Research Report

Comparison of *Cis* and *Trans* Tat Gene Expression in HIV LTR-Based Amplifier Vectors

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ABSTRACT

The long terminal repeat (LTR) of the human immunodeficiency virus (HIV) drives highly efficient gene expression in the presence of the transactivator, Tat. Thus, tat-containing vectors may be very useful tools in gene therapy. However, information about the optimal way of delivering the tat gene is limited. In this study, we compared the effects of *cis* and *trans* expressions of the tat gene and its effects on HIV LTR-driven gene expression in different cell lines using non-viral vectors. The human interleukin-2 (IL-2) gene was used as a reporter gene under the control of the HIV2 LTR (pHIV2-IL-2). The tat gene, driven by a cytomegalovirus (CMV) promoter, was either co-transfected separately (pCMV-Tat) or inserted downstream of the IL-2 gene (pHIV2-IL-2-neo-C-Tat). Our results showed that HIV2 LTR-Tat-based vectors were much more potent than CMV promoter-based vectors in transient expression. The co-transfection of both plasmids was comparable to a single transfection of pHIV-IL-2-neo-C-Tat in both high and low transfection efficiency cells. In conclusion, the co-placement of HIV2 LTR and tat genes on a single plasmid allows for gene expression as efficiently as a two-plasmid system, suggesting that HIV2 LTR-Tat-based vectors may be attractive tools for gene therapy.

INTRODUCTION

Gene therapy has become a very promising way to treat human diseases. However, insufficient gene expression is one of the major obstacles to its application because high-threshold levels of gene products are often required to obtain significant therapeutic effects (8,17). Extensive efforts have been made to refine the current gene expression approaches to increase gene expression, including the use of efficient gene delivery systems (11), modification of *cis*-acting elements [such as promoters/enhancers, introns, or poly(A) signals (9)], and use of an amplifier strategy (15). The amplifier strategy consists of two genes. The first gene encodes for a transcription activator protein that can activate the promoter controlling the second gene. A well-studied strong transactivator is the Tat protein from the human immunodeficiency virus (HIV). It can dramatically increase the basal gene expression initiated by the HIV long terminal repeat (LTR) (1). Tat protein performs its function by binding to the transactivation response RNA element to enhance the efficiency of the elongation or processivity of RNA polymerase II (5,10). Our laboratory has developed a set of plasmid amplifier vectors (15) that combine the HIV *tat* gene (1) driven by a cytomegalovirus (CMV) promoter, along with the HIV2 LTR in the same construct (15). These constructs have been shown to produce 28 times more gene product than the CMV promoter-based control vectors (15). Since the CMV promoter is generally considered to be the most powerful promoter currently used in gene therapy (9), these amplifier constructs are very promising candidates for gene therapy.

An important question that has yet to be addressed is whether placing both transcriptional units (i.e., HIV LTR and the *tat* gene) on the same plasmid (*in cis*) is an equally efficient way to achieve high-level transactivation as the co-transfection of two transcriptional units on separate plasmids (*in trans*). Conventionally, co-transfection is the most commonly used approach to deliver multiple genes into cells. The problem is that there is no assurance that multiple genes will be expressed proportionally and effectively. Since co-transfection may be limited by low rates of membrane and nuclear entry, inefficient gene expression may arise when the coordinated expression of multiple subunits is required. Our laboratory has previously reported the construction of a mammalian expression vector with two multiple cloning sites for the expression of two foreign genes (16). Using such a vector, multiple genes can be arranged at a fixed ratio in the one-plasmid system, thus ensuring the certainty of co-expression. Another disadvantage of using a two-plasmid system is that the generation of stable "co-transfectants" is laborious and time-consuming because it requires the sequential integration of plasmids containing two separate components. However, the use of a one-plasmid system would facilitate the process by producing stable co-transfectants in a single selection step.

There are at least two potential problems associated with the use of multi-genic vectors. The first such problem is promoter interference, which often occurs between internal promoters and the 5' LTR in retrovirus vector backbones. Promoter interference has been reported to be a potential problem in achieving efficient gene expression in

virus vector systems when heterologous promoters are used (12,14). In our one-plasmid system, the CMV promoter is located downstream of the HIV2 5' LTR to direct *tat* gene expression. Whether the HIV LTR interacts with the CMV promoter in non-viral vector backbones is a meaningful question to answer. Another concern when using multigenic vectors is their larger size compared to plasmids containing a single gene of interest. Whether plasmid size affects transfection efficiency also needs to be addressed. Despite the inherent inefficiency of the two-plasmid system, no study to date has clearly compared the effectiveness of the co-transfection of two plasmids to the transfer of a single plasmid containing both genes when using cationic lipids.

