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Different Simian Virus 40 Genomic Regions and Sequences Homologous with SV40 Large T Antigen in DNA of Human Brain and Bone Tumors and of Leukocytes from Blood Donors

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BACKGROUND. Many studies found only a small fragment of the large T-antigen coding sequences in human tumors, raising doubts on authenticity of SV40 sequences detected in these samples.

METHODS. Five different regions of SV40 DNA were investigated in 106 fresh human tumor biopsies (25 brain, 69 bone, 12 Wilms' tumors), 71 tumor-derived cell cultures (38 from brain and 33 from bone tumors) and normal tissues (5 fresh bone biopsies and 38 buffy coats) by polymerase chain reaction (PCR) techniques and filter hybridization with specific oligoprobes. Expression of SV40 Tag sequences was analyzed in human tumor specimens by RT-PCR.

RESULTS. SV40 large T-antigen sequences were detected at high prevalence, in human biopsies of primary brain (37–44%) and bone (21–37%) tumors, in cell cultures derived from brain (30–54%) and bone (53–80%) tumors. SV40 Tag sequences were detected in 29% of buffy coats of blood donors. However, only four brain tumor cell lines showed all the five regions of the SV40 genome investigated. Expression of SV40 Tag sequences was found in 11 of 27 (41%) human tumor samples. DNA sequence analysis indicated that the PCR-amplified products belong to the SV40 wild type. Polymerase chain reaction products of Tag middle portion from 20 of 78 (26%) samples showed a 97% homology with telomeric sequences of human chromosomes 10 and 11.

CONCLUSIONS. Authentic SV40 sequences were detected in human samples. The expression of SV40 Tag sequences indicates that SV40 could play a role, as a cofactor, in the onset/progression of specific human cancers. The inability to detect some regions of the virus genome may suggest that those regions are not required for tumor persistence or growth and have been lost or, alternatively, may be the result of assay conditions that were unable to PCR-amplify those regions in the tumors. ***Cancer* 2002;94:1037–48.** *© 2002 American Cancer Society.*
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KEYWORDS: SV40, tumor, leukocyte, DNA homology.

The accession numbers at Gene Bank for the human sequences homologous with the middle portion of SV40 Tag coding sequences are the following: no. Z96177.1, *H. sapiens* chromosome 10 telomeric DNA sequence, clone 10QTEL040; no. Z96184.1, *H. sapiens* chromosome 11 telomeric DNA sequence, clone 11PTEL014.

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SV40 Tag sequences have been detected at different prevalence by polymerase chain reaction (PCR) followed by filter hybridization in human brain tumors,[1–5] pleural mesotheliomas,[6–10] bone tumors,[11–15] pituitary[16] and thyroid neoplasms,[17] and in lymphoproliferative disorders.[18–20] Southern blot hybridization experiments with bone and papillary thyroid tumor DNAs confirmed the SV40 specificity of these sequences and assessed that the viral DNA can be present in a free episomal form as well as integrated into the human genome.[13,17] Moreover, SV40 Tag DNA sequences were detected in normal lung and pituitary tissues,[8,16] in peripheral blood mononuclear cells (PBMCs) of patients affected by osteosarcomas,[14] in PBMCs from blood donors,[2,3,14,18,20] and in sperm fluids of healthy individuals.[3] SV40 DNA was detected in PBMCs and brain tissue of monkeys,[21] and SV40 neutralizing antibodies were found in human sera.[22] In most studies, SV40 sequences were detected by PCR amplification, suggesting that SV40 infection in human tumors and normal tissues commonly results in a low viral load. Indeed, by using a semiquantitative PCR assay we determined 10^{-2} to 10^{-4} SV40 genome equivalents per cell in DNA samples from human lymphoproliferative disorders and from PBMCs of blood donors.[18] SV40 reactivation, by transfection of SV40 positive human DNA into permissive monkey cells, was reported in only a single case,[23] in agreement with the observation that in human cells that are semipermissive for SV40, this virus replicates poorly,[24] reaches low viral titers,[18,24,25] and generates at high-rate heterogeneous defective genomes,[26] which may interfere with the production of a complete, infectious viral progeny.

SV40 transforms to the neoplastic phenotype cells from various species, including human cells[27,28] and induces in rodents specific neoplasms such as ependymomas, choroid plexus papillomas, osteosarcomas, soft tissue sarcomas, lymphomas,[27] and mesotheliomas.[29] SV40 immortalization, transformation, and oncogenicity are mediated by Tag, a nuclear multifunctional phosphoprotein of 94 kilodaltons, which displays ATPase and helicase activities, induces viral and cellular DNA replication, and binds to tumor suppressor proteins p53, p105RB1, and p130RB2[30] as well as to transcriptional coactivators p300 and p400,[31,32] abolishing their functions. SV40 Tag displays clastogenic and mutagenic activities by inducing numeric and structural chromosome aberrations and gene mutations in human cells.[33–35] Complexes of SV40 Tag with p53 and pRb were detected in human mesotheliomas[36,37] and brain tumors,[5] thus adding further support to a role of SV40 in human tumorigenesis. It has been shown that antisense to SV40 Tag sequences induces growth arrest and apoptosis in SV40 Tag positive human mesothelioma cells.[38] Moreover, in SV40-transformed canine MDCK cells and human mesothelial cells Tag induces production and secretion of the hepatocyte growth factor/scatter factor that activates by phosphorylation its receptor, the c-*met* oncogene product, generating an autocrine loop.[39,40] This new mechanism of cell transformation triggered by SV40 Tag would explain why in most human tumors SV40, which is present only in a fraction of neoplastic cells as reported by different investigators,[41] is able to direct SV40 negative cells toward malignant transformation.[40]

