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Durability of Transgene Expression and Vector Integration: Recombinant SV40-Derived Gene Therapy Vectors

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Many applications of gene delivery require long-term transgene expression. In dividing cells, this result necessitates vector genome persistence, usually by integrating into cellular DNA. Since recombinant gene delivery vectors derived from *tag*-deleted, replication-incompetent simian virus-40 (SV40) provide for long-term transgene expression in resting and dividing cells, we tested whether such enduring transgene expression reflected integration into cellular genomes. Several lines of evidence suggested this likelihood. After transduction *in vitro,* continuously dividing cell lines and continuously stimulated primary cells uniformly showed transgene expression for many months. Mice whose livers were transduced *in vivo,* partially resected, and allowed to regenerate showed comparable levels of transgene expression in regenerated and preoperative livers. Thus, replication-incompetent SV40 vectors (rSV40) persist *in vitro* and *in vivo* despite extensive cell division. We tested the possibility that this persistence reflected integration directly. Southern blot analyses of genomic DNA from transduced 293 cells showed that vector genome incorporation into cell DNA happened within days of transduction. Episomal vector DNA was barely detectable 96 hours post-transduction. Inverted PCR, used to characterize vector integration points, showed vector DNA integrated randomly into the cell genome. The circular rSV40 genome opened at different points in each integrand. A significant proportion of the integrands did not contain the entire vector sequence, but rather only portions thereof. Quantitative Southern blot analysis showed approximately 3.05 transgene copies per cell. Therefore, recombinant SV40 gene delivery vectors integrate into the cellular DNA of both resting and dividing cells, and do so randomly and within days of transduction. This integration may explain long-term transgene expression.

Key Words: gene delivery, gene therapy, SV40, integration

INTRODUCTION

The longevity of a transgene's expression is a characteristic of the vehicle that is used to deliver that transgene. What types of cells can be transduced, the fate of delivered genetic material within cells, and the duration of transgene expression are dependent on the vector. Some vectors facilitate gene delivery to resting cells well, but are less effective when used for cells that are actively cycling [1]. Other vectors require cell division or are more effective in transducing dividing cells [2]. Clearly, if transgene expression is to persist in actively dividing cells and their progeny, a vector genome must either replicate with the cell or be incorporated into the cellular genome.

The latter phenomenon is assumed to occur virtually always for some viral vectors and almost never for others, and has been difficult to substantiate for some. Thus, the ability of DNA copies of retroviral and lentiviral genomes to integrate into cellular DNA is unquestioned. Adenoviral vectors and Herpes simplex viral vectors integrate rarely, if ever [3,4]. Wild type adeno-associated virus (AAV) integrates into the human genome [5], but whether recombinant AAV-derived vectors do so is still being debated. AAV vectors can persist for long times as episomes in nondividing cells [6], but the AAV integrase gene is deleted in making most AAV vectors. Because some investigators report evidence that supports the integration of rAAV

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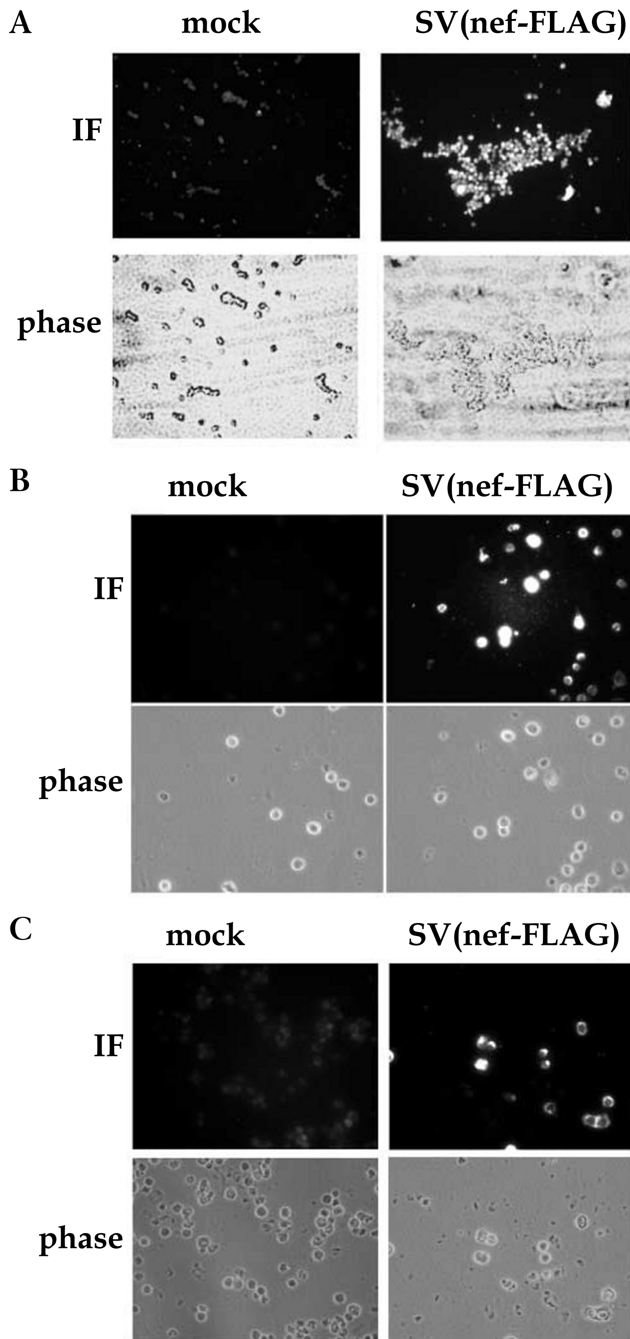

FIG. 1. Expression of rSV40-delivered transgene in unselected cultured cells. SupT1 cells were treated with SV(nef-FLAG) at MOI = 10, 3, and 3 on sequential days (right panels). As a negative control, SupT1 cells were treated the same way but untransduced (mock, left panels). (A) 1 week later, (B) 5 weeks later, and (C) 9 weeks later, unselected cells were immunostained using anti-FLAG antibody (upper panels). Phase contrast photomicrographs of the same fields (lower panels) are shown for comparison.

vector over the course of months [7,8], it seems likely that these vectors may integrate in some circumstances [6,9].

Unlike adeno-associated viruses, the double-stranded DNA genome of recombinant gene delivery vectors derived from SV40 is circular. Wild-type SV40 has been repeatedly shown to integrate into the cellular genome, but it does so only occasionally; it apparently prefers to complete a lytic cycle [10]. Because the large T antigen (Tag) protein is necessary both for SV40 DNA replication and for SV40 capsid gene expression, *tag*-deleted rSV40 vectors cannot complete a lytic cycle. These vectors provide long-term transgene expression in both dividing and resting cells, both *in vitro* and *in vivo.*

Thus, it was of considerable interest to test whether this longevity of transduction reflected rSV40 genome incorporation into cellular DNA. These studies used several different rSV40 vectors, involving several transgenes and different promoters, in both resting and dividing cells, *in vitro* and *in vivo*. Among the parameters tested were the longevity of transgene expression in dividing cells and the extent and time course of rSV40 integration.

