REVIEW

Cell and Molecular Biology of Simian Virus 40: Implications for Human Infections and Disease

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Simian virus 40 (SV40), a polyomavirus of rhesus macaque origin, was discovered in 1960 as a contaminant of polio vaccines that were distributed to millions of people from 1955 through early 1963. SV40 is a potent DNA tumor virus that induces tumors in rodents and transforms many types of cells in culture, including those of human origin. This virus has been a favored laboratory model for mechanistic studies of molecular processes in eukaryotic cells and of cellular transformation. The viral replication protein, named large T antigen (T-ag), is also the viral oncoprotein. There is a single serotype of SV40, but multiple strains of virus exist that are distinguishable by nucleotide differences in the regulatory region of the viral genome and in the part of the T-ag gene that encodes the protein's carboxyl terminus. Natural infections in monkeys by SV40 are usually benign but may become pathogenic in immunocompromised animals, and multiple tissues can be infected. SV40 can replicate in certain types of simian and human cells. SV40-neutralizing antibodies have been detected in individuals not exposed to contaminated polio vaccines. SV40 DNA has been identified in some normal human tissues, and there are accumulating reports of detection of SV40 DNA and/or T-ag in a variety of human tumors. This review presents aspects of replication and cell transformation by SV40 and considers their implications for human infections and disease pathogenesis by the virus. Critical assessment of virologic and epidemiologic data suggests a probable causative role for SV40 in certain human cancers, but additional studies are necessary to prove etiology. [J Natl Cancer Inst 1999;91: 119–34]

SHIFTING PARADIGMS

Evidence is mounting that simian virus 40 (SV40) infects humans and is associated with certain types of human tumors. These observations were unexpected, because SV40 generally has been considered to be a monkey virus that rarely infected humans and played no role in human disease. In this era of revolutionary advances in biology, well-accepted biologic concepts have undergone change. Recent findings suggest that the concepts of the rarity of SV40 infection in humans and the innocuousness of those infections are also in need of re-evaluation. This review will present selected aspects of the cell and molecular biology of SV40 and will evaluate those properties for their possible implications for human infection and disease pathogenesis. More comprehensive reviews of SV40 have recently been published *(1–3)*.

HISTORY OF SV40: CONCERN TO COMPLACENCY AND BACK TO CONCERN

The history of SV40 has its origins interwoven with the development of the polio vaccine. Both the inactivated and live attenuated forms of the polio vaccine, as well as several other viral vaccines, were prepared in primary cultures of rhesus monkey kidney cells, some of which were derived from monkeys that were naturally infected with SV40. The contaminating virus escaped detection until African green monkey kidney cells were used and the presence of the virus was recognized by the development of cytoplasmic vacuolization *(4)*. Some residual infectious SV40 survived the inactivation treatments used for the preparation of the vaccines, and millions of people were inadvertently exposed to live SV40 from 1955 through early 1963 when they were administered SV40-contaminated virus vaccines, with the major source being polio vaccines *(5–8)*. Adenovirus 3 and 7 vaccines used from 1961 through 1965 contained hybrid particles that had SV40 sequences in recombinant genomes *(6)*.

Soon after its discovery, SV40 was shown to be tumorigenic in rodents and to be able to transform many types of cells in culture *(5,9–11)*. Because of the potential public health risk due to the previous distribution of contaminated vaccines, SV40 became the object of intensive investigation. It became a favored laboratory model that was exploited in a variety of molecular biology studies. Among the major advances stemming from studies with SV40 are the determination of the first complete nucleotide sequence of a eukaryotic viral genome, the recognition of enhancers involved in transcriptional regulation, the phenomenon of alternative splicing, identification of steps in eukaryotic chromosomal DNA replication, the requirement for continued expression of a viral nonstructural protein for maintenance of transformation, identification of tumor suppressor protein p53, elucidation of viral effects on cell cycle regulation, and identification of a protein nuclear localization signal [*(1)* and references therein].

Concerns about adverse effects on human health due to SV40 exposure from contaminated polio vaccines lessened with time. No acute illnesses in individuals who received the SV40-contaminated vaccines were attributed to SV40 *(5,7)*, and 20 years later, individuals exposed to SV40-contaminated vaccines

appeared not to be at higher risk of developing cancer than those who received SV40-free vaccines *(3,7,8)*. Those studies included a 17- to 19-year follow-up of 1073 newborns who received the vaccine, and no excess risk of mortality was observed *(12)*. Although it was recognized that the length of observation may not have been sufficient to detect increased cancer risks, the study was terminated due to difficulties in follow-up. Those studies focused only on the recipients of contaminated vaccines. In a study of more than 50 000 pregnant women (from 1959 through 1965), it was noted that the rate of cancer in children born to mothers who received inactivated polio vaccine during pregnancy was about twofold greater than in offspring of mothers who had not, with neural tumors accounting for most of the difference *(13)*. A more recent analysis *(14)* concluded that individuals potentially exposed to SV40-contaminated polio vaccines as children were not at increased risk of developing cancer, although an independent analysis of the same database questioned whether such conclusions could be drawn *(15)*. Retrospective cohort studies have limitations, including the problems that the individuals who were actually exposed to SV40 are not known and that a small increase in the incidence of rare cancers in the database might escape detection *(3)*.

The accumulating reports of detection of SV40 DNA in human tumors brings the story full circle, reviving the original concerns that SV40 might pose a cancer risk to humans. The failure to observe detrimental effects that were attributable to SV40 infection in individuals who received the poliovirus vaccine led to a belief that SV40 was harmless in humans. The question to be considered now is whether the absence of appropriate data has resulted in a failure to recognize a human commensal or possible pathogen that is masquerading under the guise of a benign laboratory tool.

CLASSIFICATION OF SV40: SMALL DNA TUMOR VIRUS

SV40 is classified as a member of the *Polyomavirus* genus of the family Papovaviridae, based on the size and morphology of its icosahedral capsid and on the size of its double-stranded DNA genome *(16)*. Its closest relatives are two polyomaviruses recovered from humans, JC virus (JCV) and BK virus (BKV).

They share about 69% genomic similarity at the nucleotide level, with the lowest similarity in the regulatory region sequences. The large T antigens (T-ags) of the primate polyomaviruses have about 75% amino acid identity *(17)*. Although highly related, the viruses can be distinguished at the DNA and protein levels *(17–19)* and can be distinguished serologically by neutralization and hemagglutination inhibition assays *(18,20)*. Humans are commonly infected with JCV and/or BKV *(18)*, and so it is necessary to use highly virus-specific reagents in studies aimed at detecting the possible presence of SV40 in human tissues.
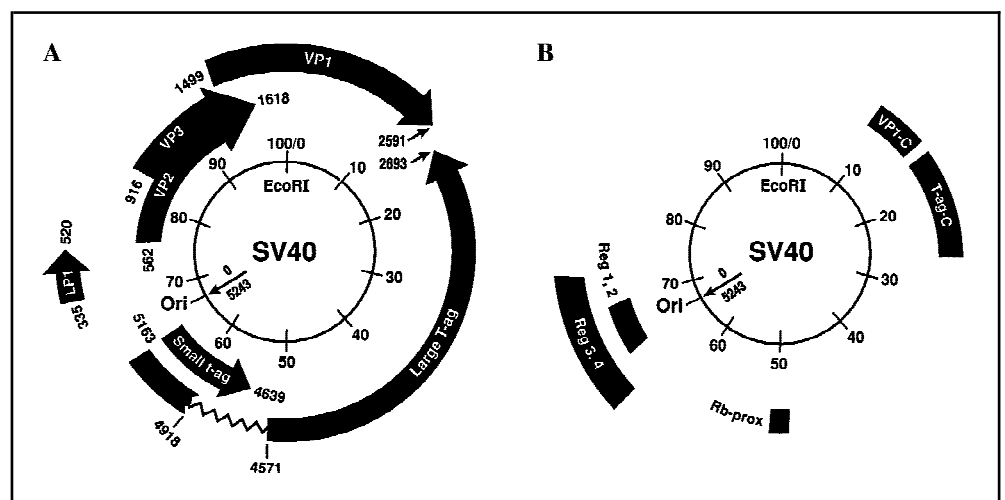
The common laboratory strains of SV40 were isolated about 1960 from contaminated vaccines or from uninoculated kidney cell cultures derived from rhesus, green, or patas monkeys. Although there is only one known serotype of SV40, different viral strains do exist and can be distinguished by variations in the structure of the viral regulatory region and in the nucleotide sequence of the extreme C terminus of the T-ag gene *(21)*. These nucleotide distinctions have been used to substantiate that human tumor-associated sequences were not the result of accidental laboratory contamination.

SV40 GENES AND GENE PRODUCTS: GENETICALLY STABLE BUT NOT IDENTICAL AMONG ISOLATES

The SV40 genome is a covalently closed, circular, double-stranded DNA (Fig. 1, A). The genome of reference strain 776 contains 5243 base pairs (bp), with other strains varying slightly in nucleotide count. Nucleotides are numbered in a clockwise direction, beginning and ending in the middle of the functional origin (Ori) of DNA replication (0/5243) and proceeding through the late region in a sense orientation and the early region in an antisense orientation.

