

Nuclear targeting of plasmid DNA in human corneal cells

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Abstract

Purpose. To characterize the mechanisms of plasmid DNA nuclear localization in primary cultures of human corneal epithelial cells and keratocytes.

Methods. Purified, supercoiled plasmid DNA was microinjected into the cytoplasm of human corneal epithelial cells and keratocytes that had been established from donor corneas two to three passages previously, and localized 8 hours later by *in situ* hybridization. To confirm the sequence-specificity of nuclear import observed in microinjected cells, liposome-mediated transient transfection experiments also were performed on human corneal epithelial cell and keratocyte cultures.

Results. Primary cultures of human corneal epithelial cells and keratocytes have the capacity to transport plasmid DNA from the cytoplasm to the nucleus in the absence of cell division. This transport activity is sequence-dependent requiring portions of the simian virus 40 (SV40) early promoter and enhancer. The majority of this nuclear transport activity resides within the enhancer domain of the SV40 DNA, a region rich in transcription factor binding sites. This DNA nuclear import sequence also manifested itself in liposome-mediated transfection experiments, causing a greater than 2-fold increase in reporter gene expression in human corneal cells in a β -galactosidase-expressing vector and up to a 1000-fold increase in a luciferase-expressing vector when compared to similar expression plasmids lacking the sequence.

Conclusion. These results demonstrate that primary, non-transformed human corneal epithelial cells and keratocytes display sequence-specific nuclear import of plasmid DNA in the absence of mitosis. The small sequence that mediates nuclear localization of plasmids is active both in microinjected and cationic liposome transfected cells, and leads to

increased gene expression. Thus, inclusion of this DNA sequence into non-viral vectors should improve the efficiency of ocular gene transfer *in vivo*.

Keywords: human corneal epithelial cells, gene therapy, gene transfer, keratocytes, nuclear transport

Introduction

Gene therapy is an attractive and novel approach to prevent and treat diseases of the eye. Inherited disorders such as congenital hereditary endothelial dystrophy, posterior polymorphous dystrophy, Fuch's corneal dystrophy, macular degeneration, and forms of glaucoma and cataracts as well as infectious diseases like herpes stromal keratitis are all candidates for gene therapy. However, inefficiency of gene transfer, immunological responses and non-specificity of cell targeting are just a few of the problems associated with the current approaches and vectors. For example, while adenovirus appears to be a desirable vector for ocular gene therapy and has gene transfer efficiencies of almost 95% *in vitro*, the values are usually much less *in vivo* and can require upwards of 10^8 to 10^9 pfu injected into the anterior chamber of the eye (1-3), commonly resulting in cell damage and inflammation (2). In contrast, no immunological responses have been detected using liposome-DNA complexes, but the efficiency of transfer is on the order of a 1% (4-6).

Under physiologically relevant conditions, the levels of gene transfer by any approach are low at best, especially in slowly and non-dividing cells. Nowhere are these cells more prevalent and important than in the eye. Many cells in the eye are post-mitotic and non-dividing while others remain in a state of quiescence. This is a major problem since several promising vectors currently being studied cannot transduce cells in the absence of cell division. While retroviruses are some of the most widely used viral vectors (7, 8), they can only transduce actively dividing cells since their genome, once reverse-transcribed into DNA, cannot enter the

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nucleus in the absence of cell division and nuclear envelope breakdown (9, 10). Thus, they cannot be used on post-mitotic or quiescent cells, including those of the corneal endothelium which cease to divide by two years after birth (11), photoreceptor cells, the vast majority of corneal epithelial cells which are non-dividing after migration from the conjunctiva and maturation, and many other ocular cell types that divide either very slowly or only upon stimulation. Clearly, techniques are needed to transduce these cells effectively without using approaches that will result in damage to the remainder of the eye.

