Role of SV40 ST antigen in the persistent infection of mesothelial cells

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Abstract

Viral DNA is maintained episomally in SV40 infected mesothelial cells and virus is produced at low but steady rates. High copy numbers of the viral DNA are maintained in a WT infection where both early antigens are expressed. In the absence of ST, cells are immortal but non-transformed and the infected cells maintain only a few copies of episomal viral DNA. We show that ST expression is necessary for the maintenance of high copy numbers of viral DNA and that the PP2A binding ability of ST plays a role in genome maintenance. Interestingly, an siRNA to the virus late region downregulates virus copy number and virus production but does not prevent the anchorage-independent growth of these cells. Furthermore, addition of virus neutralizing antibody to culture media also decreases copy numbers of viral DNA in WT-infected cells, suggesting that virus production and re-infection of cells may play a role in maintaining the persistent infection.

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Keywords: SV40 small-t; Mesothelial cells; Episomal genome; Persistent infection

Introduction

SV40 virus is known to replicate productively in monkey kidney cells in which large amounts of virus are produced and cells are killed by the infection. In contrast, the viral DNA does not replicate in rodent cells but may become integrated into the cellular genome, resulting in cellular transformation. Infection of human fibroblasts causes a third type of virus-host interaction, termed semi-permissive. Once these cells are infected, a small fraction of the cells becomes permissive at any given time. These cells show high levels of viral DNA replication and virion production and die of the infection releasing virus to culture media. Most of the cells show only a low level of viral DNA and late protein synthesis. As first described by Carbone (Bocchetta et al., 2000), human mesothelial cells define a fourth type of infection in which all cells remain persistently infected over long periods of time and none of the cells show evidence of cytopathic effect. As for other DNA tumor viruses, such as Human Papilloma Virus (HPV) and Epstein–Barr Virus (EBV) (Adams and Lindahl, 1975; LaPorta and Taichman, 1982), the SV40 DNA is maintained as an episome in mesothelial cells and very low levels of virus are chronically produced. Whereas the relationship between the expression of viral proteins and maintenance of viral DNA has been more thoroughly examined with HPV and EBV, little is known about the maintenance of the viral genome in persistently infected mesothelial cells. Examining the state and the maintenance of the viral genome in these cells is the subject of this report.

The SV40 large-T (LT) antigen is essential to the replication of viral DNA in permissive and semi-permissive primate and human cells. LT binds the origin of replication and interacts with cellular proteins necessary for the initiation of replication (Fanning, 1992; Prives et al., 1980; Stadlbayer et al., 1996). It is likely that this is essential for persistence in mesothelial cells because some mesothelial cell lines transformed by SV40 virus infection displayed reduced viability after downregulation of LT (Bocchetta et al., 2000).

Another SV40 protein, small-t (ST) antigen, can also play a critical role in the transformation of cells. This is especially the case in less proliferative cells, most likely due to the ability of ST to stimulate mitogenic and anti-apoptotic proteins and down-regulate proteins that inhibit cell cycling (Howe and Tan, 1977; Porras et al., 1999; Sontag et al., 1993; Watanabe et al., 1996). ST is essential for the transformation of several human cell types,
in conjunction with LT (Hahn et al., 2002; Yu et al., 2001), even in the absence of oncogenes such as EJ-ras. The primary effects of ST occur through its interaction with protein phosphatase 2A (PP2A) in which it displaces many regulatory subunits of this trimeric enzyme. The deregulation of PP2A activity and altered intracellular localization serve to stimulate cells to enter the cell cycle through sustained phosphorylation of key cell cycle related proteins (Howe et al., 1998; Sontag et al., 1993; Watanabe et al., 1996).

Recently, we established a series of mesothelial cell lines that were immortalized by LT and the cellular telomerase protein h-TERT. These cells have now been in culture for several years and show little evidence of transformation. When these cells express ST, introduced either by virus infection or transfection, the cells lose their contact inhibition and become anchorage independent. The differences in the behavior of these cells and the fact that ST can influence viral DNA replication (Cicala et al., 1994; Lin et al., 1998; Virshup et al., 1989) made it of interest to examine the state of the viral genome in these cells and the role ST might play in genome maintenance. In the experiments described here, we show that high levels of viral DNA are maintained in cells infected with WT SV40 while viral DNA levels in ST mutant infections are extremely low. ST is also required to maintain higher copy numbers of superinfecting genomes, and this ability is disrupted when ST does not have a fully functional PP2A binding domain. Finally, once anchorage-independent cells are obtained, high levels of viral DNA or infectious virus are not required for cells to remain transformed. These are dramatically reduced by growth of cells in the presence of SV40 neutralizing antibody, yet these cells continue to show anchorage-independent growth.

