

Factors Influencing the Production of Recombinant SV40 Vectors

Maria Vera,¹ Jesus Prieto,¹ David S. Strayer,² and Puri Fortes^{1,*}

¹Laboratory of Vector Development, Division of Gene Therapy, Foundation for Applied Medical Research, School of Medicine, University of Navarra, Irunlarrea 1, 31008 Pamplona, Spain

²Department of Pathology and Cell Biology, Jefferson Medical College, 1020 Locust Street, Philadelphia, PA 19107, USA

*To whom correspondence and reprint requests should be addressed. Fax: 34 948 425700. E-mail: pfortes@unav.es.

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Most gene therapy approaches employ viral vectors for gene delivery. Ideally, these vectors should be produced at high titer and purity with well-established protocols. Standardized methods to measure the quality of the vectors produced are imperative, as are techniques that allow reproducible quantitation of viral titer. We devised a series of protocols that achieve high-titer production and reproducible purification and provide for quality control and titration of recombinant simian virus 40 vectors (rSV40s). rSV40s are good candidate vehicles for gene transfer: they are easily modified to be nonreplicative and they are nonimmunogenic. Further, they infect a wide variety of cells and allow long-term transgene expression. We report here these protocols to produce rSV40 vectors in high yields, describe their purification, and characterize viral stocks using quality control techniques that monitor the presence of wild-type SV40 revertants and defective interfering particles. Several methods for reproducible titration of rSV40 viruses have been compared. We believe that these techniques can be widely applied to obtain high concentrations of high-quality rSV40 viruses reproducibly.

Key Words: SV40, recombinant SV40 vectors, gene therapy, production, titration, DI particles, wtSV40 revertants

INTRODUCTION

Simian virus 40 (SV40) is an icosahedral nonenveloped polyomavirus with a double-stranded circular DNA of 5.2 kb [1,2]. Several properties make SV40 a good candidate to be used as a vector for gene therapy approaches: (i) it is easily modified to be nonreplicative ([3–5], references therein, and this report); (ii) it can be produced in large quantities [3,4]; (iii) it infects almost every cell type that has been tested, both dividing and quiescent [6–8]; (iv) it is not immunogenic [9,10]; (v) it allows long-term expression of the transgene [6,7,9–13]; (vi) its molecular biology is well studied; and (vii) the effects in humans of wild-type SV40 have been documented [14,15].

The advantages of SV40 as a gene therapy vector can be explained by the SV40 replicative cycle. SV40 binds the major histocompatibility complex class I (MHC I) at the cell surface [16]. MHC I is present on most cell types, explaining SV40's wide host range. Following virus entry into the cell, MHC I is shed, which may result in poor antigen presentation by SV40-infected cells [17]. The virus enters the cell via a caveolar pathway that delivers SV40 to a microtubular network that transports the virion to the endoplasmic reticulum

[17,18]. Since SV40 traverses nuclear pores, it can infect nondividing cells productively. The SV40 genome is released in the nucleus as a nucleosome-coated mini-chromosome that can be integrated randomly into the host genome [19,20].

SV40 uses the cell machinery for replication and transcription. The SV40 early promoter drives expression of one alternatively spliced gene that encodes the large T antigen (Tag) and the small t antigen (tag) [2]. The late promoter, on the opposite strand, controls expression of the structural proteins, VP1, VP2, and VP3 (Fig. 1A). Both promoters, together with the regulatory sequences, origin of replication, and packaging signals, are located within approximately 500 bases [2]. Several of their functions are controlled by Tag. Tag is essential for genome replication and for late promoter-driven transcription. It also binds and inactivates p53 and the retinoblastoma protein, thereby immortalizing cells in culture [21,22]. Tag is mainly a nuclear protein, but it is produced in excess and inserts into the cell membrane, where it is the major virus antigen [23]. The Tag gene is removed to generate recombinant SV40 viruses (rSV40) ([3,4] and this report). This renders rSV40 replication deficient, nononcogenic, and nonimmunogenic, because the major antigen is not