Recent work from our laboratory has begun to address the nuclear targeting and entry of plasmid DNA in non-dividing cells. Using transformed cell lines, we have shown that plasmid DNA is able to enter the nuclei of cells in the absence of cell division and its accompanying nuclear envelope breakdown (12). As all other macromolecular exchange between the cytoplasm and nucleus (for a review, see ref. (13)), DNA nuclear entry appears to be mediated by the nuclear pore complex (12, 14). We have previously identified a 366 bp sequence of DNA containing the simian virus 40 (SV40) origin of replication and early promoter that is absolutely necessary for the nuclear entry of plasmid DNA in cultured cell lines derived from monkey kidney epithelium, and a human cervical carcinoma (12). Thus, at least in transformed cells, nuclear import of plasmid DNA is signal-dependent. One caveat with these previous experiments is that they were all performed in tumorigenic transformed cell lines, and if DNA nuclear targeting sequences such as the SV40 366 bp fragment are to be used ultimately in humans, it is crucial to know if these same DNA nuclear import mechanisms are present in non-transformed primary human cells. In the present report, we show that primary cells derived from the human cornea display similar sequence-specificity for plasmid DNA nuclear entry, and further, that incorporation of this nuclear targeting DNA sequence can stimulate the levels of liposome-mediated gene transfer to these cells. These experiments represent the first attempt to exploit nuclear import mechanisms to increase gene transfer efficiency in the eye.

Materials and methods

Establishment of human corneal epithelial cell and keratocyte cultures

Human corneas from three independent donors were obtained from the National Disease Research Interchange (Philadelphia, PA) and processed within 4 days of enucleation. Human corneal epithelial cells and keratocytes were established as previously described and maintained in 25 cm² flasks in Keratinocyte Serum Free Medium (KSFM; Life Technologies, Gaithersburg, MD) containing 5 µg/ml gentamicin and Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) and 1 X antibiotic-antimycotic solution (Life Technologies), respectively (15, 16). Human cor-

neal cells were a generous gift from Dr. John E. Oakes (Department of Microbiology and Immunology, University of South Alabama). Cells were plated onto etched glass coverslips on their second passage and grown to 50–75% confluency for microinjection studies or plated into 12-well plates on their second or third passage for transfection studies.

Preparation of plasmid DNAs

SV40 DNA was purified from infected TC7 cells by Hirt lysis as described (17). The SV40 minimal origin sequence (109 bp; nts 5171 to 37) was excised from plasmid pOR1 (18) by digestion with EcoRI and HindIII and replaced with a multiple cloning site (HindIII-XbaI-NcoI-SmaI-KpnI-EcoRI) to create pDD201 to facilitate cloning. pORD1 was constructed by subcloning the 366 bp HindIII-KpnI fragment from SV40 (nts 5171 to 294) containing the origin and promoter region into the plasmid pDD201. pORD6 was constructed by subcloning the 94 bp SphI-KpnI (nts 200 to 294) fragment into pDD201. pOR1 and pOR4 were kindly provided by Dr. Peter Tegtmeier (Department of Microbiology, SUNY Stony Brook) (18). To facilitate cloning steps, the 366 bp HindIII-KpnI SV40 origin region was PCR amplified using SV40 as a template and inserted into the multiple cloning site of pPCR2.1 (Invitrogen, San Diego CA) to create pDD178. pCMVβ-DTS was made by subcloning a 380 bp HindIII-SalI fragment containing the SV40 HindIII-KpnI sequence flanked by a SalI site from pDD178 into pCMVβ (Clontech, Palo Alto, CA) that had been digested with HindIII and SalI. Plasmid pCMV-lux was made by exchanging the NheI-BamHI fragment of pRL-CMV (Promega, Madison, WI) for that of pGL3-basic (Promega) to create a plasmid expressing the firefly luciferase gene from the CMV promoter. pCMV-lux-DTS was constructed similarly, but contained the NheI-BamHI fragment from pGL3-enhancer (Promega) and contains the SV40 enhancer and GC boxes (SV40 nts 44 to 268) downstream of the luciferase gene.

All manipulations were performed as described and all constructs were confirmed by double-stranded sequencing using Sequenase 2.0 (Amersham, Arlington Heights, IL) (19). Protein-free plasmid DNA was purified by either alkaline lysis and subsequent CsCl gradient centrifugation or Qiagen midiprep columns (San Diego, CA). DNA purified in either manner displayed the same intracellular distribution after cytoplasmic microinjection.

Microinjection

Purified protein-free DNA was suspended in phosphate-buffered saline (PBS) at a concentration of 0.5 mg/ml and microinjected into the cytoplasm of human corneal epithelial cells or keratocytes grown on etched coverslips as described (17). Assuming that 1 to 3 × 10⁻¹⁰ ml is delivered by microinjection (DAD, unpublished), this corresponds to approximately 8,000–20,000 molecules of plasmid injected per cell (20).

