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SV40 As an Effective Gene Transfer Vector *in Vivo**

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SV40 was used to transduce gene expression *in vitro* and *in vivo*. Using cloned SV40 genome, we replaced large T antigen gene (Tag) with a polylinker, and inserted firefly luciferase, controlled by SV40 early promoter. Transfection into Tag-expressing cells yielded Tag-deficient virus, SVluc. SVluc was Tag-deficient and therefore replication-deficient in cells that did not supply Tag. SVluc transduced functional luciferase expression *in vitro*. BALB/c mice were inoculated with SVluc, and their tissues were assayed 3–21 days post-inoculation (dpi) for luciferase protein production and enzyme activity. Luciferase protein was detected by immunohistochemistry throughout the experiment, from 3 to 21 dpi. There was no inflammatory reaction against SVluc-infected cells at any time, in any tissue studied. Luciferase activity was first detected by luminometry 14 dpi, and remained level through day 21. Thus, replication-deficient recombinant SV40 can mediate gene transfer *in vitro* and *in vivo*.

Currently available viral gene transduction agents have both strengths and weaknesses. Some are more useful in one setting, some in another. Retroviral vectors are most frequently used. They are potentially useful for gene transfer to dividing cells, but are limited by their loss of activity on concentration, inability to infect resting cells and other undesired side effects (1–5). The former problem may be addressed in part by altering retroviral packaging (6). Adenoviral transfer agents infect a wide range of cells and may infect and express their genes well in resting cells from diverse tissues. They can also be concentrated to high titer. However, adenovirus elicits a strong immune response that eliminates infected cells and limits the longevity of expression, often to 10 days or less in immunocompetent hosts (7, 8).

Several newer agents have been proposed for gene transduction, but are not understood well enough to allow definitive conclusions as to their utility. For example, adeno-associated virus is a small DNA virus that may infect both resting and dividing cells. In the latter, it may integrate into host genome at a single site, at a point of considerable genomic instability on chromosome 19 in humans (9), but it may also transduce gene expression without integration (10). Considerable additional work will probably be needed before adeno-associated virus is useful as an efficient transduction agent, particularly for normal cells (11). Other recently described viral agents include a bovine papilloma virus (12), and Herpes simplex virus (13) and, very recently, HIV (14). Analysis of these viruses as potential gene transfer vectors is just beginning. Currently available agents do not, therefore, satisfy the range of likely clinical needs. Additional vectors and strategies are necessary.

We thus sought a viral agent that: (i) could be concentrated to high titers to allow treatment of large organs or cell pools, particularly *in vivo*; (ii) could infect and express its genes in a wide variety of cells for maximum flexibility in application; (iii) was relatively nonimmunogenic; and (iv) could be made replication-deficient, and handled and packaged with relative ease. Consequently, we devised a gene transfer system based on simian virus-40 (SV40)[1] as a vector.

The rationale for this approach was based on several important aspects of SV40 virus and its activity. SV40 infects a wide range of cell types from humans and other mammals, and expresses its genes in them. Plasmids incorporating SV40 genes and/or promoter may express either transiently or stably in cell lines and in primary cultures. SV40 may integrate into the host genome (15), permitting transmission of expression to daughter cells. The virus is stable to manipulation, and can be concentrated to high titer. Furthermore, the lack of adverse effects was documented in people who received the Salk polio vaccine, early preparations of which contained wild type (wt) SV40 (16, 17). Replication-deficient SV40 can be produced with relative ease. SV40 large T antigen (Tag) is required for virus replication (18, 19). However, Tag expressed by packaging cell lines can support virus replication in trans (20).

