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Identification of Retroviral Vectors Producing High Viral Titer

Yong-Jae Shin[1], Michael J Lenardo[2], Tae-Kyu Park[1] and Kwang-Ho Lee[1]*

Department of Molecular Biology[1], Konkuk University, Chung Ju 380-701, Korea,
Laboratory of Immunology[2], NIAID, NIH, 9000, Rockville Pike,
Bethesda, MD 20892, U.S.A.

=Abstract=

Retroviral vector provide a highly efficient method for gene transfer into eukaryotic cells. This vector system can be divided into two components; the retroviral vector itself and the retroviral packaging cell line. The key improvement in the design of these two components are focused on two aspects; the reduction of helper virus production and high titer-virus. We used PA317 for retrovirus packaging cell line, for its high producibility of viral titer. To test the ability of the vectors to generate high titer-virus, we have chosen four different retroviral vectors; LN, LNSX, LNCX and LXSN. To test easily the viral titer, we have made recombinant construction with CD4 and CD8, checked their viral titer and stained their surface expression. LXSN which contain SV40 early promoter in front of *neo* gene showed best results in viral transient transfection assay, dot blot assay and surface expression. In addition, recombinant containing CD8 generally showed much higher viral titration and surface expression than CD4.

Key Words: Retroviral vector, Viral titer, CD4, CD8

INTRODUCTION

Retroviral vectors have many advantages as gene transfer vehicles, including high efficiency of gene transfer and colinear insertion of transferred genes into host cell chromosomes. However, the utility of retroviral vectors is often limited by the low and variable titer of the recombinant viruses and low efficiency of expression of the transduced gene in the infected cells. A key improvement in the design of murine virus-based retroviral vectors was the discovery that the signal for packaging of viral RNA into virions extends into the *gag* region of the virus [1,2,3]. Inclusion of this region in vectors provided about a 10-fold increase in vector titer and a corresponding increase in gene transfer efficiency compared with earlier vectors. However, homologous overlap of *gag* sequences present in packaging cells led to frequent generation of helper virus in early packaging cell lines [8], and can still lead to infrequent helper virus generation in improved packaging cell lines [5]. To solve these problems, Miller *et al* have designed a set of retroviral vectors which cannot yield helper virus by homologous recombination with the retroviral genome present in the packaging cells. They have inserted unique cloning sites and strong viral promoters to facilitate expression of inserted cDNAs [7]. CD4 and CD8 are nonpolymorphic T lymphocyte surface protein, thus these molecules are easily detected by surface

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* Corresponding author: Kwang-Ho Lee, Department of Molecular Biology, Konkuk University, Chung Ju 380-701, KOREA. Tel: 0441) 840-3613, Fax: 0441) 851-5235, e-mail: kwangho@kcucc.cj.konkuk.ac.kr 소속기관명: Kon Kuk University

staining. We have designed to insert human CD4 or CD8 into these vectors and analysed the surface expression of inserted molecules to examine their viral titers. PA317 amphotropic cells were used in this study for retrovirus packaging, for its generation of helper-free virus. In this experiment, we demonstrate the vectors producing high viral titer between examined constructions and the effects of inserted molecules for high viral titer.

MATERIALS AND METHODS

Cells and culture conditions

Cells were grown in Dulbecco's modified Eagle's medium (DMEM) with high glucose (4.5 g/L) supplemented with 10% fetal bovine serum (PA317 cells; American Type Culture Collection #CRL 9078) or 10% calf serum (NIH 3T3). After transfection of viral construction into PA317, 750 μg/ml of G418 were added to culture medium to make stable transfectant. Cells were grown at 37℃ in a humidified incubator with 5% CO_2.

Vectors and construction of virus expressing CD4 or CD8

Viral vectors such as LN, LNCX, LNSX and LXSN were described [6]. Human CD4 (3 kb) and CD8 (1.5 kb) in pMV7 vectors were digested with *EcoR1*. LNCX and LNSX were digested with *HpaI* and *StuI*, respectively. Phosphorylated *EcoRI* linkers (Stratagene) were ligated to these enzyme digested vectors by using T4 DNA ligase. LXSN has *EcoRI* site in multicloning sites. *EcoRI* fragments of CD4 or CD8 were ligated into each vector. Direction of inserted CD4 or CD8 in these constructs were confirmed with restriction enzyme map.