RESULTS

Long-Term Transgene Expression in Dividing Cells Assessed by Immunostaining

To evaluate the durability of transgene expression in continuously dividing cells, we transduced SupT1 cells as described (for 3 days at MOI of 10, 3, and 3 on sequential days with an rSV40 vector carrying a FLAG epitope tag (SV(nef-FLAG))). We measured transgene expression in unselected cells 1 and 5 weeks later. Over 95% of cells were positive for transgene expression (Figs. 1A and 1B). Because SupT1 cells divide rapidly and continuously in culture, with an approximate doubling time of 24–36 hours, we reexamined transduced cultures for persistent transgene expression 4 weeks subsequently. Despite continuous cellular proliferation in the interim, greater than 95% of unselected transduced cells continued to express the transgene (Fig. 1C).

Primary cultures of human T lymphocytes yielded similar results. We transduced normal human peripheral blood lymphocytes (PBL) with SV(Aw), a vector that encodes a single chain Fv (SFv) antibody against HIV-1 integrase, at MOI of 10, 3, and 3 on sequential days, without stimulation. We then cultured the cells for 7 weeks with continuous stimulation with interleukin 2 (IL-2) and concanavilin A (conA). Control cultures were mock-transduced. After 7 weeks, immunostaining demonstrated that almost all cells continued to express the Aw transgene (Fig. 2).

Long-Term Transgene Expression in Dividing Cells Assessed by Flow Cytometry

To corroborate these data, we tested for transgene expression by cytofluorimetry. SupT1 and AA2 cells were transduced with SV(Aw) using MOIs of 10, 3, and 3, on

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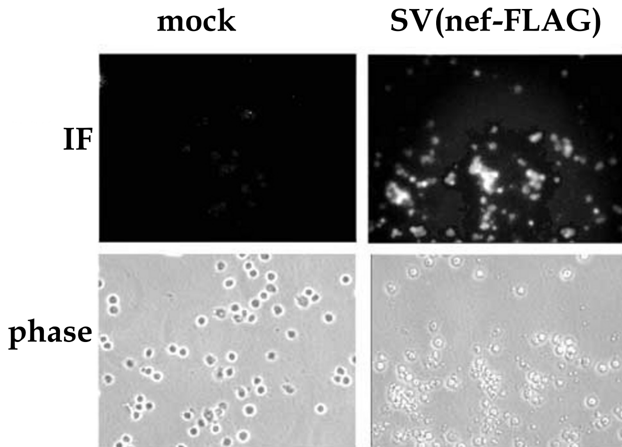

FIG. 2. Long-term expression of rSV40-delivered transgene in continuously stimulated primary human lymphocytes. Primary human peripheral blood lymphocytes were prepared, transduced with SV(Aw) at MOI = 10, 3, and 3 over 3 days without stimulation, then cultured continuously for 7 weeks thereafter in the presence of added conA and human recombinant IL-2. They were tested at that time point by immunostaining to detect Aw (murine IgG-κ SFv anti-HIV-1 IN) expression. The upper panel illustrates typical immunostaining patterns for Aw in SV(Aw)- and mock-transduced cells; the lower panels show the phase contrast micrographs of the same fields for comparison.

consecutive days, or a single exposure at MOI = 100, respectively. We analyzed unselected cells for Aw expression 1 week (for AA2) or 4 months (for SupT1) after transduction. The cytoplasmic transgene product was detectable in permeabilized cells. Flow cytometric analysis showed that almost all cells at both assay points were positive for transgene expression (Fig. 3).

Transgene Expression in Regenerated Liver Tissue

The ability of rSV40 vectors to deliver long-term transgene expression to dividing cells in culture led us to test whether transgene expression persisted in cells that were transduced in a quiescent state *in vivo* and then stimulated to divide. We transduced the liver directly by way of the hepatic portal vein, performed partial hepatectomy to stimulate hepatocyte division, and compared transgene expression in the lobectomy specimen with that in the lobe of the liver that had regenerated following lobectomy. Thus, we infused SV(AT), a vector that carries the cDNA for human α1-antitrypsin (α1AT; encoded by *SERPINA1*), into mouse liver by way of a catheter in the hepatic portal vein. We resected the two right lobes, approximately 2/3 of the liver, 2 weeks after transduction. The regenerated liver, containing cells that had proliferated in response to the partial hepatectomy, was harvested 2 weeks after the partial hepatectomy. Control mice did not receive partial hepatectomy. One control group received the rSV40 (positive control), and the other group received no vector (negative control). We extracted RNA from experimental and control livers, and analyzed it by northern blot analysis for transgene expression. (The human *SERPINA1* transgene mRNA product is sufficiently distinct from the mouse *Serpina1* mRNA that the human *SERPINA1* cDNA probe did not cross-hybridize with mouse *Serpina1* mRNA under stringent conditions.) To control for RNA loading, we hybridized the same filter with a cDNA probe for albumin to visualize the albumin transcripts. We normalized the relative levels of mRNAs to the loading controls (albumin mRNAs). Densitometric analysis showed that the ratios of human *SERPINA1* mRNA:albumin mRNA in the regenerated liver were comparable to