***In situ* hybridization**

In situ hybridizations were performed as described (12). Briefly, after microinjection and incubation for 8 hours in the appropriate growth medium, the cells were rinsed in PBS, permeabilized with 0.5% Triton X-100 in PBS at 23°C for 45 seconds, fixed in acetone:methanol (1:1) at -20°C for 5 minutes, and incubated in 70% formamide in 2X SSC (0.3 M NaCl, 30 mM sodium citrate, pH 7.0) at 70°C for 2 minutes to denature the DNA. The cells were then hybridized overnight at 37°C with a fluorescently-labeled probe in hybridization buffer (2 mg/ml bovine serum albumin, 0.25 mg/ml sheared salmon sperm DNA, 0.25 mg/ml Torula yeast tRNA, 10% dextran sulfate, 2 X SSC, and 50% deionized formamide). All samples were treated with RNaseH (8U/ml) after hybridization to degrade any RNA-DNA hybrids, and the subsequent washing steps, and the cells were mounted with DAPI and the anti-bleaching reagent DABCO. Fluorescently-labeled probes were prepared by nick translation of pBR322 and SV40 DNA as described (21) except that fluorescein-12-dUTP (Molecular Probes, Eugene, OR) was incorporated directly into the DNA. All photographs were taken with an Olympus BMAX50 epifluorescence microscope equipped with a PM20 photodocumentation system on 400 ASA Kodak Ektachrome or TriX-PAN film. Confocal microscopy was performed on an ACAS 570 laser-scanning confocal microscope.

Transfections, β -galactosidase assays, and luciferase assays

Transfections of confluent human corneal epithelial cells and keratocytes were performed using Lipofectin (Life Technologies) essentially as described by the manufacturer. Briefly, pCMV β , pCMV β -DTS, pCMV-lux, or pCMV-lux-DTS (0.25 μ g/well of a 12-well plate) were complexed with Lipofectin, diluted into serum-free Opti-MEM (Life Technologies; 2.5 μ g lipofectin/well) and added to cells in the absence of serum. Four hours after DNA-lipofectin addition, 1 ml of KSFM (corneal epithelial cells) or DMEM containing 10% FBS (keratocytes) was added to the cells. In indicated experiments, 50 μ M aphidicolin was added to the cells 24 hours before transfection, and maintained in the medium throughout the course of the transfections. At the indicated times, the medium was removed and the cells were washed with PBS and lysed in 100 μ l lysis buffer (100 mM potassium phosphate pH 7.8, 1 mM dithiothreitol, and 0.2% Triton X-100). The samples were homogenized by pipeting and the particulate matter was removed by centrifugation for 5 minutes at 16,000 \times g at 4°C. β -galactosidase and luciferase activities were measured by luminometry using the Galactolight Plus kit (Tropix, Bedford, MA) or the Luciferase Assay System (Promega), respectively. Statistical analysis was performed by combining time points of all experiments for a cell type and using a Wilcoxon Signed Rank Test (non-parametric) approach.

Bromo-deoxyuridine incorporation and detection

Cells grown on coverslips were pulsed at various times with 10 μ M bromo-deoxyuridine (BrdU) for 15 minutes in their growth medium. The cells were then fixed for 10 minutes in ethanol/acetic acid (3:1), rinsed in 30% ethanol followed by PBS, and incubated in 4 N HCl for 30 minutes to remove chromosomal proteins from the DNA. The cells were rinsed twice in PBS and blocked for 20 minutes in PBS containing 1 mg/ml bovine serum albumin. Immunofluorescence was performed using a monoclonal antibody against BrdU followed by a fluorescein-labeled secondary antibody as described (22). Cells were observed and counted for nuclear BrdU incorporation.

