

Different Susceptibility of Human Mesothelial Cells to Polyomavirus Infection and Malignant Transformation¹

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Abstract

SV40 has been associated with mesothelioma development. The possible role of the closely related human polyomaviruses JC virus (JCV) and BK virus (BKV) in mesothelioma remained unclear. We found that JCV did not infect human mesothelial cells. BKV and SV40 infected mesothelial cells, expressed viral oncoproteins, and caused similar alterations of key cell regulatory genes. BKV replicated faster than SV40 and caused mesothelial cell lysis, not cellular transformation. SV40 did not lyse mesothelial cells and caused a high rate of transformation. These findings provide a rationale for the observation that SV40 is found in mesothelioma, rather than the ubiquitous human JCV and BKV.

Introduction

SV40 has been frequently detected in MM³ (reviewed in Ref. 1), and HM cells are unusually susceptible to SV40-mediated transformation (2–4). The high rate of malignant transformation observed in SV40-infected mesothelial cells has been linked to telomerase activation (5, 6) and to the inhibitory binding of the SV40 Tag to p53 (7) and Rb-family proteins (8). Moreover, SV40 induces hepatocyte growth factor (HGF) release and *met* activation in SV40-infected HM cells and in SV40-positive MM (3). *met* is a transmembrane tyrosine-kinase receptor involved in signal transduction through the *Ras* oncogene pathway (3). Notch-1 is a key regulator of cell fate and differentiation, and it is required for Ras-mediated oncogenicity (9). SV40 induces Notch-1 activity as early as 72 h after infection and Notch-1 activity is required for the growth of SV40-transformed HM cells (10). Thus, in HM cells, SV40 induces all of the cellular changes required for malignant transformation of human cells (9, 11). These findings provide a model to account for the high rate of malignant transformation observed on SV40 infection of HM cells (the model is illustrated in Ref. 1).

When SV40 was found in some human tumor types, concerns were raised that some of these studies might have mistaken sequences belonging to the human polyomaviruses JCV and BKV for SV40 (1). In fact, in medulloblastoma, a type of brain tumor, both SV40 and JCV can be detected in the same specimens, although JCV is more prevalent (12). To address these concerns most laboratories reporting SV40 in human tumors have performed DNA sequence analyses in areas of the viral genome that would be able to distinguish among these three DNA tumor viruses (1, 13). JCV and BKV are human

polyomaviruses closely related to SV40 (14). Their Tags share about 70% homology with SV40 Tag, and appear to use similar mechanisms to cause cellular transformation (14). For example, like SV40 (15), both JCV and BKV Tags bind and inactivate pRb and p53 (14). JCV has been linked to human medulloblastoma and other brain tumors (12, 14). The role of BKV in human tumors is less clear, and BKV was detected in many different tumor types and normal tissues (14). Neither virus has been detected in MM. Here we asked whether there was a biological reason that could account for this apparent paradox, *i.e.*, that SV40, a monkey virus, was frequently found in MM and that the related human polyomaviruses JCV and BKV were not.

Materials and Methods

Cell Culture, Viruses, and Infection Experiments. Primary HM cells (SV40-negative as determined by immunostaining and PCR analyses) were cultured and characterized as described previously (2). We used four independent primary HM cultures: HM5 and HM16, from male donors; HM10 (the sex of the donor has not been determined); and HM17, obtained from a female donor. Infections were induced as described previously (2). We used SV40 strain 776 (stock solution 10^8 pfu/ml; virus preparation and titration was in African green monkey kidney cells, CV-1). We used BKV virus strain DUN (stock solution 7.5×10^6 TCID₅₀/ml; virus preparation and titration was in human lung fibroblasts WI38; a generous gift of Dr. J. Lednicky, Loyola University, Chicago), and JCV virus strain MAD-4 (stock solution 6×10^6 TCID₅₀/ml; this virus was purchased from ATCC). Infection of different HM cells with either SV40 or BKV was induced using various amounts of viral stock to achieve ~30% positive cells at 72 h after infection by immunostaining with specific antibodies. SV40 and BKV are prepared in different cell types, and their titer is expressed in pfu for SV40 and TCID₅₀ for BKV. To compare the biological effects of these two viruses, we had to infect similar numbers of cells with a similar amount of input virus. Thirty % positive cells appeared a sufficient number of cells to detect biological effects early after infection without overloading the system, which might have impaired our ability to detect possible differences during long-term follow-up. The infectivity of JCV was tested in human glial cells SVG (ATCC) measuring the amount of viral DNA produced, as described previously (16). JCV infection of HM cells consistently failed to produce evidence of JCV DNA replication or JCV Tag expression over a period of 2 months.