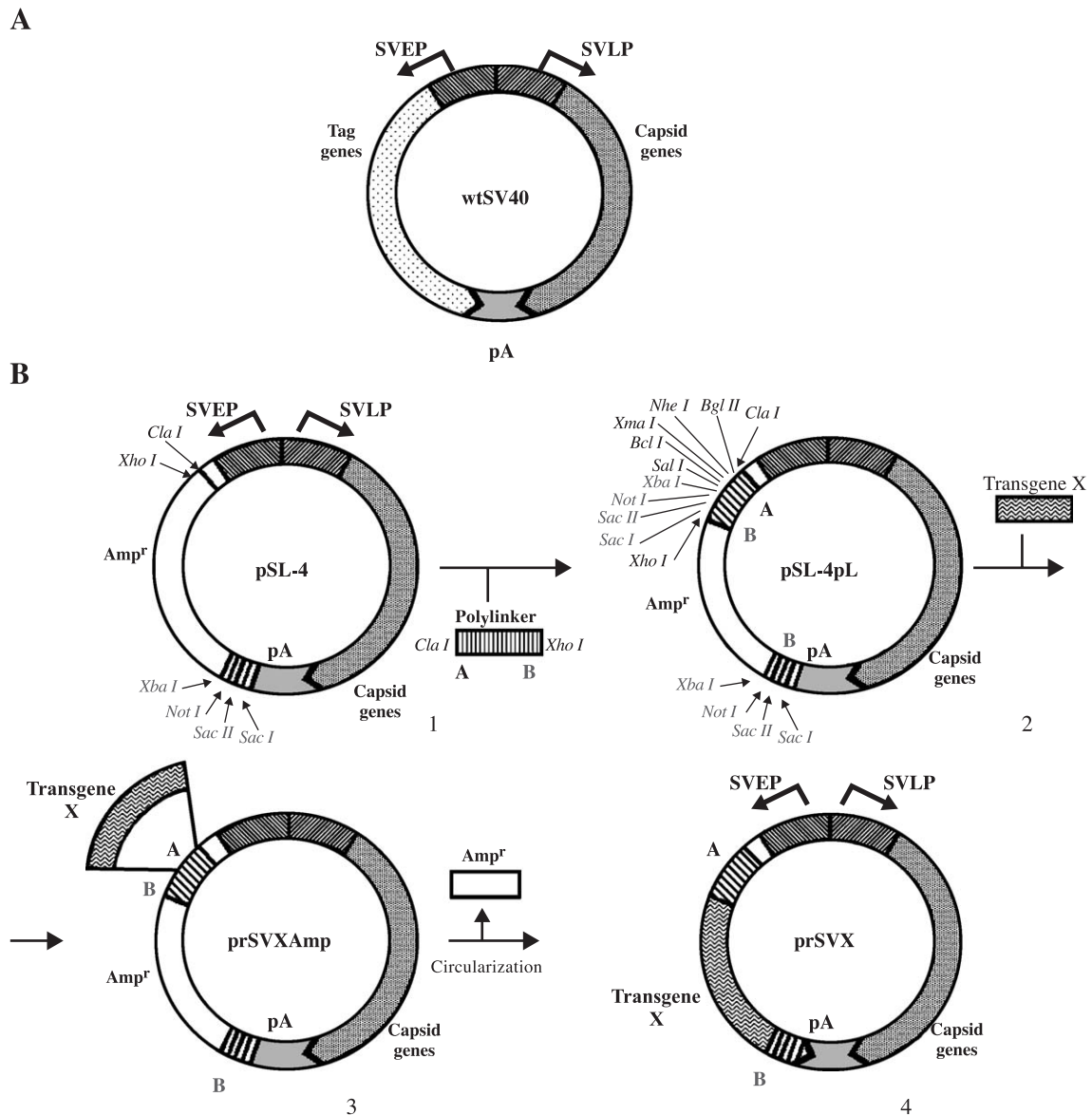


FIG. 1. Schematic view of wtSV40 genome and strategy to construct recombinant SV40 plasmids. (A) The wtSV40 genome is a 5.2-kb double-stranded DNA with an origin of replication that overlaps with the SV40 early promoter (SVEP) and a SV40 late promoter (SVLP). SVEP drives the expression of Tag genes that encode Tag and tag proteins. SVLP controls VP1, VP2, and VP3 protein expression (Capsid genes). Polyadenylation sequences are indicated (pA). (B) Generation of recombinant SV40 virus genomes. (1) Tag gene has been replaced by an ampicillin-resistance (Amp^r) gene, a bacterial origin of replication, and a small polylinker ($ClaI$ and $XhoI$) in pSL-4 [37]. (2) A longer polylinker has been introduced under the SVEP of pSL-4 to construct pSL-4pL. The polylinker has unique sites (sites A indicated in black: $ClaI$, $BglII$, $NheI$, $XmaI$, $BclI$, $SalI$, and $XhoI$) and sites that can be found at both sides of the Amp^r gene (sites B indicated in gray: $XbaI$, $NotI$, $SacII$, and $SacI$). (3) A transgene can be easily cloned with restriction sites A under the control of SVEP. (4) To produce recombinant SV40 genomes (prSVX), the Amp^r gene is removed with restriction sites B and the plasmid is circularized.

produced and capsid protein expression is not activated [2]. Removal of the Tag gene also generates approximately 2.5 kb of free space in the SV40 genome to clone transgenes. Removing the capsid genes creates approximately 2.5 kb of additional space. Thus, recombinant SV40 vectors can accommodate as much as 5 kb of inserted DNA [24].

The advantages of rSV40 as a gene therapy vector have encouraged several groups to study its efficacy in animal models. *Ex vivo* infection of hematopoietic stem cells with rSV40 has permitted expression of multidrug resistance gene 1, β -globin [8,25,26], and the surface antigen of hepatitis B virus [7,27]. rSV40 vectors have been used successfully in animal models for liver

diseases like Crigler–Najjar syndrome type 1 [10]. In addition, rSV40 has been employed for vaccination and immunostimulation purposes ([9,28], and our unpublished results or to block HIV replication using different strategies [27–35].

Despite the great potential of rSV40 in gene therapy protocols, few methods for rSV40 vector production, purification, quality control, and titering have been described. For example, published methods for wild-type SV40 production and purification need to be tested to see if they can be applied to rSV40 vectors [36]. Recently, methods to produce rSV40 vectors that do not analyze the factors influencing vector production have been proposed [3,4]. We have compared and modified several protocols to define a method that yields high titers of Tag-deleted nonreplicating rSV40 viruses. The presence of contaminating wild-type (wt) SV40 or defective interfering (DI) particles was analyzed to control for quality of vector preparations. We have also compared new or already described methods for rSV40 titering. We believe that these protocols could be widely used both in laboratories that already work with rSV40 vectors and in groups that are tempted to exploit the strengths of SV40 as a gene delivery vehicle.

RESULTS

Manipulation of the Viral Genome

Generation of recombinant SV40 viruses lacking the Tag gene is represented in Fig. 1B. We started with pSL-4, in which the Tag open reading frame has been replaced by the ampicillin-resistance gene and bacterial origin of replication (Amp^r ; Fig. 1B1) [37]. We first introduced a polylinker with seven unique restriction sites after the SV40 early promoter (EP) of pSL-4 and four restriction sites found at both sides of the Amp^r gene, to generate pSL-4pL (Fig. 1B2). The Amp^r gene, which is required only for bacterial selection, can thus be removed easily once the desired transgene has been inserted (Fig. 1B3). Also, the rSV40 genome can be efficiently circularized (Fig. 1B4). We used this strategy to produce several recombinant SV40 genomes. Luciferase and GFP DNAs were cloned into pSL-4pL to produce prSVLUC and prSVGFP as described under Materials and Methods. These plasmids were used to produce SVLUC and SVGFP recombinant viruses (rSVLUC and rSVGFP, respectively).

rSV40 Virus Production

Our recombinant SV40 genomes do not replicate in cells that lack Tag, since Tag is essential for virus genome replication and for transcription of capsid genes. Therefore we analyzed the ability of different Tag-expressing cell lines to produce rSV40s. The packaging cell lines used were COS-1, COS-7, CMT4, and COT2, which were all derived from CV-1 cells, and 293T, derived from 293

cells (see Materials and Methods for details). In all of the cell lines tested Tag expression is constitutive except for CMT4 and COT2, in which Tag is under the control of an inducible metallothionein promoter [5,39].

We tested different transfection methods to introduce rSV40 genomes into packaging cell lines: calcium phosphate precipitation and coupling DNA to lipids like Fugene and Lipofectamine. We used different amounts of prSVGFP to transfect subconfluent cells and 48 h after transfection monitored GFP expression by FACS and visualized it by fluorescence microscopy. In all cases, calcium phosphate precipitation gave greater than or equal to fourfold more GFP-expressing cells than did the other methods tested (data not shown). Efficiencies of transfection with calcium phosphate for the different packaging cell lines were comparable, except for 293T cells, which were transfected three- to fivefold more efficiently (data not shown). However, since 293T cells did not amplify recombinant viruses as well as the simian cell lines tested (see below), we used COS-1 cells to standardize rSV40 virus production.

To check whether prSVLUC and prSVGFP plasmids were able to produce rSV40 viruses, we transfected COS-1 cells with these plasmids. We collected media and cells 3, 5, or 7 days after transfection. We pooled both cells and media and subjected them to three cycles of freezing and thawing to break cell membranes and liberate the viruses. Then we used these lysates to infect CV-1 cells. Forty-eight hours postinfection we visualized GFP or lysed the cells to measure luciferase activity. Luciferase expression was higher in CV-1 cells infected with rSVLUC viruses collected on day 3 than on day 5 or 7 (data not shown). The rSVLUC viruses obtained were amplified by infection of fresh COS-1 cells. Again, we observed luciferase activity in CV-1 cells infected with rSVLUC viruses collected on day 3, 5, or 7 post-COS-1 infection (Fig. 2A). We observed the highest expression with day 3-collected viruses, which is in agreement with the time required for wtSV40 to complete an infectious cycle in tissue culture monkey cells.

We never observed GFP expression, however, when we performed the same experiments with prSVGFP. For reasons that are so far unknown, other groups working on rSV40 have also failed to detect GFP expression from rSVGFP viruses (A. Oppenheim, personal communication).

