

# The pathway of uptake of SV40 pseudovirions packaged *in vitro*: from MHC class I receptors to the nucleus

## Research Article

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**Key words:** Gene delivery; SV40 *in vitro* packaging; pathway of SV40 pseudovirions; MHC I receptors

**Abbreviations:** 5-Aza-2'-deoxycytidine, (DAC); bovine albumin, (BSA); Brefeldin A, (BFA); central polypurine tract sequence, (cPPT); central polypurine tract, (CPPT); cholera toxin, (CT); Dulbecco's modified Eagle medium, (DMEM); elongation factor 1(EF1); endoplasmic reticulum, (ER); enhanced green fluorescent protein, (EGFP); fetal bovine serum, (FBS); green fluorescent protein, (GFP); multidrug resistance gene, (MDR1); nuclear extracts, (NE); nuclear localization sequences, (NLS); paraformaldehyde, (PFA); phosphate-buffered saline, (PBS); pigment epithelium derived factor, (PEDF); polyethyleneimine, (PEI); polyethyleneglycol, (PEG); Propidium iodide, (PI); Trichostatin A, (TSA); trichostatin A, (TSA)

Received: 14 October 2004; Revised: 27 October 2004

Accepted: 16 November 2004; electronically published: November 2004

## Summary

SV40 vectors packaged *in vitro* are an efficient delivery system *in vitro* and *in vivo* using plasmids up to 17.7 kb, with or without SV40 sequences. Using confocal microscopy, we followed the pathway of SV40 pseudovirions in human lymphoblastoid cells, which are rich in MHC I receptors, using fluorescence-tagged DNA and an antibody against the main capsid protein, VP1. The wild-type SV40 virus as well as the pseudovirions enter the cells after binding to MHC I. However, the MHC I route is not the only way that SV40 pseudovirions enter cells. From the cell surface, the vectors progress through the Golgi to the ER, where they are unpackaged. Only the reporter DNA proceeds to the nucleus; VP1 remains at the ER. Results indicate that some of the reporter DNA, carried by these vectors, is trapped in the ER. Delivery of DNA plasmids which harbor nuclear localization sequences, such as the enhancer of wild-type SV40 or the cPPT sequence from the HIV-1 virus upstream from the GFP cDNA, did not improve GFP expression. However, improved expression from the EGFP reporter gene carried by SV40 vectors was achieved using the histone deacetylase inhibitor, TSA.

## I. Introduction

Packaging of SV40 pseudovirions *in vitro* results in a non-viral delivery system which satisfies the criteria for a successful gene transfer system: high efficiency, short-term expression with no integration, non-immunogenic, and relatively safe (Kimchi-Sarfaty et al, 2004b). The SV40 wild-type virus capsid is composed of three viral proteins: VP1, VP2, and VP3 (Tooze, 1981). The SV40 *in vitro* packaging system uses nuclear extracts from Sf9 cells, transduced with VP1 baculovirus, to form SV40 capsids around any reporter gene up to 17.7 kb in length.

The efficiency of the system is very high, as almost every cell is transduced. The expression is transient, and relatively low compared to retroviral transduction (Kimchi-Sarfaty et al, 2003). SV40 pseudovirions can deliver DNA plasmids to a variety of cell lines (non-dividing as well as cycling cells), and appear to be non-immunogenic. SV40 pseudovirion vectors very efficiently deliver reporter genes such as green fluorescent protein (GFP), ABC transporter genes such as the multidrug resistance gene (MDR1), a suicide gene (the *Pseudomonas* *exotoxin*) and antiangiogenic genes (the pigment

epithelium derived factor, *PEDF*) (Kimchi-Sarfaty et al, 2004b). Although the pseudovirions are an excellent vehicle for gene transfer, it is important to understand how DNA packaged in SV40 capsids is delivered to the nucleus in order to improve expression levels.

The entry of wild-type SV40 is thought to begin with the virus binding to major histocompatibility complex class I molecules that cover the cell surface (Norkin, 2001). The virus then enters via caveolin-1-containing vesicles, and is transported to the endoplasmic reticulum (ER). This pathway is similar to that taken by cholera toxin (CT), which enters the Golgi via caveolae and is then transported to the ER (Norkin, 1999, 2001, 2002; Parton and Lindsay, 1999). However, it is possible that this pathway bypasses the Golgi (Pelkmans et al, 2001; Pelkmans and Helenius, 2002). Tsai and colleagues (2003) showed that wild-type SV40 enters the cell using specific gangliosides as receptors. Most other viruses enter through the clathrin-coated, pit-mediated endosomal pathway. Viruses which enter cells by endocytosis generally disassemble in endosomes, where the pH is low. However, since the SV40 wild-type entry pathway does not lead to endosomes (Colomar et al, 1993; Khalili and Stoner, 2001), SV40 disassembly is not dependent on low pH in the endosomal compartment. For a number of years it was believed that SV40 virions enter the nucleus and disassemble there, but more recently it has been shown that disassembly occurs in the ER. However, most of the SV40 wild-type DNA does not enter the nucleus (Parton and Lindsay, 1999; Norkin, 1999, 2001; Khalili and Stoner, 2001; Norkin et al, 2002; Pelkmans et al, 2001, 2002).

Some viral delivery systems overcome low efficiency and expression using viral sequences which can target the nucleus, such as nuclear localization sequences of wild-type SV40 or the cPPT sequence from the HIV-1 virus. In a non-viral delivery system, the addition of polyethyleneimine (PEI) or polyethyleneglycol (PEG) increased delivery, mostly through the cell membrane, but also to the nucleus (Ross and Hui, 1999).

In this study, we examined the pathway of entry of SV40 pseudovirions packaged *in vitro* in human lymphoblastoid cells. We tested different stages of the pathway to find the limiting step responsible for the relatively low expression found with SV40 pseudovirions for gene delivery. Our findings indicate that disassembly of the pseudovirions is not the rate-limiting step for gene expression. We suggest that two steps in the pseudovirion's pathway are rate-limiting: DNA is trapped in the ER so that it does not reach the nucleus, and inefficient transcription from the DNA histone complex.

## II. Materials and methods

### A. Cell lines and cell culture

.45 cells, human lymphoblastoid cells with high levels of MHC I, .221 cells, human lymphoblastoid cells with low MHC I receptors, and K562 human erythroleukemia cells were maintained in RPMI media (Invitrogen, Carlsbad, CA). HeLa cells and the HeLa subclone, KB-3-1 (Akiyama et al, 1985), were maintained in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA). Bone marrow stem cells

from Cambrex (East Rutherford, NJ) were plated in HMSGM medium with 10% FBS from Cambrex, but were grown in DMEM, and were a gift of Louis Scavo, NIDDK, NIH. Mesenchymal stem cells from teeth were grown in MEM (Invitrogen) with 20% fetal bovine serum (FBS) and were a gift of Pamela Robey, NIDCR, NIH. All other media were supplemented with 10% FBS (Hyclone, Logan, UT), 5 mM L-glutamine, 50 µg/ml penicillin, and 50 µg/ml streptomycin (Quality Biological, Gaithersburg, MD). All cell lines were cultured at 37°C, in 5% CO<sub>2</sub>.