SV40 makes maximal use of a small amount of genetic information by utilizing overlapping genes and a single regulatory region (Fig. 1, A). The single Ori is embedded in a nontranslated regulatory region that contains elements controlling transcription and replication and spans about 400 bp. The regulatory region can be divided into the Ori, the G + C-rich domain containing Sp1 binding sites and comprising part of the early promoter (referred to as the 21-bp repeat region), the enhancer area containing a segment referred to as the 72-bp element,

Fig. 1. Genetic map of simian virus 40 (SV40). **A)** The circular SV40 DNA genome is represented with the unique *Eco*RI site shown at map unit 100/0. Nucleotide numbers based on reference strain SV40-776 begin and end at the origin (Ori) of viral DNA replication (0/5243). The open reading frames that encode viral proteins are indicated. Arrowheads point in the direction of transcription; the beginning and end of each open reading frame are indicated by nucleotide numbers. Large T antigen (T-ag), the essential replication protein, as well as the viral oncoprotein, is coded by two noncontiguous segments on the genome. t-ag = small t antigen. (Reproduced from Brooks GF, Butel JS, Morse SA. *Medical Microbiology,* 21st ed, 1998, with permission from Appleton & Lange.) **B)** Location of SV40 genome regions analyzed by polymerase chain reaction amplification. Polymerase chain reaction products are shown as black boxes. Two products (Reg 1, 2 and Reg 3, 4) are from the regulatory region, Rb-prox is a product that abuts the DNA sequences encoding the pRb-binding site of T-ag, T-ag-C is from the C-terminal sequence of T-ag, and VP1-C refers to a section of the viral protein 1 (VP1) gene near the C terminus.

and a region containing the late promoter/initiator. Recent studies *(21–24)* have revealed that different strains of SV40 possess variations in the structure of the regulatory region. Among the three commonly used laboratory strains, there is a duplication of the 72-bp element (''nonarchetypal'') (Fig. 2, A). Viruses that have been freshly isolated from monkey tissues *(25–27)* or found to be associated with many SV40-positive tumors *(21)* usually contain no duplications in the enhancer (''archetypal''). Besides these changes, several single-nucleotide differences (polymorphisms) have been detected in the regulatory region that are useful in distinguishing different strains.

We recently studied two laboratory strains of SV40, Baylor and VA45–54, by comparing modern versions of each strain with early-passage stocks that had been held in storage for more than 25 years *(23)*. In both cases, the low-passage stocks contained mixtures of viruses having archetypal and nonarchetypal regulatory regions, and the contemporary laboratory-adapted viruses were the same as the nonarchetypal genomes present in the low-passage stocks. Analysis of different lineages of a labora-

Fig. 2. Regulatory region of simian virus 40 (SV40). **A**) DNA sequence profiles of regulatory regions of SV40 virus isolates from monkeys and humans and of human tumor-associated DNAs are shown. The viral origin of DNA replication from nucleotides 5192 to 31 is represented by the box labeled Ori; the G + C-rich 21-base-pair (bp) repeat region between nucleotides 40 and 103 is shown; the boxed number 72 is the 72-bp sequence within the enhancer region that is duplicated in some laboratory-adapted strains. Nucleotide numbers are based on that of SV40-776. Laboratory-adapted strains (SV40-776, Baylor, and VA45-54), human isolates of SV40 (SVCPC/SVMEN and SVPML-1), and viral sequences found associated with human brain (CPT, CPP, CPC, and Ep) and bone (Ost) tumors are shown. [Reproduced from Stewart et al. *(21)* with permission from *Journal of NeuroVirology.*] **B**) Schematic representation of SV40 regulatory regions of viral clones derived from brain tissues of simian immunodeficiency virus-immunocompromised monkeys (clone designations are on the right) is shown. The G + C-rich region is indicated by a box labeled G/C, and the major late promoter start site is identified by a vertical line labeled MLP(+1). Other designations are as above. The heavy horizontal arrow represents the agnoprotein coding sequence. Numbers to the left or right of vertical lines identify the junction position within a duplicated regulatory region sequence, based on nucleotides in SV40-776. The posterior 33 or 40 nucleotides derived from the enhancer 72-bp element in viruses 6593-5, H388-2, and H388-16 are identified by boxes labeled 33 and 40. The designations ori-T or ori-C designate the type of polymorphism at nucleotide 5209 of the SV40-776 Ori sequence. [Reproduced from Lednicky et al. *(26),* with permission from *Journal of Virology.*]
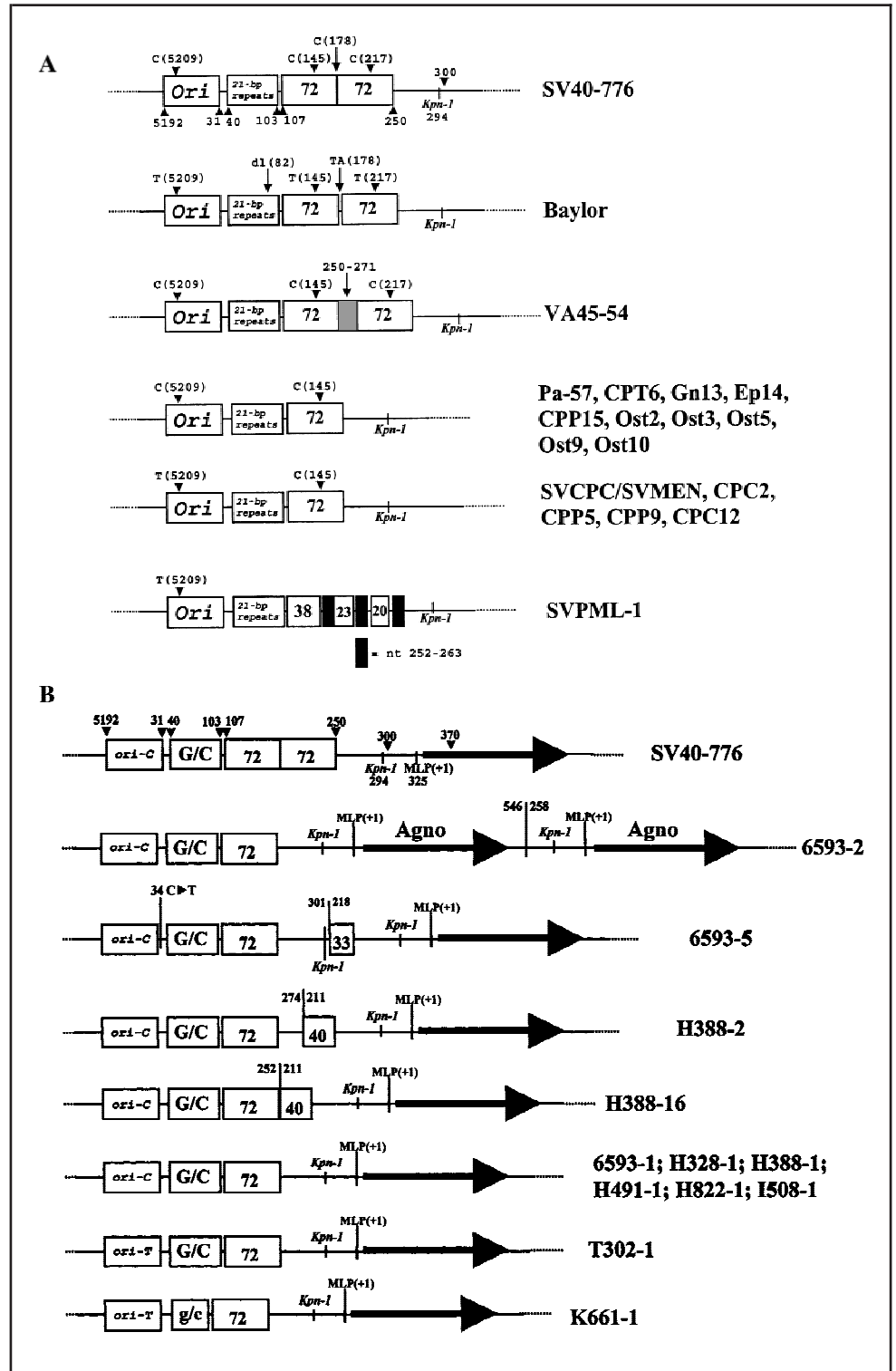
tory-adapted virus showed that, once a virus with a duplicated enhancer was selected in cultures of permissive cells, the structure thereafter was genetically stable. In a recent study of immunocompromised monkeys with disseminated SV40 infections, both archetypal and nonarchetypal viruses were recovered *(26)* *(described below)*. Considering that an enhancer duplication demonstrably improves growth of virus in tissue culture *(28),* it is not clear what effect faster-growing nonarchetypal viruses may have on persistence of SV40 infections *in vivo.* It is possible that slower or more inefficient replication by viral archetypes may promote establishment and maintenance of persistent infections by failing to elicit strong immune responses by the infected host. Low-grade ''smoldering'' infections are presumably controlled by the host immune response, a supposition supported by the fact that SV40 pathology has been observed only in immunocompromised monkeys. The generation of nonarchetypes may result in more abundant growth that, together with an impaired host immune system, may allow SV40 to escape immune control.

SV40 encodes three structural proteins (viral proteins VP1–3). The major capsid protein, VP1, contains 362 amino acids and forms the pentameric capsomeres that make up the surface of the virus particle. Little information is available concerning the epitopes of VP1. Seven different strains of SV40 recovered from humans and monkeys have now been sequenced *(21,26),* and the VP1 gene is highly conserved. Five isolates differed from SV40-776 at nucleotide 1756, which would change amino acid 86 of VP1 from glutamic acid to aspartic acid. Amino acid 86 is located in the loop between the B and C β sheets predicted in the three-dimensional structure of VP1 *(29).* From studies of JCV, it has been speculated that the BC loop is an antigenic region and

that changes in this loop may result in epitope changes *(30).* If variation at this position were to make an antigenic difference with SV40, this difference might complicate serologic assays used to determine the frequency and distribution of SV40 antibodies in human populations. However, all of the SV40-infected monkeys that recently yielded several new viral strains produced antibodies that neutralized the Baylor strain of SV40 *(26),* an independent isolate from 1961. One fragment of the VP1 coding sequence has been analyzed in the SV40 DNAs associated with 11 human brain and bone tumors (nucleotides 2288–2450) (Fig. 1, B) *(22,24).* In each case, there was an exact match with the sequence of SV40-776, except for a silent substitution at nucleotide 2384. Perhaps restrictions imposed by symmetry of the capsid permit negligible deviation in the amino acid sequence of VP1, making most changes unfavorable for the virus and, thus, inhibiting antigenic variation. If this is true, we would predict that antigenically distinct mutants of virus would rarely emerge.