Wild-type SV40 infection is usually lytic in permissive cells. If cell lysis is also very efficient in producer cells infected with rSV40, most of the viruses should be found in the supernatant. However, viruses that have been released from lysed cells may also be attached to neighboring cell membranes. To see if infectious viruses can be found in both cell pellet and media, we collected these fractions 3 days after infection of COS-1 cells with rSVLUC. We used comparable amounts of both fractions to transduce CV-1 cells and measured luciferase activity

(Fig. 2B). Even if luciferase activity was higher in CV-1 cells infected with the fraction containing the cell pellet, both fractions yielded high levels of luciferase activity.

We also wanted to identify the most advantageous packaging cell line. We amplified viruses produced in COS-1 cells twice in COS-1, COS-7, CMT4, COT2, or 293T

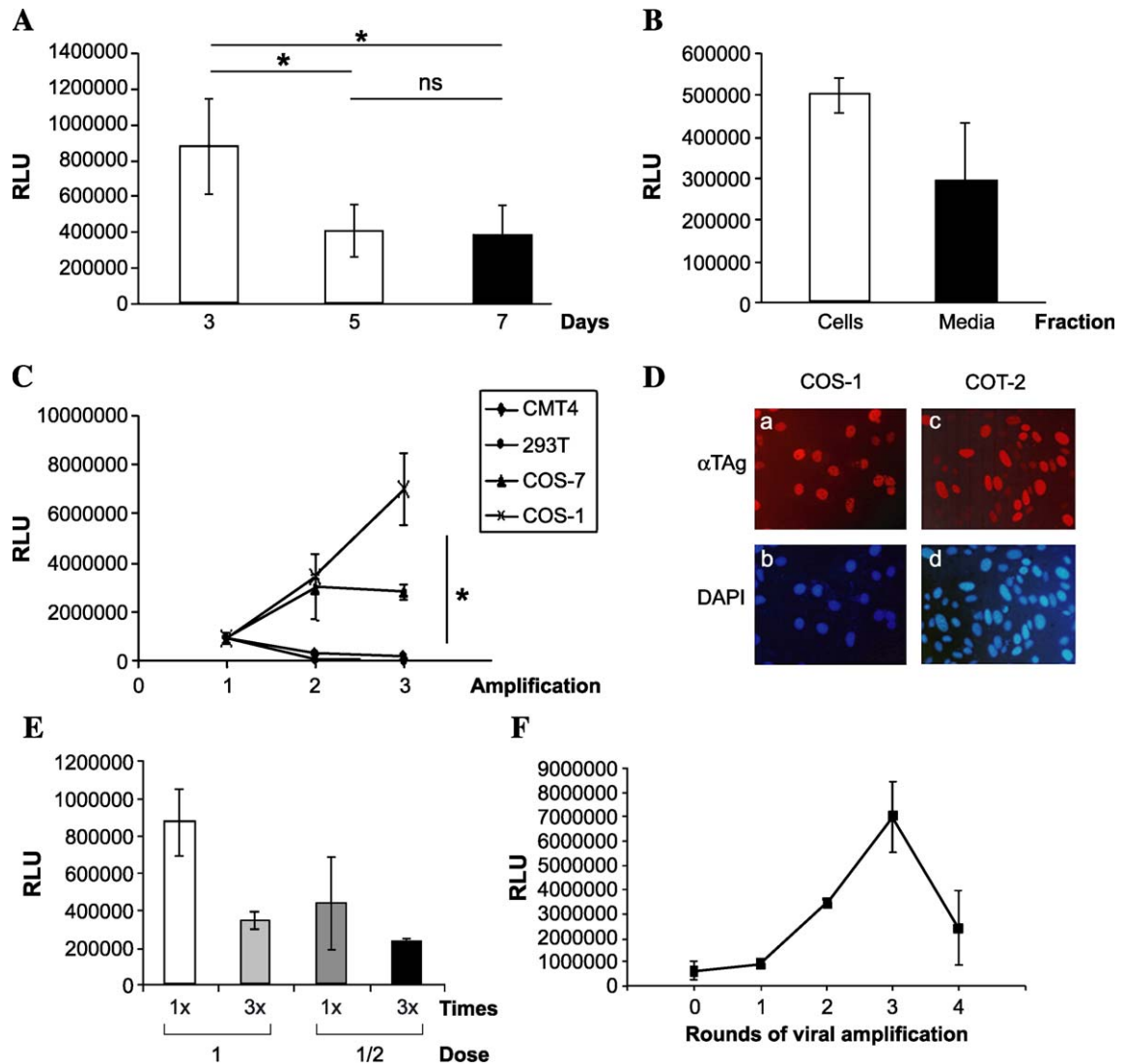


FIG. 2. Development of a method to produce rSV40 viruses. (A) Quantitation of luciferase-expressing rSVLUC viruses produced in COS-1 cells for 3, 5, or 7 days. rSVLUC viruses were used to infect COS-1 cells and recombinant virus amplification was allowed for 3, 5, or 7 days. Then, cells and supernatants were collected and lysed. The activity of recombinant viruses was quantified by infection of CV-1 cells in which luciferase activity was measured in relative luciferase units (RLU). Statistical analysis shown at the top of the graphic indicates significant (*) and nonsignificant (ns) differences. (B) Quantitation of luciferase-expressing rSVLUC viruses that accumulated 3 days after infection in COS-1 cells or supernatant. COS-1 cells were infected with rSVLUC. 3 days after infection the cellular pellet was separated from the supernatant fraction. Both fractions were lysed and comparable amounts were used to infect CV-1 cells in which recombinant viruses were titrated by measurement of luciferase activity. (C) Quantitation of luciferase-expressing rSVLUC viruses amplified in COS-1, COS-7, 293T, and CMT4 cells. rSVLUC viruses were amplified for two rounds in COS-1, COS-7, CMT4, and 293T cells. After each round of infection, viruses were titrated by infection of CV-1 cells in which luciferase activity was measured. The significant difference found between 293T and CMT4 cells and COS cells is indicated with an asterisk. (D) Tag expression of COS-1 and COT-2 cells. COT-2 cells were induced with heavy metals as described [39]. COT-2 cells (c, d) or uninduced COS-1 cells (a, b) were fixed and an immunofluorescence assay with anti-Tag antibody was carried out (a, c). Staining with DAPI of the same fields is shown (b, d). (E) Quantitation of luciferase-expressing rSVLUC viruses produced in COS-1 cells infected once or three times consecutively with two different viral doses. COS-1 cells were infected once or every 12 h three times with the regular amount or half the amount of rSVLUC virus. The viruses produced were titrated by infecting CV-1 cells in which luciferase activity was measured. (F) Quantitation of luciferase-expressing rSVLUC viruses produced after four rounds of amplification in COS-1 cells. Luciferase activity was measured in CV-1 cells infected with virus produced after each round of amplification. All the experiments were done in triplicate and repeated twice. Error bars indicate standard deviations.

cells using identical conditions. Amplification was done as described under Materials and Methods. After each round of viral amplification, we titrated viruses able to express the transgene by infection of CV-1 cells in which luciferase activity was measured. The results indicate that COT2 (data not shown), CMT4, and 293T cells are not as effective as COS cell lines in producing luciferase-expressing rSV40 vectors (Fig. 2C). Also, even though COS-1 and COS-7 amplified rSVLUC viruses to similar extents in a first round of infection, COS-1 cells produced more luciferase-expressing virus in a second round of amplification (Fig. 2C). Therefore we used COS-1 cells for rSV40 virus production.