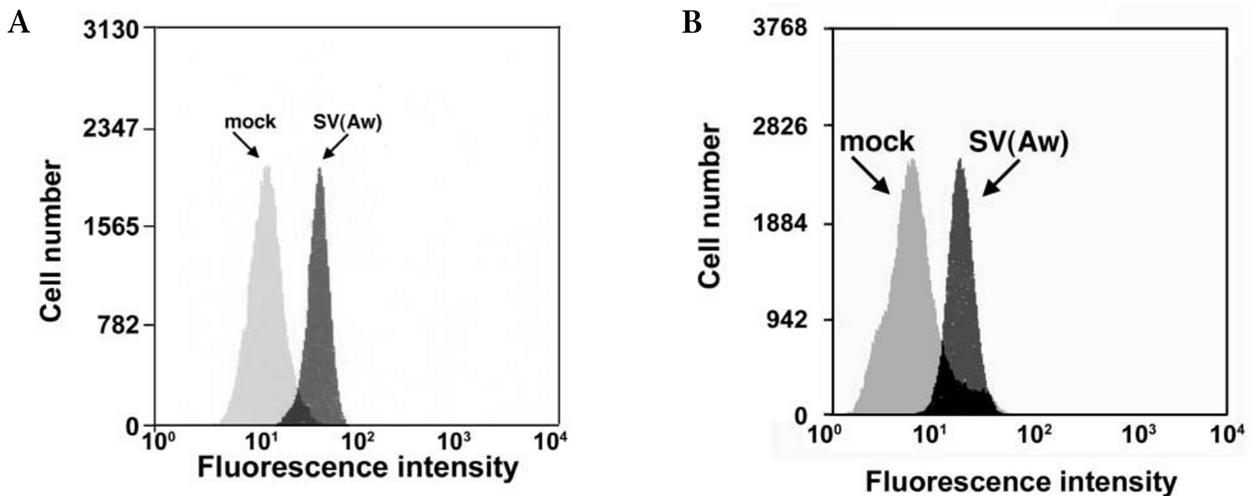

FIG. 3. Flow cytometric analysis of rSV40-delivered transgene expression in unselected cells analyzed 1 week and 4 months after transduction. Human T-cell lines were transduced with SV(Aw), which carries Aw, a single chain Fv antibody against HIV-1 integrase. These cells were fixed and permeabilized, and immunostained with FITC anti-mouse IgG, then analyzed by flow cytometry after 1 week (A) or 4 months (B) of continuous post-transduction culture. Mock transduced cells, shown side-by-side, are the negative controls for these studies. The FACS profiles for the SV(Aw)-transduced and mock-transduced cultures are superimposed to facilitate interpretation.

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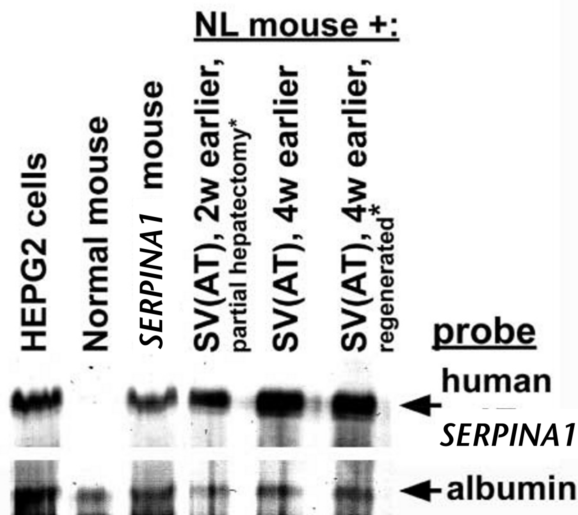

FIG. 4. Continued expression of rSV40-delivered transgene in mouse liver *in vivo,* with and without partial hepatectomy and regeneration. Normal mice were treated with SV(AT) with a portal-vein catheter. Some mice received partial hepatectomy 2 weeks after transduction, whereas some were left alone. RNA was prepared from hepatectomy specimens. Mice that had received partial hepatectomy or control treatment were sacrificed 4 weeks after transduction and RNA was extracted from their livers. Northern blot analysis was performed to detect expression of mRNA for the human *SERPINA1* transgene. All lanes received 10 μg of total RNA from: (left to right) HEPG2 cells (positive control); normal mouse liver (negative control), liver from mice transgenic for human *SERPINA1* (positive control); mouse liver tranduced 2 weeks previously, partial hepatectomy specimen; mouse liver transduced 4 weeks previously; regenerated liver from the mouse whose partial hepatectomy specimen is shown in the second lane previously. The location of the human *SERPINA1* mRNA is indicated. Under the stringent conditions used here, mouse and human *SERPINA1* transcripts do not cross-hybridize. The bottom panel shows the mRNA level of a control transcript (albumin), as a loading control.

those in the resected lobe of the SV(AT)-transduced mice and to those in the liver from mice transduced with SV(AT) but not lobecetomized (2.37:2.45:2.86, respectively). Levels of human *SERPINA1* mRNA in hepatic lobectomy specimens excised 2 weeks after transduction were comparable to those seen in regenerated liver from the same animals (Fig. 4).

Direct Demonstration of Integration: Analysis of Genomic DNA by PCR and Southern Blot Analysis

We first assessed integration in cultured cells using PCR analysis following SV(Aw) transduction. We designed PCR primers to amplify the region of the vector genome near the early and late polyadenylation signals. This region is present in all of our vectors. We isolated high-molecular weight DNAs from transduced and control AA2 cells, amplified them by PCR, electrophoresed and blotted them, and hybridized the filters under stringent conditions with an SV40 genomic DNA probe. A band of the predicted 220-bp size that hybridized with the vector DNA probe was present in preparations from the high-molecular weight DNA from SV(Aw)-transduced AA2 cells (Fig. 5), but not in control cellular DNA. Use of PCR in this setting represented the application of a highly sensitive technique to the detection of vector DNA within the cellular genome. Although unlikely, it is possible that the observed bands resulted from a small amount of contaminating episomal vector genome.

Accordingly, we used straightforward Southern blot analysis to test for vector insertion into genomic DNA. We treated 293 cells with SV(BUGT), a vector that carries the cDNA for human bilirubin-uridine 5′-diphosphate-glucuronysyl transferase (BUGT), and mock-transduced the controls. We isolated genomic DNA 4 days later and either left it uncut, or restricted it with *Not*I ± *Eco*RI. We electrophoresed, blotted, and hybridized these DNAs with a ^{32}P-labeled, full length SV(BUGT) probe (Fig. 6). The uncut DNA migrated only slightly from the origin. Three faint bands in the lane with the undigested DNA from SV(BUGT)-transduced cells probably represent small amounts of relaxed and supercoiled episomal circular

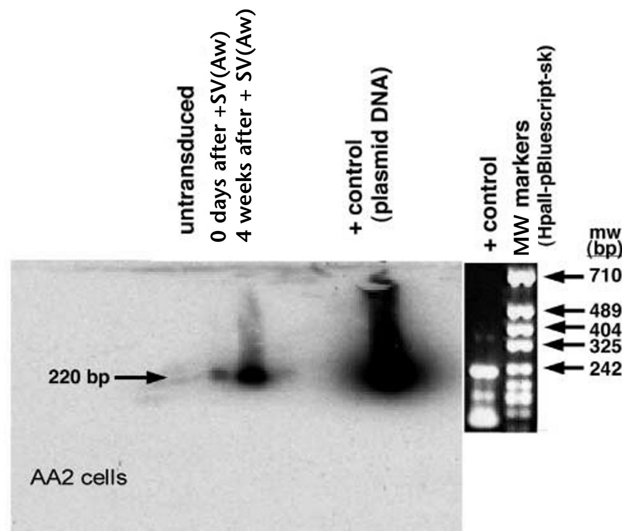

FIG. 5. PCR and Southern blot analysis of genomic DNA from rSV40-transduced cells. AA2 human T lymphoma cells were transduced with SV(Aw), then cultured for 4 weeks. Their genomic DNA was extracted, and subject to PCR using primers specific for sequences from the SV(Aw) genome. Control (+) DNA was the plasmid from which the SV(Aw) genome was prepared; control (–) DNA was from mock-transduced cells. After PCR, the DNAs were electrophoresed, blotted to nitrocellulose, and hybridized under stringent conditions to the fragment of the SV(Aw) genome that was amplified. The positions of the molecular size markers (*Hpa*II-digested pBluescript-SK) are shown on the right, as is the PCR product from the positive control plasmid. The very small DNA species at the bottom of the ethidium bromide-stained gel represent primer dimers.