SV40 encodes two ''early'' nonstructural proteins that share 82 N-terminal amino acids as a result of alternative splicing of viral transcripts. The T-ag of SV40-776 contains 708 amino acids; it is a multifunctional protein that is chemically modified in several ways (Fig. 3). The T-ag is an essential replication protein required for initiation of viral DNA synthesis that also stimulates host cells to enter S phase and undergo DNA synthesis. Because of this ability to subvert cell cycle control, it represents the major transforming protein of SV40. T-ag forms complexes with several cellular proteins, interactions that are involved in T-ag functions in viral DNA replication and induction of cellular DNA synthesis. Fundamental to T-ag effects on host cells are binding to
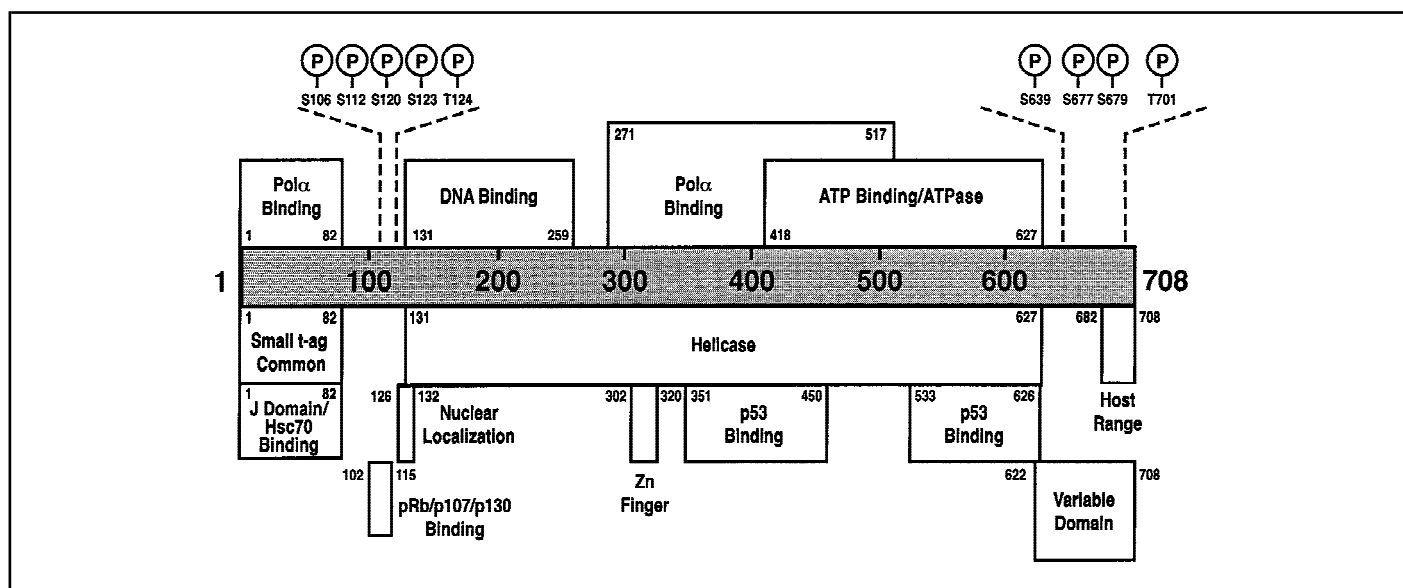
Fig. 3. Functional domains of simian virus 40 (SV40) large T antigen (T-ag). The numbers given are the amino acid residues; the numbering system for SV40-776 is used. Regions are indicated as follows: ''Small t-ag Common,'' region of T-ag encoded in the first exon (the amino acid sequence in this region is common to both T-ag and small t antigen); ''Polα Binding,'' regions required for binding to polymerase α-primase; ''J Domain/Hsc70 Binding,'' region required for binding the heat shock protein hsc70; ''pRb/p107/p130 Binding,'' region required for binding of the Rb tumor suppressor protein and the Rb-related proteins p107 and p130; ''Nuclear Localization,'' contains the nuclear localization signal; ''DNA Binding,'' minimal region required for binding to SV40 Ori DNA; ''Helicase,'' region required for full helicase activity; ''Zn Finger,'' region that binds zinc ions; ''p53 Binding,'' regions required for binding the p53 tumor suppressor protein; ''ATP Binding/ATPase,'' region containing the adenosine 5′-triphosphate binding site and adenosine 5′-triphosphatase catalytic activity; ''Host Range,'' region defined as containing the host range and Ad helper functions; ''Variable Domain,'' region containing amino acid differences among viral strains. The circles containing a P indicate sites of phosphorylation found on the T-ag expressed in mammalian cells. S indicates a serine and T indicates a threonine residue. [Reproduced from Stewart et al. *(31),* with permission from *Virology.*]

cellular tumor suppressor proteins (p53, pRb, p107, and p130/pRb2).

Most of the T-ag coding sequence is absolutely conserved among viral isolates. However, studies *(21,22,24,25,31)* have identified a variable domain at the extreme C terminus of T-ag (T-ag-C), defined as the last 86 amino acids of the molecule (residues 622–708) (Fig. 1, B). Laboratory-selected strains can be distinguished on the basis of nucleotide differences in this region (Fig. 4); these differences consist of short polynucleotide insertions and deletions, as well as single-nucleotide changes. Among the known polyomaviruses, only SV40, JCV, BKV, and SA12 (from baboons) contain the T-ag C-terminal domain; all other T-ags terminate after the adenosine 5'-triphosphatase domain (at residue 625 in SV40 T-ag) *(17)*.

In a study *(23)* comparing the regulatory region structures of early-passage archived stocks with modern-day stocks of two laboratory strains of SV40, the T-ag-C sequences were examined to determine whether genetic variation occurred over time. The variable region at the T-ag C terminus did not undergo any change with extended viral passage and thus does not represent a mutable hotspot in the viral genome. On the basis of this knowledge, we have proposed that the T-ag-C region and the structure of the viral regulatory region may serve as useful sites for identification and classification of different strains of SV40 *(21,23)*.

The function of the T-ag-C in natural infections remains to be determined. Embedded within the variable region is the host range/adenovirus helper function domain *(31–34)* (Fig. 3). This region of T-ag can relieve a block to human adenovirus replication in monkey cells, and its deletion affects the ability of SV40 mutants to grow in different monkey kidney cell lines. This same region also appears to play an undefined role in addition of VP1 during virion assembly *(35)*. It is not known whether the T-ag-C influences the replication of SV40 in dif-ferent tissues in susceptible hosts, but it remains a theoretical possibility that some variants of SV40 may be better adapted to replicate in specific types of human tissues. In addition, the C terminus of T-ag contains two epitopes involved in the antibody response of BALB/c mice to T-ag, with the epitope at the very end of the molecule (amino acids 690–708) also able to produce partial immunity to a tumorigenic challenge. Neither peptide appeared to contribute to antibody or antitumor response of C57BL/6 mice *(36)*. These observations suggest that the T-ag-C may play a role in modulating the ability of individual hosts to respond to infection and tumor formation by SV40.

SV40 DNA sequences found in human brain and bone tumors displayed sequence variation in the T-ag-C among the tumors *(21,22,24)*, ruling out the possibility of laboratory contamination of the tumor samples (Fig. 4). There is to date no evidence for human-specific strains of SV40 or for tumor type-specific associations, suggesting that SV40 has a broad host range. However, relatively small numbers of tumor-associated sequences have been analyzed and, as more samples are studied, some strain-specific associations may become apparent.

These recent observations have established that not all SV40 isolates are identical. Although biologic functions *in vivo* in primate hosts have not been attributed to the observed regulatory region and T-ag-C variations, the existence of multiple different SV40 strains raises the possibility that viral variants may differ in tissue tropism or disease potential in humans.