Results

As we have shown previously (Yu et al., 2001), TERT-expressing mesothelial cells become immortalized, but not transformed, when persistently infected with DL888, a virus that does not produce ST. In contrast, cells infected with WT virus were both immortalized and transformed as indicated by anchorage independence. The pools described in this report are referred to as 5ADL and 5AWT, respectively; additional pools have also been made and show behaviors identical to those of 5ADL and 5AWT. Both the 5ADL and 5AWT cell lines express LT at similar levels, as determined by Western blotting (data not shown), so differences in anchorage-independent growth reflect only the absence of ST in 5ADL cells.

It has been reported that the SV40 genome is maintained as an episome in mesothelial cells infected with WT SV40 (Carbone et al., 2003) and low levels of virus are continuously produced. We have found that the levels of virus produced in the absence of ST are far lower than levels produced from WT-infected cultures. For example, a 6 cm dish of 5AWT cells (over 10^6 cells) produces a total of 10^3–10^4 pfu in a 1-week period. In contrast, a 6 cm dish of 5ADL cells produces fewer than 10 pfu, although virus can always be detected by plaque assay or co-cultivation with permissive monkey kidney cells (data not shown).

The state of the SV40 genome was determined in 5ADL and 5AWT cells that had been in culture for several months. Total genomic DNA was isolated from 5ADL and 5AWT cells, linearized with EcoRI and analyzed by Southern blotting. The state of the SV40 genome was determined in 5ADL and 5AWT cells expressing mesothelial cells become immortalized, but not transformed, when persistently infected with DL888, a virus that does not produce ST. In contrast, cells infected with WT virus were both immortalized and transformed as indicated by anchorage independence. The pools described in this report are referred to as 5ADL and 5AWT, respectively; additional pools have also been made and show behaviors identical to those of 5ADL and 5AWT. Both the 5ADL and 5AWT cell lines express LT at similar levels, as determined by Western blotting (data not shown), so differences in anchorage-independent growth reflect only the absence of ST in 5ADL cells.

Table 1

<table>
<thead>
<tr>
<th>Cells</th>
<th>SV40 copies/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
</tr>
<tr>
<td>5AWT pool</td>
<td>217.9</td>
</tr>
<tr>
<td>5ADL clone 4C10</td>
<td>1.5</td>
</tr>
<tr>
<td>5ADL clone 5C2</td>
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<tr>
<td>5ADL clone 4B5</td>
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</tr>
<tr>
<td>5ADL clone 4E2</td>
<td>0.5</td>
</tr>
<tr>
<td>5ADL clone 4C4</td>
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</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
</tr>
<tr>
<td>5AWT pool</td>
<td>622.0</td>
</tr>
<tr>
<td>5ADL pool</td>
<td>4.4</td>
</tr>
<tr>
<td>5AWT/si</td>
<td>1.7</td>
</tr>
</tbody>
</table>

* SV40 low molecular weight DNA was isolated with a Genelute Plasmid Miniprep Kit (Sigma) and analyzed as described in Materials and methods.

Results

As we have shown previously (Yu et al., 2001), TERT-expressing mesothelial cells become immortalized, but not transformed, when persistently infected with DL888, a virus that does not produce ST. In contrast, cells infected with WT virus were both immortalized and transformed as indicated by anchorage independence. The pools described in this report are referred to as 5ADL and 5AWT, respectively; additional pools have also been made and show behaviors identical to those of 5ADL and 5AWT. Both the 5ADL and 5AWT cell lines express LT at similar levels, as determined by Western blotting (data not shown), so differences in anchorage-independent growth reflect only the absence of ST in 5ADL cells.