MATERIALS AND METHODS

Cell Lines—TC7 monkey cells were the kind gift of Dr. Janet S. Butel (Baylor College of Medicine). COS-7 cells were obtained from ATCC. These cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Hyclone), glutamine, and antibiotics (Life Technologies, Inc.).

pBSV(ΔT′)—The system reported here is based on pBSV-1 (kind gift, Dr. J. Butel), in which the SV40 genome had been cloned as a *Bam*HI fragment into pBR322 (see Fig. 1). To make our plasmid, pBSV(ΔT′), pBSV-1 was digested with *Avr*II and *Bcl*I, to excise the 2.4-kilobase Tag gene, just upstream from its transcriptional start site, almost to the end of its coding region. A multiple cloning site modified from pGEM7 to delete the *Bam*HI site, was inserted. The pBSV(ΔT′) multiple cloning site included unique *Bst*XI, *Xho*I, and *Xba*I sites flanked 5′ (toward SV40 early promoter) by Sp6 promoter and 3′ by T7 promoter. The inclusion of these promoters facilitates sequencing of cloned DNAs by application of standard Sp6 and T7 primers.

pBSVluc—To evaluate this system, we produced SV40 containing firefly luciferase (Luc) as a reporter. pBSV(ΔT′) was opened with *Xho*I and *Xba*I. Luc cDNA, + SV40 early promoter, was excised from pGL2-Control Vector® (Promega) as an *Xho*I-*Dra*I fragment. The *Xho*I sites of the Luc cDNA and the opened pBSV(ΔT′) were compatible. After ligation of the *Xho*I-digested end of Luc cDNA into the pBSV(ΔT′) *Xho*I site,

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[1] The abbreviations used are: SV40, simian virus 40; Luc, luciferase; m.o.i., multiplicity of infection; pBSV(ΔT′), transfer plasmid to produce recombinant replication-deficient SV40; pBSVluc, plasmid used to generate SVluc; SVluc, SV40 variant that contains luciferase; Tag, large T antigen, SV40; wt, wild type.