Dot blots and Southern Blots

Lysed cell extracts prepared for β -galactosidase assays were also used for dot blot and Southern blot analysis of transfected DNA. For dot blots, dilutions of each sample were spotted onto a nylon membrane (Zetaprobe GT, BioRad Laboratories, Richmond CA) using a 96-well vacuum blotting apparatus (Life Technologies). The membrane was washed with 1X SSC and UV irradiated to immobilize the DNA. The membrane was pre-hybridized for 4 hours at 42°C with 0.05 mg/ml sheared salmon sperm DNA, 5X SSC, 25 mM potassium phosphate (pH 7.5), 5X Denhardt's solution, and 50% formamide. Plasmid pCMV β was nick-translated with ³²P-dATP to a specific activity of 1.7 to 2.8 \times 10⁸ cpm/ μ g and 10⁶ cpm were added and allowed to hybridize to the blot for 16 hours at 42°C. The blot was washed with 25 ml 2X SSC containing 0.1% SDS, followed by 25 ml 1X SSC/0.1% SDS, and finally 25 ml 0.5X SSC/0.1% SDS. All washes were for 30 min with two changes of buffer and were carried out at 25°C, except the last wash which was performed at 42°C. The blots were dried and exposed to X-ray film at -80°C. After exposure, the dots were excised from the blots and counted by liquid scintillation to quantify the bound DNA. Southern blots were performed identically, except that samples were separated on a 1% TAE agarose gel and then transferred to nylon instead of using the blotting apparatus. The autoradiograms of the Southern blot were digitized and analyzed using NIH Image version 1.61 (NIH, Bethesda, MD).

Results

Sequence-specific nuclear import of plasmid DNA in non-dividing human corneal cells

There are two ways that plasmid DNA can enter the nucleus: [1] during mitosis when the nuclear envelope has broken down and no longer presents a barrier, and [2] through the nuclear pore complexes of an intact nucleus. To ask the question of whether plasmid DNA can enter the nuclei of non-transformed cells without the need for cell division, protein-free supercoiled SV40 DNA was microinjected into the cytoplasm of

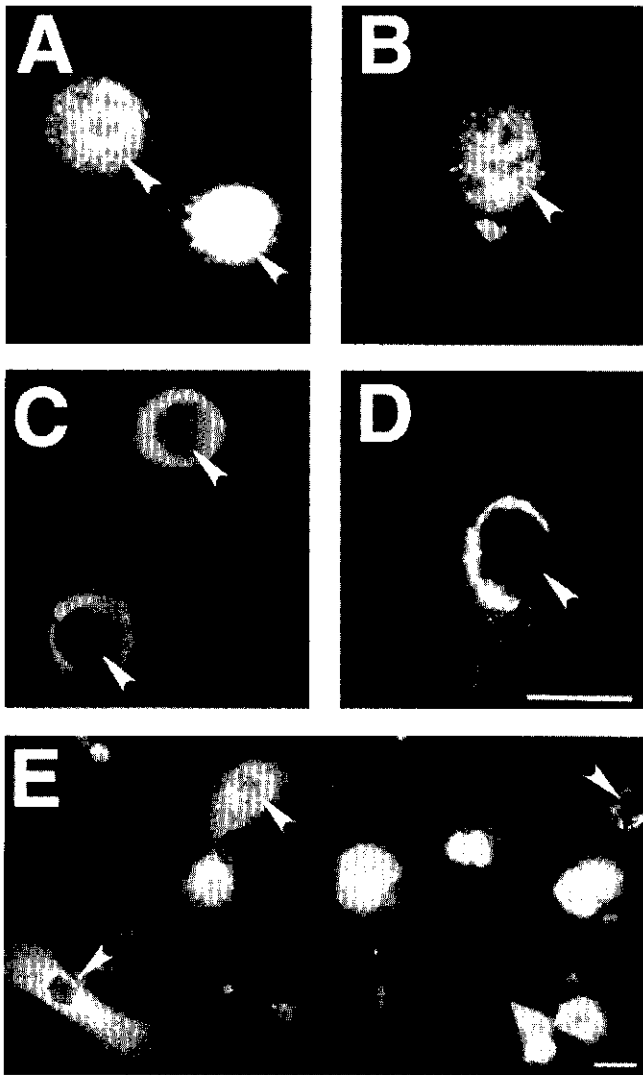


Figure 1. DNA nuclear import in human corneal epithelial cells and keratocytes. Cultures of human corneal epithelial cells and keratocytes were cytoplasmically injected with 0.5 mg/ml SV40 DNA (A, B, and E) or pBR322 (C and D) in PBS. Cells were fixed 8 hours after injection and *in situ* hybridizations were performed with fluorescein-dUTP-labeled SV40 or pBR322 DNA as probes as described in Methods. After hybridizations, RNaseH was used to destroy any hybridized RNA. Human corneal epithelial cells (A, C, and E) were used at the second passage in culture and keratocytes (B and D) at the third passage. Arrows in A-D point to cell nuclei while those in E point only to nuclei of cells showing little nuclear accumulation. Bar = 10 μ m.

