A Multicenter Evaluation of Assays for Detection of SV40 DNA and Results in Masked Mesothelioma Specimens

The International SV40 Working Group[1,2]

Abstract

This nine-laboratory multicenter investigation was designed to assess the sensitivity, specificity, and reproducibility of previously described assays for detection of SV40 DNA with three goals, *i.e.,* (*a*) to compare methods for testing human tissues, (*b*) to examine the ability of these methods to detect SV40 in human mesotheliomas, and (*c*) to uncover assay differences that could explain conflicting findings in some past investigations. Each laboratory received, in a masked fashion, paired replicate DNA samples extracted from 25 fresh frozen mesotheliomas (50 samples) and one from each of 25 normal human lungs. Interspersed were masked positive (titrations of the SV40 genome) and negative control samples. Preliminary studies confirmed the adequacy of the samples for testing high molecular weight double-stranded linear DNA targets. All 15 PCR-based assays detected 5,000 copies or less of the SV40 genome spiked into 2 μg of WI-38 DNA. A high level of specificity and reproducibility was found among the PCR assays performed in most laboratories. However, none of the selected normal human lung tissue or the 25 mesothelioma tumor specimens obtained from archival samples at a single center was reproducibly positive for the presence of SV40 DNA. Further studies are needed to reconcile these results with previous reports of detection of SV40 DNA in tumor specimens.

Introduction

Several sources of evidence indicate that SV40, an infectious agent for Asian macaques, may be a human tumor virus. SV40 is a double-stranded DNA polyomavirus that shares substantial nucleotide sequence homology with the known human polyomaviruses, JC virus and BK virus (1, 2). DNA sequences indistinguishable from wild-type SV40 have been reported in several rare human tumors by an increasing number of laboratories (3). Most of these reports have involved the following tumors: osteosarcoma (4–6), a bone malignancy that occurs mainly in teenagers and young adults; brain tumors, including meningiomas, ependymoma, and choroid plexus tumors (7–12); and pleural mesothelioma (13–19), an asbestos-associated malignancy most commonly arising in the elderly. DNA sequences from several different SV40 open reading frames and regulatory elements have been amplified from tumor samples, suggesting that the virus genome is substantially conserved in infected cancer cells (9, 20). Expression of SV40 T-ag[3] has been demonstrated in human tumor cells by immunohistochem-

Received 12/20/00; revised 3/8/01; accepted 3/15/01.
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[3] The abbreviations used are: T-ag, large T antigen; HTLV-I, human T-cell lymphotrophic virus type I.

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istry (7, 13, 19, 21). Moreover, infectious SV40 has been isolated from one human choroid plexus carcinoma and one meningioma and was shown to be distinct from common laboratory strains (9, 22).

The biological plausibility of SV40-induced tumorigenesis in humans has been partly demonstrated (3, 23). SV40 can transform human cells *in vitro* (24, 25), apparently through the action of T-ag (1). T-ag binds to and interferes with the functions of host cell tumor suppressor proteins p53 and RB, as well as the RB-related family members, p130 and p107 (reviewed in Ref. 1). Evidence of binding between SV40 T-ag and these human proteins *in vivo* has been reported in mesothelioma tissue specimens (19, 26) and in human brain tumors (21). Injection of SV40 DNA can induce tumors in rodents, notably including osteosarcoma (27), ependymoma (28), and mesothelioma (29).

Opportunities for the introduction of SV40 into humans have been well documented (30, 31). Widespread human exposure to live SV40 occurred as a result of the inadvertent contamination of a substantial proportion of lots of poliovirus vaccines during 1955–1963. Worldwide, hundreds of millions of people, most immunized as young children, were exposed to live SV40 through these contaminated vaccines. In addition, in parts of Asia human contact with the simian host of SV40 occurs regularly. Evidence also has been reported suggesting that humans can become infected with SV40 (reviewed in Refs. 3 and 30). In one study, infectious SV40 was isolated from throat swabs days after research volunteers were inoculated with an investigational aerosol inadvertently contaminated with live SV40 (32). Antibody seroconversion was detected in several of these subjects. Similarly, infants fed contaminated poliovirus vaccine intermittently excreted SV40 in their stools for up to 5 weeks (33). Furthermore, SV40 DNA has been detected in allografts from renal transplant patients (34).

Epidemiological studies in the United States and Europe, however, have generally failed to detect an increased risk of cancer in birth cohorts born in the 1950s and early 1960s, many of whom were exposed to SV40 through contaminated poliovirus vaccine (reviewed in Ref. 31; Refs. 35–37). These studies have involved decades of observation and many millions of person-years of data in several different countries. Similarly, 20-year follow-up studies of subjects inoculated during the first week of life with contaminated vaccines also failed to uncover any effect on cancer rates, despite the high titers of live SV40 later demonstrated in these vaccines (38). It could be that longer observation of these cohorts is required to detect a relationship of cancer risk with vaccine-associated SV40 exposure, especially for tumors that occur in late adulthood, such as mesothelioma. It also must be noted that no effect on cancer risk with vaccine exposure would be detected if SV40 has always been and continues to be widespread among humans. There exist, however, additional reasons for uncertainty regarding the role of SV40 in human cancer. In one recent investigation, none of 48 mesotheliomas was positive for SV40 DNA, and no neutralizing antibodies to SV40 were detected in additional patients with either mesothelioma or osteosarcoma (39). In a study of immune-compromised homosexual men infected with human immunodeficiency virus, a high prevalence of JC and BK viruria was detected, but the urines were negative for SV40 (40). Even among studies that have detected SV40 in human tissues, the prevalence of SV40 DNA in different types of tumors and especially in normal human specimens has varied greatly (8, 13, 18, 41). Moreover, two reports have proposed that the risk of laboratory contamination with SV40 DNA is potentially high because of the pervasive use of SV40 DNA sequences in construction of mammalian expression vectors, transformed cell lines, and other laboratory reagents (17, 42).

