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# Investigation of requirements for efficient gene delivery using the HIV-1 based lentiviral transduction system

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ABSTRACT

The specific recognition and selection of the HIV-1 packaging signal psi ($\Psi$) sequence which is mediated by Gag protein is believed to be pivotal for selective viral genomic RNA packaging and has been a basis for the development of HIV-based transgene delivery systems. However, the requirement of the psi sequence has been questioned recently by a report postulating that the psi element is not absolutely required for transgene transduction. Here, we used a four-plasmid transgene delivery system and analyzed the results by HIV p24 antigen assay, MT4 infection assay, HT1080 colony assay, and reverse transcription-PCR (RT-PCR). The results clearly demonstrate that the psi sequence must be present for efficient transgene encapsidation and transduction.



## Introduction

The Human Immunodeficiency Virus-1 (HIV-1) belongs to a lentivirus subfamily of *Retroviridae* and contains ~10 kb of plus-strand RNA [1]. The full-length unspliced viral RNA generated and exported from the nucleus by the viral Rev–RRE (Rev Responsive Element) interaction [2,3] can serve as a template for translation of Gag or Gag/Pol proteins as well as a genomic viral RNA encapsidated into progeny virions. In this encapsidation process, recognition and selection of the viral genomic RNA mediated by the specific interaction between the Nucleocapsid (NC) domain of Gag protein and a packaging signal called the psi ($\Psi$) sequence located downstream of the 5′ Long Terminal Repeat (LTR) [4–7] are required for selective viral genomic RNA packaging and for generation of functional infectious virus [8–10]. A complex of viral genomic RNA and Gag protein has been observed to co-localize in the perinuclear region [11] and to be transported to the plasma membrane by intracellular trafficking to encapsidate the RNA into virions [12–14]. Mutations in the NC domain and the psi sequence have been shown to affect their specific interaction and to result in generation of virus particles with packaging defects [10,15], including the incorporation of cellular tRNA or mRNA, or spliced viral RNAs instead of full-length viral genomic RNA [8].

The specificity of viral RNA packaging has been exploited as the basis for a number of lentivirus-based transgene delivery systems [16–18]. These systems generally employ several distinct engineered plasmids, including a transgene plasmid in which the transgene transcription unit is placed downstream of the psi sequence, along with other plasmids expressing either Gag/Pol protein or Env protein.

However, the necessity of the psi sequence for viral packaging, and thus transduction of a transgene, has recently been challenged. Previous studies have suggested that a portion of the Gag gene sequence, in addition to the psi sequence, is required and increases the encapsidation level of viral genomic RNA when it is placed between the psi sequence and a transgene [16–20], thus constituting an extended packaging signal.

More recently, Laham-Karam and Bacharach have shown that even when the entire psi sequence including SL1–4 was deleted, the transduction efficiency of transgene RNA was not greatly affected. Transduction efficiency was only 2- to 5-fold less than wild type, arguing strongly that the psi element is not absolutely required for transgene transduction [21].

To clarify the role of the psi sequence and the partial Gag gene sequence in the efficient packaging and transduction of transgenes, we examined the effect of systematic deletions of these elements using a four-plasmid transgene delivery system. Our results clearly indicate that the psi element must be present for efficient transgene encapsidation and transduction, and that a portion of the Gag gene sequence also makes a minor contribution.

## Materials and methods

*Plasmid construction.* HIV-based lentiviral transgene plasmids were constructed as follows. pLenti/EGFP (Enhanced Green