Antibodies and Western and Southern Blot Hybridization. We used the mouse monoclonal pAb-419 specific for the SV40 Tag (Calbiochem) and the mouse monoclonal NCL-JCVBKV clone 3.1.1. specific for both BKV and JCV (Novocastra Laboratories). The viral antigen recognized by clone 3.1.1. has not been determined. However, this antibody is considered a standard reagent to detect BKV (17) and JCV (16). We also used the rabbit polyclonal Val1744 (specific for human-activated Notch-1 protein; Cell Signaling Tech.), rabbit polyclonal Anti-c-*Met* [pYpYpY^{1230/1234/1235}] antibody (specific for phosphorylated human *met*; Biosource), goat polyclonal antibody C20 (specific for the intracellular portion of human Notch-1; Santa Cruz Biotechnology) and mouse monoclonal MAB374 (specific for human G3PD; Chemicon). Immunohistochemistry was performed as described previously (2). Western blot analysis was performed on 100 μ g of total cell lysate/sample as described previously (10), with the exception that 6% polyacrylamide gels were used for samples to be tested for *met* phosphorylation. After electrophoresis, samples were transferred onto reinforced nitrocellulose membranes (Hybond-c extra; Amersham), which were cut in half; the top half was hybridized with either

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³ The abbreviations used are: MM, malignant mesothelioma; Tag, SV40 large tumor antigen; JCV, JC virus; BKV, BK virus; HM, human mesothelial; pfu, plaque-forming unit(s); TRAP, telomeric repeat amplification protocol; ATCC, American Type Culture Collection; G3PD, glyceraldehyde-3-phosphate dehydrogenase; TCID₅₀/ml, tissue culture infections dose end point of a dilution assay that infects 50% of cells.

Notch- or *met*-specific antibodies, and the bottom half was hybridized with the anti-G3PD antibody as normalization for sample loading.

Total DNA was extracted from cells (and viral stocks) using the standard SDS/Proteinase K/phenol extraction followed by ethanol precipitation (2). Twenty μg of total DNA from each sample were digested with *Bam*HI and loaded on an 0.8% agarose gel. After electrophoresis, samples were capillary-transferred to a nylon membrane (Hybond-N, Amersham) and hybridized as described (2). The probe for SV40 and BKV DNAs was the degenerate oligonucleotide 5'-AAAATGAAGARAATGAATACTYTGTA-3', 100% homologous to both SV40 and BKV. Normalization for sample loading was performed using a 2934-bp *Bam*HI fragment of the human *Notch-1* cDNA as probe (*Notch-1* is a single copy gene with no pseudogenes). All of the enzymes used for DNA labeling and cloning were from MBI Fermentas. After hybridization and washes, membranes were exposed to X-ray films to visualize results. Quantitative measurement of the radioactivity associated with each band was performed using a phosphorimager FLA-2000 (Fuji Film).

Telomerase Assays. TRAP assays were performed on crude cell extracts using the TRAPeze kit (Serologicals Corp.) according to the manufacturer's instructions. Samples were run on 12% nondenaturing polyacrylamide gels, stained with SYBR green I, and photographed.

Results and Discussion

We compared the biological effects caused by SV40 on infection in four independent primary HM cell lines (HM5, HM10, HM16, and HM17) with those caused by JCV and BKV in the same cells. We tested the amount of viral DNA and proteins produced in HM cells by these three closely related viruses and determined the effects of these viruses on *met*, Notch-1, and telomerase, *i.e.*, the activities that have been linked to the malignant phenotype caused by SV40 in HM cells. Infected cells were monitored for viability and focus formation, and transformed foci were grown in tissue culture to test for their ability to grow in low-serum, soft agar and whether they had an immortal phenotype (>100 doublings without undergoing cell crisis).