In this paper, we directly compared the co-transfection of the *tat* gene and HIV2 LTR plasmids to the transfection of a plasmid harboring both HIV2 LTR

and *tat* genes. Because transfection efficiency may be an important factor that affects the outcome of comparisons as mentioned above, both high and low gene transfection efficiency cell lines were used. To determine whether the *tat* gene expression under the control of a CMV promoter in either pHIV2-IL-2-neo-C-Tat or pCMV-Tat is equivalent, intracellular Tat production was determined by flow cytometry. These studies should serve as a model system for the effects of *cis* and *trans* gene placement in other gene therapy approaches.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

The human lung cancer cell A549, murine melanoma cell B16, human fibroblast HS68, and human T cell leukemia cell line Jurkat were pur-

chased from the ATCC (Manassas, VA, USA). All cell lines were cultured in RPMI 1640 media (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine and grown at 37°C in 5% CO₂.

Plasmid Construction

The pHIV2-IL-2-neo-C-Tat plasmid contains both HIV2 LTR and *tat* genes on the same construct. The human IL-2 and *tat* genes were controlled by HIV LTR and CMV promoters, respectively. The pCI-IL-2-neo plasmid contains the IL-2 gene driven under a CMV promoter derived from pCI-neo (Promega, Madison, WI, USA). These two plasmids were constructed and described by Tsang et al. (15). The pHIV2-IL-2 plasmid was constructed by replacing the CMV promoter with the HIV2 LTR. pCMV-Tat was derived from pHIV2-IL-2-neo-C-

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Table 1. IL-2 Production after Transient Transfection of the pHIV2-IL-2-neo-CMV-Tat Plasmid or Co-transfection of pHIV2-IL-2 Plus pCMV-Tat Plasmids

Cell Lines	Transfection Efficiency (%)	IL-2 Production (Arbitrary Units)							
		pCI-IL-2-neo (1 µg)	pHIV2-IL-2 (1 µg)	pHIV2-IL-2-neo-C-Tat (1 µg)	pHIV2-IL-2 + pCMV-Tat (1:1)	pHIV2-IL-2-neo-C-Tat (0.5 µg)	pHIV2-IL-2 + pCMV-Tat (1:3)	pHIV2-IL-2-neo-C-Tat (0.2 µg)	pHIV2-IL-2 + pCMV-Tat (1:10)
A549 (<i>n</i> = 6) ^a	40–83	1	0.4 ± 0.2	9.3 ± 2.7	7.8 ± 1.3	3.7 ± 1.6	4.3 ± 1.5	1.8 ± 0.4	2.7 ± 0.7
B16 (<i>n</i> = 5)	6–20	1	0.7 ± 0.5	8.4 ± 4.4	8.9 ± 4.9	3.4 ± 1.8	4.4 ± 3.4	0.9 ± 0.5	2.9 ± 1.5
Jurkat (<i>n</i> = 3)	5–10	1	1.1 ± 0.6	68 ± 30	51 ± 20	39 ± 17	28 ± 15	15 ± 7	7.2 ± 1.4

To keep the total amounts of DNA in the range of optimal protocols, the amount of pHIV2-IL-2-neo-C-Tat was reduced to 0.5 or 0.2 µg when the molar ratio of *tat* gene:HIV2 LTR was adjusted to 1:3 or 1:10. Results of *cis* and *trans tat* gene expression are positioned side by side. pCI-IL-2-neo was used as a positive control. Due to great variations among experiments, the IL-2 expression level was represented in results as relative values (i.e., IL-2 expression after transfection of 1 µg pCI-IL-2-neo was arbitrarily defined as 1 arbitrary unit in each experiment). The reported values are data from several experiments and presented as $\bar{x} \pm \text{SD}$. The percentage of GFP-positive cells was determined by flow cytometry after the transfection of 1 µg pCMV-GFP into the same batch of cells used in the transfection or co-transfection experiments.

^aNumber of experiments.

Pairs of values shown in bold are significantly different ($P < 0.05$; P values were determined by Student's *t* test).