The potential role of SV40 in human tumorigenesis was discussed at an international meeting of SV40 researchers in January 1997, a workshop entitled "Simian Virus 40 (SV40): A Possible Human Polyomavirus," attended by over 200 interested scientists (43). One major outcome of the meeting was the formation of the "International SV40 Working Group," which includes the majority of laboratories that presented data at the meeting (positive and negative) regarding the detection of SV40 in human tissues. Overall, nine independent laboratories participated in the current investigation.

To assure an unbiased evaluation, this multicenter collaboration was the first to use masked replicate tumor and control specimens to study the sensitivity, specificity, and reproducibility of different methods for detection of SV40 DNA. Moreover, the study included laboratories that did and those that failed in the past to detect SV40 DNA in human tumors. Each laboratory used their own assays described previously. The purposes of this study were: (*a*) to compare methods for detection of SV40 DNA in human tissues; (*b*) to examine the ability of these methods to detect SV40 in human mesotheliomas; and (*c*) to understand the reasons for conflicting findings in some past investigations.

Materials and Methods

Overview. The study organizers (H. D. S. and J. J. G.) designed the study with input from all collaborators, but they did not perform any of the laboratory work and had no direct access to specimens or to the codes used in masking the specimens. All specimens were obtained and prepared through third parties under strict guidelines agreed to in writing by all investigators, and all data were circulated through a non-participating study supervisor (T. W.). Study administration, specimen tracking, and data handling, although monitored by the study organizers, were controlled by outside contractors.

To reduce uncertainty regarding the interpretation of results, a series of pilot studies were conducted to confirm that: (*a*) the DNA extraction method obtained low molecular weight (*e.g.*, extrachromosomal) as well as genomic DNA; (*b*) low molecular weight DNA was present in actual test specimens; (*c*) inhibitors of PCR were not present in test specimens; and, (*d*) human β-globin could be amplified from test specimens, each of which contained 2 μg of genomic DNA. In addition, only fresh frozen mesothelioma tumor specimens shown to have high content of cancer cells were selected. Replicate samples of DNA from these tumor specimens were then interspersed among normal human lung specimens prepared from fresh frozen tissues, as well as laboratory-prepared positive and negative controls that were made indistinguishable from tumor samples. Each laboratory used its own assay methods, and eight of the nine laboratories also used one approach agreed to by all participants.

Specimens. The specimens tested in this investigation are summarized in Table 1.

Tumor Tissues. Mesothelioma tumor blocks were the kind gift of investigators at Brigham and Women's Hospital (D. S., R. B., W. R.). Within 30 min of resection, these tumors were embedded in OCT compound and fresh frozen on powdered dry ice in the pathology laboratory. They were then stored at −70°C until examined for possible use in this investigation. Sections from the top and bottom of each tumor block were stained with H&E and evaluated to verify the diagnosis and measure tumor content (J. C.). From these archived frozen

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Table 1 Summary of specimens tested in each laboratory

Positive controls (SV40 DNA, titrations in WI-38 cells)	Negative controls (WI-38 cells)	Human mesothelioma	Normal human lung
Five dilutions, each in replicate $n = 10$	10 replicate samples $n = 10$	25 samples, each in replicate $n = 50$	25 samples $n = 25$

tumor blocks, 25 were selected based only on sufficient size and tumor content. Mesothelioma specimens with the common epithelial histological pattern ($n = 23$) were required to comprise 50% or more of each tumor block. For sarcomatoid mesotheliomas ($n = 2$), specimens were selected to contain >30% tumor component. Six additional sections from each specimen were prepared on slides and immediately fixed in 100% ethanol for future studies. The remainder of each tumor block was used for extraction of DNA. The mesothelioma patients were 76% male, and all were Caucasian except for one patient of Asian descent. To help preserve patient anonymity, age was rounded to the nearest 5 years. On the basis of this, the median age of cases was 60 years (range, 45–80 years). Chart review (R. B.) revealed that 60% of cases had exposure to asbestos, with the certainty of exposed/non-exposed status considered high in 52%, medium in 16%, and low in 32% of cases.

Normal Human Lung Specimens. Tissue specimens were obtained from non-cancer patients undergoing thoracic surgery at Brigham and Women's Hospital ($n = 11$). These specimens were fresh frozen in OCT compound and stored as above. A second set of normal human lung specimens were provided by investigators at the Department of Human Carcinogenesis, National Cancer Institute, NIH (B. G., C. H.). These specimens ($n = 14$) were obtained from accident victims during autopsy and frozen in vapor phase liquid nitrogen within 3 h of death. The median age of these patients was 50, 67% of them were male, and 73% were Caucasian.

Laboratory-prepared Control Specimens. Negative control specimens were WI-38 cells (ATCC #CCL75) directly obtained and grown by the contract laboratory (M. C.) using previously unopened reagents. Positive control specimens were titrations of the whole SV40 genome strain 776 (Life Technologies, Inc.; 15251-010) spiked into DNA that had been extracted from WI-38 cells. Stock specimens containing fixed amounts of the SV40 genome were mixed to distribute virus DNA and then aliquoted to prepare samples containing on average 50,000, 5,000, 500, 50, and 5 copies/tube of the virus genome and 2 μg of cellular DNA (*i.e.,* "X" SV40 copies/2 μg of human DNA). Thus, these specimens were a rough gauge of PCR assay sensitivity (*e.g.,* accurate within an order of magnitude). To verify the adequacy of the control samples, a single masked aliquot from each of the positive SV40 titrations and two random negative control aliquots were tested in one of the participating laboratories. SV40 DNA was detected in the positive control but not the negative control specimens.

