Transduction of multiple cell types using improved conditions for gene delivery and expression of SV40 pseudovirions packaged in vitro

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This comprehensive study demonstrates highly efficient transduction of a wide variety of human, murine, and monkey cell lines, using a procedure for in vitro packaging of plasmid DNA in recombinant simian virus 40 (SV40) capsid proteins to form pseudovirions. The pseudovirions are encapsidated by the VP1 major capsid protein, with no SV40 sequence requirement, and are able to carry up to 17.7 kb of supercoiled plasmid DNA. We developed a procedure to scale-up production of SV40 pseudovirions, as well as an efficient protocol to concentrate the virions with no loss of activity. We also developed a method that allows transduction of 10 times more cells than the original protocol. This protocol was tested using supercoiled in vitro-packaged plasmid carrying the human multidrug-resistance gene (MDR1 encoding P-glycoprotein; P-gp), or the enhanced green fluorescent protein reporter gene (EGFP) in .45 human lymphoblastoid cells and in K562 human erythroleukemia cells. Multiple transductions at 24-h intervals were shown to increase expression using the EGFP reporter gene. The protocols developed in this study establish in vitro-packaged SV40 pseudovirions as one of the most efficient gene delivery systems.

INTRODUCTION

The polyomavirus simian virus 40 (SV40) has been developed in recent years as a vector for gene transfer (1–8). SV40 virions prepared in a packaging cell line, which harbors the large T-antigen or other SV40 wild-type sequence, have been studied extensively (4–6). However, SV40-based vectors can also be prepared in vitro without helper DNA, from the recombinant SV40 capsid protein VP1 (prepared in Sf9 insect cells) and supercoiled plasmid DNA (produced in bacteria) (1,2). Using the SV40 in vitro packaging system, we have demonstrated efficient delivery of multidrug-resistance transporter genes (such as ABCB1, ABCC1, and ABCG2) and the green fluorescent protein (GFP) gene into human lymphoblastoid, erythroleukemia, and stem cells (7,8). Previously, we showed that the in vitro-packaged vectors can encapsidate and efficiently deliver supercoiled DNA up to 17.7 kb in length (7,8). All types of SV40-based vectors (1–8) have demonstrated highly efficient gene transfer, with almost 100% of transduced cells expressing the transferred gene.

Optimization of gene transfer using large-scale virion production, purification and concentration of the virions, and scaling up the transduction process, have been previously described only in those SV40 virions that harbor some of the SV40 wild-type sequence and that were prepared using a packaging cell line (see References 4–6). In this type of SV40-based virion, these optimizations produce very efficient vectors with excellent potential to deliver genes in vitro as well as in vivo.

For in vitro-packaged SV40 pseudovirions, previous work has been done to optimize reaction conditions and volume as well to define optimal cell number (7,8). Here, we test four new protocols: (i) one for more efficient, scaled-up preparation of the in vitro packaging reaction; (ii) a second for transducing 10 times more cells than was previously possible; (iii) a third for concentrating pseudovirions; and (iv) a fourth for multiple transductions at 24-h intervals. Furthermore, we examine the efficiency and expression of delivery of the SV40 in vitro packaging system for many different cell types—nondividing as well as dividing cells—some of which no other delivery system has been able to transduce.