Infections. All four of the HM cultures were readily infected by SV40 and BKV. Cells were infected with amounts of virus sufficient to achieve ~30% of Tag-positive cells by 72 h after infection, as determined by Tag immunostaining.

JCV infections using JCV nonarchetypal strain MAD-4 were performed using different ratios of virus per cell. The highest concentration used was 3×10^5 TCID₅₀, administered to 10^5 HM cells. We did not detect any evidence of JCV DNA replication in these cells. No positive cells were detected using the anti-JCV (16) monoclonal 3.1.1 (see "Materials and Methods") over a 2-month period, and throughout this time, these cells appeared morphologically identical to the uninfected controls. In parallel experiments, JCV readily infected human glial cells as shown by DNA extraction and Southern blot hybridization with JCV-specific probes. We further infected HM cells with archetypal JCV strains JCV-CY and JCV-JAL, both kindly provided by Dr. J. Lednicky at a titer of 8 hemoagglutination units (HAU) in 50 μl each. We used the entire amount in parallel experiments to attempt to infect 10^5 HM cells, and cells were scored for evidence of JCV infection as described above for the JCV-MAD-4 strain. Again, we found no evidence of infection. These findings indicate that HM cells are not susceptible to JCV infection *in vitro*, further confirming the restricted cell-type specificity of JCV infection (14).

Effects of BKV and SV40 on Notch-1 Expression and *met* Phosphorylation. Both BKV virus and SV40 infection caused Notch-1 induction early after infection (Fig. 1A). Different HM cultures displayed variable levels of the Notch-1 receptor expression (Fig. 1A, Lanes 1, 4, 7, and 10). Notch-1 protein expression levels were increased from ~2-fold after BKV or SV40 infection in HM17 (Fig. 1A, Lanes 11 and 12) to more than 10-fold in HM5 (Fig. 1A, Lanes 2 and 3). Increased Notch-1 receptor expression levels were paralleled by increased amounts of cleaved (activated) Notch-1 pro-

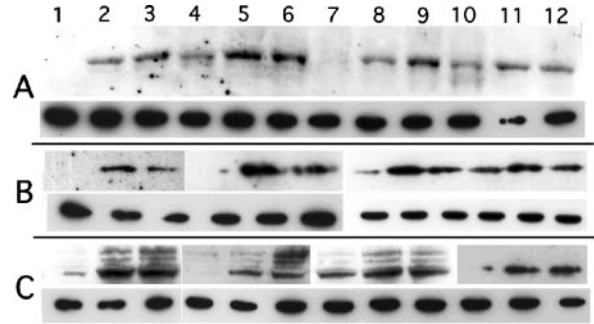


Fig. 1. Both SV40 and BKV virus infection cause Notch-1 up-regulation and activation and *met* phosphorylation early after HM infection. A, top, Western blot hybridization using antibody C20 specific for the intracellular portion of Notch-1; A, bottom, G3PD. B, top, Western blot hybridization using antibody Val1174, specific for cleaved (active) Notch-1. B, bottom, G3PD. C, top, Western blot hybridization using antibody Anti-c-Met [pYpYpY^{1230/1234/1235}], specific for phosphorylated *met*. C, bottom, G3PD. Lane 1, HM5; Lane 2, HM5 at 72 h after BKV infection; Lane 3, HM5 at 72 h after SV40 infection; Lane 4, HM10; Lane 5, HM10 at 72 h after BKV infection; Lane 6, HM10 at 72 h after SV40 infection; Lane 7, HM16; Lane 8, HM16 at 72 h after BKV infection; Lane 9, HM16 at 72 h after SV40 infection; Lane 10, HM17; Lane 11, HM17 at 72 h after BKV infection; Lane 12, HM17 at 72 h after SV40 infection.

tein (Fig. 1B). HM17 already contained biologically active Notch-1, and SV40 and BKV infection appeared to cause only a slight increase in active Notch. Active Notch was not detectable in the other 3 HM cultures. This was the first indication that HM17 had some unique characteristics that eventually influenced their susceptibility to BKV infection (see below). Both SV40 and BKV induced *c-met* phosphorylation 72 h after infection in all four of the HM cultures (Fig. 1C). These results indicated that SV40 and BKV cause similar alterations of the cellular oncogenes that have been linked to the malignant transformation of HM cells.