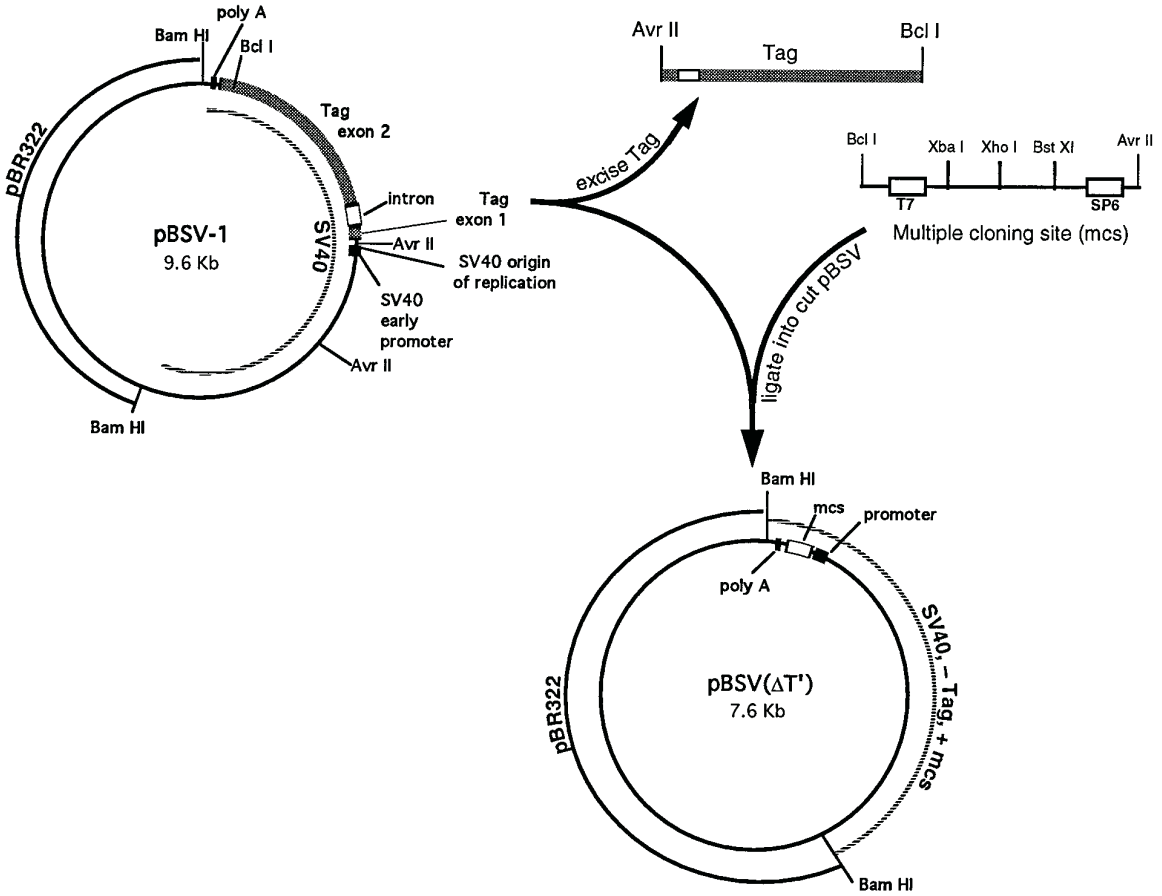

FIG. 1. **Production of pBSV(ΔT′).** pBSV(ΔT′) was made from pBSV-1, in which the complete SV40 genome was cloned as a *Bam*HI fragment into pBR322. Partial digestion with *Avr*II and complete digestion with *Bcl*I, removed the first exon and intron, and almost all of the second exon of the Tag gene. A polylinker with 3 unique restriction sites, *Bst*XI, *Xho*I, and *Xba*I, flanked by Sp6 promoter beyond *Bst*XI and T7 promoter beyond *Xba*I, replaced the excised fragment. The polylinker had an *Avr*II site at the Sp6 end and a *Bcl*I site at the T7 end. The bacteriophage promoters were included to facilitate sequencing using standard Sp6 and T7 primers.

the linearized plasmid was treated with Klenow and religated to yield pBSVluc. This plasmid contains two copies of the SV40 early promoter. Preliminary data in this system suggested that this double promoter configuration elicited higher levels of transgene expression.[2]

Recombinant, Replication-deficient Virus—To produce virus, the viral genome was excised from pBSVluc with *Bam*HI, gel purified, religated, and transfected using calcium phosphate into COS-7 cells (21). Two weeks later, when >50% of the cells showed evidence of infection, virus was harvested by freezing, thawing, and sonicating the cells.

The time course of SVluc production following transfection of COS-7 cells was measured. Culture dishes (60 mm, Falcon Plastics) containing approximately 10^6 COS-7 cells were transfected with 1 μg (0.5 pmol) of recircularized viral DNA following excision from plasmid and gel purification as described. Cultures were harvested and titered for the generation of infectious SV40 derivative virus every 3–4 days thereafter until 14 days. Results, shown in Fig. 2, indicate that maximum virus yield was obtained at 7 days post-transfection: 3.5×10^5 infectious units MV/0.5 pmol of transfected virus.

Subsequent stocks of replication-deficient SVluc were prepared by infecting COS-7 cells. Virus preparation was carried out in Dulbecco's modified Eagle's medium, 2% fetal bovine serum. When virus from such primary cultures is used to generate a working stock of virus, the 2 ml of lysate derived from the transfected 60-mm dish cultures were used to infect two 75-cm^2 tissue culture flasks of subconfluent COS-7 cells. Cultures were allowed to proceed for 2 weeks thereafter, and harvested. Cultures were harvested by removing all but 2 ml of culture medium from the flask, freezing and thawing three times, and sonication. In general, these procedures reproducibly yield virus stocks of 10^9 plaque-forming units/ml. Resulting virus (SVluc) may be band-purified by discontinuous sucrose density gradient ultracentrifugation, and titered using neutral red-agar overlay staining of COS-7 cells, according to standard protocols (22). This increases virus titer 10–100-fold in our hands.

To ascertain that SVluc was replication-deficient, virus was plated, as for titering, on COS-7 and TC7 cells. The latter lack Tag. Titers were determined by plaque-forming units, and compared to titers of wt SV40 (kind gift, Dr. Butel). Each stock of presumed replication-deficient SVluc used in these studies has been tested at least twice for the presence of replication competent virus.

Luciferase—Luciferase was detected in two different ways: by measuring its enzymatic activity and by visualizing Luc-containing cells by immunochemical analysis using affinity-purified anti-Luc antibody (Promega). Luc activity was assayed in frozen tissue samples or cell culture homogenates using a Luciferase Assay Kit (Promega), according to package insert instructions. Frozen tissues (or cultured cell lysates) were weighed and homogenized in luciferase assay buffer (Promega). Light emission was measured in arbitrary units using a Chem-Glo luminometer (Aminco, Inc.). Statistical comparisons of light emission data were performed using Wilcoxson nonparametric comparison.