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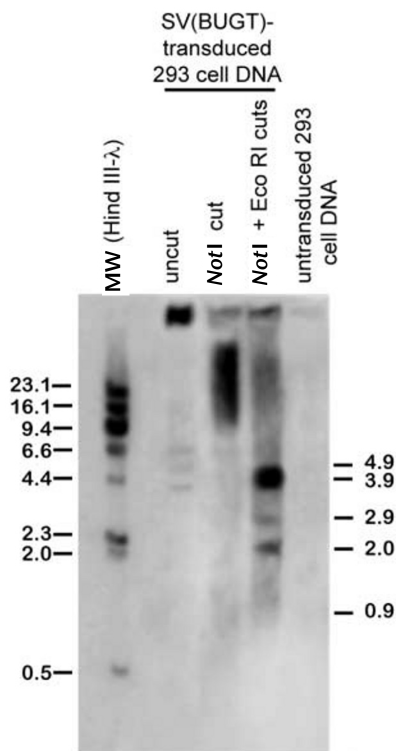

FIG. 6. Southern blot analysis of genomic DNA from 293 cells treated with SV(BUGT). Human 293 cells were treated once with SV(BUGT), and their high-molecular weight DNA harvested 4days later. DNA from the transduced cells was restricted with *Not*I ± *Eco*RI, or was left uncut. These DNAs were electrophoresed, blotted, and hybridized to a ^{32}P-labeled full-length SV(BUGT) DNA probe. Labeled molecular size markers are shown at left (*Hin*dIII digestion of λ phage DNA). An equal amount of unrestricted DNA from mock-transduced 293 cells was electrophoresed in the right lane.

SV(BUGT) genome, flanking a 4.9-kb band that most likely corresponds to linearized (or perhaps nicked) episomal virus genome. *Not*I, which cuts the vector genome once, yielded a high-molecular weight smear. It is significant that the *Not*I digestion did not liberate any detectable 4.9-kb SV(BUGT) genome as would be expected if episomal concatamers had formed, or if significant levels of single copy episomal vector DNA had remained within the cell. When DNA from transduced cells was cut with both *Not*I and *Eco*RI, a major 3.9-kb fragment, corresponding to the distance between the *Not*I site and one of the two *Eco*RI sites, is seen. We observed two smaller bands, 2.9 and 2.0 kb long, that correspond to the two SV(BUGT) genome fragments between the two *Eco*RI sites. The sizes of these bands reflect some incompleteness in the digestions by both *Not*I and *Eco*RI, but these bands are consistent with the release of viral DNA from the genomic DNA. DNA from mock-transduced 293 cells did not hybridize with the probe.

Direct Demonstration of Integration: Sequencing of Integration Points

We extracted DNA from livers of Gunn rats that had received SV(BUGT), and amplified by inverted PCR. This technique is designed to allow identification of unknown DNA sequences adjacent to known DNA sequences. In this case, we applied this approach to characterizing the DNA sequences at SV(BUGT) integration sites. The DNA sequences of both the virus DNA at the integration points and of the cellular DNA into which the virus genome inserted, could thus be identified. We identified scores of such junction points and sequenced them. Representative sequences at vector–cellular genome junction points are in Table 1.

These sequences demonstrate that rSV40 vectors integrate. They also show that integration occurs randomly with respect to the cellular DNA and with respect to the virus genome. That is, both the cellular DNA sequences and the viral DNA sequences were different at each cloned integration point. The restriction enzyme cut-site used was in the targeted DNA. We did not anticipate that we would see the entire vector sequence. Rather, if an integrand contained the complete virus genome, at least one of the sequences elucidated by the primers (pointing in opposite directions) would demonstrate vector sequences abutting the restriction site. In some cases, sequencing reactions from both primers reached cellular genomic sequences before they reached the recognition site of the restriction enzyme used. This indicates that some integration sites contain incomplete vector sequences. As expected, the random integration process occasionally interrupted the transgene or the promoter, and some of the sequences that were selected for presentation demonstrate this.

TABLE 1: Representative sequences of cellular genomic integration sites for SV(BUGT)

Clone 1	AGCCTCCTCACTACTTCT 56	TTAGGCCTACTGAGAAGTA
Clone 2	GCGGGATGGGCGGAGT 171	TGTACAGTTGGGCAATCAT
Clone 3	TTTGCATACTTCTGCCT 479	CACGATTGGCTCTGGGCAT
Clone 4	TAACTGAGAGGTGGGA 1743	TTTGCAGCAAGGGAATGCA
Clone 5	CAAAGAACAACTGCCTT 2095	AGCTAGGCCTTTAGGTAAA
Clone 6	AAAAAAATGCTTTATTT 2938	TTCCTTACACCTTCAAAGA

Livers of Gunn rats were transduced with a portal-vein catheter with SV(BUGT). Their genomic DNA was extracted, separated from low-molecular weight DNA by the method of Hirt [39], and amplified by inverted PCR. This approach is intended to identify the cellular genomic DNA sequences abutting the viral DNA sequences at the viral insertion points into the cellular genome. Left hand sequences represent SV(BUGT) DNA, and the positions of the terminal nucleotide within the SV(BUGT) genome is as follows: the SV(BUGT) sequences at the integration sites in clones 1 and 2 are within the SV40-EP. Those in clones 4, 5, and 6 are in the SV40 late genes. By this approach, > 40 cloned integration points were characterized. Representative sequences at integration points are shown here for 6 of those SV(BUGT) integrands. Sequences within the boxes are from cellular genomic DNA at the integration sites. We sequenced all DNA by automated sequencing.

