatory region) lead to acute infection (Shadan & Villareal, 1993). This conclusion is based on the fact that papovavirus infections are characteristically subclinical and persistent (Shah, 1996) and we detected archetypal SV40 genomes in each low-passage viral sample tested.

In contrast to the regulatory region DNA sequence changes that provide a selective growth advantage in tissue culture, changes at the variable region of T-ag were not detectable by

our assays. Considering the lack of detectable changes at the T-ag-C, we favour the interpretation that isolates with variable T-ag-C sequences reflect different SV40 strains present in natural infections. The polymorphic T-ag-C region may provide a handy site with which to distinguish and classify different strains of SV40. It is still possible that changes at the T-ag-C do occur *in vivo*, but animal testing will be required to resolve that question. It remains to be determined how such variation may influence the pathogenesis of SV40 infections in primate hosts.

This work was supported in part by grants CA22555 (J.S.B.) and CA61703 (J.A.L.) from the National Cancer Institute. We thank M. J. Tevethia and P. Tegtmeyer for providing information about the history of strain VA45-54.

References

Cole, C. N. & Stacy, T. P. (1987). Biological properties of simian virus 40 host range mutants lacking the COOH-terminus of large T antigen. *Virology* **161**, 170–180.

Cole, C. N., Crawford, L. V. & Berg, P. (1979). Simian virus 40 mutants with deletions at the 3′ end of the early region are defective in adenovirus helper function. *Journal of Virology* **30**, 683–691.

Diamandopoulos, G. T. & Enders, J. F. (1965). Studies on transformation of Syrian hamster cells by simian virus 40 (SV40): acquisition of oncogenicity by virus-exposed cells apparently unassociated with the viral genome. *Proceedings of the National Academy of Sciences, USA* **54**, 1092–1099.

Fiers, W., Contreras, R., Haegeman, G., Rogiers, R., Van de Voorde, A., Van Heuverswyn, H., Van Herreweghe, J., Volckaert, G. & Ysebaert, M. (1978). Complete nucleotide sequence of SV40 DNA. *Nature* **273**, 113–120.

Frisque, R. J., Bream, G. L. & Canella, M. T. (1984). Human polyomavirus JC virus genome. *Journal of Virology* **51**, 458–469.

Ghosh, P. K., Piatak, M., Mertz, J. E., Weissman, S. M. & Lebowitz, P. (1982). Altered utilization of splice sites and 5′ termini in late RNAs produced by leader region mutants of simian virus 40. *Journal of Virology* **44**, 610–624.

Girardi, A. J. (1965). Prevention of SV40 virus oncogenesis in hamsters. I. Tumor resistance induced by human cells transformed by SV40. *Proceedings of the National Academy of Sciences, USA* **54**, 445–451.

Girardi, A. J., Sweet, B. H., Slotnick, V. B. & Hilleman, M. R. (1962). Development of tumors in hamsters inoculated in the neonatal period with vacuolating virus, SV40. *Proceedings of the Society for Experimental Biology and Medicine* **109**, 649–660.

Hara, K., Oya, Y., Kinoshita, H., Taguchi, F. & Yogo, Y. (1986). Sequence reiteration required for the efficient growth of BK virus. *Journal of General Virology* **67**, 2555–2559.

Hay, R. T., Hendrickson, E. A. & DePamphilis, M. L. (1984). Sequence specificity for the initiation of RNA-primed SV40 DNA synthesis in vivo. *Journal of Molecular Biology* **175**, 131–157.

Herr, W. & Gluzman, Y. (1985). Duplications of a mutated simian virus 40 enhancer restore its activity. *Nature* **313**, 711–714.

Hirt, B. (1967). Selective extraction of polyoma DNA from infected mouse cell cultures. *Journal of Molecular Biology* **26**, 365–369.

Illyinskii, P. O., Daniel, M. D., Horvath, C. J. & Desrosiers, R. C. (1992). Genetic analysis of simian virus 40 from brains and kidneys of macaque monkeys. *Journal of Virology* **66**, 6353–6360.