NATURAL INFECTIONS IN MONKEYS: BOTH BENIGN AND PATHOGENIC

The natural hosts for SV40 are species of Asian macaque monkeys, especially the rhesus (*Macaca mulatta*). In captivity, several related species are easily infected, including the cyno-
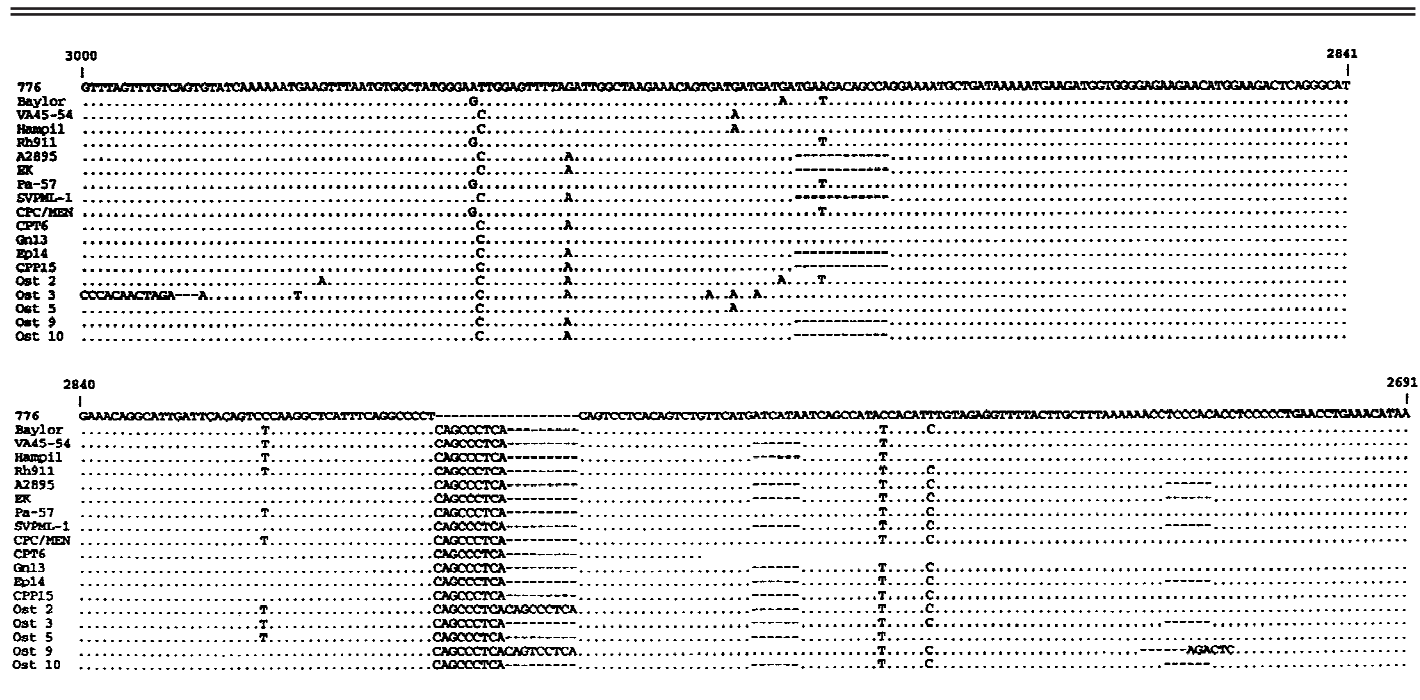

Fig. 4. Nucleotide sequences of the large T antigen (T-ag) C-terminal variable domains of simian virus 40 (SV40) isolates and human tumor-associated sequences compared to SV40-776. Each sample is identified at the left. The sequence given is for the coding strand; the numbering system of SV40-776 was used. Dots indicate identity and dashes indicate a deletion compared to the sequence of SV40-776, which is given on the top line. [Reproduced from Stewart et al. *(21)*, with permission from *Journal of NeuroVirology*.]

molgus macaque and the African green monkey. SV40 establishes persistent infections in the kidneys of susceptible monkeys, and the level of virus present may be very low. Both viremia and viruria occur in infected animals, and virus shed in the urine is probably the means of transmission among these hosts. Susceptible animals can be infected by the oral, respiratory, and subcutaneous routes (7,37).

SV40 infections in healthy monkeys appear to be asymptomatic (7,37), although SV40 has been associated with a fatal case of interstitial pneumonia and renal disease (38). SV40 can cause widespread infections in monkeys with simian acquired immunodeficiency syndrome, having been detected in brain, lung, kidney, lymph node, and spleen (25–27,39). Viral DNA has also been detected in circulating peripheral blood mononuclear cells (26). SV40-induced progressive multifocal leukoencephalopathy, a demyelinating disease, has reportedly developed in simian immunodeficiency virus-immunocompromised macaques (25,26,39,40), as has an astrocytoma containing SV40 DNA (41). The presence of SV40 in the brain of immunodeficient monkeys demonstrates that SV40 is neurotropic in addition to being kidney tropic, and the presence of viral DNA in spleen and peripheral blood mononuclear cells suggests that virus may spread within the host by hematogenous routes.

Genetic studies of SV40 recovered from natural infections in monkeys that were immunodeficient due to simian immunodeficiency virus infections revealed extensive genetic heterogeneity (25–27). Mixtures of viruses with either archetypal or nonarchetypal regulatory regions were found in some animals (Fig. 2, B). Several natural isolates of SV40 displayed variation at the C terminus of the T-ag gene, indicating the existence of multiple virus strains in the primate colony, presumably introduced by animals imported from various sources. Similar studies have not been carried out on immunocompetent hosts. The animals that contained large amounts of SV40 were all severely immunodeficient due to simian immunodeficiency virus infection, as reflected by coincident opportunistic infections with organisms such as *Mycobacterium avium* and *Candida* species (26). Whether natural infections in normal monkeys would display comparable viral genetic variation and tissue distributions of infection remains to be determined.

These observations from infected monkeys have implications for human disease. Polyomaviruses may be involved in a broader spectrum of disease than commonly believed, as the catalog of tissues recognized to be capable of harboring the viruses continues to expand. It can be predicted that in human infections SV40 could be found in various tissues, including blood cells [as has been reported by Martini et al. (42)]. Hematogenous dissemination could spread the virus throughout the body, seeding different tissues. Viral infections in healthy immunocompetent hosts would probably be subclinical and benign in most cases. However, in immunocompromised hosts, SV40 infections might flourish and become pathogenic; lesions might be produced in a number of different tissues with expected involvement of the central nervous system. It remains to be determined whether certain virus variants have a higher propensity to infect particular tissues or to cause more severe disease.

SV40 as an Experimental Tumor Virus: Potent With Broad Tissue Tropism

The oncogenic potential of SV40 was demonstrated originally by inoculation of neonatal hamsters (3,5,9–11); sarcomas usually developed at the site of subcutaneous inoculation. Intracerebral inoculation produced ependymomas (43), whereas intravenous injection of weanling hamsters resulted in lymphomas, osteogenic sarcomas, poorly differentiated sarcomas, and, rarely, lymphocytic leukemia (44). Intracardial and intrapleural injections have produced mesotheliomas in 60%–100% of animals (3,45). SV40 under the control of its natural regulatory region induces choroid plexus papillomas in transgenic mice (46), but when T-ag is placed under the control of appropriate foreign promoters, it can induce tumors in practically any target tissue (47,48). However, it appears only certain cell types in hamsters become transformed after virus exposure. As described below, human tumors found to harbor SV40 DNA sequences are among the tumor types produced in rodents by virus injection.

The SV40 transforming protein T-ag disrupts cell growth control mechanisms, primarily by binding to and abolishing the normal functions of tumor suppressor proteins p53 and pRb family members (1,49–51). Three essential regions of T-ag have been identified as required for transformation. The N terminus (amino acids 1–82) contains a J domain that binds the hsc70 molecular chaperone protein and is presumably involved in assembly and disassembly of protein complexes (52–55). A separate domain (amino acids 102–115) proximal to the N-terminal J domain is required for binding to pRb-related tumor suppressor proteins (pRb, p107, and p130/pRb2). T-ag binds the hypophosphorylated form of pRb and thus disrupts the role of pRb in coordinating cell cycle progression. pRb normally binds transcription factor E2F in early G_1 phase of the cell cycle. When pRb is phosphorylated by cyclin-dependent kinases, E2F is released and functions to activate expression of growth-stimulatory genes (56). T-ag causes unscheduled dissociation of pRb-E2F complexes, releasing active E2F. The third region contains the p53 binding sites (amino acids 350–450 and 533–626). Wild-type p53 is believed to sense DNA damage and either cause the cell to pause in late G_1 phase for DNA repair or direct the cell to commit suicide through the apoptotic pathway if repair is not possible (57,58). One way p53 functions is to transcriptionally induce p21 cyclin-dependent kinase inhibitor, which blocks the activity of G_1 cyclin-cdk complexes, arresting cell cycle progression in late G_1 phase. T-ag binding sequesters p53, abolishing its function and allowing cells with genetic damage to survive and enter S phase. This leads to accumulation of T-ag-expressing cells with genomic mutations that may promote tumorigenic growth.

Studies with tsA mutants of SV40 (having temperature-sensitive mutations in T-ag) showed that T-ag was required for both the initiation and maintenance of the transformed state (59–61). SV40 T-ag is exceptional in that it is able to mediate both immortalization and transformation of cells, in contrast to other oncogenes that display only one of these activities (62,63). This is presumably related to the ability of T-ag to negate both the pRb and the p53 regulatory pathways in the cell.

Less is known about the role of SV40 small t antigen in transformation, but it appears to potentiate the function of T-ag. Small t antigen binds protein phosphatase-2A; this binding results in activation of the mitogen-activated protein kinase pathway and growth stimulation of quiescent cells. Small t antigen is important for T-ag transformation of resting cells (49,64,65).

Many types and species of cells have been transformed *in vitro* by SV40 (11,62,66), including those from mice, humans, hamsters, rats, cattle, and guinea pigs. Various assays can mea-

sure the effects of SV40 on growth properties of cultured cells in which the virus does not replicate. In a focus-forming assay, dense foci of altered cells that have lost contact inhibition pile up on background monolayers of normal cells; however, this assay is not useful with epithelial cells, which, unlike fibroblasts, tend not to form foci after transformation. Some, but not all, morphologically transformed cells may produce colonies when suspended in semisolid medium, an indication that the cells have acquired the ability to grow in an anchorage-independent manner. Cells are considered to be neoplastically transformed if they form tumors when injected into susceptible animals (e.g., syngeneic mice, syngeneic hamsters, or nude mice). The frequency of transformation by SV40 is low when quantitated in a focus-formation or colony-formation assay. Generally, cells that are obtained from an *in vitro* transformation assay are not easily transplantable in an immunocompetent animal, presumably because additional genetic changes must accumulate to allow progression of the morphologically transformed cell to a tumorigenic phenotype.