SV40 is a monkey virus, which was believed to be transmitted to humans only under exceptional situations in natural infection.[42] SV40 contaminated vaccines,[42,43] in particular, antipolio vaccines, were administered to millions of humans worldwide between 1955 and 1963.[44,45] However, the presence of this viral agent in humans, before the introduction of SV40-contaminated vaccines, cannot be discarded.[46] Together with SV40, other simian viruses were transmitted to humans through early contaminated polio vaccines.[47] Moreover, a new polyoma virus, named CPV, was detected in Cynomolgus monkeys. This virus turned out to be closely related to SV40, because CPV Tag NH_2-terminal sequences are highly homologous with SV40 Tag NH_2-terminal conserved region and CPV Tag reacts with a MAb specific for SV40 Tag.[48] The discovery of this new monkey polyoma virus and the finding that most of the previous studies were focused only on a small region of SV40 DNA, i.e., the Tag NH_2-terminal sequence, prompted us to investigate whether the presence of SV40 sequences from different regions of the viral genome can be detected in human samples. The objectives of this study were to search by PCR, in a new series of human brain and bone tumor specimens of different histotypes, Wilms' tumors, normal bone tissue, and buffy coats from blood donors, the SV40 genomic regions corresponding to the Tag NH_2 terminus, middle portion, and COOH terminus, the VP1 structural protein and the regulatory sequences. The expression of the Tag gene was analyzed by reverse transcription (RT)-PCR, and the SV40 specificity of different regions, amplified by PCR, was investigated by DNA sequencing.

MATERIALS AND METHODS

Fresh Biopsies

Specimens under analysis were obtained from various Institutions. Twenty-five primary brain tumors from 16 glioblastoma multiforme (G1, G2, G5, G15, G28, GB7, GB28, GB33, T11, T14, T24, T45, T58, T60, T63, T77) and 9 astrocytomas (G3, G10, G23, T15, T17, T54,

T57, T72, T76) were from the Neurologic Institute "Carlo Besta," Milan. Sixty-nine primary bone tumors, namely, 30 osteosarcomas (1–12 OS; 1–18 B [bone tumor]), 11 small cell osteosarcomas (1–11 SCO), and 28 Ewing sarcomas (EW 1–28) together with 5 normal bone tissues (1–5 NBT) were obtained from the Orthopedic Institute "Francesco Rizzoli," Bologna. DNA from 12 primary Wilms' tumors (1–12 WT) was kindly obtained from Dr. Delattre, Istitut Curie, Paris. Thirty-eight buffy coat samples (1–38 BC) from blood donors were obtained from the blood bank of the "Sant' Anna" General Hospital in Ferrara.

Short-Term Cell Cultures and Cell Lines

Thirty-eight short-term cell cultures from brain tumors, 20 glioblastoma multiforme (G32, G56, G63, G82, G88, G93, G95, G98, G100, G103, G112, T4, T7, T21, T38, T49, T60, T62, T63, T69), 13 astrocytomas (G61, G86, G91, G101, G110, G111, G114, T1, T12, T13, T26, T27, T29), 2 oligoastrocytomas (G58, G90), 1 oligodendroglioma (T10), 1 gangliosarcoma (G87), and 1 ganglioglioma (G122) were from the Department of Biology and Genetics for Medical Sciences, University of Milan. Brain tumor–derived short-term cell cultures were obtained, during the period 1994–2000, from fresh biopsies. Tumor specimens, approximately 0.1–0.5 cm^3, were mechanically ground in a Petri dish with a sterile pestle, resuspended in 5 mL of complete medium, RPMI-1640 with 10% heat-inactivated fetal calf serum (FCS), 100 IU/mL penicillin G, and 100 μg/mL streptomycin and layered on the bottom of 25-cm^2 flasks to avoid detachment of tissue fragments. All cell cultures were incubated at 37 °C in 5% CO_2 humidified atmosphere. When cells reached the confluence, they were detached after treatment with 0.05% trypsin and 0.02% ethylenediamine tetraacetic acid (EDTA) at 37 °C for 5 minutes and transferred into new flasks with fresh medium. Mycoplasma contamination was investigated by mean of culturing in agar-broth mycoplasma medium and of fluorescent Hoechst 33258 staining of DNA. Cell cultures were considered established because they consistently expressed the astrocyte cell marker, the glial fribrillary acidic protein (GFAP), thus demonstrating unequivocally their astrocyte origin. Brain tumor–derived short-term cell cultures were grown at 37 °C. in RPMI-1640 or Dulbecco's modification of Eagle's medium F12 supplement with 10% FCS in a 5% CO_2 humidified atmosphere and used at the passage 3-10.[49]

Thirty-three bone tumor cell cultures derived from 15 osteosarcomas (HOS, 1-7 IOR/OSC, IOR/OS10, IOR/OS17, KHOSNP, KHOS240, KHOS312, MG63, MNNG), 13 Ewing sarcomas (1–13 EWC), and 5 giant cell tumors of bone (TCG 23–24, TCG 32–33, TCG 49) were from the oncology laboratory of the "Francesco Rizzoli" Orthopedic Institute, Bologna. Eight cell cultures from bone tumors were cell lines (HOS, IOR/OS10, IOR/OS17, MNNG, KHOSNP, KHOS240, KHOS312, MG63), whereas the other 25 were short-term cell cultures. They were obtained from surgical specimens of bone tumors. Biopsies were immediately placed in Iscove's modified Dulbecco's Medium (IMDM) supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin and stored at 4 °C or kept on ice until the treatment. Surgical samples were minced in a Petri dish with a small volume of IMDM. Tumor fragments were then digested by incubation with collagenase type I (Sigma) at 37 °C for 1–3 hours. After blocking of the enzymatic digestion with IMDM 10% FCS, we seeded collagenase-released cells in 25-cm^2 flasks with IMDM 10% FCS. Both cell lines and short-term cultures of primary bone tumor cells were cultured in IMDM 10% FCS and maintained at 37 °C in a humidified 5% CO_2 atmosphere.