It has been reported that the SV40 genome is maintained as an episome in mesothelial cells infected with WT SV40 (Carbone et al., 2003) and low levels of virus are continuously produced. We have found that the levels of virus produced in the absence of ST are far lower than levels produced from WT-infected cultures. For example, a 6 cm dish of 5AWT cells (over 10^6 cells) produces a total of 10^3–10^4 pfu in a 1-week period. In contrast, a 6 cm dish of 5ADL cells produces fewer than 10 pfu, although virus can always be detected by plaque assay or co-cultivation with permissive monkey kidney cells (data not shown).

The state of the SV40 genome was determined in 5ADL and 5AWT cells. Total genomic DNA was isolated from 5ADL and 5AWT cells, linearized with EcoRI, then analyzed by Southern blotting. 5AWT cells contain easily detectable genome-length copies of SV40 DNA (Fig. 1A). The intensity of the epimonal DNA from 5AWT was consistent with these cells containing at least 100 copies per cell. More accurate quantitation was obtained using
When genomic DNA from clone 4B5 was digested with supercoiled or nicked circular DNA in Southern blots (Fig. 1B). Interestingly, 5ADL clones showed little or no unit-length presence of some integrated copies cannot be fully ruled out. DNA in low molecular weight fractions suggest that 5ADL cells.

No clone contained the high levels of viral DNA found in 5AWT clones shown in Table 1 represent the range in low molecular weight DNA from the 5AWT pool contained over 10^3 copies per cell. Several independent pools and derived clones of 5AWT have been analyzed. These have been found to contain varying levels of episomal DNA, ranging from 50 to 200 copies per cell (data not shown).

In contrast to ST positive 5AWT cells, the ST negative 5ADL pool did not show readily detectable viral DNA in initial exposures. After a longer exposure (3 weeks rather than 2 days), a faint linear SV40 DNA band was detected (inset, Fig. 1A). To determine how much variability there was within the 5ADL pool, several individual clones of 5ADL were isolated. All of these clones continue to produce infectious virus, albeit at very low levels like the original pool (data not shown). The five clones shown in Table 1 represent the range in low molecular DNAs found by real-time PCR analysis, with most clones containing barely 1–2 copies per cell. An occasional clone contained 10–15 copies per cell, but this was the exception, and no clone contained the high levels of viral DNA found in 5AWT cells.

The production of infectious virus and the detection of viral DNA in low molecular weight fractions suggest that 5ADL cells all contain unintegrated viral DNA in some form, although the presence of some integrated copies cannot be fully ruled out. Interestingly, 5ADL clones showed little or no unit-length supercoiled or nicked circular DNA in Southern blots (Fig. 1B). When genomic DNA from clone 4B5 was digested with XbaI or BglII, enzymes that do not cut SV40, only high molecular forms of viral DNA appeared in Southern blots. These may represent concatameric viral DNAs. When cut with EcoRI to linearize genomes, unit length linear molecules were observed. Similar observations were made with the other 5ADL cloned lines.

When the TERT+ mesothelial cells were originally infected with WT SV40 or DL888, not all cells immediately became immortal; rather, a subset of the cells grew out over a 1- to 2-week period to form the 5AWT and 5ADL pools. Thus, the immediate fate of the viral DNAs could not be monitored. To do this, cells were re-infected with either WT SV40 or DL888 to monitor the effect of ST on maintenance of a superinfecting genome. Although it is difficult to superinfect a fully permissive cell, such as CV1 cells, superinfection of the mesothelial cells which contain far lower levels of viral DNA occurs readily. This was proven by superinfection with an SV40 derivative containing a BglIII site at nucleotide 5171. All viruses isolated after superinfection contained the BglIII site as shown by sequencing and restriction mapping (data not shown).

5ADL cells were superinfected at a multiplicity of infection of 10 for 2 days and then passaged weekly at a one to ten dilution. Total genomic DNA was isolated from the re-infected cells 2 days post-infection and at various passages thereafter. The superinfecting genome was readily detected 2 days post-infection with either mutant or WT virus (Fig. 2A). Upon passage, there was a gradual decrease in the number of viral episomes present in cells infected with the ST negative virus. In the experiment shown in Fig. 2A, the DL888 genome was nearly undetectable by the third week in culture. In contrast to cells infected with DL888, levels of episomal DNA in WT-infected cells were even higher after 3 weeks of passaging than they were 2 days post-infection (Fig. 4, lanes 5–8). This experiment has been repeated many times, and the loss of the DL888 is always gradual with the copy number reducing to only a few per cell by 3–7 weeks after infection.