Luciferase protein was visualized in individual cells using affinity-purified rabbit anti-Luc antibody (anti-Luc antibody, Promega) to treat acetone-fixed cultured cells or frozen tissue sections mounted on glass slides. Slides were treated with anti-Luc (or control normal IgG) antibody, followed by avidin-conjugated goat anti-rabbit Ig, then avidin-peroxidase with a biotin bridge (23). Reactivity was visualized using diaminobenzidine + H_2O_2.

Northern Analysis—Cultured TC7 or COS-7 cells were infected with SVluc (m.o.i. = 10), or mock infected. RNA was extracted 18 h later (RNAzol, Cinna-BioTex, Friendswood, TX), electrophoresed in formaldehyde gels, blotted to nitrocellulose, and hybridized with ^{32}P-labeled Luc cDNA (19) (hybridization: 50% formamide, 5 × SSC, 0.1% SDS, 42 °C; final washes: 0.1 × SSC, 0.5% SDS, 42 °C). Hybridization was visualized by autoradiography. As a control probe, rDNA was used (kind gift, Dr. E. Mercer, Jefferson Medical College).

[2] D. Strayer, unpublished data.

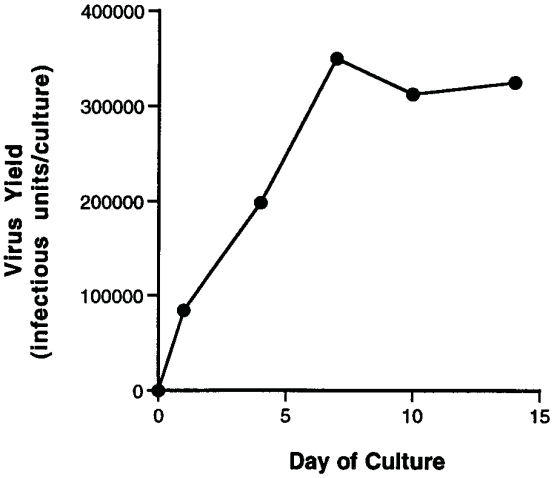

FIG. 2. **Generation of SV40 derivative virus following transfection with viral DNA.** Cultures of subconfluent COS-7 cells (approximately 10^6 cells) were transfected with 1 μg (0.5 pmol) of recircularized viral DNA following excision from plasmid and gel purification as described under "Materials and Methods." Cultures were harvested and titered at predetermined times thereafter to assess the time course of generation of infectious SV40 derivative virus. Results are expressed in infectious viral units/0.5 pmol of transfected viral DNA.

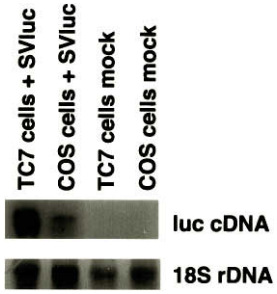

FIG. 3. **Expression of Luc in cultured TC7 cells following infection with SVluc.** Northern analysis of Luc expression in TC7 and COS-7 cells infected (m.o.i. = 1) with SVluc or mock-infected, and harvested 18 h. later. Whole cell RNA was electrophoresed, blotted to nitrocellulose, and hybridized to ^{32}P-labeled Luc cDNA. After autoradiography, the filter was boiled, and rehybridized to ^{32}P-labeled rDNA to control for the amount of RNA blotted.

Mice—BALB/cJ mice (retired breeders, Jackson Laboratories, Bar Harbor, ME) were given SVluc, 10^9 plaque-forming units/mouse, in 0.1 ml of saline via intravenous or transoral intratracheal instillation. Mice were sacrificed by cervical dislocation on days 3, 7, 10, 14, 17, and 21. Day 21 was the end of the experiment. Control mice received saline only. Experimental groups consisted of 3 to 5 animals. Control groups consisted of 2 mice per time point. Lungs, liver, kidney, spleen, heart, esophagus, and skin overlying the intravenous inoculation site in the tail were sampled. Half of each organ was used for immunohistochemical analysis to visualize Luc protein using anti-Luc antibody, and half was snap-frozen in liquid nitrogen, to be homogenized for Luc enzyme assay (see above). Negative controls for these assays included tissues from control mice, treated with the anti-Luc antibody, and, as well, experimental and control mouse tissues treated with normal IgG instead of anti-Luc antibody.