Nucleic Acid Purification Methods

All samples under analysis were never investigated before for the SV40 DNA sequences. Specimens were quickly frozen in liquid nitrogen and kept at −80 °C until the time of analysis. DNA from brain and bone tumor specimens was extracted with a commercial kit (ORCA Research Inc., Bothell, WA) as indicated by the supplier. Buffy coats of normal individuals were digested with 1% sodium dodecyl sulfate and 500 μg/mL proteinase K. DNA was extracted with a mixture of phenol-chloroform-isoamyl alcohol (25:24:1) and dialyzed for 24 hours with TEN buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 1 M NaCl) and for a further 24-hour period with TE buffer, which is identical to TEN buffer but without NaCl. DNA from buffy coats in this study, from PBMCs and lymphocytes in previous studies,[2,3,18] were purified with the phenol-chloroform-isoamyl alcohol solution, because DNA extracted with commercial kits or quick methods gave negative PCR results. Negative data were probably caused by the residual presence of hemoglobin, a well known inhibitor of PCRs. To verify whether cross-contaminations occurred during the DNA extraction procedure, we purified each sample simultaneously with a specimen of salmon sperm DNA and a mock specimen lacking DNA, and then subjected it to PCR analysis. Total cytoplasmic RNA was obtained digesting the cells using a mixture of 10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 1.5 mM $MgCl_2$, and 0.5% NP40, together with 10 mM vanadyl ribonucleoside complex (GIBCO-BRL, Life Technology, Milan, Italy) to avoid RNA degradation. RNA was purified by three phenol extractions and precipitated with cold ethanol/0.2 M NaCl.

TABLE 1
Oligonucleotides Used as Primers in PCR and as Probes in Filter Hybridization

SV40 DNA regions	Oligonucleotides[a]	Reference[b] position (nt)	PCR annealing temperature (°C)	Size (bp)
Tag NH_2	SV.for2: 5′-CTTTGGAGGCTTCTGGGATGCAACT-3′	4945-4921	55	575
	SV.rev: 5′-GCATGACTCAAAAAACTTAGCAATTCTG-3′	4372-4399		
	SV.for2: 5′-CTTTGGAGGCTTCTGGGATGCAACT-3′	4945-4921	58	543
	PYV.rev: 5′-GAAAGTCTTTAGGGTCTTCTACC-3′	4403-4425		
	SV Probe: 5′-ATGTTGAGAGTCAGCAGTAGCC-3′	4452-4473		
Tag COOH	TA1: 5′-GACCTGTGGCTGAGTTTGCTCA-3′	3070-3048	58	441
	TA2: 5′-GCTTTATTTGTAACCATTATAAG-3′	2630-2652		
	T Probe: 5′-AACCTCTACAAATGTGGTATGGCT-3′	2741-2764		
Tag middle	SV.for 7: 5′-TGAGGCTACTGCTGACTCTCAACA-3′	4476-4452	58	1847
	TA2: 5′-GCTTTATTTGTAACCATTATAAG-3′	2630-2652		
	T Probe: 5′-AACCTCTACAAATGTGGTATGGCT-3′	2741-2764		
VP1	VPA: 5′-AACTGGAGTAGACAGCTTCACT-3′	1621-1642	58	409
	VPB: 5′-AGCAGGATATTTGGTCCTGTAG-3′	2029-2008		
	VPC: 5′-TTACAGATGACTCTCCAGACA-3′	1731-1751	68	211
	VPD: 5′-TGAATGGGTTTTCCAGCACCA-3′	1941-1921		
	VP probe: 5′-TGATGTGGGAAGCTGTTACT-3′	1830-1849		
Regulatory	RA3: 5′-GCGTGACAGCCGGCGCAGCACCA-3′	358-336	52	483
	RA4: 5′-GTCCATTAGCTGCAAAGATTCCTC-3′	5119-5142		
	RA1: 5′-AATGTGTGTCAGTTAGGGTGTG-3′	266-245	55	314
	RA2: 5′-TCCAAAAAAGCCTCCTCACTACTT-3′	5195-5218		
	R probe: 5′-TTAGTCAGCCATGGGGCGGAGA-3′	29-50		

PCR: polymerase chain reaction; nt: nucleotide.

[a] Used as primers in PCR, seminested PCR, nested PCR and as probes.

[b] reference nucleotide positions in SV40 strain 776.[63]

Oligonucleotides, PCR, and Filter Hybridization

DNA extracted from different human specimens was first assessed for suitability to PCR analysis by a control reaction designed to amplify β-globin gene sequences, as described previously.[3] Then, DNA samples were investigated for different SV40 DNA sequences. To confirm the reproducibility of PCR assays and to avoid possible contaminations, DNAs were extracted with the procedure recommended for PCR investigation in laboratory equipped with PCR facilities. The distinct phases of PCR procedures were performed in separate rooms by different operators at the Section of Histology and Embryology and Center for Biotechnology (BL3/P3 laboratories), University of Ferrara. SV40 DNA wild-type strain 776 (GIBCO BRL), and the recombinant plasmid pSV3x72, also known as pSV3E (obtained from Dr. Lednicky),[50] containing the regulatory region with three 72-base pair (bp) repeats in the enhancer domain, were used as controls in PCR amplification and sequence analysis. DNA samples were analyzed in triplicate by three different operators in a blind fashion. The results obtained by the three different operators did not show discrepancy. All oligonucleotides, their nucleotide positions, size of PCR-amplified products, PCR conditions, and probes used for hybridization are summarized in Table 1. SV40 Tag NH_2-terminal sequences were investigated by seminested PCR (snPCR) using the primer sets SV.for2-SV.rev and SV.for2-PYV.rev,[1] yielding amplification products of 575 and 543 bp, respectively. These primers allow amplification of an NH_2-terminal Tag coding sequence, which contains the pRb pocket binding domain and the Tag intron.[1,11] The SV40 specificity of PCR-amplified products was assessed by filter hybridization with the internal SV oligoprobe.[1] All samples positive for the Tag NH_2-terminal region were further analyzed for the Tag COOH-terminal sequences. For this purpose, the PCR was conducted with the oligonucleotides TA1-TA2.[12] The SV40 specificity of these amplified products was verified by filter hybridization with the internal T oligoprobe.[12] The Tag middle region of 1847 bp (Table 1), which contains helicase and ATPase activities, and the binding sites to DNA, DNA polymerase α, and p53 as well as the Zn finger motif, was investigated by PCR with primers SV.for7-TA2[12] (this study), followed by filter hybridization with the internal T oligoprobe.[12] The primers VPA-VPB and VPC-VPD (this study) were used in nPCR to amplify the VP1 sequences. The PCR products were hybridized with the internal VP probe (this study). The regulatory region was investigated by nPCR with the oligonucleotide pairs RA3-RA4 and RA1-RA2.[12] The SV40 speci-

ficity of these sequences was assayed by filter hybridization with the internal R probe.[51]