### B. Infection of Sf9 cells with baculovirus, preparation of nuclear extracts (NE) from Sf9 cells, and preparation of *in vitro* packaging vectors

Infecting Sf9 cells, preparing NE and preparing *in vitro* packaging vectors were as previously described (Kimchi-Sarfaty et al, 2002, 2003). The nuclear extract contained VP1, one of the four viral late proteins (VP1, VP2, VP3, and agno). Packaged DNA in this study included the pEGFP-C1 construct (4.7Kb; Clontech, Palo Alto, CA), the pLUC construct (6.7Kb, Gene Therapy Systems, Inc., San Diego, CA), and pGeneGrip Fluorescein/ Luciferase (Gene Grip) (6.7Kb; Gene Therapy Systems, Inc., San Diego, CA). *In vitro* vector titers were calculated to be  $5 \times 10^4$ - $5 \times 10^5$  particles per 1 ml, using CMT4 cells as previously described (Sandolan et al, 1997). In all the experiments empty capsids, DNA only, and non-transduced cells were used as controls.

### C. Construction of plasmid DNAs carrying the SV40 enhancer element or the central polypurine tract (cPPT) sequence of HIV-1 as a nuclear localization signal

To compare the effectiveness of the SV40 enhancer sequence in translocating the plasmid, we used the pViro2-GFP/LacZ (InvivoGen, San Diego, CA) plasmid encoding the enhanced green fluorescent protein (EGFP) cDNA under transcriptional control of a human ferritin heavy chain (hFerH) promoter in which the 5'UTR had been replaced by the 5' UTR of mouse elongation factor 1(EF1). This plasmid also contained a 72 bp repeat from the SV40 enhancer upstream from the hFerH promoter to enhance gene expression and nuclear localization of plasmid DNA. For comparison, we constructed a plasmid with a similar backbone but devoid of the SV40 enhancer (pViro2-GFP NLS). To construct pViro2-GFP NLS, we deleted the SV40 enhancer sequence by digesting the pViro2-GFP/LacZ vector plasmid with NotI/PacI restriction enzymes. The *E. coli* origin of replication (pMB1 ori) released from the pViro2-GFP/LacZ plasmid during NotI/PacI digestion (as ~720 bp PacI/PacI fragment) was reinserted into the vector by blunt end ligation. To generate the plasmid containing cPPT, a 118-bp fragment of the central polypurine tract was amplified from plasmid pCMV R 8.91 (Naldini et al, 1996) utilizing the primers cPPT 5'(5'-GCGGGGATCCTTTTAAAAGAAAAGGGGGG-3') and cPPT 3' (5'-GCGGAGATCTAAAATTTTGAATTTTGTGAATTTG-3'), digested with *Bam*HI and *Bgl*III, and inserted at the *Bam*HI site upstream of the internal CMV promoter used to drive the transcription of GFP cDNA in the lentiviral vector plasmid pCS-CG (Miyoshi et al, 1998).

### D. Transduction of .45, .221, and K562 cells with *in vitro*-packaged vectors and transfection of HeLa and KB-3-1 cells with Lipofectamine-Plus

At concentrations indicated in each figure, cells were transduced in suspension with the *in vitro*-packaged SV40

vectors in 10 tubes ( $10^4$  cells each) or in a 60 mm culture dish ( $10^5$  cells in each). The dishes were then placed on an orbital shaker at a constant speed for 2.5 h (at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$ ), after which the infection was stopped by the addition of RPMI medium supplemented as before (Invitrogen, Carlsbad, CA) (Kimchi-Sarfaty et al, 2004a). Every *in vitro* packaging transduction experiment was done 3-6 times, and all the results were comparable. Control transfections of HeLa and KB-3-1 cells (Akiyama et al, 1985) with the plasmid DNAs using lipofectamine-plus were done according to the protocol provided by 'Lipofectamine-Plus' (Invitrogen, Carlsbad, CA) without modification. Every transfection experiment was done 4-6 times, each with a similar resulting pattern.

### **E. GFP and multidrug resistance (MDR1) expression detection**

The GFP reporter gene that was used in this study was EGFP-C1 from Clontech (Palo Alto, CA). Two to forty days post-infection,  $2 \times 10^5$  cells were washed and suspended in 200  $\mu\text{l}$  phosphate-buffered saline (PBS) (Invitrogen, Carlsbad, CA), 0.1% bovine albumin (BSA) (Sigma-Aldrich, St. Louis, MO) at  $4^\circ\text{C}$  and analyzed by FACS (FL1) for GFP as previously described (Cormack et al, 1996) or studied by confocal microscopy (detailed in *Collection of confocal images* below). pHaMDR1 plasmid DNA, 15.2 kb in size, carried the multidrug resistance gene (*MDR1*). Detection of the MDR protein was done using a specific cell surface monoclonal antibody, MRK16, as described previously (Kimchi-Sarfaty et al, 2003).

### **F. Brefeldin A (BFA), 5-Aza-2'-deoxycytidine (DAC), and Trichostatin A (TSA) treatments of .45 human lymphoblastoid and HeLa cells**

BFA, which inhibits transport into the ER from the Golgi, was used at 0.5-2.5  $\mu\text{g}/\text{ml}$  24 hours and 2 hours prior to transduction, at the same time as transduction, and 2 1/2 hours after transduction, to determine whether the pathway of entry of pseudovirions is exclusively through the ER. TSA was added to cells at a concentration of 0.1, 1, 10, 100 and 1000  $\text{ng}/\text{ml}$  prior to transduction. 5-Aza-2'-deoxycytidine (DAC) (Sigma, St. Louis, MO) was added to cells at a concentration of 1-10  $\mu\text{M}$  24-72 hours prior to transduction.

### **G. Preparation of cells for confocal imaging**

Prior to immunostaining and between each immunostaining step, transduced cells were washed twice with PBS (Invitrogen, Carlsbad, CA) supplemented with 0.1% BSA (Sigma-Aldrich, St. Louis, MO). Transduced cells were first fixed for 0.5 h with 4% paraformaldehyde (PFA) (Sigma-Aldrich, St. Louis, MO) or with additional fixation for 0.5 h with 70% ethanol at room temperature. Ethanol fixation could not be performed when it was necessary to observe GFP in cells. The presence of MHC I was detected using FITC - Anti-Human HLA - A,B,C (1:100, Becton, Dickinson, and Co., Franklin Lakes, NJ). The Golgi was detected using monoclonal antibody, #G2404 (Sigma, St. Louis, MO). For ER staining, fixed cells at  $37^\circ\text{C}$  were treated with 10% normal donkey serum (Sigma-Aldrich, St. Louis, MO). Cells were then washed once with PBS / 0.1% BSA and stained with a primary antibody (calregulin, goat, 1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) against the lumen endoplasmic reticulum (also called calreticulin). Primary immunostaining was done with a polyclonal VP1 antiserum (rabbit, 1:40) to detect the presence of the VP1 protein. Following each primary immunostain, cells were washed and incubated with an appropriate secondary immunostain. For VP1, Alexa 568 (red) conjugated goat anti-rabbit IgG (Molecular Probes, Inc., Eugene, OR) and for the ER or the Golgi antibodies,

Alexa 488 (green)-conjugated donkey anti-goat IgG (Molecular Probes, Inc., Eugene) were used as secondary antibodies. All secondary antibodies were used at a dilution of 1:250. In all experiments, cells were stained using a secondary antibody alone to determine non-specific staining. All serum, antiserum, and antibody incubations were performed for 1 h at room temperature. After the last antibody incubation, the cells were washed with PBS/ 0.1% BSA as before, dropped onto lysine-coated microscope slides (Erie Scientific Co., Portsmouth, NH), and allowed to dry. Fluorescent mounting medium (DAKO Corp., Carpinteria, CA) was then used to affix a glass coverslip to the microscope slide, and the slides were stored in the absence of light at  $4^\circ\text{C}$ .