MATERIALS AND METHODS

Cell Lines and Cell Culture

.45 human lymphoblastoid cells with high levels of major histocompatibility complex class I (MHC I), and K562 human erythroleukemia cells were maintained in RPMI media (Invitrogen, Carlsbad, CA, USA). Lines OCI-Ly3 and OCI-Ly10 [kindly provided by Louis Staudt and Eric Davis, National Cancer Institute, National Institutes of Health (NIH), Bethesda, MD, USA] were maintained in Iscove’s modified essential medium with 55 μM β-mer-
captoethanol, 50 U/mL penicillin, 50 µg/mL streptomycin, and 20% heparin-
zized normal human plasma (Invitrogen). Melanocytes were kindly provided by
Vincent Hearing and Werner Berens (National Cancer Institute, NIH). Pri-
mary keratinocytes, keratinocytes, and SP1, a tumorigenic mouse keratinocyte
cell line, (kindly provided by Stuart Yuspa and Luowei Li, National Can-
cer Institute, NIH) were kept in Eagle’s minimal essential medium (MEM) sup-
plemented with 0.05 mM CaCl₂ and 8% chelexed fetal bovine serum (FBS; Invit-
rogen). Human GM08402 fibroblasts (kindly provided by Curt Harris and
Elisa Spillare, National Cancer Institute, NIH) were maintained in Dulbecco’s
modified Eagle medium (DMEM; Invitrogen). WIF-B cells, polarized liver
derived cells (kindly provided by Irwin Arias and Yoshiyuki Wakabayashi, Na-
tional Institute of Child Health and Human Development, NIH), were grown at
37°C in modified F12 medium (Sigma, St. Louis, MO, USA) supplemented
with 5% FBS and HAT (10 mM hypoxan-thine, 4 mM aminopterin, 1.6 mM
thymidine; Quality Biological, Gaithersburg, MD, USA) as described by Sai
et al. (9). Liver cells, human telomerase reverse transcriptase (hTERT)-immor-
talized cells, were kindly provided by Lyuba Varticovski, National Cancer In-
stitute, NIH. All other media were supple-
temented with 10% FBS (HyClone,
Logan, UT, USA), 5 mM L-glutamine,
50 mg/mL penicillin, and 50 mg/mL
streptomycin (Quality Biological). All
cell lines were kept at 37°C, in 5% CO₂.

**Transduction of Sf9 Cells with
Baculovirus**

A baculovirus construct containing the VP1 gene encoding SV40 capsid
protein (1,2) was added to cells for 1 h
with occasional mixing at a multiplicity of infection (MOI) of 10. Cells were
then resuspended in fresh media. Four or
days later, the cells were harvested.

**Preparation of Nuclear Extracts
from Sf9 Cells**

Extracts were prepared by shaking the nuclei, which were isolated with
10% Nonidet™P-40 (NP40) in a buff-
er containing 20 mM HEPES, pH 7.9
(Cellgro, Herndon, VA, USA), 0.4 M
NaCl, 1 mM EDTA, 1 mM EGTA (all
from Digene, Beltsville, MD, USA),
1 mM dithiothreitol (DTT; rpi, Mt.
Prospect, IL, USA), and 1 mM phenyl-
methylsulfonylfluoride (PMSF; Fluka,
Basel, Switzerland). DTT and PMSF
were added before use (1,2).

**Preparation of In Vitro Packaging
Vectors**

Packaged DNA in this study included the pEGFP-C1 construct (4.7 kb; BD
Biosciences Clontech, Palo Alto, CA,
USA), or the phaMDR1 construct (8).
One hundred micrograms of nuclear ex-
tract from Sf9 cells, together with the
100 µg DNA construct in the presence
of 5 mM ATP, and 8 mM MgCl₂ form
viral-like particles during incubation at
37°C for 6 h in a 600-µL reaction vol-
ume (referred to hereafter as 1 reaction
volume). The reaction tube was then in-
cubated on ice for 1 h with 1 mM CaCl₂
(60 µL). The pEGFP-C1 construct was
also packaged using scaled-up produc-
tion methods. This involved mixing of
all the components of 20 reactions in
a 15-µL tube and separating the mix-
ture into 20 individual microcentrifuge
tubes before the 6-h incubation period.

**Comparison of Transduction Methods**

Cells were transduced in suspension with volumes of 660 µL in vitro-
packaged SV40 vectors in 10 tubes
(10⁵ cells each), in 60- and 100-mm
culture dishes, and in T-25 flasks (10⁸
cells in each). The dishes and flasks,
which include a volume of 1 mL cells
in medium and 660 µL of in vitro
packaging reaction—a total volume of
1.66 mL, were then placed on a rotary
shaker at 30 rpm for 2.5 h (at 37°C in
5% CO₂), after which 3.5 mL RPMI
medium, supplemented with 10%
FBS, 5 mM L-glutamine, 50 mg/mL
penicillin, and 50 mg/mL streptomycin
were added. Every in vitro packaging
transduction experiment was done 3–6
times, and all the results were repro-
ducible. Transduction was done in .45
and K562 cells using enhanced green
fluorescent protein (EGFP) or a plas-
mid DNA (pHaMDR1) carrying the
multidrug resistance gene (MDR1), as
described previously (8).