Effects of BKV and SV40 on Telomerase Activity. BKV infection induced telomerase activity in different HM cell lines (Fig. 2), and BKV was found to be a stronger inducer of telomerase activity compared with SV40. Whereas a DNA ladder indicative of telomerase activity was clearly seen in all four of the HM cultures after BKV infection, the same cells showed only a very faint ladder after SV40 infection. Moreover, in separate experiments, we were unable to detect any telomerase activity in HM10 after SV40 infection (Fig. 2B). When we increased the amount of input SV40 to match that used by Foddis *et al.* (5), we increased the number of SV40 Tag-positive HM cells from 30% (Fig. 2B) to 40–70%, and telomerase activity became detectable (Fig. 2C). These results suggested that a higher number of infected SV40 HM cells are required for the detection of telomerase activity in TRAP assays compared with BKV-infected cells. Thus, BKV, compared with SV40, is a stronger inducer of telomerase activity in HM. Cell extracts obtained from the HM17 culture displayed telomerase activity before and after BKV or SV40 infection (Fig. 2A, Lanes 15 through 20). This finding, along with the observation that these same cells contain active Notch-1, suggest that these primary HM cells are unusual and that they may represent a premalignant mesothelial cell population.

Transformation Rate and Immortalization. Long-term follow up of BKV and SV40 infections revealed profound differences in cell viability and transformation rate as assayed by focus formation (see Table 1). Overall, BKV infections caused a high rate of cell death in all of the HM cultures. HM5 and HM10 were particularly susceptible to BKV-induced cell lysis: ~50% of the cell population was lysed 7–10 days after infection and 100% of cells were lysed between 3 and 4 weeks after BKV infection. BKV caused cell lysis in 50–70% of HM16 and HM17 by 3–4 weeks after infection. No foci were observed in the sparse surviving HM16 cells. BKV induced focus formation in HM17 in only one infection experiment (Table 1). As

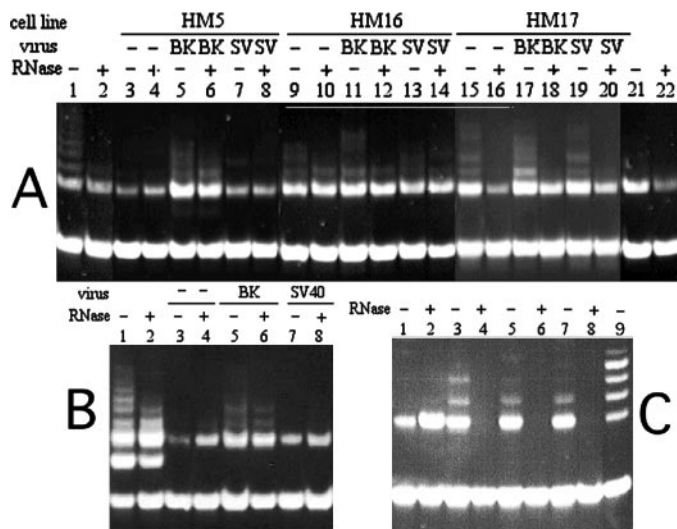


Fig. 2. Both SV40 and BKV virus infection cause telomerase activation early after HM infection. TRAP assays comparing infections with BKV or SV40 of different HM cell lines (A and B), or the effects of increasing amounts of input SV40 virus in the same HM culture (C). A, comparison of BKV (Lanes 5, 6, 11, 12, 17, and 18) and SV40 (Lanes 7, 8, 13, 14, 19, and 20) infections of HM5 (Lanes 3–8), HM16 (Lanes 9–14) and HM17 (Lanes 15–20). Lanes 3, 4, 9, 10, 15, and 16 are uninfected cells; Lanes 1 and 2 are positive controls provided in the TRAPeze kit (Serologicals Corp.); Lanes 21 and 22 are negative controls (WI38 human fibroblasts cell extract). B, telomerase activity in HM10 cell extracts before (Lanes 3 and 4) and 72 h after BKV (Lanes 5 and 6) or SV40 (Lanes 7 and 8) infection. C, effects of increasing levels of SV40 infection on telomerase activation in HM10. Lanes 1 and 2, ~18% Tag-positive HM10 at 72 h after SV40 infection; Lanes 3 and 4, about 35% Tag-positive HM10 at 72 h after SV40 infection; Lanes 5 and 6, ~57% Tag-positive HM10 at 72 h after SV40 infection; Lanes 7 and 8, ~60% Tag-positive HM10 at 72 h after SV40 infection; Lane 9, positive control.