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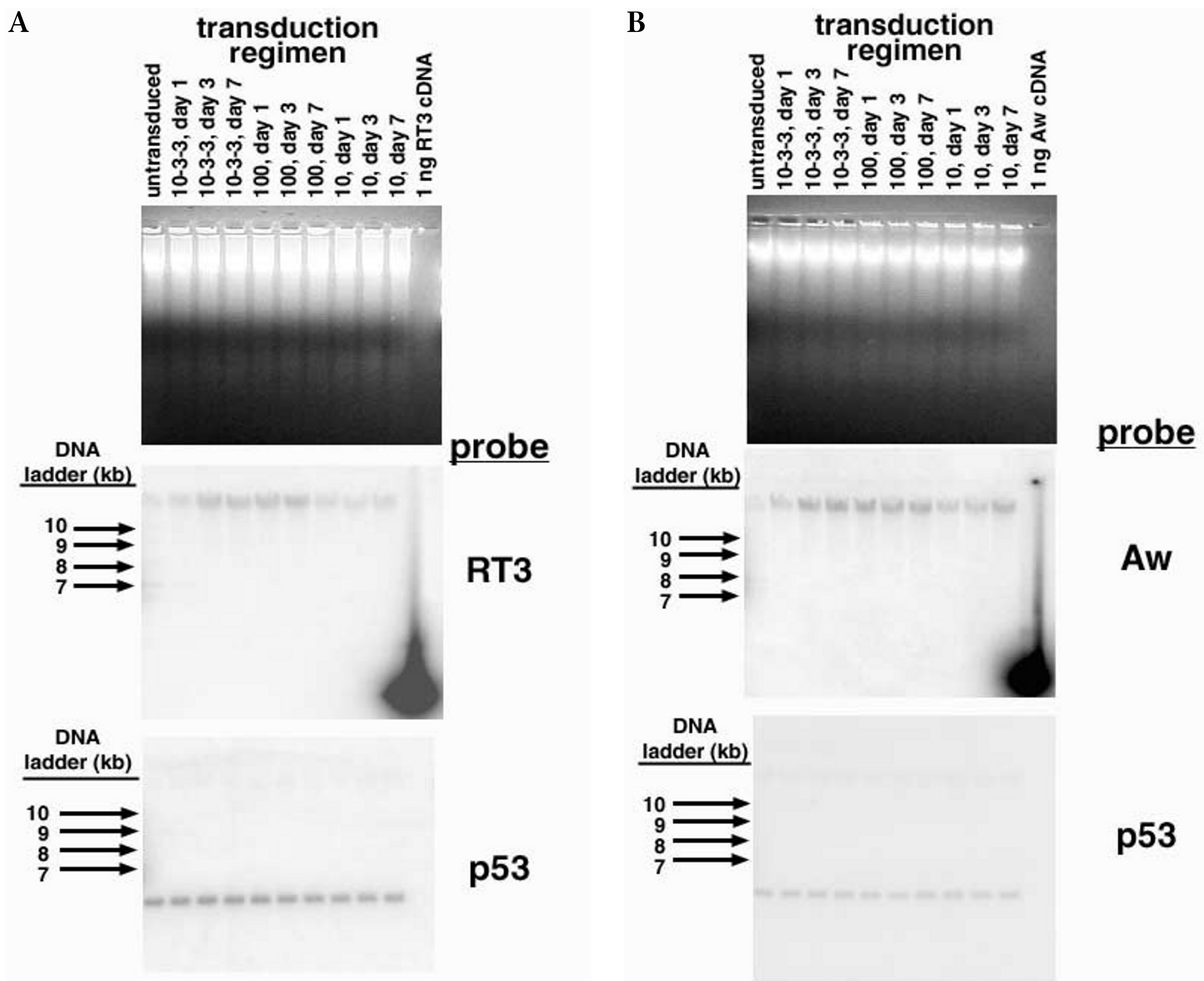

FIG. 7. Quantitating rSV40 integration: Southern blot analysis of genomic vector DNA as a function of time after transduction, using different transduction moi. SupT1 cells were treated with (A) SV(RT3) or (B) SV(Aw), according to one of three different protocols: MOI = 10, treatment once (designated 10 ×1); MOI = 10, 3, and 3, for treatments on 3 consecutive days (designated 10,3,3); MOI = 100, treatment once (designated 100 ×1). Control cells were mock-transduced. DNA was harvested from 107 cells 1, 3, and 7 days thereafter, and digested with *Sal*I, which does not cut either rSV40 genome. 20 μg of DNA was electrophoresed per lane. The top frame for each figure shows the ethidium bromide-stained gel before transfer. After Southern blot transfer and hybridization with a transgene-specific cDNA probe, and visualization and quantitation using a Phosphoimager (middle frame), the filter was stripped and reprobed with a genomic probe for human p53 (lower frame). Binding of the radiolabeled probe was visualized and quantitated as indicated. The locations of the molecular size markers are indicated on the side. (The SV(RT3) and SV(Aw) genomes are ~ 4.5 kb.) These data are representative of three independent experiments.

Direct Demonstration of Integration: Quantitating Viral Integrands

We measured the numbers of virus genome copies/cell based on quantitative Southern blot hybridization. These studies used HeLa cells treated with SV(RT3), a vector that carries the cDNA encoding single chain Fv antibody (SFv) directed against reverse transcriptase, or SV(Aw) in three different ways: thrice with MOI = 10, 3, and 3, for consecutive days, for a total cumulative MOI of 16, once with MOI = 10, or once with MOI = 100. We extracted genomic DNA 1, 3, or 7 days later, restricted it with *Sal*I, which does not cut the viral genomes, electrophoresed, blotted to nitrocellulose, and hybridized with transgene-specific cDNA probes (0.7 kb long), under highly stringent conditions (final wash in 0.1× salt sodium citrate (SSC), 0.1% sodium docecyl sulfate (SDS), at 55°C). This hybridization pattern was a high-molecular weight smear, most of which was greater than 10 kb (the largest molecular size marker used; Fig. 7).

As can be seen, after correction for loading (that is, the strength of the p53 hybridization signal), the strength of the hybridization signal was not greatly different in the DNA preparations from the cells transduced differently

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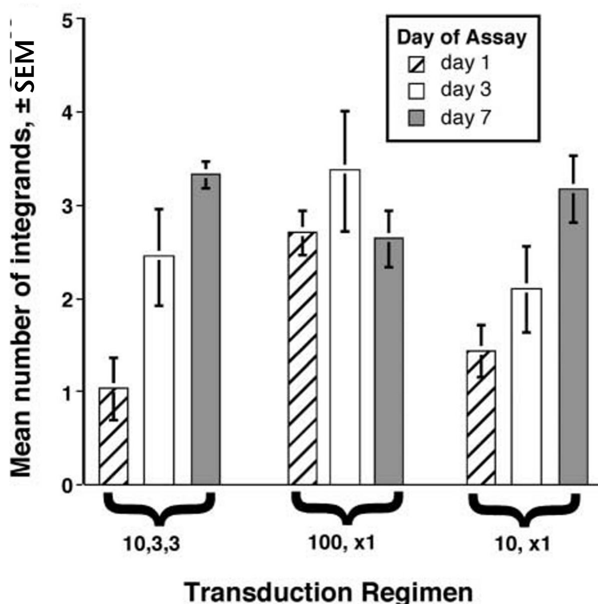

FIG. 8. Quantitating rSV40 integration: measurement of the relative strengths of genomic DNA hybridization to probes for the rSV40 vector and for p53. Ratios of hybridization to the SV(RT3) or SV(Aw) cDNA probes and the genomic p53 DNA probes were determined for each sample shown in Fig. 7, as well as one additional independent study. Background from the mock-transduced cells was subtracted, the difference in specific activity of the two probes accounted for, and the numbers of integrated transgene copies determined for each transduction condition and time point. These data include all data from three independent experiments.

but harvested on the same post-transduction day, but did appear to increase somewhat between days 1 and 3 post-transduction.