RESULTS

SVluc Is Replication-deficient—To ascertain that the virus produced is replication-deficient in cell lines that do not supply Tag, TC7 cells were used. In contrast to wt SV40, a Tag-deficient SV40 mutant should not yield a lytic, productive infection in cells lacking Tag. That is, SVluc should not produce plaques in TC7 cells. SV40 and SVluc titers using TC7 and COS-7 cultures were compared at multiplicities of infection (m.o.i.) from 10^{-3} to 10. Inocula of up to 10^7 plaque-forming units were used. SVluc did not produce plaques in TC7 cells at any m.o.i., while wt SV40 did. Both viruses produced plaques on COS-7 cells. Our current stocks of SVluc have been passed 11 times in culture, without evidence of replication competence.

Luciferase Gene Transfer to Cultured Cells—Infection of cultured COS-7 and TC7 cells was done to determine whether Luc could be detected. SVluc infected (m.o.i. = 1), cultured cells 18 h post-infection were assayed for Luc gene expression by Northern analysis and for Luc enzyme activity using a standard Luc assay kit (Promega) and a luminometer (Aminco, Inc.). Luciferase mRNA was found in both COS-7 and TC7 cells, but not in mock-infected control cultures (Fig. 3). Luciferase enzyme activity was also detected in SVluc-infected cells: SVluc-infected TC7 and COS7 cells averaged 116 ($\pm$49) light emission units/50-μl aliquot of culture protein ($\approx$75 μg), while mock-infected cells averaged 10 $\pm$ 14 light emission units/culture ($p = 0.05$). Therefore, SVluc successfully transferred Luc production to cultured cells.

The transduction efficiency of the SV40-derivative virus was examined. At m.o.i. = 1, 52% of cultured cells (either TC7 or COS-7) cells stained positively for transgene expression. At m.o.i. = 0.1, the percentage of positive cells was 4.1%. Interestingly, at m.o.i. = 10, only 57% of cells were positive for transgene expression, suggesting that for these cells, that percentage represents the maximum proportion of these cell types that will express the transgene.

Transfer of Luc Activity to Mice—To test the ability of SVluc to act *in vivo* as a gene transduction agent, BALB/c mice received SVluc (or saline only) as described under "Materials and Methods." The animals were sacrificed on days 3, 7, 10, 14, 17, and 21. Selected tissues were tested for Luc expression using anti-Luc antibody and luminometry.

Cells producing immunohistochemically detectable Luc were seen (Fig. 4) in the liver, kidney, spleen, in the large conducting airways of the lung, and in the skin at the inoculation site. The periphery of the lung and the heart were negative. Cells bound by anti-Luc antibody were first noted by day 3, but Luc protein production was most pronounced on day 21. Intravenous and intratracheal instillation yielded similar patterns of Luc expression in the tissues examined, save that intratracheal treatment produced many Luc-expressing cells in conducting airways and esophagus, while intravenous inoculation did not do so in the course of this 21-day study. In the liver, hepatocytes and Kupffer cells contained Luc protein. In the spleen, both lymphocytes and mononuclear phagocytes produced it. Splenic megakaryocytes also produced Luc (*insets*, Fig. 4). In the skin at the inoculation site, Luc was detected principally in keratinocytes, while in the kidney it was mainly found in glomeruli.

No inflammatory reaction was found in any organ that was positive for Luc, at any time point studied (days 3–21). Thus, SVluc-infected cells, as recognized by anti-Luc antibody, were normal in appearance and were not associated with any inflammation (Fig. 4).

We used several negative controls for immunochemical studies: control mouse tissues treated with anti-Luc antibody, and both experimental and control mouse tissues treated with normal IgG instead of anti-Luc antibody. All these controls yielded negligible staining (Fig. 4).