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Fluorescence Protein) was generated by removing the β-globin Intron II and tetR regions with EcoRI treatment with pLenti6/TR (Invitrogen, Carlsbad, CA) and replacing them with an EGFP open reading frame from pEGFP-N1 (Clontech, CA). To create the pLenti/PBS plasmid, PBS primers (PBS.EcoRV_F: 5′-AGGCGCCCGAACAG GGACTTGAAAGCGAAAGGGAAACCAGAGGAGCTCTCTCGGATATCCC TGGAGGAGGC-3′, PBS.EcoRV_R: 5′-GCCTCCTCCAGGGATATCCGAG AGAGCTCCTCTGGTTTCCCTTTCGCTTTCAAGTCCCTGTTCGGGCGCCT-3′) flanked by the NarI site at the 5′-terminal, and EcoRV (underlined) followed by EcoNI sites at the 3′-terminal were chemically synthesized (Genotech, Republic of Korea), and annealed together. This double-strand DNA fragment was digested with NarI and EcoNI and inserted into pLenti/EGFP in which the region from PBS to MAp was deleted by treatment with the same restriction enzymes. To generate pLenti/PBS.Map plasmid, the MAp region was amplified from pLenti/EGFP (F-MAp_EcoRV: 5′-CCCGATATCAGC GGGGGAGAA-3′, MAp_R: 5′-GCCTCCTCCAGGTCTGAAGATC-3′) and ligated into pLenti/PBS after digestion of both insert and backbone plasmids with the same EcoRV and EcoNI restriction enzymes. pLenti/PBS.psi was cloned by inserting a DNA fragment containing PBS and the psi sequence, which was obtained by PCR from pLenti/EGFP (Lenti_PBS_F: 5′-CGGCGCCCGAACAGGGACTT-3′, Lenti_psi_R: 5′-TCCTCCTCCAGGTAATACTGACGCTC-3′), into pLenti/EGFP in which the PBS to MAp region had been deleted by NarI and EcoNI treatment.

The MAp region of pLenti/EGFP was replaced by a multiple cloning site (MCS) sequence to test the effect of MAp substitution. Three hundred fifty base pairs of MCS was separated from pSE380, digested with XmnI, and inserted into EcoNI- and Klenow-treated pLenti/PBS.psi to produce pLenti/MCS.

pLP1, pLP2, and pLP/VSVG that express Gag/Pol, Rev, and VSVG, respectively, were purchased from Invitrogen. Every plasmid constructed was verified by enzyme digestion and sequencing.

*Transfection of 293FT cells.* 293FT cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% of fetal bovine serum, 1% of penicillin/streptomycin, and 1% of non-essential amino acids at 37 °C and 5% $CO_2$. One day before transfection, $1 \times 10^6$ 293FT cells were seeded into 6-well plates and incubated for 24 h. Three micrograms of protein-expression plasmids (pLP1, pLP2, and pLP/VSVG) and 1 μg of lentiviral transgene plasmids plasmids were used for virus packaging. Most of the transfection procedure was performed according to the manufacturer's instructions. Briefly, packaging plasmids and lentiviral transgene plasmid were mixed with 250 μl of opti-MEM (Gibco, MD) and incubated for 5 min at room temperature. Lipofectamine 2000™ (Invitrogen) transfection reagent was then dropped onto the plasmid mixture. The DNA–reagent mixture was incubated for 40 min at room temperature and then added to 293FT cells. After 5–6 h, the DNA–reagent mixture was removed, and fresh DMEM with 100 mg/L sodium pyruvate was added to cells. After 30 h, 293FT cells and the culture media were harvested and analyzed.

*Infection of MT4 cells.* MT4 cells were cultured in RPMI 1640 media with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C and 5% $CO_2$. To determine viral infectivity, $1 \times 10^5$ MT4 cells were suspended in 20 μl of RPMI and seeded in 96-well plates. Two hundred microliters of viral supernatants obtained from transfected 293FT cells were filtered with 0.2-μm pore filters, added to MT4 cells, and incubated at 37 °C for 3 h. Then, viral supernatants were removed, and 200 μl of RPMI were added to wells and incubated for 48 h. Forty-eight hours postinfection, GFP-positive MT4 cells were observed by fluorescence microscopy (Axiovert 200, Zeiss, Germany) and quantitatively analyzed by FACS.