### **H. Propidium iodide (PI) nuclear staining of .45 and KB-3-1 cells for confocal imaging**

KB-3-1 cells were seeded on glass coverslips in wells of a 6-well plate, while .45 cells were grown in suspension in a T-25 flask. .45 cells were transduced with *in vitro*-packaged pGeneGrip Fluorescein/Luciferase (pGeneGrip) (GeneTherapySystems, San Diego, CA) DNA and KB-3-1 cells (Akiyama, 1985) were lipo-transfected with the same construct using the transduction protocols described above. Cells were washed three times with PBS/ 0.1% BSA and then fixed with 70% ethanol for 15 minutes at  $-20^\circ\text{C}$ . Cells were washed again three times in PBS/ 0.1% BSA and then stained for 1 hour at room temperature with 100  $\mu\text{l}$  PI staining solution, which was composed of 5  $\mu\text{l}$  PI stock (100  $\mu\text{g}/\text{mL}$ ), 2  $\mu\text{l}$  of RNase (10mg/ml), and 5 ml of PBS without Ca or Mg. Cells were then washed three times with PBS/ 0.1% BSA. Coverslips with KB-3-1 cells were dried, inverted, and mounted on lysine-coated microscope slides. .45 cells were applied to slides as described above.

### **I. Collection of confocal images**

Confocal fluorescent images were collected with a Bio-Rad MRC 1024 confocal scan head mounted on a Nikon Optihot microscope with a 60X planapochromat lens. Excitation at 488 nm and 568 nm was provided by a krypton-argon laser. Emission filters of 598/40 and 522/32 were used for sequentially collecting red and green fluorescence, respectively, in channel one and two while phase contrast images of the same cell(s) were collected in the third channel using a transmitted light detector. Z-sections were taken at  $\sim 0.7 \mu\text{m}$  intervals at each wavelength, where applicable, and after sequential excitation, red and green fluorescent images of the same cell were merged for colocalization using LaserSharp software (Bio-Rad, Hercules, CA), and animation sequences were produced.

## **III. Results**

### **A. Entry of VP1 does not always correlate with levels of MHC I receptors**

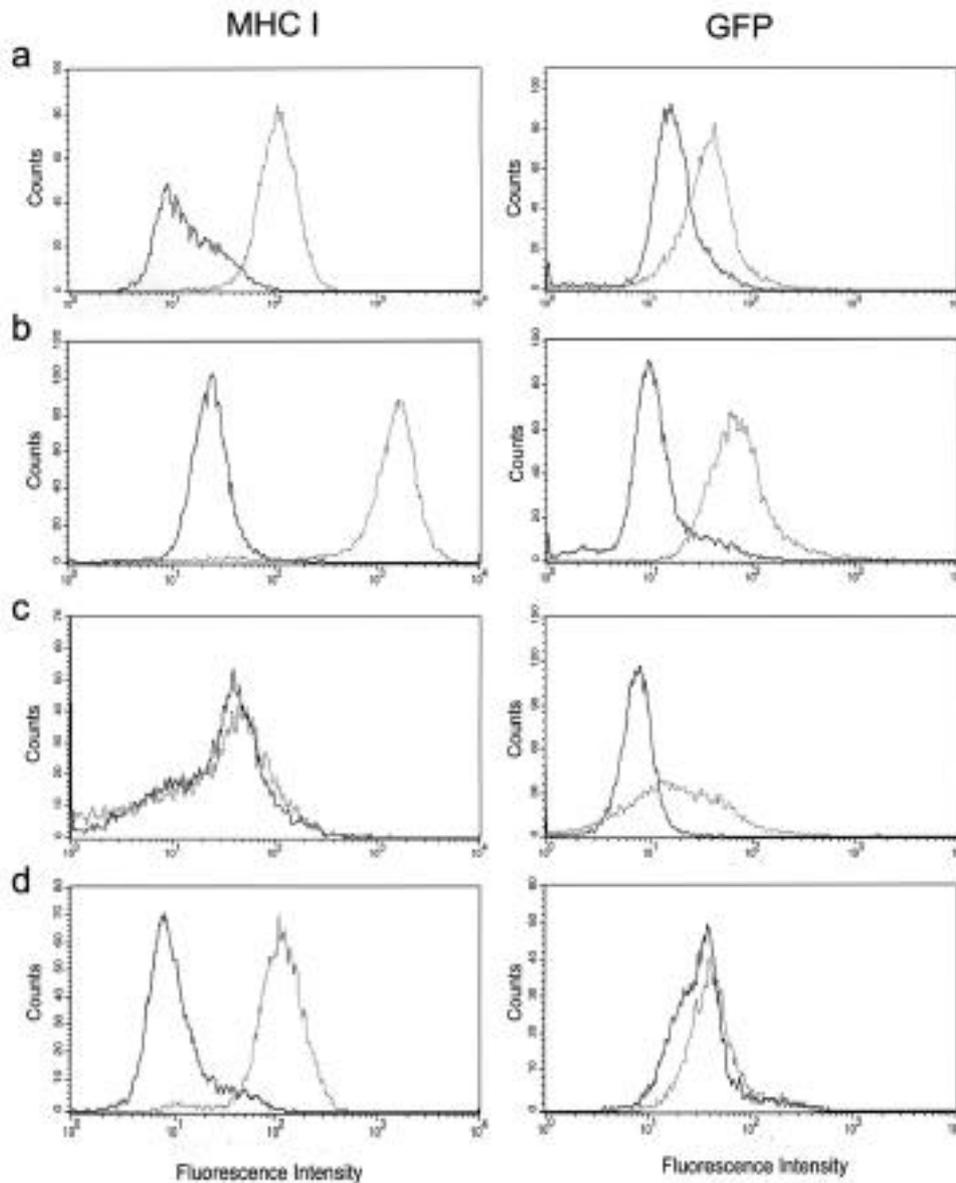
It has previously been shown that SV40 wild-type binds MHC I receptors (Norkin, 1999). We investigated whether the level of MHC I expression is a limiting factor in gene expression in different cell lines, using the SV40-based pseudovirion delivery system after *in vitro* packaging. The results shown here, and our extensive experience with other cell lines (data not shown), do not demonstrate a direct correlation between MHC I levels and GFP expression.

Four different cell lines expressing different levels of MHC I (Figure 1, a,b,c,d left column) as detected by FACS were tested for transduction using GFP DNA

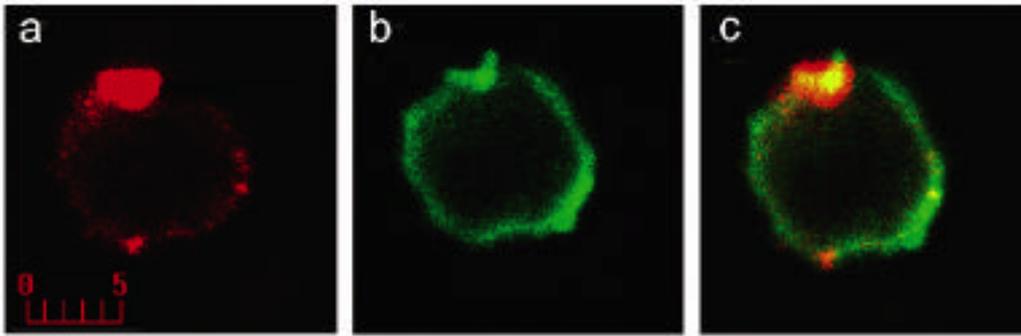
encapsidated in VP1 (right column). We used one full reaction of 660  $\mu$ l (as defined by Kimchi-Sarfaty et al, 2004a), which saturates the cellular receptors (multiplicity of infection of 0.5-5). This is the maximum volume of pseudovirions enough to transduce cells without reducing their viability. As can readily be seen, there was no correlation between the levels of MHC I and GFP expression (compare **Figure 1**, left and right columns). Some cells with high MHC I levels (**Figure 1d**, left column) had little or no GFP expression (**Figure 1d**, right column), while other cells with low MHC I levels (**Figure 1c**, left column) showed strong GFP expression (**Figure 1c**, right column).