**EGFP Gene Expression Detection**

One to seven days posttransduc-
tion with EGFP-C1, 2 × 10⁵ cells
were washed and suspended in 200 µL
phosphate-buffered saline (PBS; Invit-
rogen), 0.1% bovine serum albumin
(BSA; Sigma) at 4°C, and analyzed by
fluorescence-activated cell sorter (FACS;
FL1) for EGFP as previously described
(10). The FL1 wavelength was used on
the FACS machine, and the window
placement was selected for each cell line. Cells only were set on the left side of the logarithmic scale, and any treated cells were
measured using these settings. The same
settings were kept in different experiments.

**Rhodamine-123 Dye Exclusion Assay**

Transduced cells (2 × 10⁵) were incu-
bated with 150 ng/mL rhodamine-
123 for 20 min at 37°C and then washed
and allowed to efflux the dye for 2 h at
37°C. As a control for this assay, we
used untransduced cells. Immediately
thereafter, the cells were analyzed by
FACS using CellQuest™ software (BD
Biosciences Immunocytometry Sys-
tems, San Jose, CA, USA).

**Concentration of In Vitro Packaging Vectors**

Using the microconcentrator filtering
system (Centricon® centrifugal filter de-
vices for volumes up to 2 mL, YM-100
MW membranes; Millipore, Bedford,
MA, USA), the in vitro-packaged vec-
tors were concentrated from 1, 2, and
10 reaction volumes to 100 µL, 400 µL,
and 1.5 mL, respectively. Once concen-
trated, they were used to transduce .45
cells. Both the concentrate and the fil-
trate were used to transduce cells.

**Multiple Transductions of .45 Cells**

In vitro-packaged EGFP was also used
for multiple transductions of the
.45 cell line. Twenty-four and forty-
eight hours after the initial transduc-
tion, the same number of cells was re-
transduced with the packaged vectors.
Aside from the multiple transductions, a
sample of cells was tested for EGFP
expression every 24 h and analyzed by
FACS using CellQuest software.
RESULTS

More Efficient Preparation of the In Vitro Packaging Reaction

Previous methods of producing SV40 in vitro packaging vectors required mixing all the components, such as the DNA, Mg<sup>2+</sup>, Ca<sup>2+</sup>, ATP, and VP1 nuclear extract, in individual microcentrifuge tubes before incubating them for 6 h in a 37°C water bath (2). Although this was an effective process, it was cumbersome and time-consuming. Therefore, we tried methods in which the different components were first mixed together in 15-mL tubes before separating the mixture into 5, 10, 15, or 20 individual microcentrifuge tubes. This new altered method of mixing in one tube before separating into separate ones not only led to the same or better expression than the original method (data not shown), but it was faster and increased the homogeneity of the final SV40 in vitro-packaged vectors. Incubation in the 37°C water bath of larger volumes in the 15-mL tubes was also tested, but did not result in efficient in vitro packaging (results not shown).

High Efficiency Transduction with SV40 Pseudovirions Using a Scaled-Up Protocol

Our goal was to scale-up the transduction without loss of efficiency. The original SV40 in vitro-packaged EGFP transduction protocol for both .45 cells and K562 cells used a series of transduction experiments that used 10 tubes with 10⁴ cells in each. In order to scale-up the transduction procedure, we compared 10 tubes with 10⁴ cells in each, with 60- and 100-mm culture dishes and T-25 and T-75 flasks with 10⁵ cells in each. The dishes and the flasks were placed in the incubator for 2.5 h on a rotary shaker (30 rpm), whereas the tubes were placed in the incubator and vigorously shaken every 15 min for a period of 2.5 h. After the transduction with the 10 tubes was completed, the tubes were pooled together into one flask for analysis 24 h to 4 days posttransduction. FACS analysis indicated that better expression was observed for 10⁵ of either .45 cells and K562 cells in the experiments conducted in the 60-mm dishes rather than in the tubes or the T-25 flasks (Figure 1A for .45 and Figure 1B for K562). The experiment also confirms that transduction with SV40 pseudovirions is close to 100% efficient. For the .45 cell line, the T-75 flasks and the 100-mm dishes had less expression than in the tubes (results not shown for T-75 flasks and 100-mm dishes). FACS results of GFP expression revealed the median fluorescence intensity (arbitrary units) to be 24 for control-transduced cells, 56 for 10⁴ cells transduced in tubes, 68 for 10⁵ cells transduced in 60-mm dishes, and 54 for 10⁵ cells transduced in T-25 flasks. The controls for both cell lines were tested under all the various conditions. Since they were all the same, their results were superimposed on each other. Comparisons of dishes, flasks, and tubes were also done with .45 cells transduced with the pHaMDR1 plasmid DNA packaged in vitro. Dishes (60-mm) carrying 10⁵ cells in each had similar efficiency compared to the original tube protocol. FACS results of MDR1 efflux using the rhodamine-123 efflux assay (in which the lower the expression of the drug efflux pump, the higher the level of fluorescent substrate retained in the cell) revealed that the median fluorescence intensity (arbitrary units) of control transduced cells was 543; for 10⁴ cells transduced in tubes, the value was 382, and for 10⁵ cells transduced in 60-mm dishes, the intensity was 403.