Preliminary Laboratory Studies

Evaluation of DNA Extraction Methods. Prior to extracting DNA from test specimens, the efficiency of the planned DNA extraction method (QIAamp DNA kit; Qiagen, Santa Clarita, CA) was assessed in comparison with standard phenol/chloroform DNA purification. We were particularly interested in confirming the efficient extraction of low molecular weight

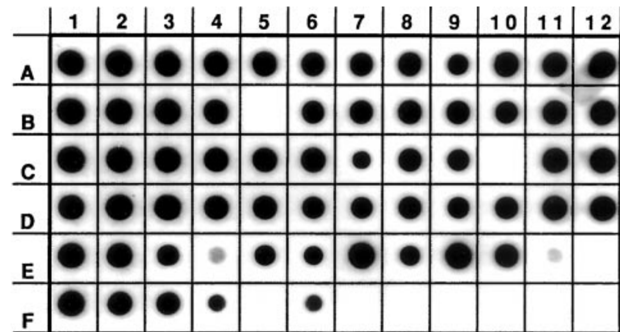

Fig. 1. Detection of human β-globin DNA amplification in test specimens. An aliquot from each of 56 specimens (DNA from 25 mesotheliomas, 25 normal lung tissues, and the negative and five positive SV40 controls) was tested by PCR amplification using primers GH20/PC04 and dot blot hybridization with probe PC03. The blank positions were all laboratory controls, as were positive positions E3 and E11. F1–F4 were replicates of a single test specimen, and F6 was a replicate of the test specimen in A1. The remaining test specimens were assayed once each. All test specimens were β-globin positive.

DNA, because the SV40 genome might be extrachromosomal in tumors. In brief, DNA was extracted from either 350,000 or 700,000 WI-38 cells spiked with various amounts (0, 25, 50, 75, 100, or 150 ng) of a low molecular weight plasmid (HB1-HB2/pCRII; Ref. 44). Of note, 25 ng of this plasmid correspond to approximately 6×10^9 copies, or 16,000 copies/WI-38 cell equivalent. The DNA concentration was determined with a spectrophotometer, and the relative amounts of low molecular weight DNA obtained were approximated using a 1% agarose gel. The findings showed that the Qiagen kits obtained greater amounts of DNA, including low molecular weight DNA, than did phenol/chloroform DNA purification (Appendix A). The same Qiagen column system, adapted for tissues (DNeasy Tissue kit), was used for the mesothelioma and normal lung specimens.

Evaluation of Test Specimens. Several steps were taken to further verify the adequacy of DNA samples for hybridization studies:

(*a*) The total amount of DNA extracted from each specimen was estimated by spectrophotometry, and replicate aliquots of fixed volume were produced, each containing 2 μg of DNA.

(*b*) Each DNA sample, including the positive and negative laboratory-prepared specimens, was tested for amplification of a 268-bp region of human β-globin (J. P.) using PCR with primers GH20/PC04 and probe PC03. All samples were β-globin positive (Fig. 1). In addition, amplification of human DNA in each test specimen was independently confirmed by Lab 5. In Lab 9, using unamplified Southern blot assays, appropriate β-globin signal was detected for all masked samples in which there was sufficient remaining material after testing for SV40.

(*c*) The presence of low molecular weight DNA in samples extracted from human tissue specimens was demonstrated in aliquots from 6 (12%) of the 50 tissue specimens (4 tumors and 2 normal lung specimens) by electrophoresis of the sample through a 0.7% agarose gel, stained with ethidium bromide. Sheared DNA was observed in the range of 1–20 kb for all samples, indicating that they contained linear low molecular weight DNA (Fig. 2).

(*d*) The absence of PCR inhibitors in the DNA extracted from human tissue specimens was demonstrated using aliquots from the above 6 specimens, prepared as undiluted, diluted

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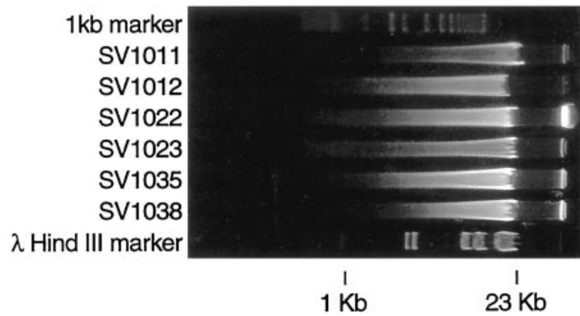

Fig. 2. The presence of linear low molecular weight DNA in samples extracted from human tissues using the Qiagen column purification system. Shown is DNA from six specimens (four mesotheliomas and two normal human lung specimens).

1:10, and diluted 1:100 (D. W.). Each sample dilution was then spiked with one of three amounts of an HTLV-I proviral DNA plasmid (500, 50, or 5 copies). The ability to amplify the HTLV-I proviral DNA was evaluated using a quantitative PCR assay as described previously (45). The data revealed the expected sensitivities after dilution, indicating the absence of PCR inhibitors (Appendix B).

SV40 DNA Hybridization Assays

Table 2 summarizes the detection methods in each of the nine laboratories. One laboratory conducted Southern blot on DNA extracts without prior amplification; all others used PCR protocols. The participants agreed that the results of this study would be presented in a way to prevent assignment of specific results to any of the PCR laboratories. The eight PCR laboratories agreed to test specimens using a PCR approach adapted from a recent study that found high prevalence of SV40 DNA in human mesotheliomas (Ref. 16; the Common PCR assay). In addition, five of the nine laboratories used their previously developed assays, which with one exception (see below) have been described in the literature (7, 9, 13, 17, 39, 42).