Virus production by transient transfection

Virus was generated from the plasmid forms of retroviral vectors by transient transfection of packaging cells. DNA was purified by filtration before use in a plasmid purification columns (Qiagen Co). PA317 retrovirus packaging cells were 5 X 10^5 cells per 60 mm dish on day 1. On day 2 the culture medium was replaced with 4 ml fresh medium, and viral plasmid DNA was then transfected onto the cells using the calcium phosphate precipitation procedure [4]. For each plasmid sample, a DNA-$CaCl_2$ solution was made by mixing 25 μl 2 M $CaCl_2$, 10 μg plasmid DNA (in 10 mM Tris-Cl, pH7.5) and water to make 200 μl total. Precipitation buffer was freshly prepared by mixing 100 μl 0.5 M HEPES-NaOH (pH7.1), 125 μl 2 M NaCl, 10 μl 150 mM Na_2HPO_4-NaH_2PO_4 (pH7.0), and water to make 1 ml total. DNA-$CaCl_2$ solution (200 μl) was added dropwise with constant agitation to 200 μl precipitation buffer. After 30 min at room temperature the resultant fine precipitate was added to a dish of cells. Cells were exposed to the DNA precipitate until day 3 when the medium was aspirated and fresh medium was added. On day 4 the virus containing medium was removed, centrifuged at 3,000 X g for 5 min to remove cells and debris, and used to infect cells. After virus harvest, the transfected cells were trypsinized and split 1:20 into medium containing 1.5 mg/ml G 418 (Gibco BRL) to determine the transfection efficiency. To generate stable transfectant, remove medium, wash cells with PBS and add 5 ml of prewarmed fresh medium containing 1.5 mg/ml of G418 (about 50% active) at day 2. Change medium at next day. After day 3 change medium at every 2~4 days. Amount of G 418 was changed to 750 μg from day 3.

Virus assay

For assay of virus, recipient NIH3T3 TK$^-$ cells were seeded at 5 X 10^5 per 60-mm dish on day one. On day 2 the medium was changed to fresh medium containing 4 μg/ml Polybrene, and various amounts of test virus were added. On day 3 the cells were split 1:20 into medium containing 1.5 mg/ml G418. Colonies were counted on day 8. The transfection efficiency was calculated as number of drug resis-

Table 1. Recombinant retrovirus titers produced after transient transfection. Vector titers were measured following transient transfection of PA317 cells as described in Materials and Methods.

Recombinant	Transfection efficiency (Colonies/μg DNA/10^6 cells)	Transient virus Titer (cfu/ml)
LNSX-CD4	3.51 X 10^2	4.4 X 10^3
LNSX-CD8	1.37 X 10^3	1.7 X 10^4
LNCX-CD4	6.6 X 10	1.2 X 10^3
LNCX-CD8	8.86 X 10^2	1.2 X 10^3
LXSN-CD4	9.81 X 10^2	3.6 X 10^3
LXSN-CD8	2 X 10^3	9.6 X 10^3

tant colonies/μg DNA/10^6 transfected cells, assuming that about 10^6 cells were present at the time of transfection.