Kelley, T. J. & Lewis, A. M. (1973). Use of nondefective adenovirus–simian virus 40 hybrids for mapping the simian virus 40 genome. *Journal of Virology* **12**, 643–652.

Krieg, P. & Scherer, G. (1984). Cloning of SV40 genomes from human brain tumors. *Virology* **138**, 336–340.

Lanford, R. E. & Butel, J. S. (1984). Construction and characterization of an SV40 mutant defective in nuclear transport of T antigen. *Cell* **37**, 801–813.

Lanford, R. E., Jacob, J. R. & Butel, J. S. (1986). Genomic organization of the simian virus 40–adenovirus 7 hybrid virus, PARA(cT), that encodes a nuclear transport defective simian virus 40 T antigen. *Virology* **155**, 271–276.

Lednicky, J. & Folk, W. R. (1992). Two synthetic Sp1-binding sites functionally substitute for the 21-base-pair repeat region to activate simian virus 40 growth in CV-1 cells. *Journal of Virology* **66**, 6379–6390.

Lednicky, J. A., Garcea, R. L., Bergsagel, D. J. & Butel, J. S. (1995*a*). Natural simian virus 40 strains are present in human choroid plexus and ependymoma tumors. *Virology* **212**, 710–717.

Lednicky, J. A., Wong, C. & Butel, J. S. (1995*b*). Artificial modification of the viral regulatory region improves tissue culture growth of SV40 strain 776. *Virus Research* **35**, 143–153.

Lewis, E. D., Chen, S., Kumar, A., Blanck, G., Pollack, R. E. & Manley, J. L. (1983). A frameshift mutation affecting the carboxyl terminus of the simian virus 40 large tumor antigen results in a replication- and transformation-defective virus. *Proceedings of the National Academy of Sciences, USA* **80**, 7065–7069.

Loeber, G. & Dörries, K. (1988). DNA rearrangements in organ-specific variants of polyomavirus JC strain GS. *Journal of Virology* **62**, 1730–1735.

Markowitz, R.-B., Eaton, B. A., Kubik, M. F., Latorra, D., McGregor, J. A. & Dynan, W. S. (1991). BK virus and JC virus shed during pregnancy have predominantly archetypal regulatory regions. *Journal of Virology* **65**, 4515–4519.

Martin, J. D. (1989). Regulatory sequences of SV40 variants isolated from patients with progressive multifocal leukoencephalopathy. *Virus Research* **14**, 85–94.

Martin, J. D. & Li, P. (1991). Comparison of regulatory sequences and enhancer activities of SV40 variants isolated from patients with neurological diseases. *Virus Research* **19**, 163–172.

Mertz, J. E. & Berg, P. (1974). Defective simian virus 40 genomes: isolation and growth of individual clones. *Virology* **62**, 112–124.

Noonan, C. A. & Butel, J. S. (1978). Temperature-sensitive mutants of simian virus 40. *Intervirology* **10**, 181–195.

O'Neill, F. J. & Carroll, D. (1981). Amplification of papovavirus defectives during serial low multiplicity infections. *Virology* **112**, 800–803.

Pipas, J. M. (1985). Mutations near the carboxyl terminus of the simian virus 40 large tumor antigen alter viral host range. *Journal of Virology* **54**, 569–575.

Rabson, A. S., O'Conner, G. T., Berezesky, I. K. & Paul, F. J. (1964). Enhancement of adenovirus growth in African green monkey kidney cell cultures by SV40. *Proceedings of the Society for Experimental Biology and Medicine* **116**, 187–190.

Reddy, V. B., Thimmappaya, B., Dhar, R., Subramanian, K. N., Zain, B. S., Pan, J., Ghosh, P. K., Celma, M. L. & Weissman, S. M. (1978). The genome of simian virus 40. *Science* **200**, 494–502.

Rochford, R., Moreno, J. P., Peake, M. L. & Villareal, L. P. (1992). Enhancer dependence of polyomavirus persistence in mouse kidneys. *Journal of Virology* **66**, 3287–3297.