Tat by removing the HIV2 LTR, IL-2 gene, simian virus 40 (SV40) promoter, and partial neomycin resistance gene (*neo*). The GFP-expressing plasmid, pCMV-GFP, was constructed with the GFP gene under the control of a CMV promoter. Figure 1 shows schematic illustrations of the plasmid structures.

Lipid-Mediated Gene Transfer

All transfections were performed in six-well plates after complexing the plasmid DNA with the cationic lipids DMRIE-C (Invitrogen), according to the manufacturer's instructions. For each six-well plate, 2×10^5 A549 cells, 1×10^5 B16 cells, or 3×10^5 HS68 cells were seeded in 2 mL media and incubated overnight. Before transfection, 1×10^6 Jurkat cells were washed with serum-free medium and resuspended in 0.2 mL optiMEM™ (Invitrogen). For transfection with DMRIE-C, an optimal ratio of DNA:lipid was used (1 µg:4 µL for A549 and HS68 cells; 1 µg:6 µL for B16 and Jurkat cells). After the formation of the lipid-DNA complexes, the lipoplexes were mixed gently with the cells and incubated at 37°C for 4–5 h in optiMEM, and then the media was replaced with complete cell media. For co-transfections, pHIV2-IL-2 and pCMV-Tat were mixed at molar ratios of 1:1, 1:3, or 1:10. The total amount (1.4 µg) of transfected DNA was kept constant by the addition of carrier DNA, pUC18 (Invitrogen). pCMV-GFP was used as a transfection control. The transfection efficiency was determined by flow cytometry.

IL-2 ELISA

Supernatants were collected 48 h after lipid transfection, and IL-2 was assayed by ELISA using a Human IL-2 ELISA kit (BD Biosciences Pharmingen, San Diego, CA, USA).

Detection of Intracellular Tat by Flow Cytometry

Intracellular Tat was detected according to a method previously described (13), with a few modifications. Briefly, cells were resuspended in 100 µL PBS, diluted 10-fold in -80°C methanol, kept at -80°C for 30 min, washed twice in PBS, and resuspended in 200 µL anti-Tat monoclonal antibody (Intracel, Rockville, MD, USA) solution (2 µg in PBS, 2% FBS). Incubations with anti-Tat or secondary antibody fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (Caltag Laboratories, Burlingame, CA, USA) were kept on ice for 60 min. The cells were washed twice in PBS between incubations. Samples were fixed in 2% paraformaldehyde and analyzed using a FACStar (BD Biosciences, San Jose, CA, USA).

Establishment of pHIV2-LTR-IL-2 Stably Transfected Clones

Linearized vector pHIV2-LTR-IL-2 (2 µg) was transfected into A549, B16, and Jurkat cells using DMRIE-C as described earlier. The cells were selected in the presence of G418 (600–800 µg/mL) after transfection, cloned by limiting dilution, and assayed by IL-2 ELISA.

Clones with different basal activity (high, intermediate, and low levels of IL-2 secretion) were chosen for analysis.

RESULTS

HIV2 LTR-Tat-Based Vectors Are Much More Potent than CMV Promoter-Based Vectors in a Transient Expression System

A CMV promoter-based vector (pCI-IL-2-neo) was used as the positive control because the CMV promoter is a commonly used and potent promoter. Previous results have shown that HIV2 LTR-Tat-based vectors were much stronger than CMV promoter-based vectors in SW480 human colon carcinoma cells (15). To study that observation further, additional cell lines with either high or low transfection efficiency were chosen. To ascertain the basal gene expression driven by the HIV LTR and the Tat transactivation effects under transient conditions, the HIV2 LTR-containing plasmid pHIV2-IL-2 was included in the experiments. As shown in Table 1, pHIV2-IL-2-neo-C-Tat was from 8- to 9-fold more potent than the CMV promoter-based vector pCI-IL-2-neo in both the high transfection efficiency cell line A549 and the low transfection efficiency cell line B16. In Jurkat cells, the difference in promoter strength between the two vectors increased by more than 60-fold. The Tat-null vector pHIV2-IL-2 was comparable to pCI-IL-2-neo in IL-2 production in all tested cell lines.