described above, HM17 must have undergone some mutational events leading to Notch-1 and telomerase activity independently from viral infection. Therefore, the HM17 genetic background may render these cells unusually susceptible to BKV-mediated cellular transformation. We tried to culture the nine foci from BKV infection of HM17. Only three foci could be established in tissue culture, but these underwent complete cell lysis before reaching passage 3.

On the other hand, SV40-induced cell lysis was minimal in all four of the HM cultures, confirming previous data indicating a characteristic resistance of HM to SV40-induced cell lysis (2–4). Moreover, SV40 infection reproducibly caused focus formation in all of the HM cultures in two independent infection experiments. The average of the frequency of focus formation obtained in the four different HM cultures was 3×10^{-4} , as in previous studies (2).

Different Rates of BKV and SV40 Replication in HM Cells Account for the Different Biological Effects Observed. We compared the rate of BKV and SV40 DNA replication in infected HM cells by Southern hybridization with an oligonucleotide probe 100% homologous to both BKV and SV40 (see “Materials and Methods”). Although the input viral DNA amounts used for SV40 or BKV infection of HM cells were substantially the same (Fig. 3A, Lanes 9 and 10), BKV-infected HM accumulated much higher amounts of BKV DNA compared with the amounts of SV40 DNA synthesized in SV40-infected HM cells (Fig. 3A). The differences in viral DNA synthesis mirrored the cytopathic effects elicited by these viruses in the different HM cultures (Fig. 3, B–E). In fact, both HM5 and HM10 synthesized BKV DNA more than 30 times faster than SV40 DNA; the same HM cultures were most susceptible to BKV-mediated cell lysis (Table 1). On the other hand, HM16 and HM17 synthesized BKV DNA only three to five times faster than SV40 DNA, and some cells in these cultures survived BKV infection (Table 1). BKV- and SV40-infected HM cultures produced complete viral particles that

were visualized by electron microscopy (data not shown). Accordingly, when supernatants from HM cultures infected with BKV and SV40 were administered to uninfected primary human fibroblasts MRC-5 (ATCC) in tissue culture, these cells stained positive with anti-BKV and anti-SV40 antibodies (see “Materials and Methods”) and developed cell lysis. DNA sequences were performed to verify the presence of the input virus (BKV or SV40) in a given cell culture and to rule out cross-contamination of the cell cultures.

Our results addressed the paradox that SV40, a monkey virus, is frequently found in MM and occasionally in HM cell lines (1), and the human JCV and BKV are not. We found that HM cell lines exhibit a very different susceptibility to infection by these polyomaviruses.

JCV did not productively infect HM cells. No positive cells were observed by immunostaining over a period of 2 months, and DNA analyses revealed no evidence of JCV replication. Instead, immunostaining with anti-BKV (17) clone 3.1.1 readily detected BKV-infected HM cells, and infectious BKV particles were produced. The rapid proliferation of BKV in HM cultures caused the same genetic alterations observed with SV40, including telomerase activation, Notch, and *met* activation; however, mesothelial cells were lysed and, therefore, could not be transformed. Accordingly, transformed foci did not appear in three of four primary HM cultures infected with BKV compared with a rate of transformation of 3×10^{-4} observed in the same four HM cultures infected with SV40. This striking difference had an apparent simple explanation. BKV lysed infected HM, SV40 did not. All of the HM5 and HM10 cultures that were infected with BKV were lysed within 4 weeks from infection. Lysis was related to the high rate of BKV replication in HM cultures; by 72 h after infection, 30 times more BKV DNA was produced compared with SV40 DNA. We have previously shown that the main reason that SV40 transforms HM cells but not fibroblasts is that SV40 replicates at low levels in HM cultures, and, thus, the cells are not lysed (2). Instead, the high levels of SV40 replication in human fibroblasts causes cell lysis; therefore, malignant transformation is not observed (1, 2). When SV40 plasmids that cannot replicate because they lack the SV40 ori region of replication are transfected into human fibroblasts, these cells are transformed (2). When SV40 replication is artificially accelerated in HM cultures by down-regulating endogenous wild-type p53, HM cells are lysed (2). These findings indicate