Real-time PCR of extracted low molecular weight DNA to detect the viral genome. In the first experiment shown in Table 1, low molecular weight DNA from the 5AWT pool contained over 200 copies per cell. Several independent pools and derived clones of 5AWT have been analyzed. These have been found to contain varying levels of episomal DNA, ranging from 50 to 1000 copies per cell (data not shown).
To determine whether ST could influence genome copy number if expressed in a manner other than by viral infection, ST positive clones were studied. 5ADL cells were serially infected with a retrovirus that encoded genes for both ST and resistance to the drug puromycin. After infection, drug-resistant clones were isolated and analyzed for expression of ST. One clone (#4) that expressed the greatest amount of ST protein (although less than the 5AWT cells) was studied in detail. As a control, a clone was isolated from 5ADL cells infected with a retrovirus encoding only the puromycin resistance gene. The ability of ST clone 4 to maintain a superinfecting genome is shown in Fig. 2B. In contrast to 5ADL cells in which levels of a superinfecting DL888 genome were dramatically reduced by week 3, the superinfecting viral DNA was readily detectable in the ST expressing cells. These experiments demonstrate a role for ST in the maintenance of the viral genome after re-infection of mesothelial cells.

Based on experiments that showed ST positive cells had more copies of the viral genome, it was possible that expression of ST protein in 5ADL cells might increase the levels of resident DL888 genome. This was not observed in any of the ST-expressing clones, even after as many as 13 weeks in culture (data not shown). This suggests that expression of ST cannot influence the levels of an already resident viral genome, once a steady state has been reached.

A critical function of ST antigen is its ability to bind and deregulate the phosphatase activity of PP2A. This function of ST has previously been shown to be important in regulating growth and transformation of human cells (Mungre et al., 1994; Sontag et al., 1993; Yu et al., 2001). To study the role of the PP2A association in the maintenance of the episomal genome in mesothelial cells, TERT-expressing primary mesothelial cells were infected with SV40 C97S. This is a mutant form of the virus in which LT is WT but ST carries a mutation at amino acid 97. A cell line was established in the same manner as 5ADL and 5AWT cells and was referred to as 5A97. The C97S mutation was used because the mutant ST protein is relatively stable and, to properly interpret experiments, it was important for ST protein to be expressed at similar levels in mutant and WT cells. As shown in Fig. 3A, levels of ST protein in the 5A97 cells were similar to

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**Fig. 4.** Depletion of genome copy number does not prevent anchorage-independent growth of the mesothelial cells infected with WT virus. 5AWT cells were infected with lentivirus expressing shRNA against the late region transcript of SV40 and the puromycin resistance gene. Following puromycin selection, surviving cells (5AWT/si) were pooled and analyzed. (A) The viral load produced by 6 cm dishes of 5AWT and 5AWT/si cells was determined by a plaque assay. CV1 cells were infected with undiluted and diluted (1:10 and 1:100) virus preparations made from equal numbers of cells. The calculated titer is shown below each column. (B) Cells were harvested and lysates were immunoblotted for the presence of VP1, LT and GAPDH. (C) To detect ST, extracts of 35S-methionine labeled cells were immunoprecipitated with pAB419, then immunoprecipitated protein was detected following SDS–PAGE by autoradiography. (D) Anchorage-independent growth was determined by plating 2.5 × 10^5 5ADL, 5AWT or 5AWT/si cells in 1.8% methylcellulose in agarose coated petri dishes.
those expressed by 5AWT. The C97S ST is less defective in PP2A interaction than other point mutant proteins. Interestingly, the C97S clone maintains intermediate levels of SV40 DNA. However, 5A97 cells did not grow well in methylcellulose, especially when reduced serum concentrations were used, indicating a defective transformed phenotype (data not shown).

Fig. 3B shows that 5A97 cells are unable to support the maintenance of high copy numbers of an ST-negative genome, suggesting that the PP2A binding ability of ST is necessary for this property. 5A97 cells were superinfected with either WT SV40 or DL888 then passaged on a weekly schedule. Real-time PCR analysis was used to quantify low molecular weight (Hirt-extractable) genomes. After several weeks in culture, levels of the superinfecting WT genome were at least 10 times those of the ST-negative DL888. The decrease in episomal copy number in re-infected 5A97 cells occurred at a slower pace than in 5ADL re-infected with DL888, which may reflect the partial defect in PP2A interaction exhibited by the C97S mutant.