DNA (0.5 μg) was PCR-amplified in a total volume of 50 μL containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatine, 150 μM of each dNTP and 25 μM of each primer, 1 unit of Taq-DNA polymerase (Roche, Milan, Italy) together with 1 unit of Platinum Taq antibody as indicated by the supplier (GIBCO-BRL). By adding the Taq antibody, the Taq polymerase activity was blocked up to 94 °C, thus avoiding the generation of aspecific amplification products at room and ramping temperatures. Polymerase chain reaction products were migrated in a 1% agarose gel and transferred to a nylon membrane (Amersham, Milan, Italy). DNA was cross-linked to filters by UV irradiation for 2 minutes. All filters were hybridized with the SV, T, VP, and R oligoprobes, which are specific for the different SV40 regions analyzed (Table 1). Oligoprobes were previously 3′ end–labeled with a tail of dUTP-fluorescein by terminal transferase (Amersham). Detection of the fluorescent DNA hybrid was conducted with antifluorescein horseradish peroxidase–conjugated antibody, as indicated by the supplier (Amersham). Exposure of the film was at room temperature for 15 minutes to 1 hour. Filter hybridization with specific SV40 oligoprobes was used both to reveal the SV40 PCR products, which were not detectable in agarose gels stained by ethidium bromide, and to prove the SV40 specificity of the amplified sequences.

RT-PCR Analysis of the Tag Expression

For RT-PCR, total cytoplasmic RNA (5 μg) was resuspended in 100 μL of a buffer containing 40 mM Tris-HCl, pH 7.5, 10 mM NaCl, and 6 mM $MgCl_2$. Contaminant DNA was removed by two repeated treatments with RNase-free DNase (50 U; Boehringer, Mannheim, Germany) at 37 °C for 20 minutes, followed by phenol extraction and ethanol precipitation. Reverse transcription was performed with a kit from Invitrogen (San Diego, CA) as indicated by the supplier. The cDNA obtained then was amplified by PCR with primers SV.for2-SV.rev,[1] which give a product of 229 bp, specific for the spliced transcript of the Tag gene, whereas the corresponding genomic DNA was of 575 bp. The filter was subjected to Southern blot hybridization with the internal SV probe as described above.

DNA Sequencing

To confirm the SV40 specificity of the amplified sequences, we directly sequenced PCR products by Sanger's technique,[52,53] or purified and sequenced by an automatic DNA sequence apparatus (ABI Prism 377; Perkin-Elmer).

RESULTS

PCR Analysis of SV40 Sequences in Human Samples

In this study, human tumor specimens of different histotypes, normal bone tissues, and buffy coats from blood donors were analyzed by PCR for sequences of five different SV40 DNA regions (Table 2). In the first step, DNA samples were analyzed by PCR for the conserved SV40 Tag NH_2-terminal region by oligonucleotide pairs, which amplify with high efficiency these sequences.[11,18] Indeed, it has been established that primers used to detect, by PCR amplification, other SV40 DNA regions in human specimens are less efficient and may give false-negative results. On this basis, only the samples tested positive for the SV40 Tag NH_2-terminal region were investigated further for additional SV40 DNA regions. The prevalence of SV40 Tag NH_2-terminal sequences in primary brain tumor samples was from 37% to 44%, whereas in derived short-term cell cultures it ranged from 30% to 54% (Table 2 and Fig. 1, A1). SV40 Tag NH_2-terminal sequences were detected in 21–37% of primary bone tumors of different histotypes, whereas in primary bone tumor–derived cell cultures, the prevalence of the viral sequences ranged from 53% to 80% (Table 2 and Fig. 1, A2). Five normal bone tissue samples were negative for SV40-Tag NH_2-terminal sequences (Table 2). In addition, 3 of 12 (25%) primary Wilms' tumors were positive (Table 2 and Fig. 1, A3). Because in previous investigations SV40 Tag sequences were detected in PBMCs from osteosarcoma patients[14] and blood donors[2,3,14,18,20] and B- and T-lymphocyte preparations from blood donors,[2,3] in this study buffy coats from blood donors were investigated for SV40 Tag NH_2-terminal sequences. These sequences were detected in 11 of 38 (29%) buffy coat samples (Table 2 and Fig. 1, A3). The Tag COOH-terminal region was detected in all 78 samples (67 neoplastic and 11 normal tissues) positive for the NH_2-terminal sequences (Table 2). The middle fragment of the Tag coding sequences of 1847 bp was chosen as next target region of our PCR investigation with the primers SV.for7-TA2 (Table 1). In reconstruction experiments, using high-purified SV40 DNA and human genomic DNA mixed in serial dilutions, the 1847-bp product was efficiently PCR-amplified, until 10^{-2} genome equivalents (data not shown) were reached. Note that in similar PCR conditions, the PYV primers set[1] was 100 times more efficient in amplifying a fragment of 172 bp of the Tag NH_2-terminal region.[18] Long PCRs to detect large SV40 DNA frag-

TABLE 2
SV40 Sequences in Human Tumors, Tumor-Derived Short-Term Cell Cultures and Cell Lines, Normal Bone Tissue, and Buffy Coats

Tissues and cell lines[a]	Positive samples/samples analyzed (%)			
	Tag NH_2- and C00H-terminal sequences[b,c]	Tag middle sequences[b,c,d]	VP1 sequences[b,c]	Regulatory sequences[b,c]
Primary brain tumors				
Astrocytoma	4/9 (44)	0/4	0/4	0/4
Glioblastoma	6/16 (37)	0/6	0/6	4/6 (67)
Brain tumor short-term cell cultures				
Astrocytoma	7/13 (54)	1/7 (14)	2/7 (29)	7/7 (100)
Glioblastoma	6/20 (30)	3/6 (50)	3/6 (50)	5/6 (83)
Oligoastrocytoma	0/2			
Oligodendroglioma	0/1			
Gangliosarcoma	0/1			
Ganglioglioma	0/1			
Primary bone tumors				
Osteosarcoma	11/30 (37)	0/11	0/3	1/2
Small cell				
Osteosarcoma	4/11 (36)	0/4		
Ewing's sarcoma	6/28 (21)	0/6		
Bone tumor cell cultures				
Giant cell tumor of bone	4/5 (80)	0/4	4/4 (100)	3/4 (75)
Ewing sarcoma	8/13 (62)	0/8		
Osteosarcoma	8/15 (53)	0/8		
Wilms' tumor	3/12 (25)	0/3		
Normal tissues				
Bone	0/5			
Buffy coat	11/38 (29)	0/11	4/11 (36)	2/3 (67)