Concentration of In Vitro-Packaged Reactions

The traditional size of an SV40 in vitro-packaged vector reaction is 660 μL, which includes 100 μg nuclear extract and 100 μg of supercoiled plasmid DNA.

![Figure 1. Enhanced green fluorescent protein (EGFP) expression in cells transduced by the standard or scaled-up protocol. (A) .45 cells and (B) K562 cells were transduced with simian virus 40 (SV40) in vitro-packaged EGFP (IVP-EGFP). EGFP gene expression was measured using fluorescence-activated cell sorter (FACS) analysis at 4 days posttransduction. The control set of experiments (mock cells) were DNA only, empty vectors, and cells only and yielded essentially identical results. These results were superimposed and are depicted as one single curve in both panels. The other curves are labeled and indicate 10⁴ IVP-EGFP-transduced cells in test tubes, 10⁵ IVP-EGFP-transduced cells in a 60-mm dish, and 10⁵ IVP-EGFP-transduced cells in a T-25 flask.](image-url)
Previous studies in which the quantity of water during the pseudovirion preparation was lowered led to less expression (data not shown). Thus, we instead investigated the effects of concentrating the pseudovirions after the in vitro packaging reaction. After using the microconcentrator filtering system, both the concentrate and filtrate were set aside and used to transduce cells, with the concentrate yielding better expression than the filtrate and comparable expression in vitro to that of the original volume (660 μL), unconcentrated, as seen in Figure 2. Using two original concentrated volumes of in vitro vectors revealed better expression than one, but not twice as much (data not shown). We conclude that concentration of the in vitro packaging system does not increase specific activity of transduction, but the same transduction activity can be achieved using smaller reaction volumes.

Transduction of a Variety of Cell Lines Using SV40 Vectors

Although the majority of the experiments discussed in this paper were conducted using the .45 human lymphoblastoid cell line, we also tested several cell lines with packaged EGFP to ascertain the variety of cell types that can be transduced with SV40 in vitro-packaged vectors. As shown in Figure 3, we observed high expression in human GM08402 fibroblasts, WIF-B liver cells, and mouse keratinocytes. Ly3 lymphocytes, Ly10 lymphocytes, and HHT liver cells revealed slightly less expression, even though the transduction efficiency was very high as observed by the shift in the curves between the control cells and the transduced cells. Primary keratinocytes along with S1 cells did not show any expression. Except for the mouse S1 cells, all of the FACS plots shown here were obtained 2 days posttransduction of one reaction volume of in vitro-packaged EGFP to 10^5 cells. For mouse S1 cells, 0.25 reaction volume was used for 10^5 cells, and FACS analysis was performed 7 days posttransduction. In all cases, cells were split or plated 1 day prior to transduction.

We compared the transduction efficiency with another nonviral delivery system (lipofection) for these cell lines. Figure 4 compares two cell lines that are very difficult to transduce, the melanocytes and the keratinocytes. Efficiency is much higher in cells transduced by the SV40-packaged vectors than in the lipofectin-transfected cells, and expression levels on average are higher in the melanocytes.