As 5AWT cells maintain and produce significantly larger amounts of SV40 virus, it was also of interest to determine whether this played a role in the transformation of persistently infected mesothelial cells. 5AWT cells stably expressing a siRNA construct against the late region of the genome (5AWT/si) maintain significantly fewer copies of SV40 DNA. The genome level in 5AWT/si was similar to the amount of SV40 detected in 5ADL cells, as determined by real-time PCR and Southern blotting (Table 1 and data not shown). This was further confirmed by plaque assay titering the amount of virus extracted from 5AWT vs. 5AWT/si cells (Fig. 4A). Interestingly, the cell lines still express similar levels of the SV40 early region (Fig. 4B, middle panel and C). The top panel of Fig. 4B shows that the siRNA specifically knocked down late gene expression in 5AWT/si cells. Cells isolated after the introduction of the siRNA construct appeared to be quite homogeneous because 10 independently isolated subclones all contained low levels of viral genomes.

Interestingly, when these cells were then grown in anchorage-independent conditions, 5AWT/si cells maintained their transformed phenotype and were still able to form large colonies similar to those made by the parental 5AWT cells (Fig. 4D). This was contrary to 5ADL cells, indicating that maintenance of high copy numbers of SV40 is not required for transformation and further highlighting the importance of ST for this role.

5AWT cells release infectious SV40 virus into the media, so a potential role of secreted virus in the persistent infection of mesothelial cells was also studied. 5AWT cells were grown for several weeks with or without the presence of SV40 neutralizing antibody in the medium. Neutralizing antibody did not affect the growth rate of the mesothelial cells (data not shown). Cells were collected once a week to extract DNA for real-time PCR and to passage in fresh media with or without antibody. Real-time PCR analysis revealed that SV40 copy number rapidly decreased in 5AWT cells grown in SV40 neutralizing antibody (Fig. 5). Furthermore, once 5AWT cells were significantly depleted of SV40 genomes, they did not recover their original levels of viral DNA after 4 weeks of growth in media without antibody. These data suggest a model where SV40 virus production and continuous re-infection are required for persistent infection.

**Discussion**

Persistent infection is a unique pattern of infection for SV40 exhibited in mesothelial cells. In mesothelial cells, WT virus is maintained as an episome, and no CPE is observed in infected cells. In this report, patterns for maintenance of the viral genome in persistent infection were explored. A new role for ST to establish and maintain high levels of viral genomes in cells was identified.

5AWT cells, mesothelial cells persistently infected with WT virus, maintain high copy numbers of the viral genome, on average greater than 500 copies per cell. In contrast, 5ADL cells, which carry the ST-defective DL888 virus, generally have fewer than 10 copies per cell. Thus, it appeared that ST might be at least in part responsible for promoting genome maintenance. When ST was introduced into 5ADL cells by plasmid transfection or retroviral infection, these cells were now able to maintain higher levels of a superinfecting ST-negative DL888 virus. This effect required the ability of ST to interact with PP2A because the C97S mutant of ST was less able to maintain genome copy numbers at a high level.

Although ST helps a superinfecting genome to persist at higher levels, it was interesting that ST did not elevate the low levels of viral DNA in parental 5ADL cells. This suggests that once a steady-state level of DNA is achieved, it is stable. When cells are re-infected, however, the early DNA replication that occurs within the first few days generates many copies of viral DNA which are then retained when ST is present.
Interestingly, anchorage-independent growth of the cells did not require high levels of viral DNA to be maintained. Transfection of ST into 5ADL cells led to their growth in methylcellulose, but as just noted, did not elevate copy numbers of the resident DL888 genome. Similarly, downregulation of virus late region expression in 5AWT cells reduced the levels of the episomal genome as well as infectious virus, but did not affect anchorage-independent growth. This may be because levels of the early viral proteins, LT and ST, were unaffected by downregulation of the late region.