*FACS analysis.* The FACSort apparatus (Becton Dickinson, NJ) was used to analyze transfected 293FT cells or infected MT4 cells. One-fifth of the 293FT cells harvested 30 h posttransfection were suspended in 1 ml of PBS and then analyzed by fluorescence-activated cell sorting (FACS). MT4 cells, which were infected with viral supernatants and incubated for 48 h, were harvested and suspended in 1 ml PBS, and EGFP-positive cells were counted by FACS.

*HIV p24 Enzyme-Linked ImmunoSorbent Assay (ELISA).* To measure the p24 concentration in viral supernatants, 100 μl of harvested viral supernatants were serially diluted (10-fold dilutions) with 1× phosphate buffer. One hundred microliters of diluted supernatants were added to wells coated with p24 antibody in the Vironostika HIV-1 antigen p24 ELISA kit (Biomerieux, France). The amounts of the viral antigen were determined by following the manufacturer's instruction.

*Colony assay.* HT1080 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% non-essential amino acids at 37 °C and 5% $CO_2$. One day before infection, $1 \times 10^5$ cells were seeded in 6-well plates and incubated at 37 °C overnight. At 30% confluence, viral supernatants were serially diluted (10-fold) four times, to a total volume of 1 ml and added to wells with a 6 μg/ml final concentration of polybrene (Sigma, MO). After incubation at 37 °C overnight, diluted viral supernatants were replaced with 2 ml of complete culture medium. The next day, blasticidin (5 μg/ml final concentration; AG Scientific, CA) was added to each well and incubated at 37 °C for 15–16 days. Every third day, the DMEM in wells was exchanged with new media containing blasticidin. After 15–16 days of selection, each well was washed with 1 ml PBS and, 1 ml of crystal violet solution [1% crystal violet (Sigma) and 10% ethanol] was added and incubated for 10 min at room temperature. Then the cells were washed with 1 ml PBS four times to remove excess crystal violet, and stained colonies were counted.

*Reverse transcription-PCR.* Viral supernatants were prepared from transfected FT cells in a 100-mm dish, according to the manual of the Lipofectamine 2000 manufacturer (Invitrogen); 9 ml of viral supernatant was harvested from each 100-mm dish and centrifuged at 50,000*g* for 2 h to collect virions. The resulting pellets of virions were suspended in 0.3 ml DMEM at 4 °C and incubated overnight. Suspended virion solution (0.25 ml) was mixed with Trizol LS (0.75 ml; Invitrogen), and a general RNA extraction protocol was followed. Fifty nanograms of RNA were treated with DNase I (Promega, WI) and used for RT-PCR. RT-PCR was performed per the protocol in the Titanium One-Step RT-PCR Kit manual (Clontech). The following two types of primers, EGFP and 5′-LTR, were used. The EGFP primers were 5′-GTGAGCAAGGGCGAGGAGCTG-3′ (forward sequence) and 5′-GGGTCTTGTAGTTGCCGTCGTC-3′ (reverse sequence). The 5′-LTR primers were 5′-GATCTGAGCCTG GGAGCTCTC-3′ (forward sequence) and 5′-CCTTTCGCTTTCAAGTCC CTGTTC-3′ (reverse sequence).

## Results

To evaluate the necessity of the psi element for packaging and transduction of a transgene using a recombinant lentiviral vector system, we examined five different lentiviral transgene plasmids, all containing CMV promoter-driven EGFP transgenes, as shown in Fig. 1. The pLenti/EGFP plasmid contains complete PBS, psi, MAp, and RRE elements, in that order, immediately downstream of the 5′-LTR. The pLenti/PBS.psi, pLenti/PBS.MAp, and pLenti/PBS plasmids harbor deletions of MAp, psi, and both MAp and psi regions, respectively. We also constructed pLenti/MCS, a plasmid in which the MAp region of pLenti/EGFP was replaced by a non-viral MCS sequence with a length similar to that of MAp to address the influence of MAp on viral packaging.

Three plasmids necessary for virus production, pLP1, pLP2, and pLP/VSVG, expressing HIV Gag/Pol, Rev, and VSVG proteins, respec-