SV40 DNA usually is found integrated in transformed rodent cells. No specific integration sites are used, but an intact early region is retained and T-ag is expressed in all transformed cells *(1)*. Such cells have allowed elegant studies of the mechanism of T-ag-mediated transformation. However, episomal polyomavirus DNAs have also been detected in various types of tumors and transformed cells (Lednicky JA, Butel JS: unpublished results). It is not clear whether episomal viral genomes are sufficient to maintain the transformed state or whether an integrated viral DNA copy is required.

Transgenic mice expressing SV40 T-ag in a particular target tissue have provided insights into mechanisms of SV40 transformation *(47,48)*. Concepts are illustrated by a transgenic mouse system that we developed for analysis of stages in liver carcinogenesis in which expression of SV40 T-ag was targeted to the liver using the regulatory elements of the human α_1-antitrypsin gene *(67)*. Members of a stable transgenic line reproducibly developed liver tumors by 10 weeks of age. The following four clearly distinguished stages were identified that occurred with predictable kinetics: normal, hyperplastic, dysplastic, and neoplastic. Nearly 100% of hepatocytes stained intensely for T-ag by immunohistochemistry at the early stages. When the livers exhibited diffuse dysplasia with no tumor nodules, or later, as hepatic carcinomas arose in a background of liver dysplasia, there was considerable variation in both the intensity of T-ag staining and the proportion of T-ag-positive cells in and between individual foci and tumor nodules. There were even some tumor nodules that were negative for T-ag. This study showed that SV40 T-ag caused widespread liver hyperplasia and dysplasia but that additional events apparently were required for cell progression to neoplasia. Immunoprecipitation analyses demonstrated that T-ag was complexed with p53 in liver tumors. In addition, the fact that some tumor nodules appeared to lack T-ag expression suggests that clonal diversification subsequent to neoplastic transformation, perhaps reflecting genetic instability in the cells due to T-ag inactivation of p53, may have made the continued presence of T-ag unnecessary for tumor progression.

In transgenic mice in which T-ag was expressed in the salivary gland under the control of an inducible promoter *(68)*, extensive ductal hyperplasia developed by 4 months of age. If T-ag expression were silenced at 4 months, the hyperplasia was

reversed, but if T-ag was not silenced until 7 months of age, the hyperplasia persisted in the absence of T-ag. This inducible system demonstrated that transformed cells may eventually lose their dependence on the initiating viral oncoprotein. Other transgenic mice have provided evidence that requirements for T-ag subdomains and related transforming functions differ among cell types. For example, the N-terminal region of T-ag is dispensable for transformation of T lymphocytes *(69)*, and the p53-binding domain is not essential for induction of choroid plexus tumors *(70,71)*.

SV40 T-ag is highly immunogenic and induces both antibody and cytotoxic T-lymphocyte responses. Five cytotoxic T-lymphocyte recognition sites have been identified and mapped *(19)*. These multiple cytotoxic T-lymphocyte determinants provide effective immunosurveillance against the outgrowth of SV40 tumor cells. Presumably, the immune response also modulates virus growth in infected hosts. It is well-known that immunosuppressed animals are more susceptible to viral carcinogenesis than normal animals and that polyomavirus infections flourish when the host is immunocompromised. It is likely that SV40 tumors develop under circumstances in which the initial transformed cells are able to evade the host immune response and continue to proliferate.

A wide variety of primary human cell types has been immortalized by SV40 via a process that differs in several respects from the general pattern described for rodent cells [*(72)* and references therein]. After introduction of intact SV40 or the SV40 T-ag gene, human cells often exhibit an extended lifespan (''lifespan extension'') but then enter a stage termed ''crisis,'' characterized by considerable cell death and decreasing cell numbers. Cells will rarely immortalize, generating a cell line able to proliferate indefinitely (immortalization). This occurs at an estimated frequency of 1×10^{-7} in fibroblasts *(73,74)* and 1×10^{-5} in mammary epithelial cells *(75)*. A study *(76)* involving human dermal fibroblasts showed that different steps in this process (escape from senescence, proliferation rate, morphologic changes, and loss of contact inhibition) are regulated by the amount of T-ag present. The T-ag gene is required for immortalization of human cells by SV40, but it is not sufficient, because recessive changes in cellular genes are also required *(77–80)*. SV40-immortalized human cells are usually not tumorigenic in nude mice, but they are sensitive substrates for subsequent oncogenic transformation by oncogenes, chemicals, and irradiation *(72)*. Clearly, SV40 T-ag can function as an oncogene in different types of human cells.

A study *(81)*, using fetal fibroblasts with a finite lifespan as the target cells and two different types of assays, compared the abilities of mutant and chimeric polyomavirus T-ags to transform and immortalize human cells. ''Transformed'' cells were recognized as dense foci of rapidly growing morphologically altered cells; clonal isolates contained T-ag. Individual transformants were subcultured to determine whether they would senesce after reaching 100 population doublings or whether an immortal line would survive and grow indefinitely (''immortalization assay''). All T-ags from SV40, JCV, and BKV were able to morphologically transform human cells, and approximately 10% of SV40 transformants became immortal. Three regions of SV40 T-ag were required for immortalization of human cells—the N terminus, a central region containing the pRb-binding domain, and the C terminus—although the T-ag C terminus was dispensable for transformation. Analysis of chimeric T-ag con-

structs revealed that T-ags from JCV and BKV contain an immortalization enhancement function at their C termini that can substitute for the SV40 functional domain. However, intact JCV T-ag did not immortalize any cells, a result that suggests that JCV T-ag lacks other functional domains important for immortalization. This study established that a large domain at the C terminus of SV40 T-ag functions in the immortalization of human cells. Because the precise C-terminal T-ag sequences involved in immortalization of human cells were not identified, it will be important to determine whether the crucial sequences include the variable domain region of T-ag that differs among human tumor-associated sequences.

This synopsis of SV40 transformation in model systems emphasizes the strong oncogenic potential of the virus and its oncoprotein in a variety of cell types, including those from humans. Observations from transgenic mice have established that, as tumors progress, the T-ag functions that were important for tumor initiation may become dispensable. Human cells are more difficult to transform than rodent cells and may not express comparable phenotypes. The observed exceptions to the common patterns of SV40-mediated transformation suggest that the association of SV40 with human cancers may take different forms, depending on factors such as host conditions, stage of disease, and cell types involved.

SV40 AND HUMAN INFECTIONS: EVIDENCE OF SUSCEPTIBILITY

SV40 can replicate productively in many types of cultured human cells, including human fetal tissues (82), newborn human kidney cells (82), and various human tumor cell lines (83), although SV40 grows poorly in human fibroblasts (84). Moreover, in vitro assays have shown that human cell extracts can support the replication of SV40 DNA, establishing that human proteins have the intrinsic ability to cooperate with SV40 T-ag to replicate viral DNA (85–87). In human fetal neuroglial cell cultures, SV40 replicated lytically and destroyed the spongioblasts, whereas astrocytes underwent morphologic transformation (88). Many human cell lines have been found to be sensitive to SV40 infection. Some cell types, classified as ''permissive,'' undergo visible cell destruction in response to SV40, whereas others, classified as ''semipermissive,'' fail to exhibit cytopathic changes and produce low levels of virus (83). It is noteworthy that many human cell lines that support lytic growth by SV40 produce significant numbers of defective viral genomes, even on low-multiplicity passage (83). General conclusions from these studies are that SV40 can replicate and produce progeny virus in human cells and that various human cell types display differences in their susceptibility to infection by SV40. The basis for the differences is unknown; T-ag functions are believed to be important (89,90), although the expression of receptors for virus attachment and uptake may also be involved.

Additional studies have provided evidence that SV40 can cause human infections and replicate in vivo. SV40 was observed to establish low-grade infections in infants fed contaminated polio vaccine, with virus excreted in the stool for up to 5 weeks (91). No antibody response to SV40 was detected in various groups of individuals who received vaccine by oral administration (7). In volunteers infected by the intranasal route with a respiratory syncytial virus stock unknowingly contaminated with SV40, SV40 caused inapparent infections and produced a low-level antibody response (92). As further evidence of

human infections, SV40 has been isolated from two patients with progressive multifocal leukoencephalopathy (93), has been associated with another case (94), and has been recovered from a child with anatomic and neurologic anomalies (95).

It is not clear how widespread SV40 infections in humans are today. In the United States, the major exposure to SV40 occurred during vaccination of the general population (mostly under the age of 20 years) with inactivated poliovirus vaccine. By 1957, more than 45 million persons under age 20 years and more than 14 million aged 20 years or more had received one or more inoculations (5,96). The overall exposure to inactivated vaccine reached 98 million by 1962; it is estimated that 10–30 million were actually exposed to live SV40. The risk of exposure to SV40 has been estimated as being high for persons born from 1941 through 1961, moderate for those born from 1921 through 1940, low for those born from 1901 through 1920, and no risk for those born in 1963 and later when polio vaccines were free from SV40 (7). Since seroprevalence studies are a standard approach to determining the frequency of viral infection, it is important to consider these patterns of possible exposure to contaminated vaccine when interpreting serology results.

Serologic surveys reported in the early 1970s detected low levels of SV40-reactive antibodies in humans whether or not they had been exposed to contaminated vaccines (Table 1). Laboratory workers who handled primary monkey cell lines, monkeys, or SV40 had a prevalence of antibodies to SV40 in the range of 41%–55% (100,101). Neutralizing antibodies to SV40 were detected in 19.8% of the sera from a group of Maryland children born from 1955 through 1957 who were at high risk of having received SV40-contaminated polio vaccine (98). Antibody responses to SV40 after exposure to contaminated vaccines were generally low titered (92,99,104). A low prevalence of serum antibodies to SV40 (3%–13%) in people not exposed to the contaminated vaccines indicated other sources of exposure to SV40 or to a related virus (97–99,101,102).