As Tag is required for virus replication, we compared Tag expression in the different packaging cell lines. While all COS cells expressed Tag to a similar extent, heavy metal-induced COT2 cells expressed different levels of Tag (compare a and c in Fig. 2D). Also, some COT2 cells showed undetectable levels of Tag (compare c and d in Fig. 2D). Heavy metal-induced CMT4 and COT2 cells produced similar amounts of Tag, as determined by immunofluorescence analysis (data not shown).

We compared rSVLUC production by infection of COS-1 cells, once or three times consecutively. We used the viruses produced to infect CV-1 cells in which luciferase activity was measured. The results indicate that the highest virus production in terms of luciferase activity was obtained with a single infection (Fig. 2E). By infecting with half the amount of virus, we reduced detected luciferase activity also by half (Fig. 2E). Increasing the amount of virus used to infect did not significantly increase the amount of luciferase-expressing vector produced (data not shown). Surprisingly, when cells were infected three times consecutively, luciferase activity in CV-1-infected cells decreased.

Finally, we wanted to know how many rounds of virus amplification could be done without altering rSV40 yields. We carried out four rounds of amplification in COS-1 cells. After each round, luciferase activity was measured in rSVLUC-infected CV-1 cells. We found that virus could be amplified for up to three rounds, as the products of the fourth round gave much less luciferase activity (Fig. 2F).

After the third round of infection viruses were purified by sucrose gradient ultracentrifugation as described [3,4,36]. We have tested several modifications of the protocol but could improve virus yield only when we used a Dounce homogenizer to break cell membranes. This increased 1.5 times the amount of luciferase produced in rSVLUC-infected CV-1 cells (data not shown).

rSV40 Titration and Quality Controls

Measurement of luciferase activity in rSVLUC-infected CV-1 cells is an easy way to quantitate luciferase-expressing rSVLUC viruses. To determine the titer of rSVLUC stocks, we infected COS-1 cells with serial 1:3 dilutions of rSVLUC and measured luciferase expression 48 h post-infection. We used the higher dilution that yielded luciferase activity to calculate virus titer as described [5], resulting in $1.2 \times 10^9 \pm 1.4 \times 10^8$ transducing units/ml (Fig. 3A).

Measurement of luciferase activity, however, cannot be applied to titrate vectors carrying other transgenes. So, we compared two techniques to titrate rSV40 viruses: *in situ* PCR, a method already described [3,4,36,43], and real-time quantitative PCR. While *in situ* PCR counts infective particles, quantitative PCR measures the number of rSV40 genomes. Titering of purified stocks of rSVLUC by *in situ* PCR after three rounds of amplification (rSVLUCp3) is shown in Fig. 3B. We obtained titers from 1×10^{10} to 1×10^{11} infectious units (iu)/ml in three different stocks. For quantitative PCR we used a pair of primers that amplify the region immediately after the SV40 late promoter, so they can be used to titer different rSV40 vectors (see Material and Methods). Using this approach, we titered six different stocks of rSVLUCp3 and obtained from 1.0×10^{10} to 1.2×10^{11} viral genomes/ml (Fig. 3C and Table 1). We obtained similar titering results with primers that amplified the luciferase gene (Fig. 3D and Table 1). The presence of viral genomes lacking the regions amplified by the two pairs of PCR primers used cannot be excluded.

DI particles may be formed by replication or recombination defects and amplified to high titers due to their advantages in replication compared to full-length viral genomes. If partial rSV40 genomes are of an appropriate size they may be encapsidated directly. Alternatively, they may be packaged together

FIG. 3. Titration of rSVLUC viruses. (A) Titration by luciferase activity measurement. Luciferase activity was measured 48 h after infection of COS-1 cells with 1:3 serial dilutions of rSVLUCp3. Titer was calculated with the highest dilution that showed luciferase activity as described [5]. (B) Titration of infective units by *in situ* PCR. CV-1 cells were mock infected (MOCK, b and d) or infected with 10-fold serial dilutions of a rSVLUCp3 viral stock. *In situ* PCR was carried out and amplified viral DNA was hybridized with a biotinylated probe and developed with streptavidin-peroxidase. Two different magnifications of a single dilution are shown (10^{-5} , a and c) as indicated at the left. The quantitation of the result indicates that the different rSVLUCp3 viral stocks tested have a titer of $5.5 \times 10^{10} \pm 4.5 \times 10^{10}$ infective units/ml as indicated at the top. (C and D) Titration of viral particles by quantitative PCR. Primers that amplify the SV40 late region (C) or the luciferase transgene (D) were used. Standards are 10-fold serial dilutions of prSVLUC as indicated at the bottom of the graphic. Amplification values of each of the standards are connected with a line. Amplification value of 2 μ l of water is shown with a triangle (C) or was not detected and therefore is not indicated (D). Amplification of 2 μ l of each viral stock is shown. The amplification values of viral stocks and water can be obtained by comparison with the standards and are indicated as particles/ml in Table 1. The amplifications were done more than three times and the averages of several stocks are indicated with standard deviations.

with full-length recombinant viruses [44]. Recombinant virus amplification is decreased in the presence of DI particles as they compete for the replication machinery.

Thus, the presence of DI particles could explain the decrease in luciferase activity produced in CV-1 cells infected with a rSVLUC that has been amplified