[a] Total specimens 220: 111 fresh biopsies, 71 cell cultures, and 38 buffy coats.
[b] SV40 DNA sequences from different genomic regions were detected by polymerase chain reaction amplification (Table 1).
[c] Only the samples found positive both for Tag NH_2- and COOH-terminal sequences were investigated further for the other SV40 regions.
[d] Only samples positive for the entire Tag middle sequences (1847 base pair) are reported.

ments in human specimens were not reported until now. Among the 78 samples found positive both for Tag NH_2- and COOH-terminal sequences, the Tag middle region of 1847 bp (Table 1) was found only in 4 samples, i.e., in short-term cell cultures from 3 of 6 glioblastomas and 1 of 7 astrocytomas (Table 2 and Fig. 2). In 20 samples, an amplification product of approximately 500 bp was detected by PCR, whereas the other 54 samples were negative for the Tag middle sequences (Table 2 and Fig. 2). An explanation for the amplification of the 500-bp short sequence is difficult on the basis of only the PCR results. Very likely, the 1847-bp-long amplicon of the SV40 Tag middle region may be difficult to obtain by PCR amplification, at the low SV40 DNA load typical of human tissues. Instead, a shorter segment of the human genomic DNA, which annealed to the primers SV.for7-TA2, can be amplified more efficiently, since present in a much higher concentration compared to SV40 DNA.

Samples found positive for both Tag NH_2-terminal and COOH-terminal regions were further investigated by PCR for SV40 VP1 coding sequences and regulatory region (Table 1). As summarized in Table 2, the prevalence of VP1 sequences analyzed in 41 samples, was from 29% to 100%, whereas in 32 human specimens the SV40 regulatory region was detected with a prevalence ranging from 50% to 100% (Fig. 1, B1, B2).

Altogether, these data indicate that, under our DNA extraction and PCR conditions, only four samples (one astrocytoma and three glioblastoma short-term cell cultures) carry all five different SV40 regions investigated. In other studies, some but not all SV40 genomic regions were detected in human tumor and normal specimens.[11,14,18] Indeed, it has been reported that: 1) the primers used vary in PCR amplification efficiency for different SV40 sequences; 2) the SV40 sequences could not be detected because of mutations, deletions, or strain variations[41]; 3) the SV40 genomes could belong to defective viral DNA molecules that may occur in human cells even when infected at low multiplicity of infection.[26] Finally, some SV40 regions, as previously described in other studies,[11,14,18] may be absent in our samples.

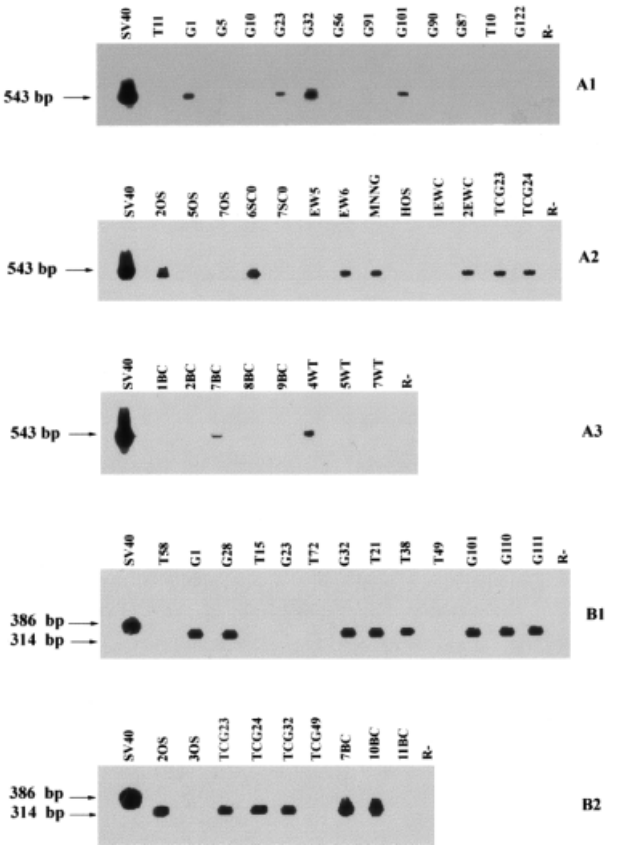

FIGURE 1. Hybridization of SV40 sequences amplified by PCR to SV40 specific oligoprobes. (A1–A3) SV40, Tag NH_2-terminal sequences amplified from SV40 wild-type strain 776 DNA (543 bp), as a control. (B1 and B2) SV40, regulatory region sequences (386 bp) amplified from plasmid pSV3E, containing three 72-bp repeats in the SV40 regulatory region, as a control. The amplified band of 314 bp indicates the SV40 wild-type regulatory region sequences detected in samples analyzed in this study. Lane R^- contains a negative control of the PCR without DNA template. (A1) and (B1) DNAs from primary brain tumors: T11, T58, G1, G5, G28, glioblastoma multiforme; T15, T72, G10, G23, astrocytoma. DNAs from brain tumor cell lines: T21, T38, T49, G32, G56, glioblastoma multiforme; G91, G101, G110, G111, astrocytoma; G90, oligoastrocytoma; G87, gangliosarcoma; T10, oligodendroglioma; G122, ganglioglioma. A2, A3, and B2, DNAs from primary bone tumors: 2OS, 3OS, 5OS, 7OS, osteosarcoma; 6SCO, 7SCO, small cell bone tumor; EW1, EW5, EW6, Ewing sarcoma. DNAs from bone tumor cell cultures: MNNG, HOS, osteosarcoma; 1EWC, 2EWC, Ewing sarcoma; TCG23, TCG24, TCG32, TCG49 giant cell tumor of bone. DNAs from buffy coats of blood donors: 1BC, 2BC, 7BC, 8BC, 9BC, 10BC, 11BC, and from Wilms' tumors: 4WT, 5WT, 7WT. The even intensity of bands in B1 and B2 is caused by the nested PCR that obscures the quantitative differences between the various samples.