To quantitate the number of integrands, we used a phosphoimager to compare total cpm/lane. Accounting for the data generated for two studies of SV(RT3) integration and one study of SV(Aw) integration, correcting for background, activities of the probes, and the fact that there are two alleles of our reference gene, p53, per cell, the average number of copies of the transgenes could be calculated for each assay point, and plotted as a function of both the time of the assay and the transduction technique (Fig. 8). Across the different transduction regimens, increases in integrands between day 1 and day 3, and between day 1 and day 7, were statistically significant ($P = 0.002$ and $P = 0.011$, respectively). There was no significant difference between the numbers of rSV40 integrands on day 3 compared to day 7 ($P = 0.30$), nor was there a difference in the final mean numbers of integrands observed comparing the three different transduction regimens (transduction with MOI = 10, 3, and 3, on consecutive days versus with MOI = 100 on one day, $P = 0.19$; transduction with MOI = 10, 3, and 3 on consecutive days, versus with MOI = 10 on one day, $P = 0.83$; transduction with MOI = 10 on one day versus with MOI = 100 on one day, $P = 0.14$). By 7 days post-transduction, we observed an average of 3.05 ± 0.16 copies of rSV40/cell: 3.33, 3.05, and 2.64, respectively, for transduction on 3 consecutive days at MOI = 10, 3, and 3, once at MOI = 10, or once at MOI = 100.

Integration of wild-type SV40 genomes has been reported to occur in tandem, one genome copy adjacent to another. Accordingly, we asked whether rSV40 vectors would integrate in this fashion. We prepared genomic DNA from HeLa cells treated once with SV(RT3) at MOI = 100, then cut it either with *Sal*I, which does not cut SV(RT3), with *Sac*II, which cuts SV(RT3) once, or with a combination of *Sac*II and *Xba*I, each of which cuts SV(RT3) once. We electrophoresed the restricted DNAs, blotted them, and probed filters with a RT3 cDNA probe. If tandem copies of SV(RT3) integrated into cellular DNA, a single band corresponding to the SV(RT3) genome size would be expected. None was seen (Fig. 9). Thus, most integration of SV(RT3) vector genomes occurred singly, rather than in tandem.

DISCUSSION

The object of gene therapy is usually to achieve an effective level of expression of a particular transgene. Some therapeutic situations require that transgene expression be brief, whereas others require that it be enduring. Sometimes transgene expression must be limited to a particular population of cells, whereas in other settings such specificity is either unnecessary or undesirable. Large numbers of cells may have to be transduced for some purposes, whereas other treatments may be effective even if only a small number of cells express the transgene. Often it would be desirable to repeat transduction, for example for dose

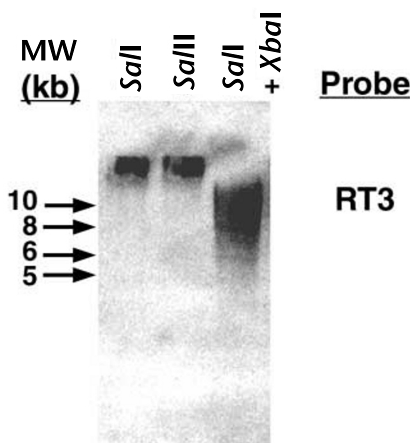

FIG. 9. Lack of detectable tandem integration by rSV40 vectors. DNA samples from SupT1 cells transduced with SV(RT3), as in Fig. 7, were restricted with *Sal*I, which does not cut the SV(RT3) genome at all; *Sac*II, which cuts the SV(RT3) genome once; or a combination of *Xba*I + *Sac*II, each of which cuts the SV(RT3) genome once. DNAs were electrophoresed, blotted, and probed with radiolabeled RT3 cDNA. The locations of the molecular size markers are indicated at the side. The SV(RT3) genomic DNA size is 4.5 kb. The ethidium bromide-stained gel, before transfer, is shown at the right.

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escalation studies or to treat recurrent tumor metastases. The principal limiting determinant of one's ability to meet most of these specifications is the gene delivery system, that is, the vector. Furthermore, the effectiveness of individual vector–transgene combinations for each target cell type and species does not necessarily follow predictable patterns. Therefore, no single vector is suitable for all needs.

Studies to date using replication-crippled recombinant gene delivery vectors derived from *tag*-deleted SV40 have focused on settings requiring long-term transgene expression by large numbers of cells, both resting and dividing. In most of these studies, rSV40 vectors have proven to be quite effective. The experiments reported here tested that conclusion and asked if it coincided with an ability of rSV40 DNAs to insert into the cellular genome.

Previous work suggested that rSV40 gene delivery could result in long-term transgene expression. For example, transplanting rSV40-transduced bone marrow cells into irradiated compatible recipients resulted in continuous transgene expression in large percentages of bone marrow and peripheral blood cells in both rodents [11] and non-human primates (D.S.S. and Robert Andrews, University of Washington, unpublished data) for many months following transplantation. The durability of transgene expression has not been limited to cycling cells; transduction of the liver led to stable and permanent correction of a metabolic abnormality [12] and provided long-lasting serum levels of transgene products (M.A.Z. and D.S.S., unpublished data).

The current studies evaluated the possibility that rSV40 vector insertion into the cellular genome could explain these observations. We also sought to extend these earlier observations using test systems designed specifically to assess the longevity of transgene expression in cycling cells *in vitro* and *in vivo*. Immunological analysis, both by direct visualization and by flow cytometry, confirmed that virtually all progeny of rapidly cycling cells lines transduced with SV(Aw) continued to express the transgene product when tested after up to 4 months of continuous cell culture. When we transduced quiescent livers *in vivo* with SV(AT), and either left them untouched or stimulated their hepatocytes to divide by partial hepatectomy, transgene expression in the regenerated liver was comparable to that seen in the specimens obtained during partial hepatectomy or in controls that were transduced but did not thereafter undergo partial hepatectomy.

These data together strongly suggested that rSV40 vectors integrate and that they do so in both cycling and non-cycling cell populations, both *in vitro* and *in vivo*. We then tested directly whether rSV40 DNAs were incorporated into cellular DNA. PCR analyses of genomic DNA in a transduced lymphocyte line yielded the predicted PCR product. That vector DNA inserted into the cell genome was demonstrated directly by two types of studies. Southern blot analysis of restricted and unrestricted high-molecular weight DNAs from cells transduced *in vitro* showed that almost all vector DNA was incorporated into chromosomal DNA within a few days of delivery. The detection of very small amounts of unintegrated vector DNA underscores the fact that practically all SV(BUGT) DNA integrated into the genome. Comparable Southern blot analyses performed on restricted DNA from livers transduced *in vivo* yielded a similar interpretation: rapid incorporation of rSV40 DNA into high-molecular weight cellular DNA (J.R.C. and D.S.S., unpublished data). A degree of incomplete digestion of the DNA notwithstanding, there is no other explanation for the observed release of SV(BUGT) restriction fragments from the genomic DNA, as seen in the smear obtained using *Not*I and in the patterns and sizes of bands obtained with *Not*I + *Eco*RI, other than that SV(BUGT) DNA integrated into the cellular genome.