In a recent study (103), we found that 16% of men infected with human immunodeficiency virus 1 and 12% of men who were seronegative for human immunodeficiency virus 1 were seropositive for SV40 with low titers of SV40-neutralizing antibodies by a plaque-reduction assay. These seroprevalence values are somewhat lower than those reported for children who were probably exposed to SV40-contaminated vaccines (≈20%) (98), although the sera from the men were collected and analyzed at least 25 years after the children's sera were collected and analyzed; the longevity of SV40 antibodies induced by the contaminated vaccines is not known. Notably, we observed that approximately 10% of the individuals born after 1962, who had no risk of exposure to contaminated vaccines, had SV40-neutralizing antibody, indicating that there must exist an alternative source of human infection by SV40. Because there are no lower mammals or arthropods known to serve as reservoirs of infection, SV40 may be transmitted among humans. One can speculate that the use of contaminated vaccines may have introduced the virus into the human population or, alternatively, may have merely broadened a pre-existing infection. The seropositivity rate (≈10%) that we observed for the group born after 1962 is higher than that reported two decades ago (98,99,105) for persons not exposed to vaccines (2%–5%). Whether this reflects a higher frequency of human infection by SV40 today, a difference in assay sensitivity, or an anomaly of sample size is not known.

Recently, we sought to estimate the frequency of SV40 in-

Table 1. Serologic evidence of simian virus 40 (SV40) infections of humans

| Serum donor group | Possible exposure to SV40 | Samples with SV40 antibody | | Reference No. |
		No. tested	No. positive* (%)	
Studies reported in the 1970s				
Elderly cancer patients (United States)	Unknown	184	7 (3.8)	(97)
Children (Maryland), 12–14 y old in 1969	Contaminated polio vaccine	141	28 (19.8)	(98)
Children (Maryland), 1–5 y old in 1969	Unknown (after vaccines SV40 free)	278	9 (3.2)	(98)
Prisoners (Ohio) 20–29 y old in 1954	Unknown (before monkey kidney-grown vaccines)	100	2 (2)	(98)
Monkey handlers (India)	Contact with rhesus monkeys	37	10 (27)	(99)
Elderly cancer patients (India)	Possible contact with monkeys	975	52 (5.3)	(99)
Laboratory workers (Hungary)	Contact with monkeys or monkey kidney cells	37	15 (40.5)	(100)
Studies reported in the 1980s				
Laboratory workers (Germany)	Contact with SV40	11	6 (54.5)	(101)
Cancer patients (Germany)	Unknown	143	42 (29.4)	(101)
Control subjects (Germany)				
Born from 1959 through 1961	Contaminated polio vaccine	72	17 (23.6)	(101)
Born from 1962 through 1964	Unknown	115	15 (13.0)	(101)
Medical students (Wisconsin)				
Sera collected in 1952	Unknown (before vaccines)	51	6 (11.8)	(102)
Studies reported in the 1990s				
HIV-infected men (Texas)†				
Born before or in 1962	Contaminated polio vaccine	219	36 (16.4)	(103)
Born after 1962	Unknown	17	2 (11.8)	(103)
HIV-negative men (Texas)				
Born before or in 1962	Contaminated polio vaccine	149	18 (12.1)	(103)
Born after 1962	Unknown	31	3 (9.7)	(103)
Children (Texas)				
Born from 1980 through 1995	Unknown	337	20 (5.9)	Butel et al.: unpublished results

*The majority of results reported in the 1970s were based on tube neutralization assays, the results reported in the 1980s detected SV40 immunoglobulin G antibodies by enzyme-linked immunosorbent assay, and the recent studies (reported in 1990s) used plaque-reduction assays to detect SV40-neutralizing antibody.

†HIV = human immunodeficiency virus.

fections in unselected hospitalized children who were born from 1980 through 1995 and, thus, would not have been exposed to SV40-contaminated vaccines. We detected low titers of SV40-neutralizing antibody in about 6% of the children (Butel JS, Arrington AS, Jafar S, Wong C, Lednicky JA, Opekun AR, et al.: unpublished results). Seropositivity increased with age and was significantly associated with kidney transplants. The only child younger than 7 years of age to have detectable SV40 antibody was a 3-year-old child with a brain tumor. Many of the children with SV40 antibodies had been treated with drugs that compromise the immune system. It is possible that the virus may not replicate abundantly enough in many immunocompetent hosts to induce the production of detectable levels of neutralizing antibody, suggesting that an antibody survey may not reflect the true prevalence of SV40 infections in humans. However, based on available data, the prevalence of SV40 infections in children is much lower than that of BKV and JCV infections (Table 2). Neutralization tests predicated on the abolition of virus infectivity are a highly specific measure of virus antibodies (108), and the SV40 plaque reduction test as a specific measure of SV40 neutralizing antibodies in human serum has been discussed [(103); Butel JS, Arrington AS, Jafar S, Wong C, Lednicky JA, Opekun AR, et al.: unpublished results]. However, the possibility that cross-reactive antibodies to the human polyomaviruses may be contributing to the apparent SV40 antibodies has not been ruled out.

To confirm the presence of SV40 infections in children, we used polymerase chain reaction and DNA sequence analysis to analyze archival tissue samples from patients identified as pos-

Table 2. Comparison of simian virus 40 (SV40) seroprevalence with reported BK virus (BKV) and JC virus (JCV) antibody prevalences in comparable age groups of children*

Age, y	SV40 antibody prevalence (Butel et al.: unpublished results)	Reported BKV antibody prevalence (106)	Reported JCV antibody prevalence (107)
<1–4	1/95 (1.1%)	44/89 (49.4%)	2/20 (10.0%)
5–9	5/88 (5.7%)	69/87 (79.3%)	19/69 (27.5%)
10–15	14/154 (9.1%)	54/58 (93.1%)	13/20 (65.0%)

*Data are number of children with viral antibodies/number of children examined (total). Numbers in parentheses are the percent of total children with viral antibodies.

sessing SV40-neutralizing antibody (Butel JS, Arrington AS, Jafar S, Wong C, Lednicky JA, Opekun AR, et al.: unpublished results). SV40 DNA was detected in tissue specimens from four children. Other investigators have detected SV40 DNA by the polymerase chain reaction in normal tissues from patients, including in peripheral blood cells (42), pituitary tissue (109), and lung/pleural samples (110). Because polyomaviruses establish long-term persistent infections, it is not surprising that SV40 DNA would be found in some normal tissues.

Several lines of evidence demonstrate that humans are susceptible to SV40. Numerous tissue culture studies have shown that SV40 can replicate in human cells, serologic surveys have detected SV40 antibodies in humans, and the molecular identification of SV40 DNA in patient tissues has proven that SV40 is able to naturally infect humans.

SV40 AND HUMAN CANCER: CASUAL ASSOCIATION OR CAUSAL RELATIONSHIP?

SV40 DNA has recently been detected by polymerase chain reaction technology and sequence analysis in human tumors (Table 3), including pediatric and adult brain tumors *(22,42,117)*, mesotheliomas *(110,118–120,122,123)*, osteosarcomas *(24,124)*, bronchopulmonary carcinomas *(110)*, pituitary tumors *(109)*, and papillary thyroid carcinomas *(125)*. These observations support earlier, frequently anecdotal, reports of the association of SV40 with human cancers *(8,111,113,114–116,126–128)*. The expression of T-ag has also been observed in some tumors (Table 3). However, there have been reports of failures to detect either viral DNA sequences or expression of T-ag in many types of tumors [reviewed in *(117);(121,129)*]. Differences in results reported by independent groups may reflect the numbers and types of tumors examined, the population or geographic origin of the tumors, the age of the patients, or the analytic methods used.

Molecular approaches have confirmed the presence of authentic SV40 in at least some of the human tumors found to contain SV40-like DNA. Four separate sections of the SV40 genome have been amplified (Fig. 1, B) and sequenced from

Table 3. Association of simian virus 40 (SV40) with human tumors

| Tumor type | No. positive/No. tested | | Year of report | Reference No. |
	SV40 DNA	SV40 T-ag*		
Brain tumors	1/7		1979	*(111)*
Brain tumors	14/53	18/68	1984	*(112)*
			1990	*(8)*
Brain tumors		2/39†	1978	*(113)*
Brain tumors	11/32‡		1982	*(114)*
Melanoma		1/1§	1976	*(7)*
Meningioma		2/2	1976	*(7)*
Meningioma	5/16§	3/16	1975	*(115)*
			1981	*(116)*
Astrocytoma	1/8		1981	*(116)*
Astrocytoma	8/17		1996	*(42)*
Oligodendroglioma	1/3		1981	*(116)*
Medulloblastoma	1/2		1981	*(116)*
Choroid plexus	10/20‖	4/5	1992	*(117)*
			1995	*(22)*
Choroid plexus	5/6		1996	*(42)*
Ependymoma	10/11	3/6	1992	*(117)*
			1995	*(22)*
Ependymoma	8/11		1996	*(42)*
Glioblastoma	10/30		1996	*(42)*
Mesothelioma	29/48	13/16	1994	*(118)*
Mesothelioma	4/9		1996	*(119)*
Mesothelioma	8/11		1995	*(120)*
Mesothelioma	0/50		1996	*(121)*
Mesothelioma	14/22‡	32/52	1997	*(122)*
Mesothelioma	30/35	4/4	1997	*(123)*
Mesothelioma	10/21		1998	*(110)*
Pulmonary carcinoma	18/63		1998	*(110)*
Osteosarcoma	40/126		1996	*(124)*
Osteosarcoma	5/10		1997	*(24)*
Pituitary tumors	26/30		1995	*(109)*
Thyroid carcinoma	3/69		1998	*(125)*

*T-ag = large T antigen.
†Positive tumors were an ependymoma and a choroid plexus papilloma.
‡Positive for SV40 messenger RNA.
§Infectious SV40 was rescued by cell fusion from one tumor.
‖Infectious SV40 was rescued by transfection of one tumor DNA.

several brain tumors and osteosarcomas *(22,24,124)*. In those cases, it is highly likely that SV40 was present rather than a new or hybrid virus with limited similarity to SV40. Infectious SV40 was isolated from one choroid plexus carcinoma *(22)*, and complete genomic sequence analysis confirmed its identity *(21)*.