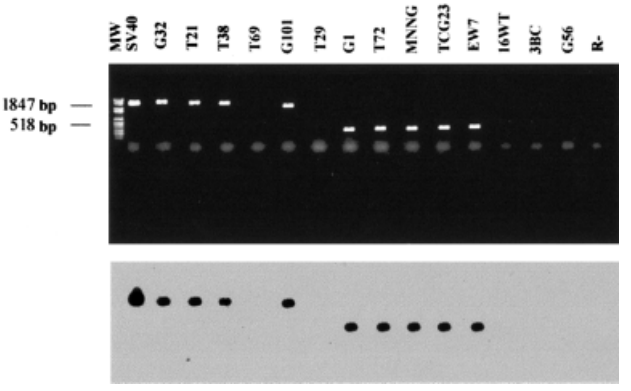

FIGURE 2. (top) Agarose gel electrophoresis of PCR-amplified products of SV40 Tag middle sequences, stained by ethidium bromide. MW: molecular weight markers (Marker VI, Boehringer, Milan, Italy); SV40, positive control (1847 bp), and DNA samples analyzed in this investigation (lanes G32, T21, T38, T69, G101, T29, G1, T72, G56, brain tumors; lanes MNNG, TCG23, EW7, bone tumors; lane 16WT, Wilms' tumor; lane 3BC, buffy coat). PCR-amplified samples exhibit either the entire SV40 Tag middle sequence of 1847 bp or a shorter product of 518 bp. Lane R^- contains negative control of the PCR without DNA template. (bottom) Hybridization of the filter, obtained by transfer of the top panel bands, to the SV40 specific T oligoprobe.

Expression of SV40 Tag Sequences

The expression of the SV40 Tag gene was investigated at mRNA level, mostly in short-term tumor cell cultures and cell lines, because the primary tumors were usually too small for the extraction of both DNA and RNA. SV40 Tag mRNA was investigated by RT-PCR. The cDNA obtained by retrotranscription then was amplified by PCR with primers SV.for2-SV.rev,[1] which give a product of 229 bp, specific for the spliced transcript of the Tag gene. Note that the corresponding genomic DNA, which includes the Tag intron sequences, is of 575 bp. The filter was subjected to Southern blot hybridization with the internal SV probe as described above.

Four of 5 (80%) primary Ewing sarcomas and short-term cell cultures from 2 of 5 (40%) Ewing sarcomas, 2 of 7 (28%) astrocytomas, 2 of 4 (50%) osteosarcomas, and 1 of 6 (16%) glioblastomas, which were positive by PCR for SV40 Tag sequences, showed expression of SV40 Tag mRNA (Fig. 3). Three other short-term cell cultures (two from glioblastomas and one from astrocytoma) negative for SV40 Tag sequences were also negative by RT-PCR (Fig. 3 shows the two negative glioblastoma short-term cell cultures).

DNA Sequence Analysis

To confirm the specificity of SV40 footprints detected in human tissues, we subjected all PCR products to DNA sequence analysis. All five SV40 genomic region amplified were sequenced: Tag NH_2-terminal region from nucleotides 4403–4945, Tag middle region from nucleotides 2630–4476, Tag COOH-terminal region from nucleotides 2630–3070, VP1 region from nucleotides 1731–1941, and the regulatory region from nu-

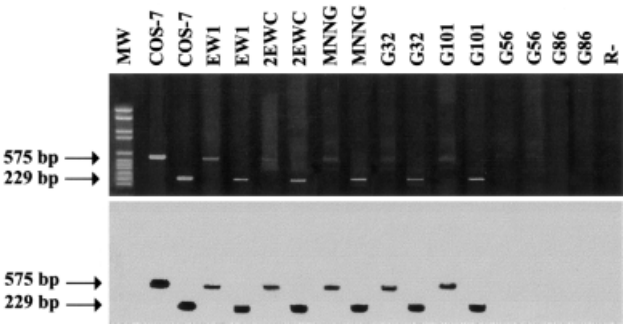

FIGURE 3. (top) Agarose gel electrophoresis, stained by ethidium bromide, of PCR-amplified DNA (575 bp) and cDNA (229 bp) from each sample. COS-7 cells were used as SV40 positive control. Bone tumor samples were represented by EW1, 2EWC, MNNG, whereas brain tumor short-term cell cultures were G32, G101, G56, and G86. Lane R^- contains a negative control of the PCR without cDNA template. The first lane on the left contains the molecular weight (MW) markers (Marker VI, Boehringer, Milan, Italy). (bottom), Filter hybridization of the samples shown in the top panel, to the SV oligoprobe, specific for SV40 Tag coding sequences.

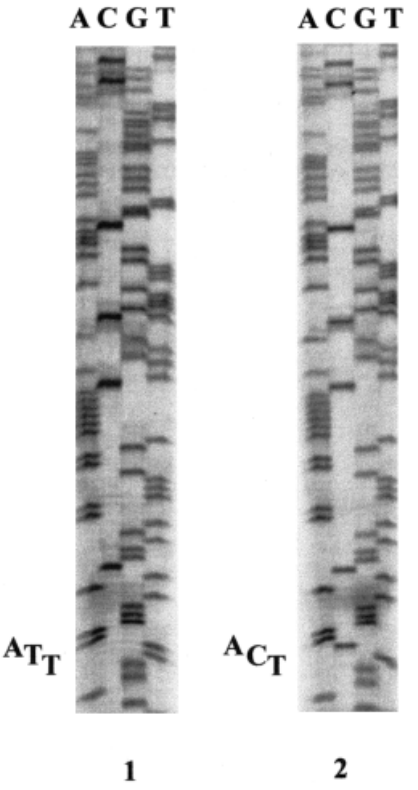

FIGURE 4. DNA sequence analysis of the PCR-amplified products from the giant cell tumor of bone cell culture TCG23 (panel 2) and from control DNA of SV40 wild-type strain 776 (panel 1). The SV40 DNA sequence is shown, from bottom to top, from nucleotides 2943–3038, corresponding to the Tag COOH-terminal sequences. The SV40 DNA sequences in the giant cell tumor of bone cell culture TCG23 and in the SV40 wild-type strain 776 were identical, except for the presence of a T to C transition at nucleotide 2950, corresponding to codon 623, which is ATT (panel 1) in the wild-type strain and ACT in the mutated Tag of the tumor sample (panel 2), corresponding to the amino acid substitution Ile to Thr.