Table 1 Focus formation in different HM cultures infected with either SV40 or BKV in two separate infections

	Infected cells ^a	No. of foci at 28 days
Experiment 1		
HM5 (SV40)	1.1×10^5	163
HM5 (BKV)	1.4×10^5	0 ^b
HM10 (SV40)	1.6×10^5	32
HM10 (BKV)	2.1×10^5	0 ^b
HM16 (SV40)	1.1×10^6	143
HM16 (BKV)	0.4×10^6	0 ^b
HM17 (SV40)	1.3×10^6	110
HM17 (BKV)	1.8×10^6	9 ^{b,c}
Experiment 2		
HM5 (SV40)	0.22×10^5	5
HM5 (BKV)	0.25×10^5	0 ^b
HM10 (SV40)	0.53×10^5	8
HM10 (BKV)	0.56×10^5	0 ^b
HM16 (SV40)	0.4×10^6	28
HM16 (BKV)	0.53×10^6	0 ^b
HM17 (SV40)	0.46×10^6	34
HM17 (BKV)	0.58×10^6	0 ^b

^a Number of positive cells by immunostaining with SV40- and BKV-specific antibodies (“Materials and Methods”) 7 days after infection (which corresponded to ~50% of the entire number of cells).

^b All of the BKV-infected HM5 and HM10 cells were lysed within 3–5 weeks after infection. Eight weeks after BKV infection, the HM16 and 17 cell population was numerically ~30–50% of the original.

^c Three of nine foci were successfully established in tissue culture, but all of the cells lysed after two doublings. Infected cells were monitored for 8 weeks after infection.