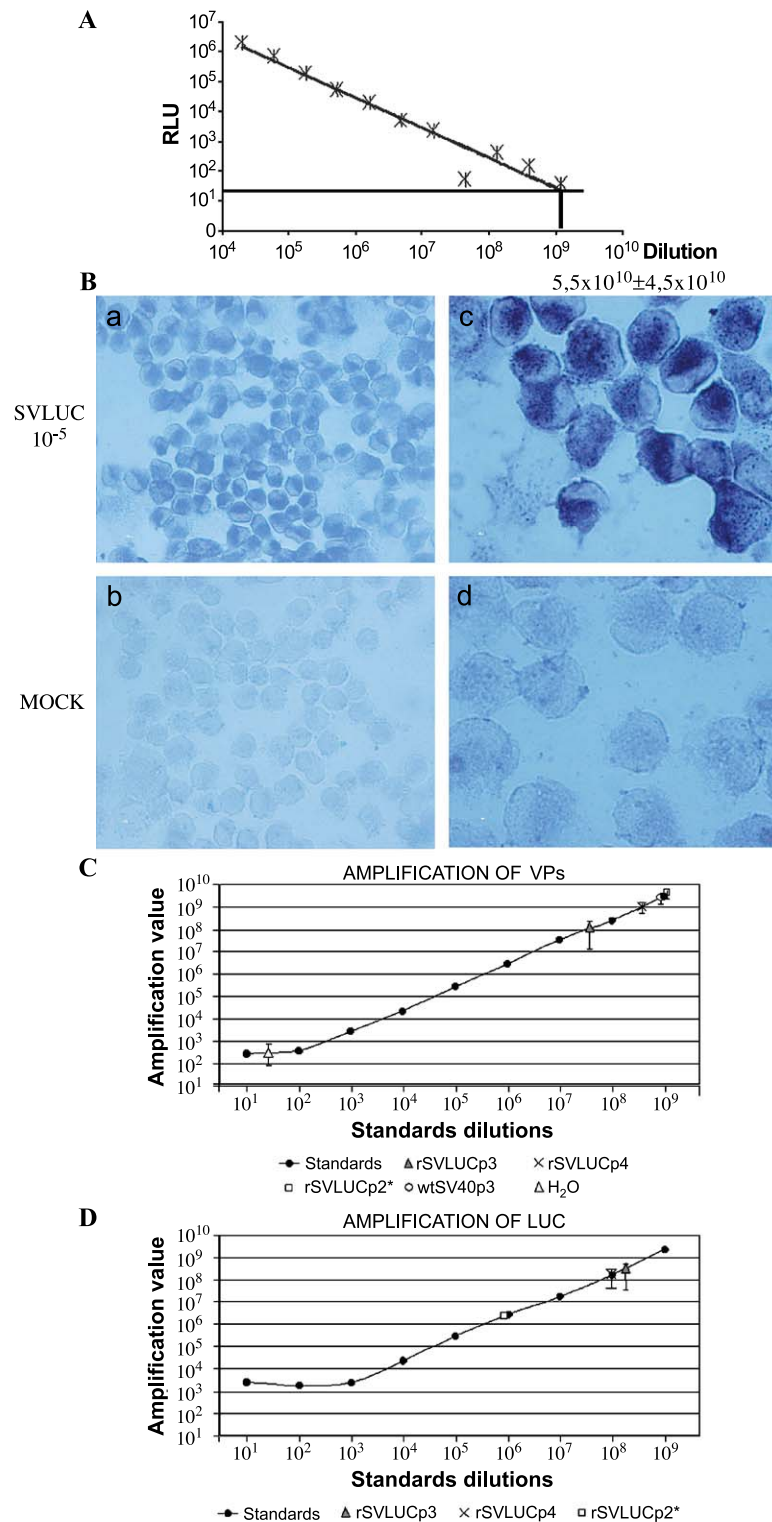


Table 1: Comparison of viral stocks titrated by quantitative PCR and luciferase activity in CV-1 cells

	VPs part/ml	LUC part/ml	LUC activity
rSVLUCp3	$6.30 \times 10^{10} + 5.63 \times 10^{10}$	$1.93 \times 10^{11} + 1.81 \times 10^{11}$	$6.99 \times 10^6 + 1.45 \times 10^6$
rSVLUCp4	$7.01 \times 10^{11} \pm 1.22 \times 10^{11}$	$9.97 \times 10^{10} \pm 6.54 \times 10^{10}$	$2.41 \times 10^6 \pm 1.55 \times 10^6$
rSVLUCp2*	$2.12 \times 10^{12} \pm 9.40 \times 10^{11}$	$1.05 \times 10^9 \pm 1.06 \times 10^8$	$8.14 \times 10^5 \pm 3.30 \times 10^5$
wtSV40p3	$1.28 \times 10^{12} \pm 5.98 \times 10^{11}$	Non detected	Non determined

Viral DNA isolated from rSVLUCp3, rSVLUCp4, rSVLUCp2*, and wtSV40 stocks was subjected to quantitative PCR using primers that amplified the SV40 late region (VPs) or the luciferase transgene (LUC). The amplifications were done more than three times with each of six, four, and two different stocks of rSVLUCp3, rSVLUCp4, and rSVLUCp2*, respectively. Averages of the results are indicated with standard deviations. Values were corrected to viral particles/ml and are plotted as a graphic. The activity of recombinant viruses was quantified by infection of CV-1 cells in which luciferase activity was measured. Measurements were done in triplicate and repeated more than twice for each stock.

four times (rSVLUCp4) compared to cells infected by rSVLUCp3 (see Fig. 2E). We analyzed DI particle contamination by Southern blot. Viral DNA isolated from four different stocks of rSVLUCp3 or seven different stocks of rSVLUCp4 was hybridized with labeled probes containing the sequences of SV40 ori-VP1 (Fig. 4) or luciferase (data not shown). DI particles that carry the SV40 ori or SV40 late region (VPs) but not the luciferase gene were initially detected in the third round of viral amplification (lanes 4 to 7) and increased significantly in the fourth round of amplification (compare lanes 4–7 with lanes 8 and 10–15). Quantitation of the Southern blot indicates that the DI particles represent $2.3 \pm 2.8\%$ of passage 3 rSVLUC stocks and $36.2 \pm 8.9\%$ of passage 4 rSVLUC preparations. Surprisingly, DI particles were not detected in passage 3 wtSV40 stocks (lane 9). The presence of DI particles lacking the sequences used as probes (SV40 ori-VP1 and luciferase) or whose difference in size is not detected by Southern blot cannot be excluded.

The quality of a recombinant virus stock can be measured by the amount of infectious units/viral genomes or the amount of DI particles and also by the amount of particles containing recombinant wtSV40 sequences (rwtSV40) that may contaminate the rSV40 viral stocks. Recombinant wtSV40 can be formed by homologous recombination between the rSV40 DNA and the COS-1 cell genome in which a full-length SV40 virus mutated in the origin of replication has been integrated [45]. We analyzed the presence of rwtSV40 DNA by Southern blotting of viral DNA from rSVLUC. We used wtSV40 DNA as positive control. The Southern blot was hybridized with labeled probes containing the

sequences of SV40 ori-VP1 (Fig. 4), luciferase (data not shown), or Tag (Fig. 5A). However, rwtSV40 was not detected in rSVLUC stocks (compare Figs. 4 and 5A, lanes 4–8). To increase the sensitivity of detection we attempted to amplify Tag from rSVLUC DNA using diluted wtSV40 DNA as a positive control. Again, we did not detect rwtSV40 viral DNA in rSVLUC stocks (Fig. 5B, compare lane 5 to lane 4). We also analyzed the presence of rwtSV40 by quantitative PCR using Tag oligonucleotides. The level of rwtSV40 contamination in passage 3 rSVLUC stocks was below the limit of detection (data not shown). Finally, rSVLUCp4 stocks or viral stocks obtained in two rounds of amplification maintained for 7 days each in COS-7 cells (rSVLUCp2*) were contaminated with replication-competent rwtSV40 viruses, as they could be amplified in CV-1 cells (data not shown). However, rwtSV40 viruses were not detected after four amplifications of 3×10^8 iu of rSVLUCp3 in CV-1 cells infected for longer than 1 week. As this technique was sensitive enough to detect a single wtSV40, we conclude that contamination is lower than 1 rwtSV40 per 3×10^8 infectious units of rSVLUCp3.