cleotides 5195–266 (Table 1). DNA sequence analysis showed that the viral sequence of our samples was indistinguishable from that of the wild-type SV40 strain 776, and contained two 72-bp repeats in the regulatory region. This result does not seem to be a consequence of contamination, because we used as control a recombinant plasmid containing three 72-bp repeats,[50] which were constantly detected in our PCR and sequence analyses, whereas none of the experimental specimens exhibited three 72-bp repeats. Only one point mutation, at nucleotide 2950[54] in the SV40 sequence present in the TCG23 giant cell tumor of bone short-term cell culture, was detected. This point mutation, a T to C transition, occurred at codon 623 of the Tag sequence,[55] determining the amino acid substitution Ile to Thr at the COOH terminus of the Tag protein (Fig. 4). Of note, in previous studies the same mutation was detected in SV40 strains from human bone tumors and monkey kidney tissues.[54] DNA sequencing confirmed that the SV40 Tag middle region of 1847 bp, detected only in 4 samples (short-term cell cultures from 3 glioblastomas and 1 astrocytoma), was wild-type. Conversely, 20 specimens (3 primary astrocytomas: G23, T15, T72; 3 primary glioblastomas: G1, G28, G58; 3 primary Ewing sarcomas: EW1, EW7, EW8; 2 primary small cell sarcomas: 1SCO, 5SCO; 2 glioblastoma cell cultures: G103, T49; 3 osteosarcoma cell lines: MNNG, OS10, OS17; 1 Ewing sarcoma primary cell culture: 2EWC; 1 giant cell tumor of bone cell culture: TCG23; and 2 buffy coats: 7BC, 28BC) contained SV40-like sequences of only 518 bp. These sequences appear to be the fusion product of 2 small DNA segments of 373 and 145 bp, located at the NH_2 terminus and at the COOH terminus of the Tag middle region, from nucleotides 4476–4104 and nucleotides 2774–2630, respectively (Fig. 5). It is plausible that in these 20 samples, which apparently carry a large deletion of 1329 bp in the Tag middle region from nucleotides 4103–2775, a smaller fragment of 518 bp from human genomic DNA, homologous with SV40 Tag sequences, was PCR-amplified efficiently instead of the Tag sequences of 1847 bp. Of note, a computer-assisted analysis by BLASTN search program (NCBI) indicated that these SV40 Tag sequences of 518 bp from nucleotides 4476–4104 (373 bp at the NH_2 terminus) and from nucleotides 2774–2630 (145 bp at the COOH terminus) show a 97% homology with human genomic sequences present in the telomeric regions of chromosomes 10 (191 of 195 bp) and 11 (340 of 348 bp;

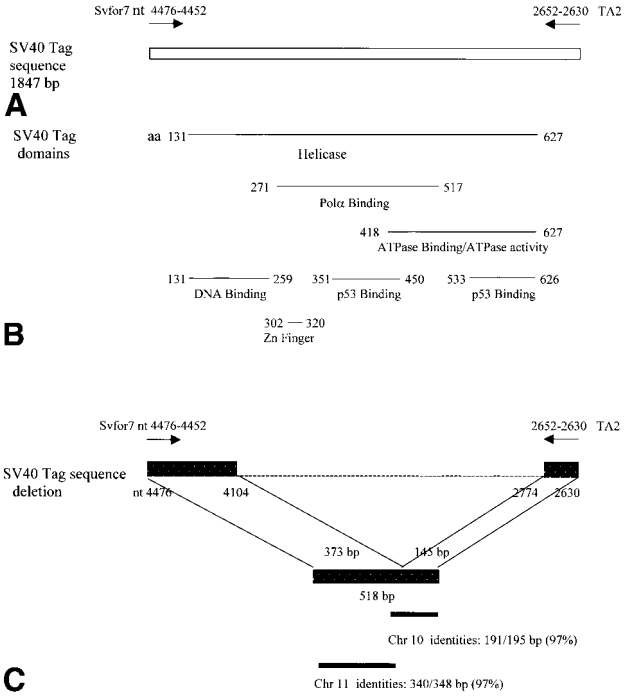

FIGURE 5. (A) Schematic drawing of SV40 Tag middle sequences of 1847 bp (open box), from nucleotides 4476–2630 (5′-3′), analyzed by PCR with the oligonucleotide pair SV.for7, nucleotides 4476–4452 (5′-3′), and TA2, nucleotides 2630–2652 (5′-3′) (arrows). (B) Functional domains (continuous lines) of SV40 Tag at the amino acid (aa) residues 131–627, contained within the SV40 Tag sequence from nucleotide 4476 to nucleotide 2630 (5′-3′). (C) SV40 sequences resulting from fusion of the NH_2-terminal segment of 373 bp and the COOH-terminal segment of 145 bp of the Tag middle portion (dotted bars), yielding a fragment of 518 bp. Sequences of chromosomes 10 and 11 homologous with SV40 DNA are indicated by filled bars.

Fig. 5). To our knowledge, this is the first evidence indicating that the human genome contains sequences with high homology with SV40 DNA.

DISCUSSION

Sarcomas of different histotypes are induced in rodents by SV40.[56] As observed in this study and in previous reports,[11–15] SV40 sequences were found in primary human osteosarcomas and Ewing sarcomas, as well as in giant cell tumor of bone cell cultures, indicating a possible role of this virus in the pathogenesis of bone tumors. SV40 footprints were found in this investigation in 29% of buffy coats, which is similar to the prevalence detected in PBMCs of blood donors (23%) investigated before,[2,3] but lower than that detected in PBMCs of osteosarcoma patients (43%).[14] SV40 sequences also were detected in PBMCs of monkeys.[54] Taken together, these data support the hypothesis that blood cells may contribute to the diffusion of SV40 to other tissues of the host. The discrepancy, in detecting by PCR SV40 sequences in blood samples, between positive results obtained by three different groups, in Italy,[2,3,18] Japan,[14] and the U.S.[20] and negative data reported by Bergsagel et al. (1992)[1] may be because of the use of different technical approaches. It is well known that residual hemoglobin present in DNA from blood specimens interferes with the PCR and it is the cause of negative PCR data. To circumvent this technical problem that we experienced in early steps of our investigations, all DNAs from blood samples, in this study and in previous investigations,[2,3,18] were extracted with the phenol/chloroform procedure.