To determine if the site of VP1 entry into cells is coincident with the location of MHC I receptors, we stained simultaneously for VP1 and MHC I in the human lymphoblastoid cell line .45, which has high MHC I levels (**Figure 2**). MHC I receptors appeared fairly uniformly around the plasma membrane (panel b), but VP1 (panel a) appeared in scattered locations around the membrane. Some colocalization of VP1 and MHC I is seen (panel c), but it is clear that the presence of MHC I (green fluorescence) does not predict binding of VP1. A similar phenomenon was observed in .221 stained cells although less MHC I staining was observed, it was also not colocalized with VP1 staining (data not shown).

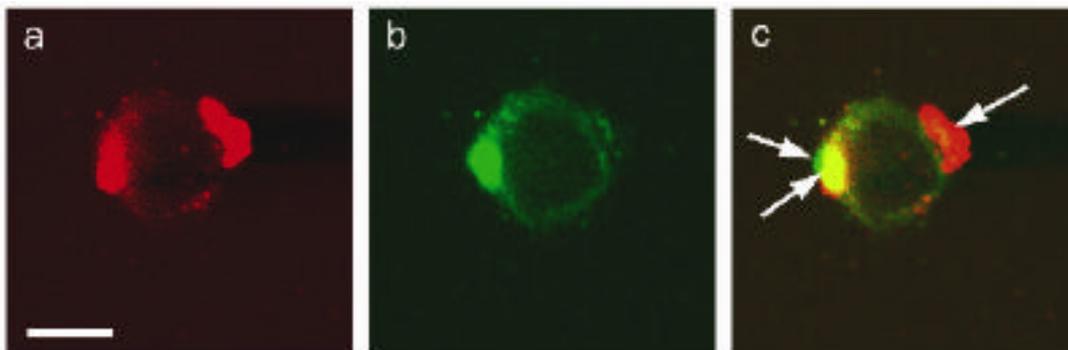
All the experiments in this section were repeated 4 times, and each resulted in a similar pattern of staining.



**Figure 1.** Expression of major histocompatibility complex I (MHC I) receptors and of Green Fluorescent Protein (GFP) using FACS analysis in different cell lines. Cell lines were: H190 stem cells (**a**), .45 human lymphoblastoid cells (**b**), .221 human lymphoblastoid cells (**c**), and Human Mesenchymal Stem Cells (**d**). Cells were tested for their MHC I receptor levels (grey) and background fluorescence was detected using control antibody IGg2a (black) (left column). Expression studies of the EGFP-C1 reporter gene were done using FACS two to four days after transduction (grey) (right column). All control cells, cells transduced with DNA only, mock-transduction without reporter DNA, and untreated cells were tested for GFP expression in the same way as the experimental cells in all the experiments described in this paper (black).



**Figure 2.** Expression of major histocompatibility complex I (MHC I) receptors using confocal microscopy. Immediately after transduction, cells for confocal analysis were fixed as described in the Materials and Methods section, and the following parallel treatments were applied to cells: (1) VP1 polyclonal antibody staining with a secondary Texas Red antibody staining; (2) MHC I antibody staining conjugated to a secondary FITC antibody; (3) VP1 polyclonal antibody staining with its secondary Texas Red antibody, together with MHC I antibody staining conjugated to a secondary FITC antibody. No background was seen in secondary antibody staining only. (a) .45 cell immunostained for SV40 VP1 using rabbit anti-SV40 VP1 using rabbit anti-SV40 polyclonal antiserum, followed by a Alexa-568-conjugated (red) goat anti-rabbit IgG secondary antiserum. (b) .45 cells immunostained for MHC-I receptors using FITC-conjugated to anti-human MHC-I (HLA-A, -B, -C). (c) Merge of panels a and b.



**Figure 3.** VP1 entry relative to Golgi apparatus in .45 cells. The following parallel treatments were applied to cells: (1) Same as in Fig. 2; (2) Monoclonal mouse anti-Golgi 58K protein antiserum staining, followed by a Alexa-488-conjugated (green) goat anti-mouse IgG secondary antiserum staining; (3) VP1 polyclonal antibody staining with its secondary Texas Red antibody, together with monoclonal mouse anti-Golgi 58K protein antiserum staining, with a Alexa-488-conjugated (green) goat anti-mouse IgG secondary antibody. No background was seen in secondary antibody staining only. .45 cells were fixed and immunostained for SV40 VP1 capsid protein using rabbit anti-SV40 polyclonal antiserum, followed by a Alexa-568-conjugated (red) goat anti-rabbit IgG secondary antiserum (a); then cells were immunostained with monoclonal mouse anti-Golgi 58K protein antiserum, followed by a Alexa-488-conjugated (green) goat anti-mouse IgG secondary antiserum (b). Panel c is a merge of panels a and b. Left top white arrow indicates Golgi staining only, Left bottom white arrow indicates costaining of VP1 and Golgi and right white arrow indicate VP1 staining only. Scale bar, 5  $\mu$ m.

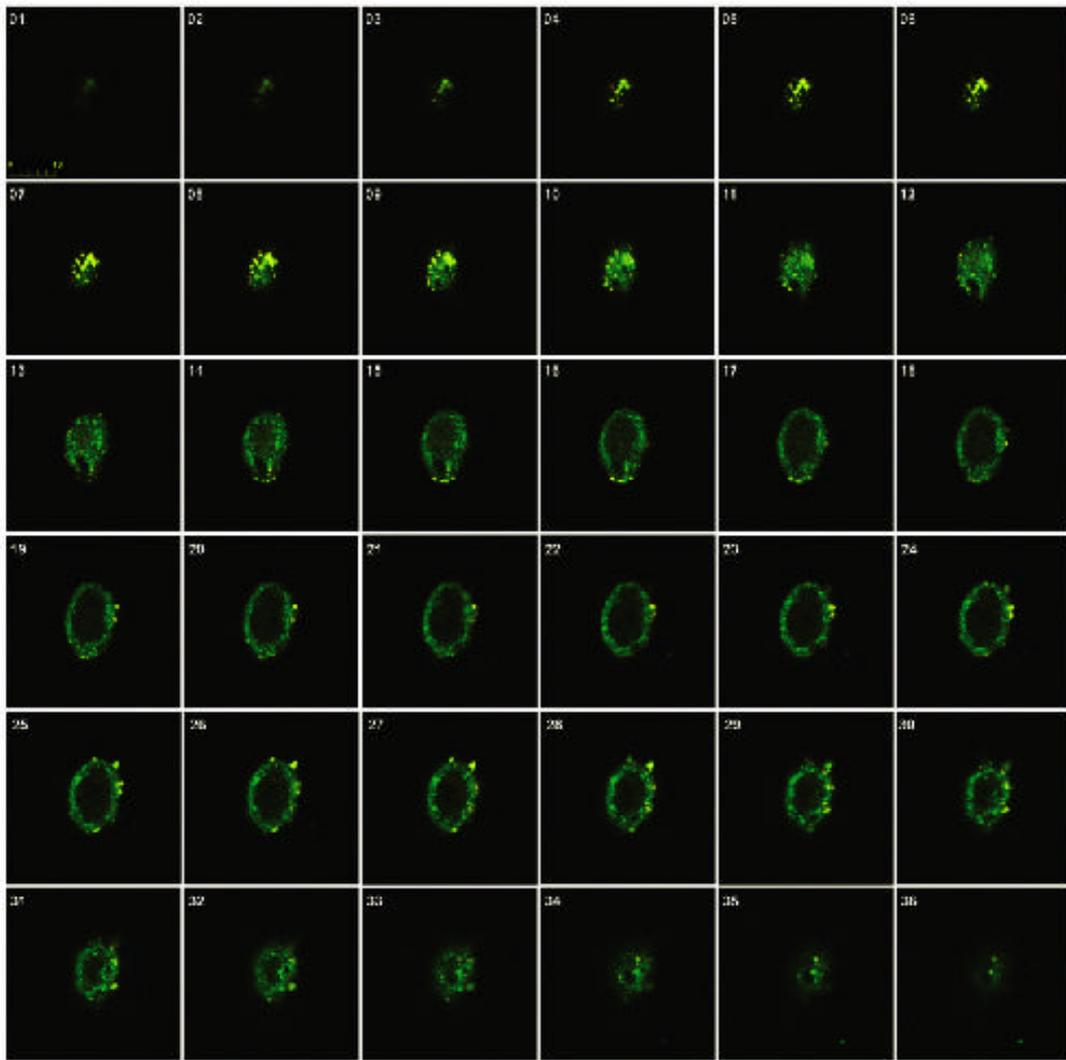
### B. Some of the VP1 capsid protein is localized to the Golgi thirty minutes after transduction