The tumor-associated SV40 DNA sequences that have been analyzed in detail usually had an archetypal regulatory region arrangement (Fig. 2, A) and a variety of C-terminal T-ag variable domains *(21)*. To date, there are no obvious tissue-specific differences among viral isolates (Fig. 5). Interestingly, one particular T-ag variable region sequence (virus isolate SVPML-1) was recovered from multiple human tumors *(21);* whether it is more common among human disease or fortuitously overrepresented in the samples analyzed is unknown.

These sequence results have allayed concerns of possible laboratory contamination of tumor samples. The tumor-associated sequences lack the enhancer duplication typical of laboratory virus and display T-ag-C sequences different from those of laboratory strains. In our own studies, positive control plasmids are based on SV40-776 and the Baylor strain of SV40, and those sequences have not been recovered from any tissue samples.

The integration state of the viral DNA has seldom been determined in studies of human tumors, due to insufficient amounts of sample available for analysis. A recent study *(125)* did provide evidence of integration of SV40 viral DNA in three different thyroid tumors. The fact that four different segments of the SV40 genome could be amplified from some brain and bone tumors *(22,24)* suggests indirectly that full-length episomal viral DNA was present in those specimens, but the data do not address whether integrated copies may have been present as well. Our attempts to rescue virus from osteosarcoma DNA samples were unsuccessful *(24)*. There are several possible explanations for the virus recovery failures, including that the viral DNA was integrated in the bone tumors or that the viral genomes were defective. As mentioned above, SV40 propagated in cultured human cells often produces many defective viral genomes.

Attempts to quantitate the viral genomes present in tumor samples have not been informative, due to limitations of available samples. The heterogeneity of tumor samples, which generally contain both nontumor and tumor cells, complicates interpretation of results. If SV40 is etiologically important in the development of the tumors, we would expect SV40 DNA to be present in the tumor cells. However, tumor progression models predict the accumulation of cellular genetic changes over time, accompanied by changes in the phenotype of tumor cells, and it is possible that the accumulation of mutations makes T-ag functions dispensable in late-stage tumors, permitting the loss of SV40 DNA from some tumor cells. Early-stage tumors would be better candidates in which to search for evidence of mandatory expression of T-ag.

The diversity of SV40 sequences detected in human tumors argues against a single source of human infection (Figs. 4 and 5). Rather, it appears that SV40 has entered the human population on multiple occasions. There are many strains of SV40 in simians, as evidenced by the distinctiveness of the known laboratory strains *(21)* and the heterogeneity observed among recent natural isolates from monkeys *(25–27)*. The contaminated polio vaccines presumably would have contained mixtures of SV40 strains; perhaps many were successful at establishing human infections.

The SV40 regulatory region sequences appear to be clonal in
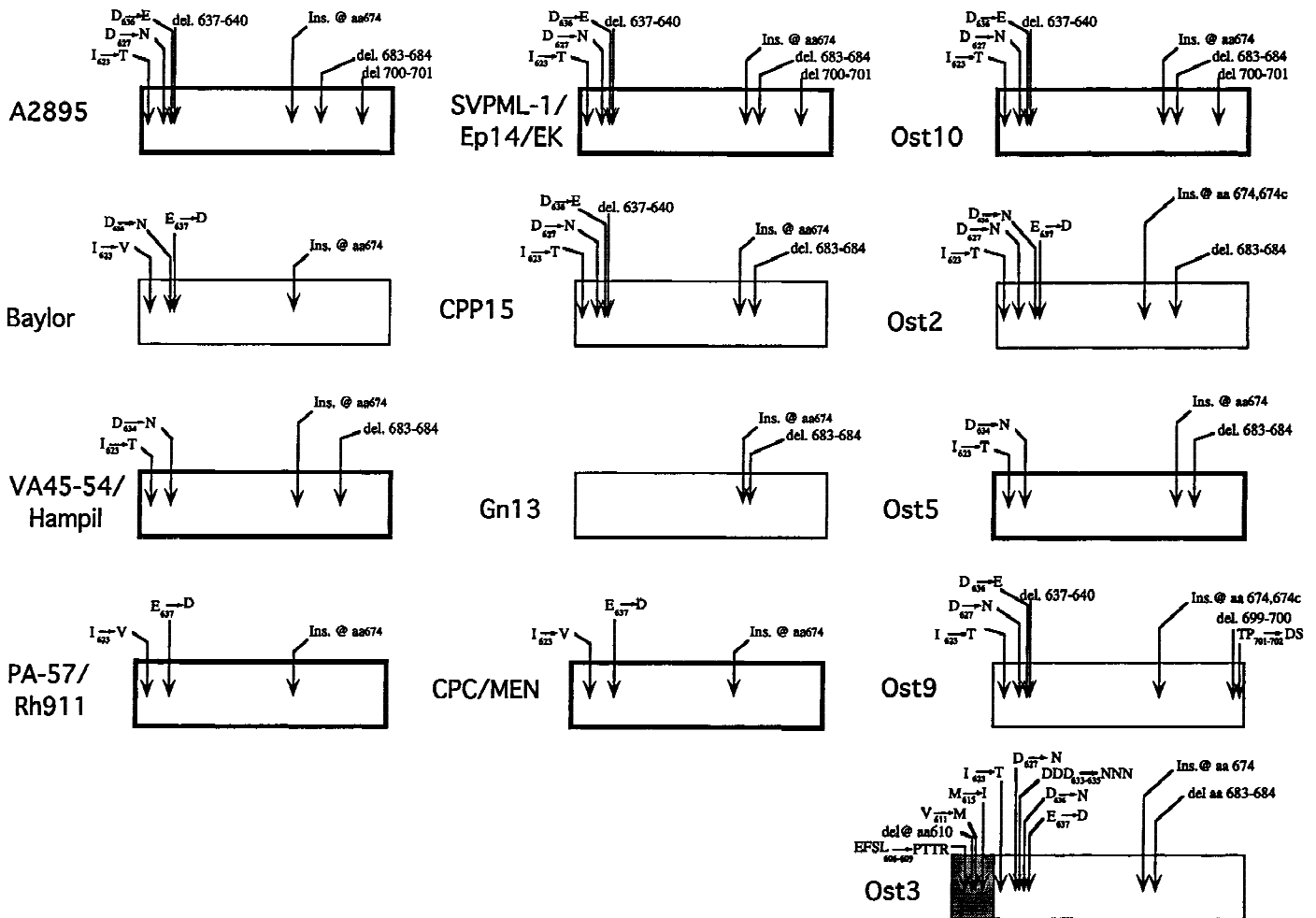
Monkey kidney   Human brain   Human Bone

Fig. 5. Schematic comparison of the large T antigen (T-ag) C-terminal variable domain amino acid sequences of simian virus 40 (SV40) isolates and human tumor-associated sequences to SV40-776. The rectangular boxes represent the T-ag C-terminal region from amino acids 622 to 708. Laboratory strains isolated from monkey kidney cells are shown on the left; human brain isolates and brain-tumor-associated sequences are in the center, and human osteosarcoma-associated sequences are on the right. Viral strains and DNA sequences are identified to the left of all boxes. Identical T-ag amino acid sequences in DNAs from different tissues are aligned horizontally and the boxes encased in bold lines. The numbering follows the system for SV40-776; insertions in the region of amino acid 674 are designated with 674 plus a lowercase letter. Arrows indicate the position and type of amino acid changes. The shaded area of the Ost3 box indicates the region immediately upstream of T-ag residue 622. aa = amino acid(s); del = deletion; Ins = insertion. [Reproduced from Stewart et al. *(21)*, with permission from *Journal of NeuroVirology.*]

each human tumor analyzed to date. This is in contrast to the samples from immunocompromised monkeys from which mixtures of viral regulatory region structures were recovered. This may suggest that the viral DNA was important in the development of the tumor, as opposed to a passenger virus model in which the tumor merely represents a susceptible site for virus replication.

Recent studies detected the expression of T-ag in mesothelioma tumor cells by immunohistochemical methods. Protein extraction approaches showed that T-ag from five different mesothelioma lysates was apparently complexed with p53 *(122)* and that T-ag from four mesotheliomas was capable of interacting with pRb family members translated *in vitro* in a rabbit reticulocyte lysate and then mixed with tumor cell extracts *(123)*. These data suggest, but do not prove, that T-ag may be functioning in human mesotheliomas to dysregulate growth control pathways by mechanisms similar to those described in rodent tumors *(130)*.

It is impressive that SV40 has been detected much more frequently in human cancers than either JCV or BKV, the ubiquitous human polyomaviruses known to be tumorigenic in rodents. One explanation may be that the human polyomaviruses have had millenia to become adapted to humans but that SV40, which may be a much more recent acquisition, may be less well adapted and more pathogenic to its human hosts. The same reasoning is used to explain the virulence of human immunodeficiency virus or herpes virus B infections in humans compared with their benign effects in their natural simian hosts. Another plausible explanation is that, just as some strains of human papillomaviruses are carcinogenic in humans and others are not,

SV40 may be a more oncogenic polyomavirus than JCV or BKV in humans.