These same selected tissues were homogenized and assayed for Luc enzymatic activity using a luminometer (Fig. 5). SVluc recipients showed strong Luc activity in the liver, kidney, and spleen beginning on day 14, and lasting through the end of the study on day 21. Heart and the lungs (excluding large airways) were negative at all times. Although slight increases in luciferase activity appeared in samples of heart and lungs toward the end of these studies, these increases were not statistically significant ($p > 0.2$). Skin at inoculation sites was strongly positive (data not shown). The level of activity observed was

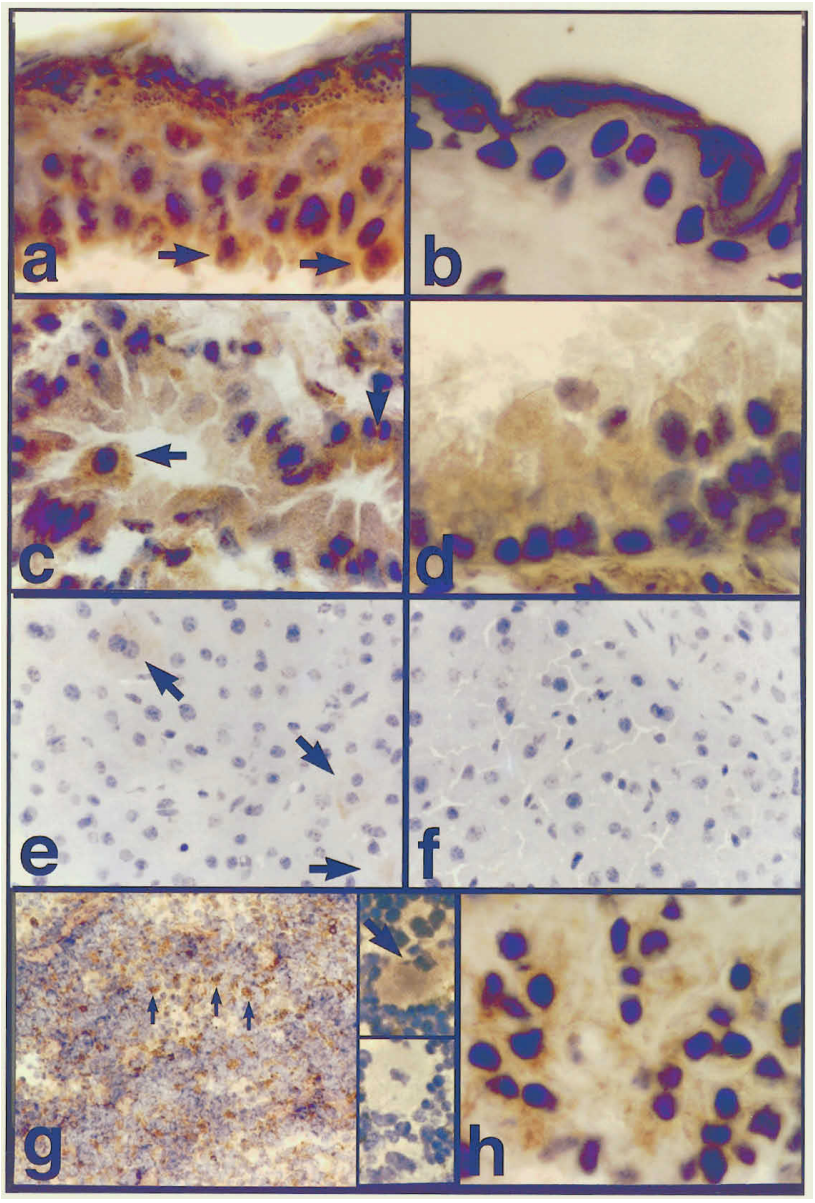

FIG. 4. **SVluc transduces Luc production *in vivo*.** BALB/c mice received SVluc intravenous or intratracheal, and were assayed for Luc production by immunochemical staining 17–21 days later. Frozen sections were examined for Luc production by immunochemical staining with anti-Luc antibody as described under "Materials and Methods": *a*, skin at the injection site (IV) (× 1100); *c*, trachea from an animal receiving SVluc intratracheal (× 600); *e*, liver from a mouse receiving SVluc IV (× 300); *g*, spleen from a mouse receiving SVluc IV (× 125); and *h*, renal glomerulus from a mouse receiving SVluc IV (× 600). Cells from skin, liver, spleen, kidney, and large airways of SVluc recipients show strong positive staining, indicative of SVluc infection and Luc production. *Arrows* in several frames highlight representative cells that show the brown color indicative of positive staining for luciferase in these studies. Note the lack of inflammation at or about any of the Luc-producing cells. In the spleen, lymphocytes were not the only positive cells. The two *insets* between *g* and *h* show megakaryocytes from the spleen of an SVluc recipient (*upper inset, arrows*, indicate positive staining) and a control animal (*lower inset*) (× 1100). Two sets of negative control studies were done: *b*, sections of skin (× 1100), and liver (*f*) (× 300) from control mice (vehicle only), were treated with anti-Luc antibody, and showed no positive staining. *d*, frozen tissue sections (trachea shown here, *middle right*) (× 1100) from the same animal shown in *c* were treated with normal IgG instead of anti-Luc antibody. Slides were counterstained with hematoxylin.

relatively constant from day 14 onwards, and did not deteriorate during the time frame studied (Fig. 5). Tissues from all organs of control mice (receiving vehicle only) were negative at all times for Luc activity.