DISCUSSION

We devised protocols to produce and purify large amounts of high-quality recombinant SV40 vectors (Fig. 6). Our starting plasmid, pSL-4pL, carries a wtSV40 genome lacking Tag, but with early promoter and polyadenylation signals intact. As effective encapsidation is possible for SV40 genomes ≤ 5.7 kb, insert sizes for these vectors should be smaller than 3.0 kb [24]. Additional modifications can be made to increase rSV40 insert

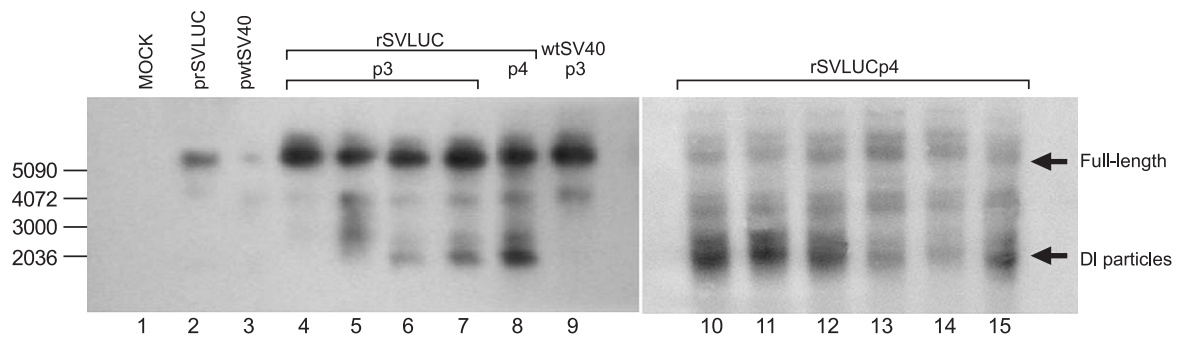


FIG. 4. Detection of DI particles. Southern blot of viral DNA hybridized with a SV40 ori-VP1 probe. DNAs tested were salmon sperm DNA (MOCK), plasmids prSVLUC and pwtSV40, and viral genomes rSVLUCp3, rSVLUCp4, and wtSV40p3 as indicated at the top. The size of molecular weight markers is indicated on the left. Arrows on the right indicate the positions of full-length viruses and DI particles.

capacity. The SV40 capsid genes may be deleted, since expression of these genes can be provided *in trans* by COS cells ([24] and data not shown). Thus, inserts up to 5 kb may be accommodated.

Once the transgene has been cloned, the ampicillin-resistance gene is deleted and the producer plasmid is circularized to transfect COS-1 cells. Transfection does not seem to be a limitation to produce high titers of rSV40 viruses: the virus stocks produced by transfection are expanded by infecting COS-1 cells for up to three rounds of amplification (Fig. 6). This means that a single transfection of 6 μ g of plasmid could produce up to 10 L of viruses, as every round of viral amplification produces a 10-fold increase in the volume of the viral mix.

We do not know why COS-1 cells were the most effective packaging cells in these studies. All the packaging cells tested are from SV40-susceptible monkey kidney origin (CV-1) except the 293T cells. Also, all cell lines but COT2 and CMT4 expressed similar levels of Tag (Fig. 2D and data not shown). It was reported

that COS-1 and COS-7 cells respectively bear 1 and 5 to 7 wtSV40 genomes, mutated at the origin of replication [38,45]. However, there may be considerable variation in these cell lines, depending on their origin. Thus, multiply passaged COS-7 cells from ATCC were shown to have only 1–2 copies of integrated wtSV40 DNA, while EACC-derived COS-7 had 3–4 copies of wtSV40 DNA, which increased to up to 12 copies upon passage (data not shown). It has been reported that such COS-7 cells have a higher probability than COS-1 cells of homologous recombination that produces rwtSV40 viruses [45]. These may contaminate viral stocks packaged in COS-7 cells so that recombinant viruses cannot be productively amplified. Using PCR, and testing for growth in CV-1 cells, we have observed heavy rwtSV40 contamination in rSVLUC viral stocks grown for 7 days and amplified twice in COS-7 cells (data not shown). These data differ from those of some other investigators using COS-7 cells from other sources ([24]; D. S. Strayer, unpublished data; L. Couture, personal communication).

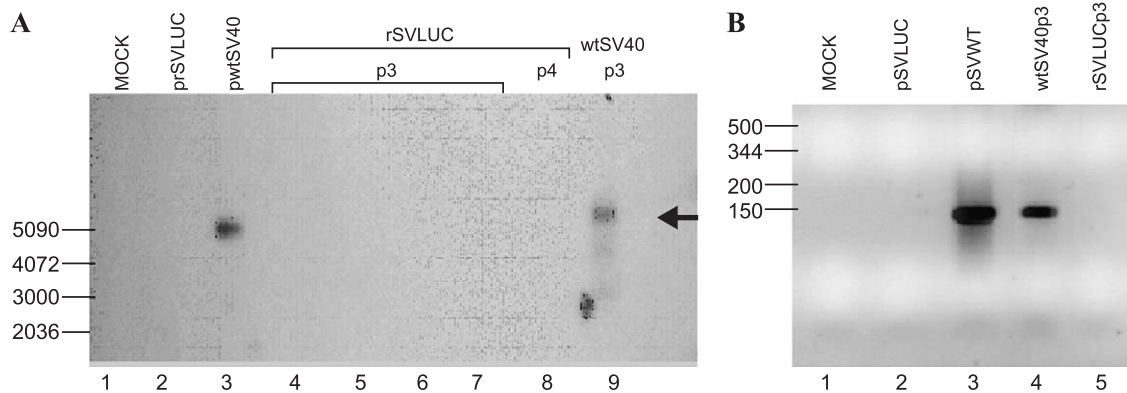


FIG. 5. Detection of recombinant wtSV40. (A) Southern blot of viral DNA hybridized with a Tag probe. Samples are as in Fig. 4. The sizes of molecular weight markers are indicated on the left. The arrow on the right indicates the position of wtSV40 viral DNA. (B) PCR of viral DNA to amplify Tag. DNAs tested were salmon sperm DNA (MOCK), plasmids prSVLUC and pwtSV40, and viral genomes rSVLUCp3 and wtSV40p3 as indicated. The sizes of molecular weight markers are indicated on the left. Tag amplified sequences are seen at approx. 150 pb.

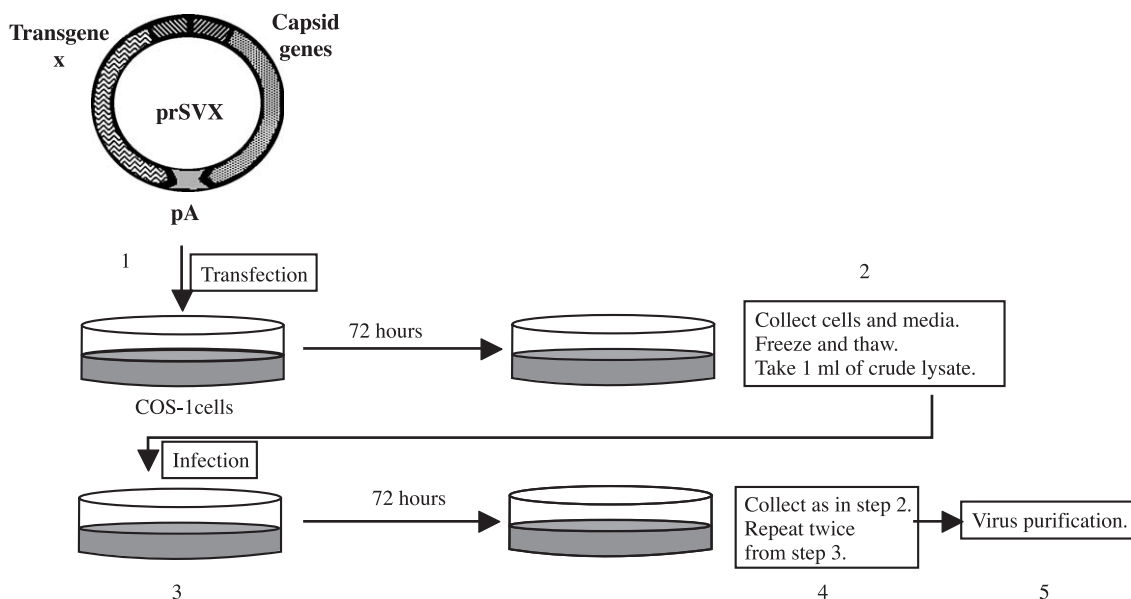


FIG. 6. Method for rSV40 vector production. (1) prSV40 is transfected into COS-1 cells by calcium phosphate precipitation. (2) Three days after transfection cells and supernatant are collected and frozen and thawed three times. (3) 1 ml of the virus mix is used to infect fresh COS-1 cells. (4) Three days after infection viruses are collected as in step 2 and a new round of infection is done as before. (5) After a final round of infection is done, viruses are collected and purified.