Multiple Transductions to Increase Gene Expression

To determine if multiple transductions would increase the EGFP expression in the .45 cell line, we retransduced the same number of cells

Figure 2. Enhanced green fluorescent protein (EGFP) expression of concentrated packaged virions. Results were recorded 24 h after transduction. Curves are labeled and indicate control, non-infected cells, in vitro-packaged EGFP (IVP-EGFP)-infected cells, IVP-EGFP-infected cells with the concentrate, and IVP-EGFP-infected cells with the filtrate.
(10^5), 24, 48, and 72 h after the initial transduction with the packaged vectors. The remaining portion was used for FACS analysis. These steps were repeated until three transductions had occurred. As seen in Figure 5, the primary transduction had less expression at 4 days than after the second, third, and fourth transductions at 72, 48, and 24 h, all of which had similar expression levels. FACS results of GFP expression revealed the median fluorescence intensity (arbitrary units) of control-transduced cells as 12, the first transduction as 30, the second transduction as 63, the third transduction as 58, and the fourth transduction as 51.

**DISCUSSION**

In this paper, we show the ability to transduce with high efficiency a wide variety of cell lines using SV40 pseudovirions, many of which are otherwise difficult to transduce using other delivery systems. We also present improved procedures for in vitro packaging of plasmid DNA and for transduction using the SV40 pseudovirion delivery system. These procedures include a scaled-up procedure for producing SV40 pseudovirions, a protocol to concentrate virion volume, and a method to transduce 10 times more cells than the original transduction protocol.

**Figure 3.** Enhanced green fluorescent protein (EGFP) expression patterns in different adherent cell lines. All the conditions for preparation of cells and transductions are described in Materials and Methods. The panels are labeled with the transduced cell lines, green curves are mock-transduced cells, and blue curves are in vitro-packaged EGFP-transduced cells. hTERT, human telomerase reverse transcriptase.

**Figure 4.** Enhanced green fluorescent protein (EGFP) expression patterns in cells transduced with simian virus 40 (SV40) vectors versus lipofection. Melanocytes and keratinocytes were plated in dishes (10^5 cells/dish) 24 h before transduction with in vitro-packaged EGFP. For transduction, the dishes were placed on a rotary shaker for 2.5 h, and then fresh media was added. They were analyzed for EGFP expression 2 days after transduction. IVP, in vitro-packaged.
Transducing different cell lines and analyzing EGFP expression by FACS showed a full shift in the curve from control cells to the transduced cells, indicating that all of the cells are expressing EGFP. This has been confirmed by confocal microscopy detection of EGFP in individual cells (data not shown). Thus, the cell lines all show high transduction efficiency except primary mouse keratinocytes and S1 cells, which did not have expression. This may be due to the low virion volume that was used for transduction of the S1 cells. Higher virion volumes than those used resulted in low viability of the S1 cells (data not shown). Comparisons with other nonviral delivery systems (such as lipofection) show that the SV40 pseudovirions are able to get into a wider variety of cell lines.

Introducing the EGFP gene on a daily basis led to greater expression than a single transduction of EGFP. These results could be applied in the future to animal studies where there is a need for transductions of packaged genes.

A new set of experiments led to more efficient production and transduction of the SV40 vectors. The scaled-up procedures for packaging allowed for homogenous mixtures of the virions, as well as faster overall production. The ability to concentrate the virion mixture to a smaller volume would facilitate injection in mouse tumors or tail-vein injections. Concentrating the virion mixture led to very similar EGFP expression compared to expression of cells transduced with unconcentrated EGFP. However, we detected a low level of expression in the filtrate, which might be explained by unpackaged DNA or small particles that pass through the filter membrane of the microconcentrator tubes. Unpackaged DNA in the regular procedure is eliminated using DNase I. Using two different cell lines and two different genes, we could demonstrate very efficient transduction of 10 times more cells as compared to the original protocol.

The new protocols examined here for SV40 pseudovirion production and transduction demonstrate the efficiency of this system to deliver genes to various cell lines and provide a much easier to use gene delivery system.

ACKNOWLEDGMENTS

We thank Ariella Oppenheim (The Hebrew University, Hadassah Medical School and Hadassah University Hospital, Jerusalem, Israel) for fruitful collaboration on the SV40 vectors. We are grateful to George Leiman for editorial assistance.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing interests.

REFERENCES


Received 19 March 2004; accepted April 26, 2004.

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