Genome levels and quantities of infectious virus tend to parallel one another in the mesothelial cell lines. When 5AWT cells were grown in the presence of anti-SV40 neutralizing antibody, quantities of the resident episomal genome were dramatically reduced. This experiment suggests that the copy number of the WT genome in 5AWT cells is maintained by a cycle of virus production and re-infection. We had also considered other possible means through which high copy numbers of viral DNA could be maintained. For example, viral genomes might be slowly diluted out from cells as they divide. Because 5AWT cells are less contact-inhibited, WT viral DNA replication might continue at a greater rate than that of cellular DNA when cells reach confluence. Thus, WT cells would be able to “replenish” copies by over-replication at confluence, much as bacterial plasmids do when bacteria reach stationary phase. However, we never found any experimental evidence to support this possibility.

At this time, replenishment by new infection appears to have the primary effect on DNA copy number. This would occur more readily in WT infections in which levels of infectious virus are much higher as well. Cells grown in the presence of neutralizing antibody retained their anchorage-independent phenotype, again suggesting that high genome levels are not required for transformation. What then is the advantage of high levels of intracellular viral DNA? One key aspect would be the ability to successfully distribute DNAs to each daughter cell. Other tumor viruses that maintain viral DNA episomally, such as HPV and EBV, have documented mechanisms for controlling where episomes are positioned during partitioning of a cell (Kanda et al., 2001; Sears et al., 2003, 2004; Serrano et al., 1995; Van Tine et al., 2004). HPV and EBV control maintenance of episomes by having a viral protein, such as EBNA-1 in the case of EBV, work in trans with the viral episome to form a physical connection between cellular chromosomes and the viral genome. No such protein is known for SV40. Some preliminary experiments to see whether viral LT could be found on mitotic chromosomes did not support the idea that this viral protein could tether viral and cellular chromosomes.

Without a controlled system for regulating where episomal DNA is located during cell division, viral genomes can be “lost” and a newly formed daughter cell could potentially not contain any viral DNA. For mesothelial cell lines like 5AWT, the probability is very high that each daughter cell will contain a viral genome because hundreds of copies are present. In 5ADL cells, there would be a greater chance that an individual daughter cell would not inherit a viral episome. In this case, that cell might eventually senesce because LT is required in addition to hTERT for immortalization of mesothelial cells (Yu et al., 2001). In preliminary experiments, we did not detect major differences in the ability to generate clones from 5ADL and 5AWT cells, but this would be difficult to detect if senescence required several generations to become apparent.

In summary, the virus–host relationship of SV40 and mesothelial cells is unusual in experimental systems used to study SV40. However, episcopal maintenance of viral DNAs is not unprecedented for the papovaviruses, either for murine polyomavirus-induced tumors or human persistent infections with BKV and JCV (for reviews, see Benjamin, 2001; Doerries, 2006). The in vitro system of SV40-infected mesothelial cells may provide a new, manipulable model for further studying persistence of papovaviruses and mechanisms through which this occurs.

Materials and methods

Cell culture

Cells were cultured in Dulbecco’s modified Eagle’s (DME) medium containing 10% FBS, 20 mM L-glutamine and 100 U/ml penicillin–streptomycin. Confluent stock cultures were split using a 1:10 (5ADL cells) or 1:20 (5AWT) dilution once a week. The isolation of 5ADL and 5AWT cell lines from TERT-expressing mesothelial cells was described previously (Yu et al., 2001). 5A97 cells derived from infection with SV40 ST mutant C97S were similarly developed after infection of TERT-expressing cells with 10 plaque-forming units (pfu) per cell of C97S virus (Munger et al., 1994). For re-infection experiments, 5ADL or 5A97 cells were grown to 80% confluence and then infected with 10 pfu/cell of either SV40 WT or ST mutant DL888.

Protein extracts

Cells were grown to confluence in DME containing 10% FBS, unless otherwise noted. Plates were washed once with cold PBS and then scraped and collected in 1 ml cold PBS. Cells were pelleted by centrifugation at 5000 rpm at 4 °C. Pellets were lysed in 0.5% NP40 lysis buffer containing 10 μg/ml aprotinin, 10 ng/μl leupeptin, and 10 μg/ml PMSF and supernates were cleared by centrifugation at 14,000 rpm at 4 °C. Protein concentration was determined using Bio-Rad protein assay reagent and reading absorbance at 595 nm.