Transfection of a Single Plasmid Harboring Two Genes Is Comparable to Optimal Co-Transfection of Separate Tat and HIV2 LTR Plasmids

To compare the effectiveness of a one-plasmid with a two-plasmid system in transient gene expression, the single plasmid pHIV2-IL-2-neo-C-Tat or the two individual pHIV2-IL-2 and pCMV-Tat plasmids were transfected into the A549, B16, and Jurkat cell lines as described. The results (Table 1) showed that the co-transfection of independent pHIV2-IL-2 and pCMV-Tat plasmids (at molar ratios of 1:1 and 1:3) was not significantly different ($P > 0.05$) in IL-2 expression from the transfection of the single plasmid pHIV2-IL-2-neo-C-Tat in each of the three cell lines. When the molar ratio of pHIV2-IL-2 to pCMV-Tat was adjusted to 1:10, IL-2 expression was significantly higher (P

< 0.05) than that obtained by transfection of equal molar pHIV2-IL-2-neo-C-Tat in A549 and B16 cells. In Jurkat cells, IL-2 levels did not increase even with transfection of a 10-fold excess of the Tat-expressing plasmid ($P > 0.05$).

Dose-Dependent Tat Transactivation

Tat is a critical component of the amplifier vector pHIV2-IL-2-neo-C-Tat. Determination of the Tat transactivation pattern is helpful to maximize the amplification. This has been achieved using pHIV2-IL-2 stably transfected clones. A dose-dependent response was observed when different amounts of pCMV-Tat (0.25, 0.5, 1, and 2 μ g) were transfected into the pHIV2-IL-2 stably transfected A549 clones (Figure 2). The transfection of 0.25 μ g pCMV-Tat led to a 3- to a 16-fold increase in IL-2 production. The inducible potential in A549 clones with

low basal LTR activity (approximately 1.5 ng/10⁶/24 h) was significantly higher than the clones with high basal LTR activity (9–28 ng/10⁶/24 h). Similar dose-response relationships of Tat transactivation were also observed in B16 and Jurkat clones (data not shown).

Differential Tat Protein Detection in pCMV-Tat and pHIV2-IL-2-neo-C-Tat Transfected Cells

Since there was no significant difference in IL-2 expression between a single transfection and individual co-transfection, it was necessary to determine whether *tat* gene expression under the control of the CMV promoter in both the pHIV2-IL-2-neo-C-Tat and pCMV-Tat plasmids was also equivalent. Intracellular Tat production was determined by flow cytometry. Interestingly, after the transfection of 1 μ g pHIV2-IL-2-neo-C-Tat plasmid into

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A549 cells, only 18% of the A549 cells expressed detectable levels of Tat protein. In contrast, more than 41% of the A549 cells produced Tat when the same molar amount of individual pCMV-Tat plasmid (0.6 μ g) was used (Figure 3). Transfection of more pCMV-Tat (1 μ g) did not significantly increase (41%–49%) Tat expression in these cells. Similar results were observed repeatedly in B16 cells (data not shown).

DISCUSSION

Lipid-mediated gene transfer has been widely used in both in vivo and in vitro studies. However, compared with viral vectors, gene transfer by lipids is inefficient (8,21). To overcome this hurdle, high-performance lipids with minimal toxicity have been developed (2,4,7, 18). An alternative strategy is the optimization of plasmid vectors for high gene expression. Basic elements for the optimization of vectors include promoters, enhancers, introns, poly(A) signals, and transactivators. The CMV promoter is one of the most commonly used and strongest promoters. However, optimization of CMV promoter-based vectors by swapping introns and poly(A) signals and adding enhancers has only led to limited improvement (19,20). A more efficient way of obtaining high gene expression is the use of transactivators.

Figure 1. Structure of the plasmids. pCI-IL-2-neo was used in transient expression experiments as a positive control. pCMV-Tat was derived from pHIV2-IL-2-neo-C-Tat by removing the HIV2 LTR, IL-2 gene, pA, SV40 promoter, and partial *neo* genes. pCMV-GFP was used to standardize transfection efficiency. pA, polyadenylation signal. SV40/neo, simian virus 40 promoter and neomycin resistance gene.

Our previous results have shown that the combination of HIV LTR with the transactivator Tat allows much more potent gene expression than CMV promoter-based vectors in a human colon cancer cell line, SW480 (15). This finding was confirmed with other human cell lines, A549, Jurkat, and human foreskin fibroblast (HS68) (data not shown). Although murine cells are less responsive to Tat (6), the pHIV2-IL-2-neo-C-Tat plasmid is also more efficient than pCI-IL-2 in the murine cell line B16. Our amplifier vectors worked as efficiently in stable and transient transfections. The average levels of

IL-2 production by pHIV2-IL-2-neo-C-Tat-modified A549 clones were up to 5- to 10-fold higher than CMV-based vector-modified clones. High-expressing cells remain high expressers after stable transfection (data not shown). Efficient gene expression achieved by HIV LTR-Tat-based amplifier vectors suggests their broad potential applications.