Similarly, our data for COS-1 cells are divergent from studies reported by some other groups [45]. A ratio of 1 rwtSV40/1000 rSV40 viruses was noted after three rounds of amplification in COS-1 cells [5]. However, we did not detect rwtSV40 until four rounds of amplification in COS-1 cells. We believe that these differences may reflect the short time (3 days) we allowed viruses to replicate, compared to other protocols (5, 7, or even 14 days). We found that such longer incubations did not increase virus titer (see Fig. 2B), but they may increase the chances for homologous recombination to generate wtSV40.

rSVLUCp4 viral stocks were less effective than stocks from earlier passages. This may be because high-passage stocks had rwtSV40 and DI particles, which may have decreased effective luciferase expression (Figs. 2F and 4 and Table 1). Also, packaging cells may progressively inactivate a transgene, e.g., by methylation. It may be for this reason that we and others have been unable to produce rSV40s expressing GFP (this work, [23], and Arad and Oppenheim, personal communication) or thymidine kinase (data not shown) from packaging cell lines. *In vitro* packaging appears to be the only way to produce GFP-expressing rSVGFP [26].

Formation of DI particles when producing rSV40 viruses has important implications. Several groups have designed Tag-expressing cell lines with a decreased risk of homologous recombination to produce rwtSV40 particles ([5] and D. S. Strayer, unpublished), but DI particles may continue to be made in these settings. Purification

protocols that can separate full-length rSV40 viruses from DI viruses may help address this problem. It is also of note that we did not detect DI particles in wtSV40p3 viruses, suggesting that the multiplicities of infection that we are using for viral production are not a direct cause of DI particle formation.

We found that rSV40 vector stocks produced according to our protocols should not be amplified more than three times, to maximize expression and to minimize the formation of DI particles or wtSV40 revertants (Figs. 2F, 4, and 5). Vector titers achieved were on the order of 10^{11} /viral particles/ml. Over 10 other rSV40s produced in the laboratory gave titers and quality similar to those of rSVLUCp3, indicating that luciferase DNA or expression does not affect viral production (data not shown and M.V. *et al.*, manuscript in preparation).

There may not be an ideal method for titrating rSV40 vectors. *In situ* PCR is the method used so far for titration of rSV40 infective particles, but the specificity of this approach for quantitating vector genomes is a function of the specificities of the PCR primers used. Therefore we tested real-time quantitative PCR, using two sets of primers, one specific for VP sequences and another that amplified transgene sequences. Combined, these analyses can provide information about the quality of the particles: similar results with VP primers and LUC primers allow accurate quantitation of rSVLUCp3 particles. If the two primer sets give different numbers, as for rSVLUCp2*, lower quality stocks are likely.

Similar results were obtained by *in situ* PCR and by quantitative PCR or when quantitative PCR was used to quantify rSVLUC genomes in infected cells (data not shown). The last technique was used after cell fractionation in nucleus and cytoplasm to reveal that 24 h after infection 70% of rSVLUC genomes are in the nuclear fraction (data not shown). However, not all rSVLUC genomes express the transgene. If a direct comparison can be made, of the 1.0×10^{10} to 1.2×10^{11} infective genomes only $1.2 \times 10^9 \pm 1.4 \times 10^8$ express luciferase as titered by measurement of luciferase activity. Silencing of the transgene, e.g., by methylation, could explain this phenomenon.

Protocols for rSV40 production and titration described here offer methods for preparation of high-titer, good-quality, nonreplicative rSV40 viruses. We have found these vectors to be very efficient in different cell types *in vitro* and *in vivo* (data not shown). Our methods may help laboratories working with rSV40s and encourage new laboratories to try this promising vector system.

MATERIALS AND METHODS

Cloning procedures. pSL-4 plasmid (kindly provided by S. de la Luna) contains SV40 sequences in which the large T antigen gene was replaced by the ampicillin-resistance gene and a small polylinker [37]. We first wanted to increase the number of restriction sites in the polylinker to facilitate easy cloning of transgenes and simple removal of the Amp^r gene. To that end, we introduced two hybridized oligos with polylinker sequences at pSL-4 sites *Clal* and *XhoI*, located between the SV40 EP and the 5' end of the Amp^r gene, to generate pSL-4pL (Fig. 1B1). The polylinker contains the unique sites *Clal*, *BglII*, *NheI*, *XmaI*, *BclI*, *SalI*, and *XhoI* close to the EP sequences and sites *XbaI*, *NotI*, *SacII*, and *SacI*, which are also found at the other end of the Amp^r gene (Fig. 1B2). Positive clones were selected and the polylinker was verified by sequencing (ABI Prism 310 genetic analyzer from Perkin-Elmer).

To generate a recombinant SV40 virus with the luciferase transgene (rSVLUC), firefly luciferase gene was extracted with *StuI* and *SalI* from pGL3 plasmid (Promega) and cloned into the same restriction sites of pSL-4pL. To generate a recombinant SV40 virus with GFP (rSVGFP), pEGFP-N1 (Clontech) was digested using *SalI* and *NotI*, and the GFP gene was cloned at the same restriction sites of pSL-4pL. The resulting plasmids, named prSVLUC and prSVGFP, respectively, were verified by restriction analysis.

Cell lines. COS-1 and COS-7 (ECACC) [38] cell lines derive from the monkey kidney fibroblast CV-1 cell line by integration of mutated SV40 genomes. CMT4 [39] and COT2 cells (kindly donated by A. Oppenheim) are Tag-expressing cells derived from CV-1 cells in which expression of Tag is under the control of the heavy-metal-inducible murine metallothionein promoter [5]. The human embryonic kidney fibroblast cell line 293T cells (donated by I. Narvaiza) constitutively express Tag. All cell lines were grown in culture using Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL/Life Technologies) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL/Life Technologies), penicillin-streptomycin, and glutamine as recommended. COS-1, COS-7, 293T, CMT4, and COT2 cells were used to expand rSV40 viruses because of their ability to express Tag.

Virus production. Transfections were done by calcium phosphate precipitation, Fugene (Roche), or Lipofectamine (Invitrogen) as recommended by the suppliers. Six micrograms of the rSV40 plasmid were used to transfect 3.5×10^6 cells previously washed with DMEM 2% FBS.