Western blots for SV40 proteins

Sample buffer containing 5% beta-mercaptoethanol was added to 25 μg of total protein extract and was boiled for 5 min. Boiled samples were run on a 12% SDS–PAGE gel and protein was transferred to PVDF membrane (Immobilon). Membranes were incubated with pAb419 monoclonal primary antibody (Harlow et al., 1981) at a 1:100 dilution to detect SV40 proteins. Rabbit polyclonal anti-VPI antibody was kindly provided by Dr. Harumi Kasamatsu (UCLA) and diluted 1:40,000. Goat-anti-mouse (Sigma, A4416) and goat-anti-rabbit secondary antibodies (Cell Signaling) conjugated to HRP were
used at a 1:1000 dilution. Membrane was incubated with chemiluminesence reagents (Pierce) and exposed to Hyperfilm (Amersham Biosciences).

**DNA extractions**

For total genomic DNA, cells were plated in 100 mm culture dishes at equal densities. When cells reached 80% confluence, DNA was collected using the DNeasy Tissue Kit (Qiagen). The concentration of DNA was determined by reading absorbance at 260 nm. Episomal low molecular weight DNA was collected by DNA extraction method of Hirt (Hirt, 1967). Confluent 60 mm plates of cells were trypsinized, cells were pelleted, washed with PBS, and then resuspended in 400 μl of 10 mM Tris, 10 mM EDTA, 0.6% SDS. One hundred microliters of 5 M NaCl was added to the solution and it was mixed and allowed to incubate overnight at 4 °C. The next day, the supernate was collected by centrifugation at 13,000 rpm for 10 min. Supernates were extracted twice with phenol and once with phenol:chloroform:isoamyl alcohol (25:24:1). DNA was ethanol precipitated and resuspended in sterile water.

**Southern blots**

An equal concentration or volume of genomic or Hirt extracted DNA was linearized overnight with EcoRI at 37 °C. Linear DNA was ethanol precipitated and resuspended in sterile water. Samples were run on a 0.8% agarose gel in 0.04 M Tris – acetate, 0.001 M EDTA (TAE) at 70 V for 4–5 h. Excess gel was removed and the remaining gel was washed for 20 min, twice, in denaturing solution (1.5 M NaCl, 0.5 M NaOH). The gel was rinsed with deionized water and then washed for 20 min. Twice, in neutralizing solution (1.5 M NaCl, 1 M Tris-HCl). Transfer of DNA to Nytran membrane (Pall) was performed overnight via capillary action.

The next day, the membrane was incubated in prehybridization solution (6× SSC, 5× Denhardt’s solution, 0.5% SDS, 50% deionized formamide) for 6 h to overnight at 42 °C. Radioactive probe was made with (γ 32P)-dCTP (Perkin Elmer) using the DecaPrime II kit (Ambion) using 35 ng of the 1.1-kilobase SV40 HindIII fragment as template. 1–2×10⁶ cpm of probe was added to the prehybridization solution and incubated with the membrane overnight at 42 °C.

The membrane was washed in 2× SSC containing 0.1% SDS for two, 30-minute incubations at 55 °C. Followed by two, 30-minute washes with 0.2× SSC containing 0.1% SDS at 55 °C before exposure to film for 1–21 days.

**Retroviruses and lentiviruses**

A retroviral vector that expressed ST cDNA was generated by cloning ST cDNA (Porras et al., 1996) into pBABE-Puro that lacked the SV40 origin of replication. The origin was inactivated by linearizing pBABE-Puro with SfiI then blunt-ending the DNA with T4 DNA polymerase prior to ligation.