primary non-transformed cultures of confluent human corneal epithelial cells and keratocytes and localized by *in situ* hybridization. By 8 hours after injection, the majority of the SV40 DNA was inside the nuclei of both corneal epithelial cells (Fig. 1A) and keratocytes (Fig. 1B). That the DNA had accumulated inside the nucleus as opposed to binding to the outside surface of the nuclear envelope was confirmed by confocal microscopy (not shown). Microscopic observation of the injected cells, during the injection and at 2 hour intervals thereafter, indicated that the cells were not undergo-

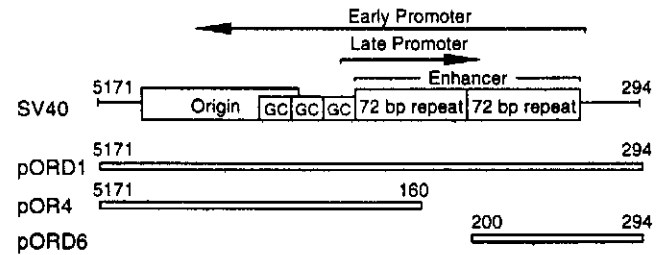


Figure 2. Schematic representation of the 366 bp SV40 DNA nuclear import sequence and plasmids used in this study. The functional elements of the SV40 origin region are shown, including the origin of replication, early and late promoters, GC boxes, and 72 bp enhancer repeats indicated. The relevant portions of the 366 bp region contained within the plasmids used for nuclear localization studies are also shown. The SV40 nucleotide numbers²⁹ flanking the different fragments are shown (nucleotide 5243 = nucleotide 0).

ing cell division during the time course of these experiments. That the vast majority of cells were not undergoing cell division over the course of our experiments was confirmed by the fact that less than 0.01% of the cells in cultures of corneal epithelial cells and keratocytes incorporated BrdU into their DNA when pulse labeled at various times, whereas non-confluent cultures did so readily. Interestingly, the nuclear localization of SV40 DNA was quantal: while roughly 60% of the cells showed near complete nuclear staining of the injected DNA (> 90% of the signal in the nucleus), the remainder of the cells showed almost no nuclear staining of the DNA (Fig. 1E). This phenomenon has also been observed with transformed cell lines (12).

In contrast to SV40 DNA which migrated into the nucleus (Figure 1 A and B), the bacterial plasmid pBR322 remained in the cytoplasm of both corneal epithelial cells and keratocytes in all injected cells (Figure 1 C and D, respectively). Further, when a mixture of SV40 DNA and pBR322 DNA were co-injected into the same cells, the pBR322 remained cytoplasmic and the SV40 DNA localized to the nucleus (not shown). This experiment also confirms that cells are not undergoing cell division, since if they were, both pBR322 and SV40 DNA would be nuclear. These results are identical to those seen with transformed cell lines and suggest that sequence-specific DNA nuclear entry is a general property of mammalian cells, both transformed and non-transformed (12).

Sequence requirements for plasmid DNA nuclear entry

To determine what sequence elements of the SV40 DNA were responsible for its ability to target to the nucleus, hybrid plasmids were constructed that contained portions of the SV40 genome inserted into pBR322 and tested for their ability to enter the intact nucleus in cytoplasmically micro-injected cells. We previously have identified the SV40 origin region as mediating nuclear entry in monkey kidney epithelial cells (Figure 2) (12). We tested whether the same

Table 1. Subcellular localization of plasmid DNA.

Plasmid	Nuclear localization (% cells) ¹
SV40	61
pBR322	0
pORD1	65
pORD6	40
pOR4	17

¹ Plasmid DNA (0.5 mg/ml) was microinjected into the cytoplasm of human corneal epithelial cells and its cellular distribution assayed 8 hours later by *in situ* hybridization. Approximately 100 cells were visualized per plasmid in two independent experiments.

sequence mediated import in human corneal cells. The plasmid pORD1, which contains the 366 bp SV40 origin of replication and early and late promoters, localized to the nucleus of human corneal epithelial cells to the same extent as the entire SV40 genome did (Table 1). Both SV40 DNA and pORD1 localized to the nucleus in over 60% of the visualized cells, and in all of these cells, roughly 80 to 100% of the hybridized signal was in the nucleus. In the remaining 35 to 40% of the cells, the hybridized signal was entirely within the cytoplasm, similar to the patterns of staining observed with SV40 DNA (Fig. 1E).