Seventy-two hours after transfection, both cells and media were subjected to three cycles of freezing and thawing to break cell membranes and liberate the virus. One milliliter of this lysate was then used to transduce fresh cells. Cells (3.5×10^6) were washed twice with PBS (Gibco BRL/Life Technologies) and incubated with 1 ml of the lysate for 2 h at 37°C. Then, the lysate was removed and cells were grown in DMEM 2% FBS for 72 h, when the cells and supernatant were harvested. This virus amplification cycle was repeated twice using identical conditions.

Virus purification. rSV40 viruses were purified as described [3,4,36]. Briefly, 3.5×10^6 infected cells were harvested in a total volume of 10 ml of cell debris and media. One milliliter of 10% Triton X-100 and 5% sodium deoxycholate was added to dissociate the virus from cell membranes, and a Dounce homogenizer was used to disaggregate cell membranes. Cell debris was removed by centrifugation at 16,000g for 20 min. The virus present in the supernatant was concentrated in a discontinuous sucrose gradient (1.5 ml of 75% sucrose and 2.5 ml of 20% sucrose) by centrifugation at 23,000 rpm for 3.5 h in an SW28.1 rotor (Beckman). Eight fractions of 0.5 ml were collected by piercing the bottom of the tube. Fractions 4 to 6 were pooled and dialyzed against PBS overnight at 4°C and using sterile conditions.

Viral DNA analysis. To isolate viral DNA, 800 µl of a standard stock of purified virus was incubated in 0.01% SDS, 25 mM EDTA at pH 8.0 and 0.84 mg/ml of proteinase K (Roche) for 3 h at 37°C. After phenol extraction, viral DNA was precipitated with ethanol.

Titration of rSVLUC was done by three different methods: *in situ* PCR, quantitative PCR, and luciferase activity measurement. *In situ* PCR was used to titrate rSVLUC infection units as described [3,4]. Briefly, 5×10^5 CV-1 cells were transduced or mock transduced with 10-fold serial dilutions of the virus stock. Twenty-four hours after transduction cells were collected, bound to slides, and fixed in paraformaldehyde. After proteinase K treatment, *in situ* PCR was done using primers that hybridize specifically with the SV40 late promoter region: SV40 4.1 (5'-ACACCTGTTGCTGACTAAT-3') and SV40 4.2 (5'-CAG-TATCTTCCCCTTCACAAA-3'). The PCR product was denatured and hybridized to a biotinylated DNA probe (5'-AAGTACACCATTCACAGCTGGTCTTTCCGCCTCAGAA-3'). *In situ* PCR was developed with a streptavidin-peroxidase solution.

Titration of rSV40 particles was done by quantitative PCR [40]. Two different pairs of primers and probes were used. To amplify the SV40 late region, the primers used were SV394S (5'-GGTTATTTGAGGCCATGGTG-3') and SV460AS (5'-GATGACCTACGAACCTTAAC-3'), and the SV probe (5'-TAACTGACACACATTCCACAGCTGGTCTTTCCGCCTCAGA-3'). To amplify the luciferase transgene we used primers LUCIF (5'-AACATAAAGAAAGCCCGGC-3') and LUC1R (5'-GCCTATGCGAGTTGCTCTCCA-3') and the probe SLUC (5'-CATTCTATCCGCTGGAAGATGGAACCG-3'). The quantitative PCR was done with 10-fold serial dilutions of the prSVLUC plasmid as standards, 2 µl of distilled water as a negative control, and 2 µl of each viral DNA. The PCR was done following the instructions of the manufacturer (Light Cycler; Roche). The following conditions were used: 95°C for 10 min and 40 cycles at 95°C for 10 s, 40°C for 1 min, and 60°C for 20 s.

PCRs were performed to test for the presence of recombinant wild-type SV40 in rSVLUC viral stocks. A pair of primers was used to amplify 100 bp between the SV40 EP and the Tag (SVEP, 5'-CTCGGCCTCTGAGC-TATTCC-3', and TAG, 5'-CCCCAGGCACTCCTTTC-3'). The wtSV40 genome (kindly provided by J. Ortin) was used as a positive control and viral DNA from rSVLUC was assayed. The following conditions were used: 94°C for 2 min and 27 cycles at 94°C for 30 s, 60°C for 1 min, and 72°C for 15 s and a final extension at 72°C for 6 min.

The presence of rwtSV40 in rSVLUC viral stocks was also tested by quantitative PCR. Conditions used were the same as described above. Tag was amplified with primers Tag3944S (5'-ACATCCCAAGCAATAACAA-CACA-3') and Tag4028AS (5'-GGAACTAAACAAGTGCTCTGGAAG-3') and quantified with the probe TTAQ (5'-CATCACATTTGTTTCCATTG-CATACTC-3').

Southern blots [41] were done to test for the presence of defective SV40 particles and rwtSV40 in rSVLUC viral stocks. The blots were hybridized with an SV40 origin of replication and VP1 probe (SV40ori-VP1) a Tag and/or a luciferase probe. SVori-VP1 DNA was obtained by *Clai* and *Bam*HI digestion of prSVLUC, Tag by PCR of wtSV40 viral DNA with Tag4228S (GGAGCAGTGGTGAATGC) and Tag3640AS (GTGTAGC-CAAGCAACTCCA), and luciferase by *Stu*I and *Sal*I digestion of pGL3 plasmid. Probes were labeled with [³²P]dCTP with a random-primed DNA labeling kit (Roche). Prehybridization was performed at 60°C for 2 h in 5 mM EDTA, 0.75 M NaCl₂, 0.5% SDS, 50 mM Tris, and 200 µg/ml tRNA and the membrane was then hybridized to the probe for 14 h at 60°C. Finally, the membrane was washed three times with 2× SSC and 0.1% SDS at 60°C and radioactivity bound to the membrane was visualized by autoradiography (Hyperfilm; Kodak) and phosphorimager analysis (Cyclone; Perkin-Elmer).

Protein analysis. GFP expression was analyzed 48 h after transfection or infection of CV-1 or COS cells with prSVGFP or rSVGFP, respectively. Cells were washed and fixed with 4% paraformaldehyde in CSK buffer as described [42]. Cells were mounted and fluorescence was observed in a microscope (Nikon) or by FACS (Becton-Dickinson). Immunofluorescence assays to detect Tag were done with a 1:500 dilution of a monoclonal anti-Tag antibody (provided by J. Ortin) and an anti-mouse secondary antibody labeled with C_γ3 and diluted 1:200 (Sigma). DNA was stained with DAPI (Vectashield; Vector Laboratories).

The Simple-Luciferase Reporter Assay System (Promega) was used to measure luciferase activity from cells transfected with prSVLUC or transduced by rSVLUC viruses. Normally, 100 µl of virus stock was used to infect 0.2 million CV-1 cells. Luciferase activity was also used to quantify rSVLuc transducing units by infecting 1.5×10^5 COS-1 cells with serial 1:3 dilutions of rSVLUC stocks. Forty-eight hours after infection cells were harvested and luciferase activity was measured in a luminometer (Berthold LB9507) following the recommendations of the supplier. In all cases dilutions of the virus or dilutions of the extracts were made to discard saturation of the luciferase activity (data not shown).

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