The sequencing of a large number of rSV40 integration points further substantiated this conclusion. These studies documented rSV40 incorporation into genomic DNA definitively, and also provided insight into the nature and practical implications of rSV40 vector integration. Cellular sequences at integration sites were all different. In addition, all of the viral sequences adjoining the cellular DNA were also different at all integration sites. These observations for rSV40 gene delivery vectors are consistent with many previously reports that characterized the integration of wtSV40 into the cellular genome: there is no favored genomic site; there is no particular site where the circular SV40 genome opens when it integrates; and there is no clear or strong homology or pattern of similarity between SV40 and cellular DNA sequences at the integration sites [13–15]. In contrast to most [16,17], but not all [18], studies of wild-type SV40 integration, however, we have found that many integrands contain only a part of the rSV40 genome.

The potential impact of the random opening of the virus genome in integrating was also noted. That is, some selected integrands showed interruption of the promoter. Others showed interruption of the transgene. The possibility of such an event is likely to be proportional to the percentage of the rSV40 genome occupied by the DNAs of the promoter + transgene. Transduction efficiency for very large transgenes (compared to vector genome size) should be lower than for smaller ones. We have studied rSV40 transduction of Fanconi anemia complementation group A (*FANCA*) cDNA (5 kb) and found it to be inefficient and incapable of rectifying the functional defect in *FANCA*$^{-/-}$ cells (unpublished data). By contrast, we have been able to express the full length 4.2-kb cDNA for human cystic fibrosis transmembrane conductance regulator (*CFTR*) in greater than 98% of a transduced *CFTR* negative cell line, and to restore the Cl^- channel defect (F.B. and D.S.S., manuscript in preparation).

We have also quantitated rSV40 integrands in transduced cells. On the average, transducing cells at an MOI of 16 over three days, or an MOI of 10 or 100 at one time,

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resulted in long-term transgene expression in almost all cells and an average number of integrands/cell of 3.05 ± 0.16. In those studies, the p53 gene was used as a quantitation control. The single-copy gene p53 has two alleles and known restriction digestion patterns. High-stringency hybridization assured specificity of hybridization.

Southern blot hybridization patterns for genomic DNA from SV(RT3)-transduced cells were identical whether the DNA was restricted with *Sal*I or *Sac*II. The former enzyme does not cut the SV(RT3) genome, whereas the latter cuts it once. The facts that hybridization with the RT3 probe was principally at very high molecular sizes in both cases, and that we saw no band corresponding to the SV(RT3) genome, indicate that concatamer formation and tandem integration do not occur detectably in this system. A weak band is present in the *Sac*II–*Xba*I digestion, of a size close to the whole genome size, and reflecting the predicted restriction fragment from these digestions. The preponderance of larger molecular sizes probably reflects the high likelihood that the vector genome does not integrate completely in many integrands.

The observation that transduction with MOIs of 10 or 100 once, or MOI of 16 over 3 days yielded similar numbers of integrands/cell may reflect a limited ability of rSV40s to enter target cells or, alternatively, saturable integration mechanism(s). If cells receive very large amounts of an rSV40 vector at once, or within a short time frame, we usually do not see higher levels of transgene expression, as compared to the same cells treated simply with enough vector to transduce every cell (~ MOI = 10, in our experiment). By contrast, if we treat the same culture with two different rSV40 vectors 1 week apart, greater than 98% of cells express both transgenes [19]. These observations suggest that there is a mechanism that limits the number of rSV40 particles that can effectively transduce cells in a short time frame. Thus, only limited integrations may occur during a short time span (that is, 1 to 3 days). Additional subsequent integrations may require longer intervals between treatments.

The mechanism by which SV40 inserts into cellular DNA is unclear. Wild-type SV40 generally integrates infrequently; when it can complete a lytic cycle it does so. It encodes only six proteins [20], none of which is known to function as an integrase. The rSV40s used in these studies cannot replicate and carry only the late (largely structural) genes of the virus. These genes are not transcribed in the absence of Tag [21].

SV40 DNA is reported to associate with nuclear matrix [22]. This association may bring it into close apposition with cellular chromosomal DNA. Tag protein that is produced by the virus after infection appears to mediate much of this association [23,24]. That mechanism of association with matrix is obviously not available to *tag*-deleted rSV40s described here. A recent report notes that the SV40 origin of replication (ori) also associates with nuclear matrix [25]. Such an ori–nuclear matrix protein interaction may mediate a close approximation of rSV40 genomes to cellular DNA. Nuclear matrix binds to actively transcribed chromatin [26,27], which may be important in the ability of these rSV40 vectors to integrate into nondividing cells.

Other authors, using different vector systems, implicate the cytomegalovirus immediate early promoter (CMV-IEP) in waning transgene expression [28,29]. Diminished transgene expression with time may be associated with promoter methylation, histone deacetylation, and other factors [30,31]. The longevity and continuity of transgene expression delivered by these rSV40 vectors, almost all of which use CMV-IEP to drive transgene expression, may also reflect in part an association with the nuclear matrix. Histone acetylases, which are important facilitators of transcription, bind to the nuclear matrix [32]. This association has been suggested to play a role in the correlation between the sites of chromosomal attachment of nuclear matrix and some genomic transcriptional activity [33].

Much work remains to be done before we understand the mechanism of rSV40 integration. However, it is now clear that these vectors do integrate and that they provide long-term transgene expression *in vitro* and *in vivo*, in cycling cells and in resting cells. These characteristics of rSV40s are potentially useful features for a gene delivery vector and make rSV40s attractive vehicles for many gene therapeutic purposes.

MATERIALS AND METHODS

Cell lines. We obtained SupT1 and AA2 lymphocyte cell lines from the NIH AIDS Research Reference Repository Program, and cultured them in RPMI-1640 (GIBCO-BRL) supplemented with 10% fetal bovine serum (FBS, Hyclone), penicillin and streptomycin, and glutamine. Under these culture conditions, these cells divide continuously, and must be split approximately twice weekly at ~ 1:10 dilutions in order to maintain uniform cell density. Thus, the approximate doubling time of SupT1 cells in culture is 24–36 hours. COS-7 and HeLa cells were from American Type Culture Collection (ATCC, Bethesda, MD). TC7 monkey kidney cells were the gift of Janet S. Butel (Baylor College of Medicine). We cultured the latter three cell lines in Dulbecco's modified Eagle's medium (DMEM), supplemented as described.