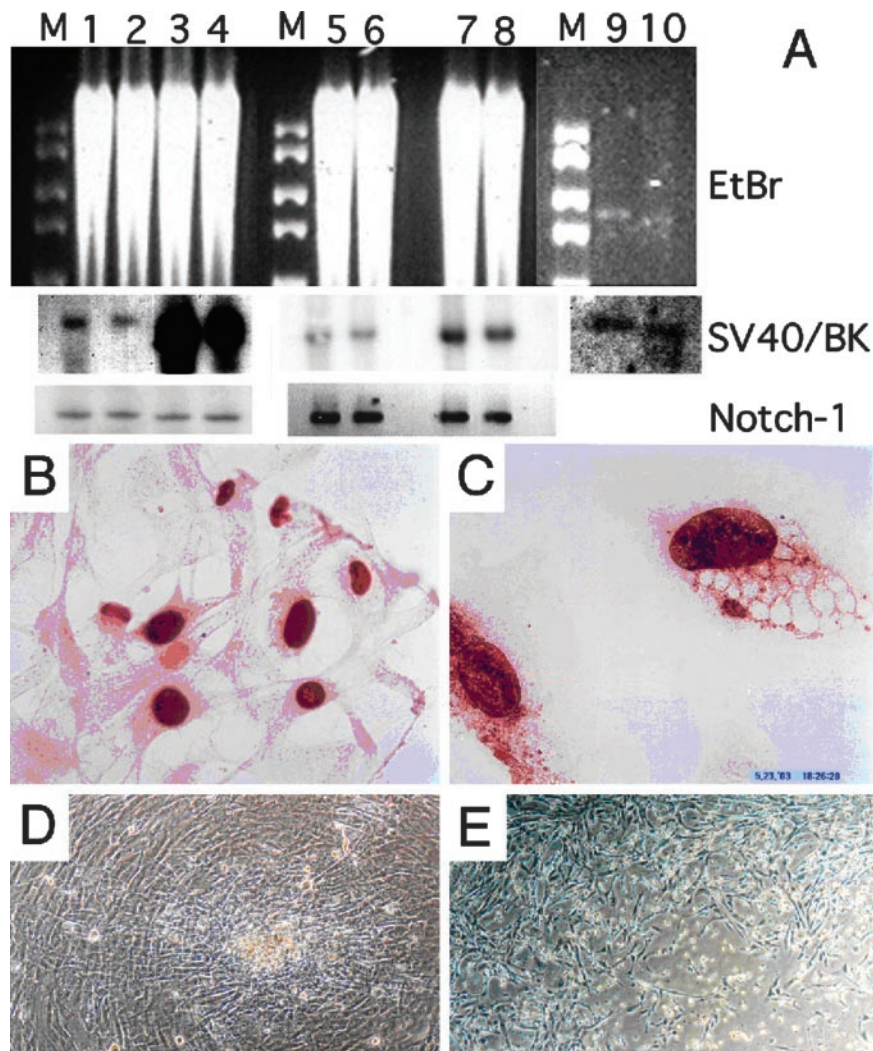


Fig. 3. BKV DNA is replicated faster than SV40 DNA in different HM cultures. *A*, Southern blot hybridization of total DNA extracted from different HM 72 h after viral infection (see "Materials and Methods" for details). *Top*, ethidium bromide (*EtBr*) staining of agarose gels; *bottom*, autoradiography after hybridization with ^{32}P -probes. *Lane M*, molecular weight marker; *Lane 1*, HM5 at 72 h after SV40 infection; *Lane 2*, HM10 at 72 h after SV40 infection; *Lane 3*, HM5 at 72 h after BKV infection; *Lane 4*, HM10 at 72 h after BKV infection; *Lane 5*, HM16 at 72 h after SV40 infection; *Lane 6*, HM17 at 72 h after SV40 infection; *Lane 7*, HM16 at 72 h after BKV infection; *Lane 8*, HM5 at 72 h after BKV infection; *Lane 9*, one-twentieth of the total SV40 input viral DNA used to infect cells in *Lane 1*; *Lane 10*, one-twentieth of the total BKV input viral DNA used to infect cells in *Lane 3*. *B*, pAb-419-Tag immunostaining of SV40-infected HM10 2 weeks after infection, showing positive nuclei and absence of cell lysis. *C*, immunostaining with clone 3.1.1 of BKV-infected HM10 at 6 days after infection, showing extensive vacuolization; *D*, same as *B*, morphology showing confluent cells and development of an early transformed focus; *E*, same as *C*, morphology shows cell lysis in ~50% of the cells. *B* and *C*, $\times 400$; *D* and *E*, $\times 100$.

that the amounts of SV40 replication in a given human cell type influence whether the cells are more likely to be lysed (fibroblasts) or transformed (mesothelial cells; Ref. 1).

Human cell transformation is associated with the induction of telomerase activity that causes immortalization and directly contributes to the malignant phenotype (11), the inhibition of *p53* and *Rb* tumor suppressor genes that prevent uncontrolled cell growth (11), and the activation of genes such as *Ras* that promote cell division (11). Down-regulation of Notch-1 activity blocks the growth of human fibroblasts containing the SV40 Tag and tag (SV40 small tumor antigen), telomerase, and oncogenic *ras*, indicating that Notch-1 play an additional important role in human cell transformation (9). When SV40 infects HM, it induces all of the activities listed above (1). Moreover, SV40-transformed HM cells inactivate the tumor suppressor RASSF1A during growth in tissue culture and *in vivo*; this causes a more aggressive tumor phenotype (18). Three independent panels, one from a SV40 and human tumor consensus meeting that convened at the University of Chicago (20), one organized by the National Cancer Institute (13), and one organized by the Institute of Medicine (20), have independently concluded that there is compelling evidence that SV40 is present in some MM (reviewed in Ref. 1). Our findings provide a biological explanation for the observation that SV40 is detected in MM (1) and that BKV and JCV are not. Our findings suggest that, in rare circumstances, BKV may provide a cocarcinogenic stimulus to premalignant HM cells. These findings underscore

the fact that viral carcinogenesis is not an all-or-none phenomenon. Rather, viral carcinogenesis is strongly influenced by host factors, such as the particular cell type exposed to the virus, the eventual presence of preexisting cellular alterations (such as those found in HM17), and, *in vivo*, by the immune status of the host. These variables will play a key role in determining the outcome of the virus-cell interaction.

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