It is informative to consider what we have learned from well-characterized rodent systems about the mechanism of SV40 oncogenesis and to relate this information to what is known for virus-related human tumors. It is well-established that 1) T-ag is the viral oncoprotein, 2) the virus can transform cells in culture and induce tumors in rodents, 3) viral DNA is integrated in most rodent tumors, 4) T-ag expression is retained in most tumor cells, 5) target proteins for T-ag binding include p53 and pRb family members, and 6) wild-type p53 rather than mutant p53 is found in SV40-transformed cells (2,11,49,50,131,132). Some comparable data have been gathered that support an association of SV40 with human tumors. SV40 DNA sequences have been commonly detected in certain tumor types (choroid plexus, ependymomas, mesotheliomas, and osteosarcomas), but few studies have identified which individual cells (tumor versus normal) in the heterogeneous mixture of cells in a tumor actually contain the viral DNA. The T-ag gene is retained, and the expression of T-ag protein has been detected in some tumors; a limited number of studies (42,117,118,122) have carried out immunostaining and have demonstrated T-ag synthesis in tumor cells per se. Infectious SV40 has been isolated from one meningioma and one choroid plexus tumor. The young age of the patients with pediatric brain tumors corresponds to that required for tumor induction in experimental animals by polyomaviruses and suggests that virus infection may have occurred transplacentally or in the perinatal period.

There are limitations to the rodent model systems as predictors for molecular details of SV40 in human cancers. One important difference is that SV40 does not set up productive infections in rodents, and so the experimental systems cannot mimic the dynamics of long-term persistent infections of the host, with the different cell types infected and virus dissemination routes involved. The possibility of virus variants arising within the infected host also is not a feature of the rodent models. Although it has been reported that the viral genome is usually integrated in rodent tumors (Lednicky JA, Butel JS: unpublished results), there is no indication of whether that is a formal requirement for tumor formation in all types of tissues.

Evans and Mueller (133) summarized the difficulties of proving a causal link between a candidate virus and a human cancer. They identified the following potential problems: 1) a long incubation period between initial infection with the virus and the cancer with which it is associated; 2) the common and ubiquitous nature of most candidate viruses and the rarity of the cancers with which they are associated; 3) the initial infection with the candidate virus is often subclinical, so that the time of infection cannot be established by clinical features; 4) the need for cofactors in most virus-related cancers; 5) the causes of cancer may vary in different geographic areas or by age; 6) the different viral strains may have different oncogenic potential; 7) the human host plays a critical role in susceptibility to cancer, especially the age at time of infection, genetic characteristics, and status of the immune system; 8) cancers result from a complex and multistage process and a virus may play a role at different points in pathogenesis in association with alterations in the host's immune system, oncogenes, chromosomal translocations, and other molecular events; 9) the inability to reproduce human cancers in experimental animals with the virus; and 10) the recognition that multiple factors (virus, toxin, chemical, and al-

tered gene) may all affect processes that result in cancers with the same histologic features. Evans and Mueller (133) then suggested types of evidence that would surmount some of those problems to support an etiologic role for a virus in a given human cancer. Suggested epidemiologic guidelines included the following: 1) the geographic distribution of viral infection should coincide with that of the tumor, adjusting for the presence of known cofactors; 2) the presence of viral markers should be higher in case subjects than in matched control subjects; 3) viral markers should precede the tumor, with a higher incidence of tumors in persons with the marker than in those without; and 4) prevention of viral infection should decrease tumor incidence. Suggested virologic guidelines included the following: 1) the virus should be able to transform human cells in vitro, 2) the viral genome should be demonstrated in tumor cells and not in normal cells, and 3) the virus should be able to induce the tumor in an experimental animal.

With the use of these guidelines, evaluation of the association of SV40 with human cancers leads to the conclusion that the virus may be an important factor in some cancers, although etiology is not yet proven beyond a doubt. The following virologic guidelines for causality from Evans and Mueller (133) are met: SV40 can transform a variety of human cells in vitro, SV40 is a potent tumor inducer in experimental animals, and the types of tumors that are induced by SV40 in laboratory animals are the same as those human cancers found to contain SV40 DNA. Furthermore, SV40 DNA has been found in tumor cells, but it has also been detected in some normal tissues, as would be expected of a virus that establishes long-term persistent infections. Limited data are available, however, to apply Evans and Mueller's epidemiologic guidelines. The geographic distribution of human infections by SV40 is unknown, no case–control study has been carried out that compares SV40 markers, and no longitudinal study has determined the time of virus infection relative to tumor development in specific individuals.

Criteria have been proposed by Sir Austin Bradford Hill (134) to help differentiate between causation or mere association when there is an observed connection between a disease and some environmental factor. The Bradford Hill Criteria are as follows: 1) strength of association, 2) consistency (Has the association been observed repeatedly by different people in different places?), 3) specificity, 4) temporal relationship (Does exposure to the presumed causative factor precede the disease?), 5) biologic gradient (Is there a dose–response curve?), 6) biologic plausibility, 7) coherence (Does the association seriously conflict with known facts of the natural history and biology of the disease?), 8) experimental evidence (Are there supportive experimental results?), and 9) analogy (Are there observations from related systems that support the proposed association?).

Several of the Bradford Hill Criteria are met by available evidence that links SV40 and human cancer. The association of SV40 with human cancer is consistent, since the presence of SV40 DNA in certain types of human tumors has been independently confirmed by multiple investigators in different geographic locations. The association is biologically plausible, based on the well-characterized properties of SV40 as a DNA tumor virus and the evidence that human infections by SV40 do occur. The association is coherent, because it does not conflict with known facts and the widespread use of contaminated polio vaccines provides a reasonable explanation of how SV40 may have become broadly seeded throughout the human population.

The following experimental evidence from studies of human samples is supportive: 1) the T-ag gene is retained in human tumors, 2) T-ag protein has been detected in tumor cells in several reports, 3) molecular markers certify that SV40 is the virus being detected, 4) infectious SV40 has been isolated from a tumor, and 5) antibody surveys and molecular data show that SV40 can and does infect humans in contemporary times. Reasoning by analogy also supports the putative association: SV40 is a proven cancer virus, based on experimental animal model systems and *in vitro* transformation studies involving rodent and human cells. Natural history studies of SV40 in monkeys and of BKV and JCV in humans provide logical explanations for possible transmission of SV40 among humans and the dissemination of SV40 *in vivo* to different tissues. However, data are not yet available to address several of the Bradford Hill Criteria. More samples need to be analyzed before the strength of association of SV40 with any particular tumor type is known, although the number of mesotheliomas analyzed is accumulating and the consistent association of SV40 with that tumor is compelling. Studies addressing temporal relationship and dose–response curves of SV40 to cancer development remain to be done. Therefore, using the Bradford Hill Criteria, it appears that the association of SV40 with human tumors must be taken seriously but that further studies are needed to prove causality.

In summary, a review of the literature from the last 30 years provides persuasive evidence that SV40 DNA can be found in human cancers. As more sophisticated technologies have been applied, simple questions of possible laboratory contamination of tumor samples and misidentification of viral type have been ruled out. Some criteria necessary to prove causality of human tumors by SV40 have been met, but others remain unanswered. Although proof of causation of specific types of tumors awaits further analysis, the association of SV40 with human cancers is currently strong enough to warrant serious concern.

FUTURE: NEW VIEWS OF AN OLD VIRUS

The totality of the cell and molecular biology of SV40 provides undeniable evidence that the virus is a potent oncogenic agent under a variety of conditions. That knowledge identifies future studies that could be carried out to establish the etiologic significance of SV40 DNA associated with human tumors. Analytical studies of additional tumor samples should examine the frequency of virus-infected cells and whether T-ag is expressed and functional in the majority of tumor cells. Because similar types of tumors may have different causes or cofactors, the challenge will be to determine the circumstances that place certain individuals at risk of infection by SV40 and of the development of cancer containing viral sequences. Sources of infection and modes of transmission for SV40 in the human population need to be determined. The incidence of viral infection in different geographic regions and among different groups of individuals remains to be established, as well as the breadth of target tissues that might support viral infection. The possibility should be investigated that SV40 may be associated with examples of nonmalignant disease in humans, as well as with cancer. Assays to readily detect both humoral and cellular immunity to SV40 would facilitate such studies.

SV40, as with other polyomaviruses, appears to replicate most robustly when the host immune response is compromised. Increased viral loads would facilitate virus spread to other tissues and the opportunity for detrimental virus–cell interactions.

This suggests that segments of the population at elevated risk of possible adverse consequences would include the very young, organ transplant recipients, patients undergoing treatment for cancer, and human immunodeficiency virus-infected individuals. People exposed to possible cofactors, such as asbestos, may also be at higher risk of developing SV40-related cancer.

It remains possible that genetic changes may be involved in SV40 adaptation to human hosts. The indications of a relatively low frequency of SV40 infections in humans ($\leq$10% seroprevalence) suggest that the virus is not highly successful at maintaining human infections or, alternatively, efficiently evades immune surveillance. The conventional virologic wisdom is that when a virus crosses into a different species, some limited genetic changes are necessary to ensure a stable host–pathogen relationship. Additional sequence studies should compare primary human and monkey isolates of SV40 to seek such putative adaptive genetic changes. The possible influences of strain differences in viral regulatory regions and C-terminal T-ag sequences on tissue tropism and disease development need to be determined. The original source of SV40 strains now present in humans is not known. Knowledge of whether they date to the use of contaminated polio vaccines would reveal the history of human infections by SV40 and whether other sources of virus exposure may continue to exist today.

The association of SV40 with human disease provides the potential for new approaches to the diagnosis and treatment of certain types of cancers and, possibly, other illnesses. It may become advisable to develop preventative measures to inhibit virus infection and transmission, including perhaps a vaccine against SV40. It is of no small irony that SV40, once having been found as an unrecognized contaminant of a widely heralded viral vaccine, might itself one day become a candidate for vaccine development.

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