To identify more precisely the sequence needed for nuclear localization, this 366 bp region was dissected into two parts (Figure 2). pOR4 contains the origin of replication, the GC boxes and 53 bp of the first 72 bp repeat of the SV40 enhancer. When injected into the cytoplasm of human corneal epithelial cells, the plasmid localized to the nucleus in only 17% of the visualized cells. The plasmid pORD6, which contains the remaining 18 bp of the first 72 bp repeat, the entire second 72 bp repeat, and the sequence up to the KpnI site, localized to the nucleus in 40% of the cells. Again, cells injected with both of these plasmids displayed two distinct staining patterns of the hybridized DNA: cells either showed near complete nuclear localization of the DNA or complete cytoplasmic localization as is seen for SV40 DNA and pORD1. Thus, multiple sequences within the 366 bp origin/promoter region appear to be capable of mediating nuclear import of plasmid DNA, although the majority of the activity seems to lie within the enhancer region.

Effect of DNA nuclear import sequence on gene expression in liposome transfected cells

Since the goal of gene therapy is to express a gene product in a desired cell, and the efficiency to do so at present is so low, we wanted to test the effect of the SV40 promoter/enhancer sequence on the expression of a reporter gene when transfected into human corneal cells. Because this region of SV40 DNA promotes nuclear entry, we reasoned that it would also lead to increased expression of a gene carried on the same plasmid since more of the plasmid would be localized

to the site of transcription (i.e., the nucleus). To test this, a β -galactosidase-expression plasmid, pCMV β , was constructed to contain the 366 bp SV40 DNA nuclear targeting sequence (DTS). The SV40 sequence in pCMV β -DTS was inserted downstream of the coding sequence for *lacZ* in an attempt to minimize the effect of the promoter and enhancer sequences on the transcription of the *lacZ* gene from the CMV immediate early promoter on the plasmid. A similar set of plasmids was constructed that expressed the firefly luciferase gene from the CMV promoter and either lacked (pCMV-lux) or contained (pCMV-lux-DTS) the 224 bp SV40 enhancer and CG boxes that contain the majority of the nuclear import activity in corneal cells. These plasmids were then used in lipid-mediated transfection experiments.

Since we predicted that the presence of the DTS would have the greatest effect on non-dividing cells, we transfected both confluent, contact-inhibited cells, as well as confluent cells that were treated with aphidicolin for 24 hours prior to and continuously during transfection to arrest the cells in G1 (23). That both conditions resulted in cells that were not actively dividing was confirmed by pulse-labeling the cells with BrdU at times during the experiment (Fig. 3). Both corneal epithelial cells and keratocytes readily incorporated BrdU into DNA 24 hours prior to reaching confluency (Fig. 3A and D), but once the cells were confluent (Fig. 3B and E) or treated with aphidicolin (Fig. 3C and F), no cells in S phase were detected out of approximately 10,000 cells observed (Fig. 3B and E).

When such non-dividing corneal epithelial cells and keratocytes were transfected with plasmids using the cationic lipid Lipofectin, significant gene expression was detected in cells that had received the DTS-containing vectors. The effect of the DTS was most obvious when the luciferase vectors were used. A typical time course of expression is shown in Figure 4. Assays were performed in quadruplicate and show that the presence of the SV40 DTS enhanced gene expression by a factor of 1000 in keratocytes and 30 in corneal epithelial cells by 36 hours. It should be pointed out that the levels of gene expression in keratocytes were 100- to 300-fold less than in epithelial cells, but were still significant compared to cells receiving no DNA (0 hours) and were in the linear range of the assays for β -galactosidase and luciferase activities. Cells arrested in G1 with aphidicolin gave virtually the same results with respect to the levels of gene expression and the positive effect of the SV40 DTS (Fig. 4). Additional transfections with the β -galactosidase vectors were performed on cells established from 4 other donors. While cells from each donor showed variation in transfection efficiency as expected (24), in all experiments the parent plasmids consistently showed lower levels of gene expression than did the SV40 nuclear targeting sequence-containing derivatives (Table 2). However, when the β -galactosidase vectors were used, the differences in gene expression levels were less striking. When the increased gene expression by pCMV β -DTS was quantified as the fold-increase over the amount of gene expression obtained with the parent pCMV β vector, the results of 6 independent ex-