DISCUSSION

The utility of SV40 or any other virus as a gene transfer agent requires that certain conditions be met. The virus must: (i) be replication-deficient under normal circumstances; (ii) be safe for its intended recipients; and (iii) transfer gene expression effectively.

The requirement for replication incompetence reflects a need for limited exposure to any virus and a concern over potential transmissibility of an infectious therapeutic agent. We studied this question using a titer assay in Tag+ and Tag− cells. Plaques require that virus complete a lytic replicative cycle in infected cells, lyse those cells, spread to adjacent cells, and repeat the process. At a range of m.o.i. from 10^{-3} to 10, SVluc did not produce plaques in Tag− TC7 cells. Wild type SV40 did. Thus, SVluc could not replicate detectably in cells lacking Tag.

Tag is the SV40 transforming protein (24). It was excised in our shuttle vector, greatly reducing potential risks of applying this vector to therapeutic systems. These findings do not rule out the possibility that SVluc may reacquire Tag during passaging. We have not yet detected SVluc replication in TC7 cells even 11 passages beyond the initial generation of the virus. The observed replication incompetence of SVluc suggests that reacquisition of Tag happens very rarely or not at all in our system. The possible occurrence of such an event indicates a need for caution in using this virus as a gene transfer agent. There have been reports of SV40 genome detection in DNA from some rare forms of human tumors (25, 26). However, the most convincing report used tumor specimens from patients who had no known contact with the virus and were too young to have received live virus during immunization for polio (25). Furthermore, the careful epidemiological studies of Salk vaccine recipients showed that inoculation of wt SV40 does not have significant harmful sequellae for humans (16, 17). As I have been unable to detect replication competent revertants, however, such concerns at this point are mostly theoretical.

Effective gene transfer using SV40 as a vehicle was first described by Gething and Sambrook (27) and by Asano and co-workers (28). These studies used replication-deficient SV40 variants to transfer gene expression to cultured cells. This article describes the first application of SV40 to gene transfer