.45 human lymphoblastoid cells ( $10^5$  cells) were transduced with *in vitro*-packaged GFP, and were harvested immediately after transduction, and 10, 30, and 120 minutes later, as described in *Materials and Methods*. **Figure 3** demonstrates partial colocalization of VP1 and the Golgi apparatus 30 minutes after transduction; some of the VP1 (red) sites are costained with the Golgi (green) and appear dark yellow (lower left arrow), while other VP1 are not in the Golgi and appear red (right arrow). Some of the Golgi staining is not covered by VP1 (upper left arrow). The same pattern is seen at the other harvest time-points full colocalization was not found. In 60% of the cells there was no costaining of VP1 and the Golgi, and in 40% there was some colocalization. All these

experiments were repeated five times with comparable results.

### C. Initial colocalization of VP1 with calregulin, an ER marker, 30 minutes after transduction

Thirty minutes after transduction, VP1 staining appears throughout the cell, but not in the nucleus. To verify the location of VP1 staining, .45 human lymphoblastoid cells ( $10^5$  cells) were transduced with *in vitro*-packaged GFP, and were harvested immediately after transduction, and at 10, 30, 120, and 240 minutes, 1, 2, 4, and 7 days later, as described in *Materials and Methods*. **Figure 4** is a panel of Z sections of a cell seen via confocal microscopy, as described in *Materials and Methods*. We demonstrated that in 60% of the cells, 30 minutes after transduction, all VP1 (red) is colocalized to



**Figure 4.** Z stacks of sections of .45 cells stained for VP1 and ER. .45 cells were harvested and fixed at 30 minutes post-transduction before immunostaining. The confocal microscope Z sections were collected at 0.3  $\mu\text{m}$  intervals using sequential excitation for each fluorophore. The following parallel treatments were applied to cells: (1) Same as in Fig. 2; (2) Monoclonal mouse ER lumen protein, calregulin staining, against calreticulin, an ER membrane protein, followed by a Alexa-488-conjugated (green) donkey anti-goat IgG secondary antiserum staining; (3) VP1 polyclonal antibody staining with its secondary Texas Red antibody, together with a monoclonal ER lumen protein, calregulin, with a Alexa-488-conjugated (green) donkey anti-goat IgG secondary antiserum staining. No background was seen with secondary antibody staining only.

the ER (green), where it appears as yellow. The same phenomena is observed in the other 40% of the cells, but later in the time course at the 120-minute time point, we could observe green and yellow staining only. The same pattern of full co-localization is seen at the other harvest time-points beginning at 120 minutes. All these experiments were repeated 6 times, and the results were all similar.

#### **D. EGFP expression is reduced in BFA-treated cells transduced with SV40 *in vitro*-packaged DNA**

Since all VP1 was co-localized with calregulin to the ER lumen, we wanted to determine whether VP1 transit through the ER is essential for gene expression. This was determined by blocking retrograde entry into the ER using BFA, and monitoring the expression of EGFP delivered by

SV40 vectors. .45 cells were treated with different concentrations of BFA (0.5-2.5  $\mu\text{g}/\text{ml}$ ) according to Norkin and colleagues, (2002), 24, and 2 hours before transduction, at the time of transduction, or at the end of the transduction process, when fresh medium is added to the cells. The effect of BFA treatment on GFP expression was monitored in transduced cells at various time points between 0-6 days post-transduction and compared with that of BFA-untreated GFP-transduced cells. We found a reduction in GFP expression in cells treated with 2.5  $\mu\text{g}/\text{ml}$  BFA, but not a complete inhibition of GFP expression. Even very high concentrations of BFA (25  $\mu\text{g}/\text{ml}$ ) did not completely inhibit GFP expression. A concentration of 2.5  $\mu\text{g}/\text{ml}$  led to a 50% reduction in GFP expression on day one. However, 48 hours after transduction, the reduction in GFP expression was less than 20% compared to untreated cells. From day three onward, decreasing the concentration of BFA (from 2.5

$\mu\text{g/ml}$  to  $0.6 \mu\text{g/ml}$ ) actually increased the GFP expression by 100% as compared to untreated cells (data not shown). BFA experiments were repeated 6 times, and the results were comparable in all experiments.

### E. Dissociation of VP1 from fluorescently labeled DNA occurs in the ER 20 hours after transduction

.45 human lymphoblastoid cells ( $10^5$  cells) were transduced with SV40 vectors carrying fluorescent pGeneGrip DNA and were harvested immediately after transduction and at 2.5, 5.5, 10, 20, 30, 120, and 240 minutes, 1, 2, 4, and 7 days later, as described in *Materials and Methods*. Several investigators (Oppenheim et al, 1986; Oppenheim and Peleg, 1989; Dalyot-Herman et al, 1999; Strayer, 1999, 2000; Strayer and Zern, 1999; Kimchi-Sarfaty et al, 2002, 2003) have determined that all types of SV40 delivery systems are able to deliver DNA which is expressed in virtually all cells of many different cell types that have been tested. In the present study, using confocal microscopy to detect fluorescent-tagged DNA, every .45 cell contains a label 3.5 hours after transduction. These results clearly indicate that entry into cells is a very efficient process using VP1 only for encapsidation in the SV40-based delivery system.

Cells shown in **Figure 5a** are 5.5 hours post transduction. The yellow staining clearly shows that VP1 (red) and the green Grip signal are co-localized, which suggests that disassembly has not yet occurred at this time point. However, 20 hours post-transduction (**Figure 5b**), some of the red and the green staining is no longer co-localized, which indicates that disassembly has occurred and the DNA is no longer trapped within the VP1. 50 randomly chosen cells were examined thoroughly for each time point.