For the HIV LTR-Tat amplifier system to work efficiently, the HIV LTR needs to have sufficiently expressed the Tat protein that is present. This co-ex-

Figure 2. Dose-response relationship in Tat transactivation effects. Different doses of pCMV-Tat (0.25, 0.5, 1, and 2 μ g) were transfected into pHIV2-IL-2 stably transfected A549 (AMs) clones. Three clones with low (AM-6), medium (AM-44), and high (AM-58) basal activity of IL-2 production (24 h; 10^6 cells) were chosen. The measured fold increase over the specific basal level for each clone is shown. The reported data was obtained from three independent experiments and presented as $\bar{x} \pm$ SD.

Figure 3. Intracellular Tat expression after transient transfection with Tat-expressing plasmids. Detection of intracellular Tat protein after the transfection of 1 μ g pHIV2-IL-2-neo-CMV-Tat, 0.6 μ g, and 1 μ g pCMV-Tat was determined by flow cytometry as described in Materials and Methods. Positive rates (shown in brackets) of intracellular Tat were determined as shifts in fluorescence intensity compared with untransfected A549 cells. One microgram of pHIV2-IL-2-neo-CMV-Tat contained the same numbers of copies of the *tat* gene as 0.6 μ g pCMV-Tat.

pression is not a problem when both the HIV LTR and *tat* gene are positioned in a single plasmid. However, when the HIV LTR and *tat* gene are on two separate plasmids and co-transfected into cells, especially cell lines with low transfection efficiency, the unpredictable distribution of two plasmids in each cell may lead to suboptimal transactivation effects and an overall lower level of gene expression. Similar concerns about the use of two plasmids in vivo, in which the gene transfer efficiency may be low, also exist.

Using HIV LTR stably transfected A549, B16, and Jurkat clones, we demonstrated that there was a dose-response relationship in Tat transactivation effects. Therefore, it was important to know whether the Tat protein production would be equivalent between pCMV-Tat and pHIV2-IL-2-neo-C-Tat. By flow cytometry, the results showed that only 18% of A549 cells transfected with pHIV2-IL-2-neo-C-Tat plasmid were positive for Tat, while equal molar transfection of pCMV-Tat plasmids produced Tat in 41% of the cells. Lower expression of Tat by the intact pHIV2-IL-2-neo-C-Tat plasmid may partially explain why, in low transfection efficiency cell lines, the effects of co-transfection were comparable to that obtained by the transfection of a single plasmid. However, it is possible that the binding form of Tat, which associates transactivation response element, may not be accessible to anti-Tat antibody. To further clarify the possibility of promoter interference, a different reporter gene than *tat* may be needed.

The pHIV2-IL-2-neo-C-Tat plasmid is 7.6 kb and significantly larger than either the pHIV2-IL-2 (6 kb) or pCMV-Tat (4.6 kb) plasmids. Thus, it was possible that the larger size of the single plasmid might have limited the lipid-mediated gene transfer. To address this issue, FITC-labeled, plasmid-transfected cells were evaluated by flow cytometry 24 h after transfection. Our results showed that plasmid sizes ranging from 4.3 to 7.6 kb did not affect DNA trafficking through the cell membranes (data not shown). However, it is still possible that size can affect the nuclear transport of the plasmids, which is generally considered to be a more important limitation than plasmid uptake.

In summary, our results revealed that a HIV2 LTR-Tat-based vector, pHIV2-IL-2-neo-C-Tat, produced from 10- to 60-fold higher gene expression compared to CMV promoter-based vectors. The co-transfection of separate Tat and HIV2 LTR plasmids was comparable to the transfection of a single plasmid harboring both genes. Although the co-placement of the LTR with the *tat* gene in the same construct (one-plasmid system) was not more advantageous than the two-plasmid system in in vitro transient gene expression, prominent features of LTR-Tat-based vectors (i.e., high gene expression and easier use) suggest that these vectors might be useful and practical tools for in vivo gene therapy.

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