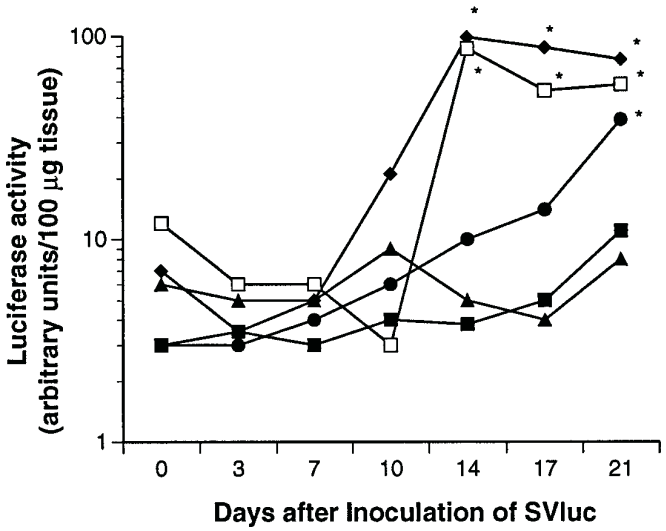

FIG. 5. **Luc activity in tissue homogenates after SVluc inoculation *in vivo*.** SVluc, 10^9/0.1 ml, was inoculated intravenously into BALB/c mice. Animals were sacrificed at various times thereafter and selected tissues were homogenized: lungs (▲), liver (♦), heart (■), spleen (□), and kidney (○). Luc activity was measured in these homogenates using a standard assay kit (Promega) and a luminometer. Activities are shown for SVluc recipients' organ homogenates. Each time point represents at least two independent determinations. Luc activity is reported as light emission (arbitrary units)/100 μg wet wt. Machine background values were subtracted. *, $p < 0.05$ compared to control values for that organ.

in live animals. The ability of a mutant SV40 to transfer Luc reporter activity to mice was demonstrated using two detection techniques: immunochemistry with affinity-purified anti-Luc antibody, and assay for reporter protein enzymatic activity. Initial studies indicated that Luc transduced by SVluc was detectable by immunochemistry, and that it was functionally active.

When we tested SVluc in animals, Luc-producing cells were detected immunohistochemically 3 days after administration, and enzyme activity was demonstrated by luminometry 14 days post-inoculation. This time lag may be needed for enzyme levels to accumulate sufficiently to be detectable in tissue homogenates. Subsequently, Luc activity persisted undiminished. Increases in Luc activity with time cannot be explained by the presence of replication-competent revertants in our stock of SVluc. Even if some such recombinants were to have occurred and escaped our detection, mice are not permissive hosts for wt SV40.

Some gene transfer agents, adenovirus, for example, elicit strong immune responses that eliminate infected cells, usually within 7 to 10 days (7, 8). In that context, we chose 21 days as the duration of these studies to test whether the uncompromised host immune system would eliminate the SV40-derived viral vector. We found no evidence that host reactivity to SVluc or SVluc-infected cells altered the course of reporter gene expression or eliminated cells infected with SVluc. That is, Luc activity did not decrease during this study, and there was no histologic suggestion of an inflammatory reaction *versus* Luc-producing cells. Time course studies of sera from SVluc infected mice to detect neutralizing anti-SVluc antibody are in progress. However, immunologic responsiveness to SV40 has been shown to be relatively limited: both humoral and cellular immune responses have been found to be focused primarily on Tag, with scant or no reactivity detected *versus* viral structural proteins (29, 30). The lack of inflammatory response in our tissue sections is in keeping with these reports, since Tag is not expressed in this system. This finding also suggests that luciferase protein does not elicit a cytotoxic immune response as produced by transduced cells. This may reflect its intracellular localization so that it does not present a cell membrane target for the immune system, or perhaps a strong susceptibility to degradation.

Clearly, additional work is needed to understand this system better. The size limitations for inserts in SV40 are not yet known. The Tag gene is 2.4 kilobases, comparable in size to the Luc cDNA. Smaller inserts (300 base pairs to 1 kilobase pair) have been packaged in recombinant SV40 (preliminary data), but the upper size limits of this system are not yet clear. We have obtained expression of β-galactosidase in this system (3.6 kilobases), but have observed that packaging does not proceed as efficiently for this as for smaller inserts.[3] Current studies in progress include excising SV40 late genes to see whether additional cloning capacity can be added in this fashion. Studies for longer time periods, involving multiple inoculations of virus, and including analysis of more organs will assess more definitively the longevity and distribution of reporter gene expression, and whether immune responsiveness may eliminate infected cells. However, stable transgene expression *in vivo* over 21 days without evidence of inflammation is encouraging. Preliminary studies using organ homogenates suggest that SVluc DNA integrates into the host genome.[4] The ability of wt SV40 DNA to integrate into the host genome is well documented (15).

In conclusion, recombinant, Tag-deficient SV40 can transfer active gene function that is stable, at least for 21 days. This system may have potential for application to therapeutic gene transfer *in vivo*.

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