The Common PCR Assay. SV40 PCR primers SV5 (5′-TAG-ATT-CCA-ACC-TAT-GGA-ACT-GAT-3′) and SV6 (5′-GGA-AAG-TCC-TTG-GGG-TCT-TCT-ACC-3′), which amplify a conserved 173-bp region of the SV40 T-ag, were used under conditions adapted from Testa *et al.* (16). Specifically, hot-start PCR was conducted using 0.5 μg of DNA (~67,000 cells) from each study sample and 0.5 mM final concentration of each primer in 2.5 mM $MgCl_2$. After 3 min at 95°C to denature DNA, 45 amplification cycles were performed. Each cycle consisted of 1 min at 95°C, 1 min at 59°C, and 1 min at 72°C. Finally, extension was conducted for 10 min at 72°C. All PCR products were tested for amplified SV40 DNA sequences using Southern blot with SV.probe (5′-ATG-TGG-AGA-GTC-AGT-AGC-C-3′). Lab 2 and Lab 6 did not use Southern blot hybridization, diverging from the intended protocol in this one consideration.

Other PCR assays included one unpublished procedure in Lab 2. A semi-nested PCR protocol was used in combination with the hot-start technique, as follows. Primers PYV.for/SV.rev at 0.5 mM each were used in reaction mixtures that contained 5 μl of DNA extract, 2.5 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate, and PCR buffer, as per the manufacturer's suggestions (Perkin-Elmer), to achieve a final volume of 50 μl. After 3 min at 94°C, 1.25 units of Taq polymerase were added, and 30 PCR cycles were performed. Each cycle consisted of 1 min at 94°C, 1 min at 52°C, and 1 min at 72°C, with a final extension step of 5 min at 72°C. A second reaction series was then conducted using primers SV.for3/SV.rev and 10 μl of the earlier amplification product. The reaction mixture contained 1.5 mM $MgCl_2$, 0.5 mM of each primer, and 200 μM of each deoxynucleotide triphosphate. After 3 min at 94°C, 1.25 units of Taq polymerase were added, and 30 PCR cycles were performed. Each cycle consisted of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, followed by a final extension step of 5 min at 72°C. The entire PCR assay volume was then loaded onto an 8% polyacrylamide gel and run for 60 min at 100 V (30 mA). The gel was stained with ethidium bromide (0.5 mg/ml) and visualized under UV light.

DNA Sequencing. All SV40-positive amplification products, as determined by DNA hybridization, were required to be sequenced, and the alignment of each sequence with that of the reference strain 776 had to be submitted. Sequencing for three laboratories (Lab 4, Lab 6, and Lab 7) was conducted at an independent central laboratory (L. R.). The other laboratories chose to be responsible for their own DNA sequencing.

Southern Blot. One laboratory (Lab 9) used Southern blot without prior PCR amplification to detect SV40 DNA in test specimens. The methods used were adapted from a recent publication (6). In brief, 1 μg of DNA from each sample was digested with 5 units of *Hin*dIII for 2 h and then separated on 1% agarose gels at 50 V. All Southern blots included COS-1 cells, which contain a single integrated copy of SV40, and normal human DNA as positive and negative controls, respectively. All samples were examined on two Southern blots. The gels were transferred to Biotrans nylon membranes and hybridized overnight at 42°C using 5×10^6 dpm/ml ^{32}P-labeled probe. The final rinse of the blots was in 0.1× SSC at 65°C. The full-length SV40 genome, isolated from pBRSV by excision with *Bam*HI, was used as a probe. The quality and quantity of DNA were checked by hybridizing with exon 2 of the β-globin probe.

Results

The sensitivity and specificity of each SV40 DNA assay were assessed using masked positive and negative controls prepared by an independent laboratory not involved in testing specimens. Positive controls were titrations of the whole SV40 genome. Table 3 shows the frequency of SV40 DNA detection in these materials.

Unexpectedly, the "negative control" samples gave positive signals in eight of nine laboratories, indicative of contamination with SV40 DNA before distribution (Table 3). Investigation revealed that 55 (92%) of 60 positive results in these samples involved the first of two separately prepared batches of negative control specimens. It was learned that the processing laboratory had aliquoted samples from this first batch immediately after aliquoting SV40 DNA-positive control samples. Although the biosafety hood used had been cleaned and laboratory personnel reported changing gloves between samples, the hood was not resterilized with UV irradiation before aliquoting the negative control samples. The second batch of negative control samples were prepared ~1 month later. Two of the five positive results in the second batch of negative control samples occurred in Lab 1, which had reported contamination of its PCR primers. The other three positive results were in Lab 3 and Lab 9. None of the second-batch negative control samples was positive in the remaining six laboratories, despite good to excellent sensitivity of their assays in the positive controls (Table 3). DNA sequencing of one of the

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Table 2 Summary of laboratory DNA amplification, hybridization, and detection methods[a]

Assay	Primers	PCR cycles, temperatures, and time	Probes	Reference
Common	SV5/SV6	Denaturing at 95°C for 5 min 45 amplifications at 95°C for 1 min, 59°C for 1 min, 72°C for 1 min Extension at 72°C for 10 min	SV.probe	(16)
2.1	PYV.for/SV.rev then (seminested) SV.for3/SV.rev	Denaturing at 94°C for 3 min 30 amplifications at 94°C for 1 min, 52°C for 1 min, 72°C for 1 min Extension at 72°C for 5 min Then Denaturing at 94°C for 3 min 30 amplifications at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min Extension at 72°C for 5 min	Ethidium bromide gel	See text
3.1	SV.for2/SV.rev	Denaturing at 94°C for 4 min 50 amplifications at 94°C for 45 s, 55°C for 45 s, 72°C for 45 s Extension (none)	SV.probe	(17)
3.2	LA1/LA2	Denaturing at 94°C for 4 min 50 amplifications at 94°C for 45 s, 52°C for 45 s, 72°C for 45 s Extension (none)	VP1.probe	(17)
5.1	RA1/RA2	Initial at 94°C for 2 min 29 amplifications at 94°C for 15 s, 63°C for 15 s, 72°C for 15 s Extension at 72°C for 7 min Add 1 aliquot of enzyme Repeat (total = 62 cycles)	Ethidium bromide gel	(9)
5.2	SV.for3/SV.rev	Initial at 94°C for 2 min 29 amplifications at 94°C for 15 s, 63°C for 15 s, 72°C for 15 s Extension at 72°C for 7 min Add 1 aliquot of enzyme Repeat (total = 62 cycles)	Ethidium bromide gel	(7)
7.1	SV.for3/SV.rev	Denaturing at 94°C for 5 min 45 amplifications at 95°C for 1 min, 59°C for 1 min, 72°C for 1 min Extension at 72°C for 10 min	SV1.probe	(13, 39)
8.1	4FG-6BG	Denaturing at 94°C for 5 min 39 amplifications at 94°C for 1 min, 50°C for 2 min, 72°C for 1 min Extension at 72°C for 6 min	P5	(42)
9.1	Southern blot	Southern blot without PCR amplification	SV40FL	(6)