Although SV40 sequences were detected in a high proportion of brain and bone tumors of different histotypes, none of the 5 normal bone tissues in this study and 13 normal brain tissues analyzed earlier[2,3] was positive for SV40 sequences. This result is surprising, because SV40 sequences were detected in buffy coats, in this investigation, and in PBMC (B and T lymphocytes) in previous studies.[2,3,14,18,20] However, note that the positive hybridization signals of amplified products from blood-derived DNA samples were always very faint, suggesting that the amount of SV40 DNA in blood is lower than in neoplastic tissues. Consequently, the SV40 DNA present in normal bone tissues caused by circulation of PBMCs is probably diluted in these samples, and it was not detectable in our conditions of PCR amplification. The detection of SV40 sequences in tumor cell cultures rules out the possibility that the SV40 positive signals were becaue of SV40 positive blood cells infiltrating the primary tumors and supports the conclusion that SV40 sequences were present in the neoplastic cells within the tumors[2,3,15,45,57] (this study). DNA sequencing of the amplification products from brain and bone tumor specimens confirmed their SV40 origin. Because we amplified and sequenced segments of the early, late, and regulatory regions of SV40 from four brain tumor short-term cell cultures (derived from three glioblastomas and one astrocytoma), it is possible that the whole SV40 genome is present in these samples.

In this and in other studies, SV40 sequences were detected in brain and bone tumors and another neoplasms, which affect early childhood such as Wilms' tumor.[56,57] Perhaps embryos infected in utero by transplacental route or newborn children are particularly susceptible to SV40 tumorigenicity. In this context, it is notable that the transplacental transmission of polyoma viruses, closely related to SV40, such as the murine polyoma virus and human BKV, occurs in mice[58] and in humans,[59] respectively. SV40 transmis-

sion from mother to newborn has been observed in monkeys.[21]

DNA sequencing of PCR-amplified products confirmed the SV40 specificity of the sequences detected in human tumor and normal tissue samples, ruling out that they belong to other simian or human polyomaviruses such as CPV, BKV JCV, or recombinant polyomaviruses. Moreover, by using RT-PCR specific oligonucleotides for the spliced Tag transcript, we detected SV40 early region mRNA sequences in 11 of 27 Tag positive samples, but not in 3 SV40 negative tumor cell lines.

Although the SV40 nucleotide sequence found in human tissues is remarkably conserved, different SV40 strains seem to infect humans, as well as monkeys.[21,54] Indeed, their genomes can be distinguished for differences in the sequence of the regulatory region and of the Tag COOH-terminal variable domain. In our study, as well as in previous investigations conducted in Italy, the sequences of the viral DNA detected in clinical specimens were indistinguishable from the nucleotide sequence of the SV40 776 wild-type strain, which has two 72-bp repeats in the enhancer domain of the regulatory region. Recently, SV40 regulatory region sequences with two 72-bp repeats have been detected in primary human mesotheliomas as well as in bone and papillary thyroid tumors[9,12,17] and in monkey tissues.[21,54] Because of their distinct advantage during viral replication, variants with two 72-bp repeats may be selected after infection of human tissues. SV40 strain variability found in the U.S.[45] could be because of the heterogeneous human population of this country, whereas SV40 wild-type strain 776, the only strain so far detected by different groups in Italian patients,[11,17,18,51] may reflect the more homogeneous population present in Italy. It have been reported that the JCV polyomavirus, which is closely related to SV40, has a geographic strains distribution.[57] For this characteristic, the JCV fingerprinting was used to study human population migrations.[60]

Of note, the SV40 DNA present in a giant cell tumor of bone cell line shows a point mutation identical to that detected in the DNA sequence of SV40 from human bone tumors and monkey kidney tissues.[54]

We also PCR-amplified SV40-like sequence of 518 bp in 20 of 78 (26%) samples, which showed 97% homology with human genomic DNA. Control PCR analysis with the primers SV.for7-TA2 conducted with SV40 negative human genomic DNA indicated that the 518-bp PCR product can be amplified in approximately 30% of the samples (data not shown). One may speculate that human telomeric regions of chromosomes 10 and 11 carry different repetitive sequences, and consequently they are present only in a fraction of the human genomic DNA pull. Note that SV40-like sequences of the regulatory region were detected in the monkey genome,[61] which is 98% homologous with the human genome and human genomic sequences homologous with the SV40 regulatory region can be amplified by PCR under low stringency conditions.[12]

This study, which searched for the simultaneous presence of multiple regions of the SV40 genome crucial for virus replication and transformation, indicates that, although authentic SV40 sequences were detected, all five SV40 regions analyzed were PCR-amplified only in four tumor short-term cell cultures. Various hypotheses may explain these results: 1) it is possible that in our PCR conditions some regions of the viral genome, although present, were not detected; 2) negative data could be because of the presence of defective SV40 genomes within the sample, as observed in early investigations[18,26]; in this instance some SV40 regions appear dispensable for tumor onset/progression; 3) it is also possible that cells harboring SV40 complete genomes, which express the highly immunogenic viral proteins VP1 and Tag, may be recognized and eliminated by the host immune system, whereas cells containing defective viral genomes/particles are maintained.

The clastogenic activities of SV40 Tag may be responsible for the induction of chromosomal aberrations early after infection of human cells by SV40.[62] These chromosomal alterations would persist and progress even if the SV40 sequences are lost or rearranged during the subsequent natural history of the tumor. In this regard, SV40 may act like chemical and physical carcinogens that, after inducing mutations in the human genome, can become dispensable for subsequent tumorigenic steps. This "hit and run" mechanism would also explain, together with the semipermissive nature of human cells for SV40, the low copy number of these viral sequences in human tumors.[41,57]

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