### F. Twenty hours post-transduction and thereafter, Grip-DNA can be visualized in the nucleus

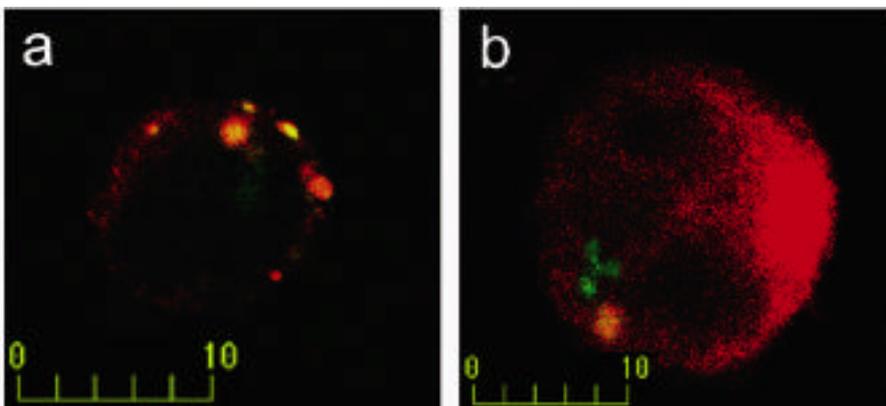
Does disassociation of the DNA from VP1, starting at 20 hours post-transduction, enable it to enter the nucleus? .45 cells were harvested 2.5, 4, 5, 20, 24, 26, 28 hours after transduction. Grip-DNA and confocal sections of cells were used to distinguish whether the green signal

was in the nucleus or just close to it. **Figure 6a-c** demonstrates 3 stages of entry of the Grip-DNA into the nucleus, taken from the animated movies, 4.5, 22.5, and 53 hours post-transduction. By 53 hours (**Figure 6c**), the DNA (green) appears to be in the nucleus, stained with propidium iodide (red).

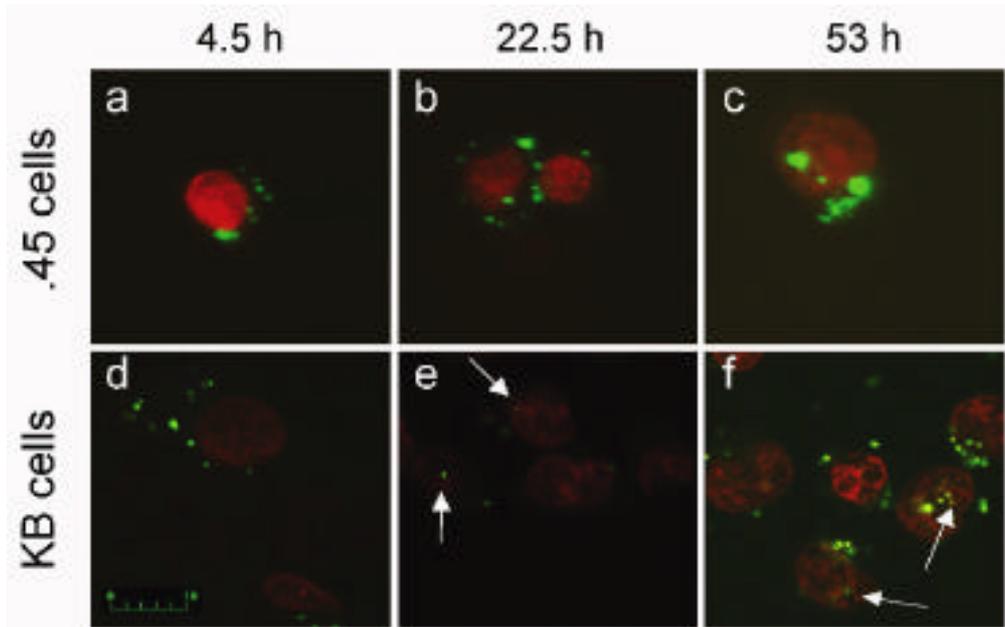
We compared DNA entry into the nucleus using *in vitro*-packaged SV40 pseudovirions and using a non-viral delivery system Lipofectamine-Plus from Invitrogen. Since lipofection of cells in suspension is not an efficient process, we transfected KB-3-1 (HeLa) adherent cells. At the same time points (4.5, 22.5, and 53 hours post-transduction) we examined GeneGrip DNA entry to the nucleus using lipofection as demonstrated in **Figure 6d-f**. It is important to note that the only valid comparison between the methods is the proportion of DNA in the cytoplasm vs. in the nucleus, since the amount of DNA used in the SV40 delivery system is approximately  $10^3$  lower compared to the Lipofectamine-Plus method. DNA is delivered to the nucleus earlier using the Lipofectamine-Plus delivery system. Based on our observation of 200 cells, 53 hours after transduction 59% of the DNA was still located in the ER.

### G. Neither nuclear localization sequences (NLS) from SV40 wild-type, nor cPPT sequences from the HIV-1 virus facilitated DNA entry into the nucleus using the SV40 delivery system

Previously it has been shown that an SV40 enhancer comprised of a 72-base pair repeat could direct nuclear localization of plasmids and allows the enhancement of gene expression in a broad range of hosts (Dean, 1997, 1999; Vacik et al, 1999; Li et al, 2001). Similarly, the 188 bp central polypurine tract sequence (cPPT), a part of the polymerase gene of HIV-1 virus, has been shown to facilitate nuclear entry of HIV-1 preintegration complexes in the context of wild-type HIV-1 virus as well as HIV-1-based replication defective lentiviral vectors (Sirven et al, 2000; Zennou et al, 2000). To examine whether the inclusion of these sequences could improve the nuclear transfer of plasmid DNAs encapsidated in SV40 pseudovirions, we constructed plasmid DNAs encoding



**Figure 5.** VP1 and SV40 IVP-DNA colocalization and disassembly in .45 cells. .45 cells transduced with IVP-pGeneGrip (green), fixed and immunostained for VP1 (red) at 5.5 hours post transduction (**a**) and at 20 hours post transduction (**b**).



**Figure 6.** pGeneGrip-DNA entry to the nucleus followed by PI staining. .45 cells transduced with SV40 IVP-pGeneGrip (panels a-c), and KB-3-1 cells transduced with the same DNA using lipofectamine-plus (panels d-f) were fixed and immunostained at 5.5, 22.5, and 53 hours post-transduction. The nucleus is labeled with propidium iodide (PI).

the *GFP* reporter gene with an SV40 enhancer or HIV-1 cPPT sequences placed upstream of the promoter used to drive transcription of *GFP* cDNA. We also constructed identical plasmids without these sequences and used these as controls for comparison.

A time course (1, 2, 3, 4, 5, and 6 days after transduction) analysis of *GFP* expression from these two constructs delivered to .45 cells by the SV40 system was carried out using flow cytometry. As clearly seen in **Figure 7a** (3 days post-transduction), there was no detectable difference in *GFP* expression from constructs in the presence or absence of the SV40 enhancer. As a control, we compared the expression of *GFP* in HeLa and KB-3-1 cells that were transfected with the same constructs using Lipofectamine-Plus reagent and analyzed at the same time points as before. Interestingly, in this case, the plasmid carrying the SV40 enhancer sequence clearly revealed higher *GFP* expression than that lacking the NLS (**Figure 7b** – 4 days post transfection). Similar experiments were carried out using two other *GFP* DNA plasmids constructed with or without the cPPT sequence from HIV-1 downstream to the *GFP* gene. As expected, neither the *in vitro*-packaged SV40 vector (**Figure 7c** – 4 days after transduction), nor the transfection delivery system carrying these DNA plasmids revealed any differences in *GFP* expression over time (**Figure 7d** – 5 days post-transfection). These experiments were repeated 8 times, with similar results.