Rubinstein, R., Schoonakker, B. C. & Harley, E. H. (1991). Recurring theme of changes in the transcriptional control region of BK virus during adaptation to cell culture. *Journal of Virology* **65**, 1600–1604.

Salzman, N. P., Natarajan, V. & Selzer, G. B. (1986). Transcription of SV40 and polyomavirus and its regulation. In *The Papovaviridae*, vol. 1, *The Polyomaviruses*, pp. 27–98. Edited by N. Salzman. New York: Plenum Press.

Shadan, F. F. & Villareal, L. P. (1993). Coevolution of persistently infecting small DNA tumor viruses and their hosts linked to host-interactive regulatory domains. *Proceedings of the National Academy of Sciences, USA* **90**, 4117–4121.

Shah, K. V. (1996). Polyomaviruses. In *Fields Virology*, 3rd edn, pp. 2027–2043. Edited by B. N. Fields, D. M. Knipe & P. M. Howley. Philadelphia: Lippincott–Raven Publishers.

Spence, S. L. & Pipas, J. M. (1994). Simian virus 40 large T antigen host range domain functions in virion assembly. *Journal of Virology* **68**, 4227–4240.

Stewart, A. R., Lednicky, J. A., Benzick, U. S., Tevethia, M. J. & Butel, J. S. (1996). Identification of a variable region at the carboxy terminus of SV40 large T-antigen. *Virology* **221**, 355–361.

Stinebaugh, S. & Melnick, J. L. (1962). Plaque formation by vacuolating virus. *Virology* **16**, 348–349.

Sweet, B. H. & Hilleman, M. R. (1960). The vacuolating virus, SV40. *Proceedings of the Society for Experimental Biology and Medicine* **105**, 420–427.

Takemoto, K. K., Kirschenstein, R. L. & Habel, K. (1966). Mutants of simian virus 40 differing in plaque size, oncogenicity, and heat sensitivity. *Journal of Bacteriology* **92**, 990–994.

Tavis, J. E., Walker, D. L., Gardner, S. D. & Frisque, R. J. (1989). Nucleotide sequence of the human polyomavirus AS virus, an antigenic variant of BK virus. *Journal of Virology* **63**, 901–911.

Tegtmeyer, P., Dohan, C., Jr. & Reznikoff, C. (1970). Inactivating and mutagenic effects of nitrosoguanidine on simian virus 40. *Proceedings of the National Academy of Sciences, USA* **66**, 745–752.

Tevethia, M. J. & Ripper, L. W. (1977). Biology of simian virus 40 (SV40) transplantation antigen (TrAg). *Virology* **81**, 192–211.

Tominaga, T., Yogo, Y., Kitamura, T. & Aso, Y. (1992). Persistence of archetypal JC virus DNA in normal renal tissue derived from tumor-bearing patients. *Virology* **186**, 736–741.

Tornow, J., Polvino-Bodnar, M., Santangelo, G. & Cole, C. N. (1985). Two separable functional domains of simian virus 40 large T antigen: carboxyl-terminal region of simian virus 40 large T antigen is required for efficient capsid protein synthesis. *Journal of Virology* **53**, 413–424.

Van Heuverswyn, H. & Fiers, W. (1979). Nucleotide sequence of the *Hin*d-C fragment of simian virus 40 DNA. Comparison of the 5′-untranslated region of wild-type virus and of some deletion mutants. *European Journal of Biochemistry* **100**, 51–60.

Watanabe, S. & Yoshiike, K. (1986). Evolutionary changes of transcriptional control region in a minute-plaque viable deletion mutant of BK virus. *Journal of Virology* **59**, 260–266.

Weiner, L. P., Herndon, R. M., Narayan, O., Johnson, R. T., Shah, K., Rubinstein, L. J., Preziosi, T. J. & Conley, F. K. (1972). Isolation of virus related to SV40 from patients with progressive multifocal leukoencephalopathy. *New England Journal of Medicine* **286**, 385–390.

Received 17 September 1996; Accepted 30 January 1997