[a] One laboratory used Southern blot without PCR amplification; all others used PCR. The eight PCR laboratories agreed to test specimens using a Common PCR assay. Five of the eight PCR laboratories also used assays that they have reported previously in the literature (as referenced). The PCR assay in Lab 2 had not been published, and it is described in the text.

amplification products from Lab 5 confirmed that the sequence of SV40 DNA detected in the first batch of negative laboratory controls was identical to that of strain 776, the strain used to prepare the SV40 genome titrations.

The unamplified Southern blot assay of Lab 9 was not consistently able to detect 50,000 or fewer copies of SV40 in the masked positive control samples. However, Southern blotting easily detected the single copy/cell of SV40 in COS-1 cells. In contrast, all 15 PCR-based assays reproducibly detected viral sequences in both samples containing 5,000 copies of the virus genome added into 2 μg of extracted WI-38 cell DNA. Of the PCR assays that correctly identified all five second-batch negative controls, the majority (7 of 13) detected the viral DNA in both samples with 500 copies of the SV40 genome added; of these, 4 detected SV40 DNA in both samples with 50 copies of the SV40 genome added, and the most sensitive 3 PCR assays (in two laboratories) reproducibly detected SV40 DNA in all dilutions down to 5 copies of the virus genome. All but one laboratory detected 5–50 viral genome copies with the Common PCR assay, which used 0.5 μg of DNA (~67,000 cells) per experiment (Table 3).

Table 4 shows the SV40 DNA hybridization results for all 25 mesotheliomas by assay in the nine study laboratories. All positive results were based on Southern blot and confirmed to have homology with SV40 by direct DNA sequencing, except as noted. Some positive results were observed in five of the nine laboratories. Lab 1 detected SV40 DNA in 10 (40%) of 25 mesotheliomas. Lab 6 reported viral sequences in 6 (24%) of the mesotheliomas. These positive findings, however, were not confirmed in the paired (same tumor extract) replicate samples, with the exception of mesothelioma H in Lab 1 and mesothelioma Q in Lab 6. Lab 1 discovered SV40 DNA contamination in its PCR primers. Lab 6 did not conduct Southern blot to confirm results on ethidium bromide gel (see "Materials and Methods"), but all positive amplification products were found to be SV40 by direct sequencing. None of the other seven laboratories had concordant positive SV40 DNA results in paired replicate mesothelioma samples. Three laboratories found 1 mesothelioma sample to be positive, with none positive in the paired replicate (involving a different tumor in each laboratory). Four laboratories did not detect SV40 DNA in any of the 50 paired replicate samples from the 25 mesotheliomas tested.

Similarly, there were few positive SV40 DNA results in normal human lung samples (Table 5). Unlike the mesothelioma specimens, the normal lung samples were not tested in replicate. Four laboratories did not detect viral sequences in any of the 25 normal human lung samples. Three laboratories reported SV40 DNA in three separate samples (L, T, and Y). Lab 6 had two positive results (normal lungs H and K). Only Lab 1 found SV40 DNA in more than 2 normal human lung samples. The low frequency of positive results in normal human lung and mesothe-

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Table 3 Positive SV40 DNA results in laboratory-prepared positive (two replicate samples for each titration) and negative (five replicate samples each) controls

Laboratory	Assay	Positive control specimens (two samples each) No. of SV40 genome copies contained					Negative control specimens (5 samples each per batch)	
		50,000	5000	500	50	5	Batch 1[a]	Batch 2
Lab 1	Common	2	2	2	2	2	5	2
Lab 2	Common	2	2	2	2	1	3	0
	Assay 2.1	2	2	2	2	1	5	0
Lab 3	Common	2	2	2	2	2	5	2
	Assay 3.1	2	2	2	0	0	0	0
	Assay 3.2	2	2	1	2	1	0	0
Lab 4	Common	1 of 1[b]	2	2	2	2	2	0
Lab 5	Common	2	2	1	2	1	4	0
	Assay 5.1	2	2	1	2	0	4	0
	Assay 5.2	2	2	1	2	0	4	0
Lab 6	Common	2	2	0	0	0	0	0
Lab 7	Common	2	2	2	2	2	5	0
	Assay 7.1	2	2	2	2	2	5	0
Lab 8	Common	2	2	2	1	2	5	0
	Assay 8.1	2	2	1	0	1	2	0
Lab 9	Southern	0	1	0	0	1	1	1

[a] Batch 1 was contaminated with SV40 when samples were aliquoted (see text). Batch 2 was prepared separately ~1 month later.
[b] The amplicon from one sample was lost and could not be tested.