The Lentivirus vector expressing shRNA for the late region of SV40 was prepared according to protocols described on the pLKO.1-TRC Cloning Vector website (http://www.addgene.org/pgvec17?f=c&cmd=showcol&colid=170&page=2) (Moffat et al., 2006). Oligos containing corresponding sequence in the VP2 region of the genome (596–615), plus the addition of an XbaI site to form a loop, and sequence to generate EcoRI and AgeI restriction enzyme overhangs were synthesized (IDT). The 5′→3′ primers used are listed below. Underlined nucleotides represent the restriction enzyme overhang sequences and non-capital letters represent the XbaI loop sequence. The following primers were used: AntiVp2-1A, 5′-CCGGTTGTCACTGTGTTCTGAAGCtctagaGCTTCAGACAGTACATTTT; AntiVp2-1B, 5′-AATTTAAAATGCTACTGTGTTCTGA-AGCtctagaGCTTCAGACAGTACAGCAAA. The oligos were annealed to each other, treated with T4 polynucleotide kinase and then ligated into the pLKO.1-puro lentiviral vector (kindly provided by W. Hahn, Harvard University, Dana Farber Cancer Institute) first digested with EcoRI and AgeI followed alkaline phosphatase treatment. The addition of a unique XbaI site was used to confirm the addition of the annealed oligos to the plasmid.

One day before transfection, gp293 cells (Clontech) were plated at a 1:5 split ratio into fresh 10 cm dishes. Cells were then transfected with 6 μg retroviral or lentiviral plasmid and 0.375 μg VSV-g plasmid with Lipofectamine Plus. Virus was collected 36–60 h post transfection and collected following passage through a sterile 0.25 μm filter.

Cells to be infected were plated at 10⁵ cells per 60 mm culture dish and infected at both 24 and 48 h after plating in the presence of polybrene (8 μg/ml). In some instances, cells were split and infected the next day for a third time. Cells were grown to confluence then subcultured for selection using 0.2 μg/ml puromycin or 30 μg/ml hygromycin. Drug-resistant colonies were either pooled or isolated with cloning cylinders and expanded in culture.

**Detection of ST in 5AWT and 5AWT/si cells**

To detect ST, 4×10⁵ cells were plated and then labeled overnight with 100 μCi of Trans35S-Label (MP Biomedical) in DME containing 1/17th the normal concentration of methionine plus 1% serum. The cells were washed with PBS and then lysed with 400 μl cold 0.5% NP40 buffer (20 mM Tris pH 8.0, 100 mM NaCl). Lysates were cleared of nuclear debris and equal cpm (counts per minute) were immunoprecipitated from each sample using monoclonal antibody pAB419 and heat inactivated and formalin fixed S. aureus cells (SAC) as described previously (Boyapati et al., 2003). Immunoprecipitated proteins were separated using a 20% Tris–sulfate gel (Rundell et al., 1977) and visualized by autoradiography.

**Plaque assay**

Virus from the mesothelial cells was prepared by freeze–thawing equal numbers of cells in fresh DME four times and then clearing the medium of all cellular debris by centrifugation. CV1 African green monkey kidney cells were grown to confluence for 3.5 cm petri dishes and infected for 2 h with different
dilutions of the viral preparations. After infection, the CV1 cells were overlaid with medium containing 1% agar for 12–14 days. The cells were then fixed with methanol and stained with 0.6% methylene blue.

**Growth in methylcellulose**

To assay for anchorage-independent growth, the bottoms of 6 cm petri dishes were first coated with 1% agarose. The coated plates were next filled with 8 ml of 1.8% methylcellulose, 10% serum in DMEM. 2.5 × 10⁶ cells were then added to each plate and colony formation was monitored after 2 to 3 weeks of incubation at 37 °C. Bright-field images were taken showing representative fields.

**Quantitative real-time polymerase chain reaction**

SV40 episomal DNA was isolated from 2.5 × 10⁶ trypsinized cells using a Genelute Plasmid Miniprep Kit (Sigma) and eluted in a final volume of 100 μl. Two microliters of this DNA was used as a template in a real-time PCR reaction with 2× SYBR Green JumpStart Taq ReadyMix (Sigma), ROX passive reference dye and 50 pmol forward and reverse primers in a total volume of 20 μl. SV.for3 and SV.rev primers (Bergsagel et al., 1992), specific to the RB binding region of T antigen, gave an amplicon of 105 bp. Reactions were run on an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems) for 40 cycles (95 °C for 15 s, 60 °C for 1 min) after an initial 2 min at 50 °C and 10 min at 95 °C. All reactions were done in triplicate and results reported as an average. A dissociation curve analysis was performed to ensure specific priming, and negative controls containing no template consistently gave negligible results. Standard curves generated from serial dilutions of SV40 episomal DNA were used to quantify the relative amounts of template per reaction. Linear regression analyses for all standard curves gave r² > 0.98.

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