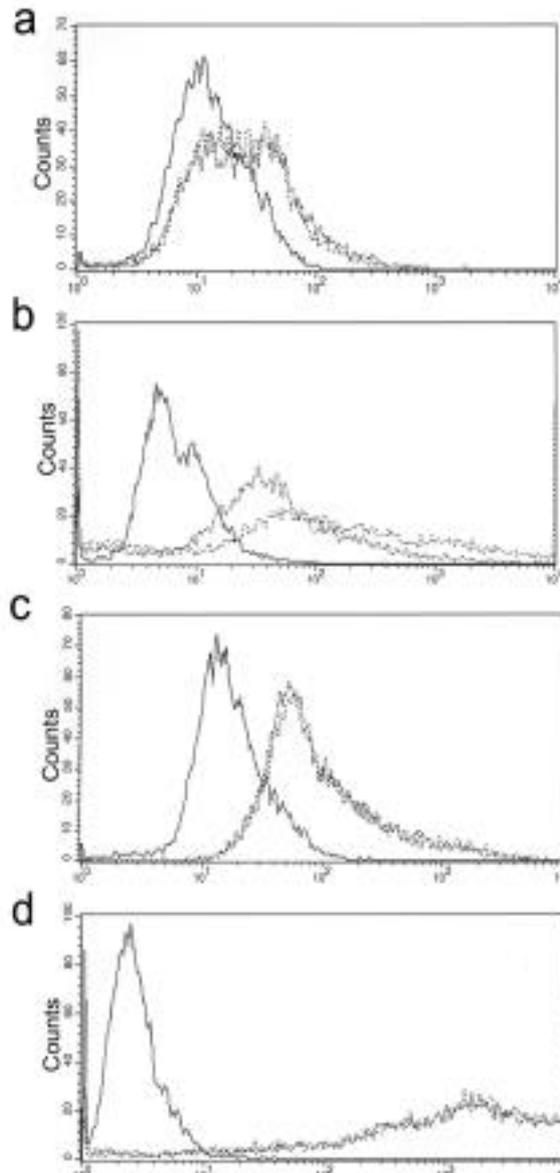
#### H. DNA histone acetylation, but not DNA demethylation, promotes DNA expression via SV40 *in vitro* vectors

In order to increase gene expression, we treated .45 cells prior to transduction using the SV40 delivery system with various concentrations of the DNA histone

deacetylase inhibitor, TSA. In order not to saturate the cells, and to see the effect of TSA, only 2/3 of a pseudovirion reaction (Kimchi-Sarfaty et al, 2004) was used. Acetylation of histones allows DNA to be more accessible to transcription factors by separating basic N-termini of histones. This makes histone-DNA interaction looser which results in gene activation. *GFP* expression was monitored every day for 6 days. Expression was higher starting 48 hours after transduction in treated cells. An 8.6-fold increase in *GFP* expression (4.39 in cells transduced with *in vitro*-packaged *GFP* as compared to 37.73 in cells treated with TSA and transduced with *in vitro*-packaged *GFP*-median fluorescence intensity, arbitrary units) was observed in cells treated with 10 ng/ml 6 days after transduction. A similar experiment using the pHAMDR1 plasmid packaged *in vitro* revealed similar results expression using the MRK16 monoclonal antibody was 30% higher 48 hours post transduction after treating the cells with 10 ng/ml 24 prior to transduction.

TSA treatment (0.1, 1, 10 ng/ml) was used on KB-3-1 and HeLa cells 24 hours prior to transfection with EGFP using Lipofectamine-Plus reagent. Measuring 24 and 48 hours post transduction, we found that treatment with 0.1 and 1 ng/ml slightly increased *GFP* expression (1627.48 in cells transduced with *GFP* as compared to 1968.52 in cells treated with 0.1 ng/ml TSA and transduced with *GFP*-median fluorescence intensity, arbitrary units), A higher concentration of 10 ng/ml did not change *GFP* expression level.

Treating cells with the DNA methylase inhibitor, DAC which incorporates into the DNA in place of cytosine but cannot be methylated, results in loss of DNA methylation, and in some cases, gene reactivation. In contrast to TSA treatment, treatment of cells with DAC prior to transduction (24, 48, 72 hours prior to



**Figure 7.** GFP expression via Lipofectamine-Plus or SV40 delivery system using NLS sequences. **(a)** – .45 cells transduced with *in vitro* packaged-EGFP-C1 plasmid DNA which carries the SV40 enhancer as NLS sequence (- - -), or with no NLS sequence (—) 3 days after transduction. Mock transduced cells are indicated with a solid line. **(b)** – HeLa cells transduced with GFP plasmid DNA which carries the NLS sequence (- - -), or with no NLS (—) sequence using lipofectamine-plus 4 days after transduction. Mock transduced cells appear as a solid line. **(c)** – .45 cells transduced with *in vitro* packaged-GFP plasmid DNA which carries the HIV-1 cPPT sequence (- - -), or with no cPPT (—) sequence 4 days after transduction. Mock transduced cells appear as a solid line. Panel **(d)** – HeLa cells transduced with GFP plasmid DNA which carries the cPPT sequence (- - -), or with no cPPT (—) sequence using lipofectamine-plus 5 days after transduction. Mock transduced cells appear as a solid line

transduction) at different concentrations (1-10  $\mu$ M) did not change the reporter gene expression (data not shown).

#### IV. Discussion

Vectors which use the SV40 major capsid protein VP1 can be used to package supercoiled plasmids up to 17.7 kb in size *in vitro* without an SV40 viral sequence (Kimchi-Sarfaty et al, 2003). Previously we have shown the high efficiency of delivery of SV40 pseudovirions, but expression from *in vitro* encapsidated DNA is lower than retroviral delivery systems (Kimchi-Sarfaty et al, 2003). One of the aims of this work was to identify the limiting

step in the pathway of entry of SV40 pseudovirions in order to improve expression of packaged DNA. Studying the SV40 pseudovirion pathway in a human lymphoblastoid cell line, we show here that the pseudovirions colocalized to MHC I receptors as does the wild-type SV40 virus, but a high level of MHC I is neither necessary nor sufficient for entry. Over a period of several hours, VP1 protein as well as packaged plasmid DNA labeled with a fluorescent tag was detected by confocal microscopy, and were shown to move from the surface of the cell into the Golgi, eventually accumulating in the ER. Initial disassembly of the packaged DNA from VP1 occurs

in the ER, with some of the tagged DNA appearing in or near the nucleus 53 hours post transduction. No staining of VP1 was observed within the nucleus. Trapping of some of the DNA in the cytoplasm might explain the known limitation in expression of *in vitro* packaged virions. To overcome these limitations, we constructed GFP reporter DNAs harboring the enhancer repeat of the SV40 early promoter or the cPPT sequence from the HIV-1 virus, but saw no effect on gene expression. However, GFP expression was elevated when cells were treated with a histone deacetylase inhibitor TSA prior to transduction.