Table 4 Positive SV40 DNA results in paired replicates of 25 mesothelioma tumor samples (A–Y) by type of assay in each laboratory

Laboratory	Assay	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y
Lab 1[a]	Common	1[b]	1			1	1		2					1			1		1			1			1	
Lab 2	Common																									
	Assay 2.1																									
Lab 3	Common																		1							
	Assay 3.1																									
	Assay 3.2																									
Lab 4	Common					1[c]							?													
Lab 5	Common																									
	Assay 5.1																									
	Assay 5.2																									
Lab 6	Common	1							1	1							1	2							1	
Lab 7	Common																									
	Assay 7.1																									
Lab 8	Common					?				·					·									¿		
	Assay 8.1																									
Lab 9	Southern											■									1	■				

[a] Lab 1 reported detecting contamination of their PCR primers (see text). Samples in Lab 1 were unavailable for DNA sequencing. However, positive results in all other laboratories were confirmed by sequencing unless indicated (see footnote c, below.)
[b] 1, one of the two paired replicate aliquots from the denoted specimen was positive; 2, both paired replicate aliquots from the denoted specimen were positive; ?, questionable weak band on Southern blot in one of the two aliquots, but the amplification product could not be sequenced because of insufficient material; ·, the amplification product in one of the two aliquots appeared as a broad smear on ethidium bromide gel and hybridized faintly with probe for SV40. The sample was not further analyzed because no specific fragment could be identified for cloning and sequencing. Thus, the sample was considered uninterpretable by the laboratory; ¿, amplification product of wrong size (600 bp) was observed on ethidium bromide gel in one of the two aliquots. It was considered a negative result by the laboratory and not further tested, although signal was present on Southern blot; ■, insufficient amount of DNA for unamplified Southern blot testing in one of the two aliquots, as indicated by the inability to detect β-globin without amplification or underloading of the SV40 DNA test. All other samples were positive for β-globin on unamplified Southern blot and had adequate amounts of DNA for testing in this laboratory.
[c] One of the two paired aliquots was positive on Southern blot. However, the amplification product could not be sequenced because of insufficient material.

lioma samples was similar. Five of 370 (1.4%) SV40 DNA assays (excluding those considered inadequate by the reporting laboratories and those reported by Lab 1) were positive in normal human lung samples, as were 10 of 746 (1.3%) assays in human mesothelioma samples [2 mesothelioma samples were found uninterpretable in the Common PCR assay in Lab 8 (see Table 4); 5 normal lung and 2 mesothelioma samples had insufficient DNA in the unamplified Southern blot assay of Lab 9].

The few positive results in human tissues all were obtained with the Common PCR assay, adapted from Testa *et al.* (16).

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Table 5 Positive SV40 DNA results in 25 normal lung samples by type of assay in each laboratory

Laboratory	Assay	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y
Lab 1	Common					+[a]						+					+					+		+	+	+
Lab 2	Common																									
	Assay 2.1																									
Lab 3	Common																									+
	Assay 3.1																									
	Assay 3.2																									
Lab 4	Common																									
Lab 5	Common																									
	Assay 5.1																									
	Assay 5.2																									
Lab 6	Common								+			+														
Lab 7	Common																									
	Assay 7.1																									
Lab 8	Common												+						¿							
	Assay 8.1																									
Lab 9	Southern									■	■			■					■		+			■		

[a] The one aliquot was positive. Only a single aliquot from each normal lung specimen was tested in each laboratory. See footnote b of Table 4 for a description of symbol meanings.

Because the primers used in this assay amplify sequences that are entirely conserved, the amplicons cannot be used to determine the SV40 strain.

Discussion

In this multi-institutional study, we assessed the sensitivity, specificity, and reproducibility of 16 assays in nine laboratories for the detection of SV40 DNA and used these assays to estimate the prevalence of SV40 in masked replicate DNA extracts from normal human lung tissue and fresh frozen mesothelioma specimens. Eight separate laboratories, including the majority of participants who presented data (positive and negative) regarding SV40 in human tissues at a recent international workshop focused on this topic (43), performed PCR assays using a common protocol agreed to by all participants (16). In addition, five laboratories used their methods developed previously for detection of SV40 DNA (6, 7, 9, 13, 17, 39, 42). A single laboratory (Lab 9) used Southern blot hybridization without PCR amplification as the sole detection method.

Understanding the performance of PCR in detecting SV40 DNA was a major goal of this study. It was hoped that these efforts would help to determine optimal methods for the detection of SV40 genomic sequences in human specimens, as well as to explain conflicting results in some earlier studies. Assessment of PCR assay specificity (as defined by correct identification of negative controls) was complicated by contamination of some contract laboratory-prepared negative control specimens, but this appeared to involve only the first of two separately prepared batches of samples. Among 75 PCR assays conducted using the second batch of negative control specimens, only Lab 1 (with reported contamination of its primers) and Lab 3 (with two reported positives in its Common assay among the five negative controls) reported any positive findings. Moreover, the specificity of SV40 PCR assays used by most laboratories in this study was demonstrated by the low frequency of positive findings reported among normal human lung samples. Excluding Lab 1, only Lab 6 (2 of 25 specimens) and Lab 8 (1 of 25 specimens) found any normal lung samples positive for SV40 DNA (each using the Common assay). These findings were not replicated in other laboratories reporting assays of equal or greater sensitivity.

The high level of specificity found among SV40 PCR assays in most of the laboratories is important. The absence of contamination among these laboratories in the current study, as well as the demonstration of variation in the DNA sequences that have been detected (4, 9), suggests that routine contamination of their PCR assays is an unlikely explanation for their previous reports of the detection of SV40 DNA in tumor specimens. By contrast, Southern hybridization (Lab 9) did not detect SV40 DNA in the 50,000 copies/2 μg of positive control and yielded positive results in two negative controls and one normal lung tissue sample (which might have been attributable to JC or BK virus, which cross-react with the SV40 probe used; Ref. 6).