Source and preparation of peripheral blood lymphocytes. We used venous blood from HIV-1 negative individuals without identifiers from the Thomas Jefferson University blood donor center. Peripheral blood mononuclear cells (PBMC) prepared by Histopaque-1077 density separation (Sigma) grew overnight in complete RPMI. After separation from plastic adherent mononuclear cells and macrophages PBMC cell preparations were enriched in PBLs. For simplicity, these adherent cell-depleted PBMC preparations are called PBLs. We maintained these PBLs in complete RPMI in the presence of natural human purified IL-2 (20 units/ml, GIBCO) at 37°C in a humidified CO_2 incubator. Under these culture conditions, PBL cultures must be split approximately 1:4 every 3–4 days to maintain cell density.

Animals. Transgenic mice expressing mutant human α_1-anti-trypsin were a gift from Strategene Corp. (LaJolla, CA). They were housed at the Thomas Jefferson University (TJU) Division of Animal Resources, in accordance with IACUC standards.

Infusion of recombinant SV40-derived viruses into mice and Gunn rats. In rats under ether anesthesia, we exposed the hepatic portal vein by a midline abdominal incision, inserted a polyethylene catheter (Helix Medical, Inc., Carpinteria, CA) into the portal vein and fixed it in position using cyanoacrylate gel. The proximal end of the catheter was tunneled to

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a subcutaneous location and filled with heparin before the wound was closed. We used this catheter to administer single or repeated doses of rSV40 vectors.

Recombinant SV40-derived vectors (rSV40) used. We used four different rSV40 vectors, constructed as described [34] from cloned SV40 genomes from which the *tag* gene was deleted. SV(nef-FLAG) carries the HIV-1 protein Nef, to which a CO2-terminal FLAG epitope tag was added. SV(AT) carries a cDNA encoding wild-type human α1-anti-trypsin. Both of these constructs use the cytomegalovirus immediate early promoter (CMV-IEP) [35]. SV(Aw) and SV(RT3) carry the cDNAs encoding single chain Fv antibodies (SFv) that are directed against HIV-1 integrase and reverse transcriptase, respectively. The latter construct also carries a carboxyl-terminal FLAG epitope tag [19,36]. Expression of both SFvs is driven by CMV-IEP. SV(BUGT) carries the cDNA for human bilirubin-uridine 5′-diphosphate-glucuronysyl transferase (BUGT), driven by two tandem SV40 early promoters [SV40-EP, 12].

Production of rSV40s. We have reported the general principles for making recombinant, replication-defective SV40 viral vectors. Briefly, we excised the virus genome from a carrier plasmid, gel-purified and recircularized it, then transfected it into COS-7 cells. These cells supply Tag protein, which is needed for producing infectious, replication-defective SV40 virus, in *trans* [37]. No helper virus is involved. We prepared crude virus stocks as cell lysates, and band-purified them by discontinuous sucrose density gradient ultracentrifugation [37]. We titered the infectivity of our replication-deficient SV40 viral vector stocks by *in situ* PCR [37]. Infectious titers were generally approximately 10^{11} to 10^{12} infectious units (IU)/ml.

Transduction of human cell lines. We treated SupT1 human T lymphoma cells with rSV40s, for 24 hours at multiplicity of infection (MOI) of 10 [37]. In some studies, we repeated this procedure twice on subsequent days, at MOI = 3. AA2 cells received SV(Aw) for a single exposure, with MOI = 100. We treated HeLa and 293 cells (ATCC) separately using both approaches. All of these studies used unselected cell populations.

Immunostaining for protein expression. Immunostaining to detect intracellularly expressed transgene products within the cells used indirect immunofluorescence. We cultured transduced SupT1 cells after infection with SV40 on glass slides overnight, fixed them with methanol at room temperature for 10 minutes, and blocked overnight with 1% phosphate-buffered saline/bovine serum albumin (PBS/BSA). To detect FLAG epitope on RT3, we incubated slides with rabbit anti-FLAG (Sigma Chemical Co., St. Louis, MO), followed by Cy3-goat-anti-rabbit IgG. To detect Aw SFv, we treated slides with goat anti-mouse IgG (Nordic Immunology, Oslo, Norway), followed by FITC-labeled rabbit anti-goat IgG (Sigma Chemical Co.). Alternatively, direct immunostaining employed FITC-goat-anti-mouse IgG (DAKO, Copenhagen, Denmark). After extensive washing in PBS, we placed coverslips on and analyzed the slides them by epifluorescence microscopy using an Olympus inverted fluorescence microscope with a digital imaging attachment attached to a G3 Power Macintosh computer (Apple Computer, Cupertino, CA).

RNA preparation. We extracted RNA from homogenized liver specimens using Trizol reagent (GIBCO-BRL), according to the manufacturer's instructions, separated it by electrophoresis on formaldehyde gels [38], transferred it to nitrocellulose filters, and probed it using [^{32}P]cDNA probes prepared by random priming [38]. We visualized binding by autoradiography or both visualized and quantitated binding with a Molecular Dynamics Storm 840 phosphoimager.

DNA separation for Southern blot analysis of episomal and genomic transgene. We prepared DNA from liver tissues and from cultured cells by standard techniques [38], separately fractionating high and low molecular weight DNAs by the method of Hirt [39]. We also used the Genomic DNA preparation kit (Promega, Madison, WI) to prepare high-molecular weight DNA from cell cultures. To digest genomic DNA, we used restriction enzymes according to manufacturers' instructions. We performed agarose gel electrophoresis as described [38], with the percentages of agarose varying according to the molecular sizes of the DNAs being separated.

Southern blot transfer used standard protocols [38]. We labeled DNA probes for northern and Southern blot analyses with [α^{32}P]-dCTP by random priming (Boehringer-Mannheim, Ridgfield, CT) according to manufacturer's instructions, and performed hybridization using denatured probes, again according to standard techniques [38]. We washed all blots twice for 30 minutes, under stringent conditions (0.1× SSC, 0.1% SDS, T ϑ 42°C).

PCR analyses. PCR analyses of genomic DNAs from transduced cells used oligomeric primers specific for a portion of the rSV40 genome, located in the 3′ end of the SV40 VP1 gene and extending beyond the SV40 late polyadenylation signal. We applied standard PCR techniques [40].

Inverted PCR. The principles of inverted PCR have been described [41,42]. We performed this analysis only on liver DNA from SV(BUGT)-transduced cells. Briefly, we prepared genomic DNA from rat livers treated with SV(BUGT) at least 1 month earlier, and restricted the DNA using one of several enzymes that cut once within the vector genome, then religated. We performed PCR on the resulting circular DNAs, using oligonucleotide primers that recognized SV(BUGT) DNA sequences, but pointed in the opposite directions. The pairs of primer binding sites used for each inverted PCR analysis were situated within the SV(BUGT) vector on the same side of the cut site for the enzyme used to digest cellular genomic DNA.

Flow cytometry. We performed flow cytometric analyses of SV(Aw)-transduced and control AA2 and SupT1 cells after alcohol fixation and permeabilization of these cells. We detected SFv expression by direct immunofluorescence staining using FITC-goat anti-mouse IgG (DAKO), and visualized using a Becton-Dickinson (Mountain View, CA) flow cytometer.

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