### A. Entry of pseudovirions into cells

The efficiency of the entry of pseudovirions was monitored here using a GRIP-fluorescent DNA, with which we were able to demonstrate a fluorescent tag in every cell. Wild-type SV40 utilizes MHC I as a receptor (Norkin, 1999). Increased SV40 wild-type entry to cells can be achieved by transfecting more MHC I molecules into these cells (Breau et al, 1992). The results shown here, and our extensive experience with other cell lines (data not shown), do not demonstrate a direct correlation between MHC I levels and GFP expression. These results indicate that the MHC I level is not the limiting factor for reporter gene expression using the SV40 *in vitro* packaging delivery system. In some cell lines we found high levels of MHC I receptors, but GFP expression was low. These observations confirm our previous conclusions about *in vitro* packaging, that enhancing MHC I receptor levels in cells using interferon- does not enhance GFP expression via the SV40 delivery system. Previously we also measured MHC I receptors of .45 cells after multiple pseudovirion transductions, and we found that even after the third transduction, more than 60% of the cells still express MHC I (unpublished data of the authors). We speculate that other coreceptor(s) are needed for the entry of the pseudovirions, and without these coreceptors even high levels of MHC I are not sufficient for the entry of the pseudovirions. SV40 vectors transit from the cell membrane to the ER in .45 cells. However, blocking the pathway to the ER did not completely inhibit GFP expression, suggesting that alternative pathways are available under these conditions.

### B. The pathway of SV40 pseudovirions from the ER to the nucleus

The process of DNA entry to the nucleus is slower using the SV40 delivery system compared to transfection using Lipofectamine-Plus, another type of non-viral gene delivery. Godbey and colleagues (1999) used poly(ethylenimine)/DNA complexes and showed that the DNA initially appeared in the nucleus 4-5 hours post-transfection. Similarly, our study showed that transfection using lipids initially delivered the DNA to the nucleus 4.5 hours post-transduction. Using the SV40 system, the DNA reporter plasmid appeared in the nucleus later, and a small amount of DNA was localized in the nucleus 20 hours post transduction. It was clear that most of the DNA did not move to the nucleus, but was trapped in the ER. For mouse polyomavirus, VP1 accumulates on nuclear membranes,

and its entry is not inhibited by BFA. The majority of the polyomavirus viral DNA is also not delivered to the nucleus, but moves back to the cytosol, and possibly degrades (Mannova and Forstova, 2003). An earlier study examining the pathway of the poly(ethylenimine)/DNA complexes also revealed similar results: some of the DNA was trapped in the cytoplasm and did not reach the nucleus (Godbey et al, 1999).

### C. The known NLS sequences, the enhancer of SV40 wild-type virus and cPPT sequence from lentivirus do not improve gene expression using the SV40 pseudovirion vectors

The function of the nuclear membrane as a barrier against macromolecules was described in the 1970s (Dingwall and Laskey, 1992). However, according to Whittaker and colleagues (2000), polyoma and papilloma virus particles (up to 60 nm) are able to pass into the nucleus. Previously, we have shown that the size of the pseudovirions did not exceed 55 nm (while SV40 wild-type is 45 nm). Therefore, it was surprising that we did not find any VP1 staining within the nucleus.

NLS were used previously as peptides delivered *in trans* to the DNA or *in cis* carried by the plasmid DNA that needed to be delivered to the nucleus (Akuta et al, 2002). In the latter, fusion protein was expressed initially in the cytosol, but moved to the nucleus under the influence of the NLS. The enhancer repeat of the SV40 early promoter has been shown to increase the nuclear transport of transfected plasmid DNAs and also enhance the expression of transgenes in several cell types (Dean, 1997; Dean et al, 1999; Vacik et al, 1999; Li et al, 2001). We could only detect a marked increase in GFP expression when the same plasmid was transfected into cells.

It has been shown that the SV40 enhancer contains binding sites for several transcription factors. Several cellular transcription factors have been demonstrated to form nucleoprotein complexes after binding to their specific DNA sequences in the SV40 enhancer. The DNA sequences could interact with NLS receptors and enter the nucleus using the normal nuclear protein import machinery (Nigg, 1997). Other SV40 delivery systems such as the one developed by Vera et al, (2004) always imprint the NLS sequence of wild-type SV40. The failure to obtain nuclear delivery of the plasmid DNA harboring the SV40 enhancer using *in vitro*-packaged SV40 vectors, and our success using a Lipofectamine-Plus delivery system in the current study suggests that the NLS sequences or binding sites of cellular transcriptional factors in the SV40 enhancer might have been blocked or inactive due to conformational changes when packaged in the SV40 delivery system. Alternatively, we could speculate that the cellular factor(s) necessary to facilitate the SV40 enhancer-mediated nuclear transport is absent in cell lines used in this study.

The cPPT sequence of HIV-1 virus pol gene virus has been shown to increase nuclear transport of preintegration DNA complexes formed after reverse transcription of wild type HIV-1 genome or replication

defective HIV-1-based vectors in infected cells (Sirven et al, 2000; Zennou et al, 2000). Although the nuclear transport function of the cPPT sequence has been well documented in the context of the wild type HIV-1 viral infection or transduction with HIV-1-based vector systems, its effectiveness in the context of plasmid DNA delivery and/or gene expression has not been studied. Not surprisingly, in the present study we were unable to detect any differences in GFP expression when we used plasmid DNA carrying or not carrying the cPPT sequence. However, these results suggest that the SV40 system could effectively package the HIV-1 based vectors and generate pseudovirions capable of delivering the vector plasmid into cells.

#### D. Inhibition of histone deacetylation increases GFP expression delivered via SV40 pseudovirions

These results led us to search for different ways to increase the reporter gene expression via the SV40 delivery system. In this work, we show that inhibition of histone deacetylation, but not DNA demethylation, dramatically improves GFP expression delivered by the SV40 *in vitro* packaging vectors. Treatment of cultured cells with trichostatin A (TSA), a specific histone 4 deacetylase inhibitor, was shown to change gene expression, probably by inducing hyperacetylation of histones. Sowa and colleagues, (1997) and others (Schuettengruber et al, 2003) demonstrated activation of genes or gene promoters using TSA, but others (Siddiqui et al, 2003) showed that TSA might repress transcription. Treating other cell lines (KB-3-1 and HeLa) prior to transduction using another delivery system-Lipofectamine-Plus-produced only very slightly higher GFP expression, as compared to delivery using the SV40 vectors. Therefore, we suggest that treatment with TSA might not be a useful method to increase gene expression for other delivery systems. White and Strayer, (2003) found that DNA methylation can occur during SV40 production in the packaging cell line and this may explain the relatively low expression of transgenes using other SV40 virions for gene delivery. Our results, however, indicated that inhibition of DNA methylation did not increase transgene expression.

The SV40 *in vitro* packaging pathway characterized in this study has many similarities to the wild-type pathway. Both pathways are very efficient, both use MHC I for entry, in both the virions are delivered to the ER, and in both the efficiency of the delivery to the nucleus is not very high. However, some differences were observed. MHC I is not an exclusive pathway for the pseudovirions, not all the pseudovirions travel through the Golgi, and a large proportion of the reporter DNA is trapped in the ER. Although we were not successful in improving the efficiency of DNA delivery to the nucleus, blocking acetylation of histone H4 appears to substantially increase expression of DNA delivered by SV40 pseudovirions, and this approach may prove useful in exploiting SV40-based delivery systems.

#### Acknowledgments

We thank Ariella Oppenheim (The Hebrew University, Hadassah Medical School and Hadassah University Hospital, Jerusalem, Israel) for fruitful collaboration on the SV40 vectors, and for providing us the VP1, and VP2/3 polyclonal antibodies. We thank Pamela Robey, and Sergei Kuznetsov, National Institutes of Dental and Craniofacial Research, NIH, and Louis Scavo, National Institute of Diabetes and Digestive and Kidney Diseases, NIH for providing us with adult stem cells, and George Leiman for insightful editorial assistance.

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