Apparent differences in sensitivity among the various assays could be explained by several factors. Some reaction conditions or primer pairs might be more sensitive than others. For example, one of the most sensitive reactions included a second round of amplification using nested primers. Several other assays (including the Common PCR assay) included a hybridization step to improve their sensitivity. The total number of cycles used in the PCR assays also varied, ranging from 39 to 60. Also, investigators may have tested different volumes of samples in their assays. Alternatively, contamination of the first batch of negative controls by the independent contract laboratory raises the theoretical possibility that inadvertent contamination of some aliquots of the positive controls (which were prepared earlier on the same day) could explain some perceived discrepancies in sensitivity among the laboratories. Any contamination could theoretically have reached levels up to 500 copies/2 μg of sample, based on: (*a*) the ability of one Lab 3 assay to detect the 500 copies/2 μg of positive control, but the inability of this assay to detect the known contamination of the negative controls; and (*b*) the inability of several other assays to reproducibly detect the 500 copies/2 μg of positive controls, despite their reported ability to detect one or both positive controls with fewer copies of SV40 DNA added. In either case, it seems unlikely that the assays in this study, performed in the

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same laboratories by the same protocols that previously detected SV40 in tumor specimens, would have been less sensitive in this study than in previous studies of these PCR methods, which demonstrated sensitivity ranging from 1 to 100 copies/reaction (17, 46).

Another finding of this investigation is that none of 25 mesothelioma specimens tested was clearly positive for SV40. Three laboratories (Lab 2, Lab 5, and Lab 7) failed to detect SV40 sequences in any tumor samples. Four additional laboratories (Lab 3, Lab 4, and Lab 8) each reported a solitary positive finding. However, none was positive in the paired replicate sample, and a different tumor was positive in each of these laboratories. Only two laboratories (Lab 1 and Lab 6) reported positive results in more than a single sample. Lab 1 discovered SV40 contamination of its PCR primers. Lab 6 reported 6 tumors positive. However, the paired replicate of the same tumor sample was positive in only one instance. Because Lab 6 was the least sensitive in detecting the positive controls (and except for Lab 1 was the only laboratory to detect SV40 DNA in more than a single normal lung specimen), its findings in examining mesothelioma specimens could have been attributable to sporadic contamination or false-positive results. Possible explanations for the sporadic positive findings reported by laboratories other than Lab 1 or Lab 6 include: the presence of SV40 DNA at a level close to the sensitivity of the assays used (although the assays in Lab 2 and Lab 7 appeared to be among the most sensitive); sporadic contamination of the specimens by the central DNA extracting laboratory; or sporadic contamination of the specimens by some of the PCR laboratories themselves. Overall, these results suggest that SV40 was not present in the mesothelioma tumor DNA samples analyzed or was present at a level below the sensitivity of these assays.

An alternative explanation of these findings is that some technical problem prevented detection of SV40 DNA in these mesotheliomas. The negative results cannot readily be ascribed to the mesothelioma tissues tested. The mesothelioma tumor blocks used were fresh frozen under optimal conditions for preservation of DNA, and each block was confirmed by a histopathologist to contain a high fraction of tumor cells. In addition, all test samples contained 2 μg of DNA from which human β-globin was successfully amplified, showing that high molecular weight DNA was present and that samples were free of PCR inhibitors. We cannot exclude the possibility that geographic differences (47, 48) or chance sampling of SV40-negative mesothelioma patients might account for the absence of SV40 DNA in the tumors tested, but the high prevalence of SV40 DNA detected in earlier studies of mesothelioma makes these observations difficult to reconcile.

Neither can these findings be easily explained by the laboratory methods used. Eight independent laboratories tested the study samples by PCR. The several PCR primers used amplify different regions of the SV40 genome. Therefore, even if certain SV40 gene region sequences were not present (*e.g.,* lost during multistage tumorigenesis), the virus genome should still have been detected by primers used by some of the laboratories. In addition, several of the assays used in this study had been used previously in investigations that reported detection of SV40 DNA in human tissues.

A third possibility is that there could have been selective loss of viral DNA during the extraction procedures. This study used the Qiagen column, which is different from the phenol/chloroform extraction procedure used in studies published previously that detected SV40 DNA in tumor specimens. The Qiagen column has been successfully used by other investigators to extract viral genomic DNA including hepatitis B virus (49) and EBV (50) from tissue or blood samples, although others have reported less efficient recovery of cytomegalovirus and adenovirus DNAs using this kit (51–53); reduced recovery of hepatitis C and G viral RNAs was also noted using a similar kit (54). In the present study, the Qiagen method used to extract tissue DNA was only confirmed qualitatively to yield high and low molecular weight DNA species. Direct evidence that the samples in fact contained some low molecular weight DNA was obtained by observing sheared low molecular weight linear DNA by agarose gel electrophoresis. The possible loss of small quantities of closed circular, double-stranded SV40 DNA with the Qiagen method was not examined directly in this study, because the positive controls were spiked after, rather than before, DNA extraction. In any case, it is not possible to satisfactorily determine the efficiency of virus-specific DNA extraction using spiked samples, because there may be differences in the ability of any method to recover added *versus* endogenous DNA. Additional differences, such as generally shorter protease digestion of cells in suspension than in tissue blocks, or other intrinsic differences between cells in culture *versus* cells in tissue blocks, make precise estimation of the sensitivity of PCR assays for endogenous SV40 DNA in tissue blocks very difficult. More research is needed to address issues associated with the extraction of these types of specimens, for example, by comparing the frequency of SV40 detection in mesothelioma DNA extracted using different approaches.

Overall, the data indicate that most of the SV40 PCR assays used were sensitive, specific, and reproducible. None of the assays reproducibly demonstrated the presence of SV40 DNA in the selected human mesothelioma or normal lung tissue samples. Nonetheless, the observed specificity of PCR assays in laboratories that previously reported the detection of SV40 DNA in tumor specimens warrants that further studies be conducted to explain the discrepancy between the findings of previous and present studies.

Appendix A

The Comparative Efficiency of DNA Purification Using Standard Phenol/Chloroform Extraction *Versus* the Qiagen Column Kit. Specimens containing 350,000 or 700,000 WI-38 cells were supplemented with specified amounts of a low molecular weight plasmid (HB1-HB2/pCRII; Ref. 44). Each specimen was digested with proteinase K in lysis buffer at 70°C for 10 min and then processed using either the Qiagen column, in accordance with manufacturer's instructions, or phenol/chloroform extraction. Cell lysates undergoing phenol/chloroform extraction were treated with equal volumes of phenol, phenol/chloroform, and chloroform. DNA was precipitated from these phenol/chloroform-treated specimens with one-tenth volume of 3 M sodium acetate and two volumes of 100% ethanol. DNA concentration was determined with a spectrophotometer, and the relative amounts of low molecular weight DNA obtained were determined using a 1% agarose gel. The findings showed that the Qiagen column obtained greater amounts of overall DNA than phenol/chloroform extraction, even when starting with half as many WI-38 cells in the Qiagen assay (Table A.1). Moreover, the Qiagen column obtained greater amounts of small molecular weight DNA than phenol/chloroform extraction in specimens spiked with similar amounts of the pCRII plasmid (Fig. A.1).

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Table A.1 Total amount of DNA obtained from WI-38 cell preparations using Qiagen column purification *versus* phenol/chloroform extraction, based on spectrophotometry

Sample	Approximate no. of WI-38 cells	Extraction method	Added plasmid DNA (ng)	Total DNA obtained (μg)
C1	350,000	Qiagen column	None	3.8[a]
C2	350,000	Qiagen column	25	4.0
C3	350,000	Qiagen column	50	4.0
P1	700,000	Phenol/chloroform	None	2.0
P2	700,000	Phenol/chloroform	50	2.5
P3	700,000	Phenol/chloroform	100	4.4

[a] The observed amount of DNA is greater than expected (~2.6 μg) because the adherence of WI-38 cells leads to underestimation of the actual number of cells present. However, this applied equally to specimens processed by each DNA extraction method.

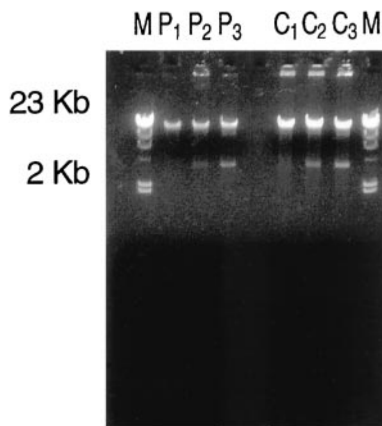

Fig. A.1. Relative amount of low molecular weight DNA in WI-38 cell specimens supplemented with specified amounts of a low molecular weight plasmid (HB1/pCRII). See Appendix A and Table A.1 for the description of the specimens.

Appendix B

Evaluation of Specimens for Inhibitors of PCR Amplification. DNA from 6 human tissue specimens were prepared as undiluted, diluted 1:10, and diluted 1:100. Each dilution was then spiked with one of three amounts of an HTLV-I proviral DNA plasmid, 500, 50, or 5 copies. The ability to amplify the HTLV-I proviral DNA was evaluated using a quantitative PCR assay. The findings showed no effect of dilution on PCR amplification (Table B.1), suggesting that these specimens did not contain inhibitors of PCR amplification.

HTLV-I PCR Methods. Quantitative proviral DNA levels were detected by a real-time automated PCR method (Science Applications International Corporation, Frederick, MD). For each sample, 10 μl of DNA were amplified for 45 cycles with AmpliTaq Gold polymerase (PE Applied Biosystems, Foster City, CA) using an ABI PRISM Sequence Detection System (PE Applied Biosystems, Foster City, CA) and TaqMan PCR Reagent (P/N N808-0230; PE Applied Biosystems) in a 96-well format. During amplification, a fluorescent signal was generated from a dually labeled hybridization probe. The signal was proportional to the number of rounds of Taq-mediated copying of the specific target template sequences, which permitted the real-time measurement of PCR amplicon generation. The probes were labeled with a 5′ reporter dye, 6-carboxyfluorescein and a 3′ quencher dye, 6-carboxy-tetramethylrhodamine

Table B.1 Number of HTLV-I proviral DNA copies detected in diluted human DNA specimens containing known amounts of an HTLV-I plasmid

Specimen and dilution	HTLV-I copies detected		
	500[a]	50[a]	5[a]
A			
Undiluted	543	54	None
1:10	1010	94	4
1:100	620	45	15
B			
Undiluted	643	50	5
1:10	388	78	8
1:100	396	56	4
C			
Undiluted	797	41	2
1:10	704	43	None
1:100	479	71	4
D			
Undiluted	786	36	6
1:10	490	26	4
1:100	501	57	8
E			
Undiluted	390	27	3
1:10	407	59	4
1:100	599	51	9
F			
Undiluted	407	69	9
1:10	530	40	10
1:100	350	88	None

[a] Number of copies of an HTLV-I proviral DNA plasmid added to each sample.

(Operon Technologies, Inc., Alameda, CA). The 5′ nuclease degradation of the hybridization probe by the AmpliTaq Gold polymerase during the course of PCR resulted in the release of 6-carboxyfluorescein quenching by the 6-carboxy-teramethyl-rhodamine and the production of signal, which was detected by the ABI PRISM 7700 instrument. This assay can detect as few as 10 copy equivalents/reaction and has a 5 log linear dynamic range. The HTLV-I/II primers used in this study were from highly conserved sequences (GenBank National Center for Biotechnology Information, Bethesda, MD) from the *tax* gene and are designated as HTV-F5 (7358–7378) and HTV-R4 (7518–7499). The HTLV-I specific probe pHTV-02 (7369–7392) is labeled with two fluorescent dyes. The primer probe set can detect and quantify either HTLV-I or HTLV-II provirus. For each assay, a well-characterized HTLV-I plasmid control template standard curve is generated over a nominal input template copy number range from 1.0×10^0 to 1.0×10^6 copy equivalents/reaction. Unknown copy numbers were then automatically calculated by interpolation from the plasmid control regression curve and reported as copy equivalents per 10^5 cells.

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