Retroviral titering by hybridization

Virus-containing medium were washed by centrifugation at 3,000 rpm for 10 min to remove cells and debris. Viral supernatant was centrifuged again at 160 k X g for 90 min at 4℃ in a Ti roter. The virus pellet was suspended in 200 μl of VLT solution containing 5 ml of TE, 5 ml of 2 X lysis buffer (1% SDS, 0.6 M NaCl, 20 mM EDTA and 20 mM Tris), 64 μl of RNasin (3.9 mg/ml) and 50 μl of tRNA (10 mg/ml). The solution was extracted twice with 200 μl of phenol-200 μl of chloroform and once with 400 μl of chloroform. The RNA was precipitated at -70℃ after addition of 1 ml of ethanol, centrifuged at 13,000 X g for 15 min at 4℃, washed with 70% cold ethanol and dried briefly. RNA was suspended in 50 μl of DEPC water. 50 μl of DEN solution containing 30 μl of SSC and 20 μl of 37% formaldehyde was added in RNA solution, and incubated at 60℃ for 15 min. Denatured RNA was blotted onto nitrocellulose filter (S & S) using dot blot apparatus (Millipore). The filter was presoaked with 100 ml of 10 X SSC buffer. After samples were applied, filter was washed with 100 ml of 10 X SSC for each well, soaked in 2 X SSC, dried, and UV cro-sslinked. The condition for hybridization and washing for this filter was described [6]. The probe for hybridization was 3' LTR fragment of MoMLV.

The surface staining of CD4 and CD8

The surface expression of transfected CD4 and CD8 in retrovirus vector was determined by immunofluorescence staining with biotin conjugated anti-human CD4 and biotin conjugated anti-human CD8 (Pharmingen), respectively. FITC-Avidin (Beckton Dickinson) was used for second antibody. FACS (Beckton Dickinson & Co) was used for the analysis.

RESULTS

Transient transfection efficiency

To determine the transfection efficiency of recombinant viral DNA into PA317 cell, transfected cells were trypsinized and split 1:20 into medium containing 1.5 mg/ml G418. Colonies were counted after day 10. As shown in Table 1, LXSN-CD8 has most efficiently transfected (2 X 10^3 colonies) among tested constructions. This might be due to the length of viral construction. Because LXSN has shortest length among tested viral vectors. Furthermore the length of CD8 (1.5 kb) is half of that of CD4 (3 kb).

Virus assay

To test the ability of viral infection of the recombinant viruses, we measured virus pro-

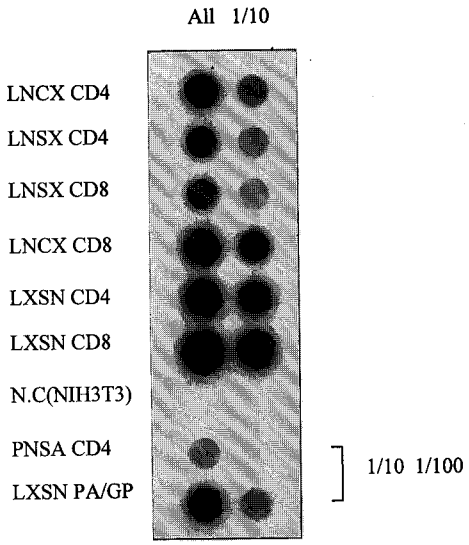

Figure 1. Dot blot hybridization analysis of transfectant. Viral RNAs from transient transfected cells were analyzed by dot blot hybridization. Each vector was inserted by CD4 or CD8. Resuspended or 1/10 diluted viral RNAs were blotted. RNA from NIH3T3 cells was used for negative control (N.C). RNAs from both PNSA CD4 and LXSN PA/GP were used for positive control. Positive control RNAs were applied after dilution to 1/10 and 1/100.

duction following transient transfection of the vectors into PA317 cells as mentioned in materials and methods. Viral titers ranged from 1.2 X 10^2 to 1.7 X 10^4 (Table 1). LNSX CD8 showed highest viral titer (1.7 X 10^4) among tested recombinant viruses, followed by LXSN CD8. This data is consistent to the test carried out with only vectors *albeit* recombinant viruses were produced much less than vector virus alone [7].

Dot blot hybridization test

To measure the virus production, we have harvested the virus from transient transfected cells and prepared RNA from virus and analysed the amount of produced virus with RNA dot blot hybridization. As shown in Figure 1, recombinant with LXSN vectors were most highly produced. Among these two, LXSN inserted with CD8 showed highest density. These data are consistent to the datas of transient transfection efficiency *albeit* LNSX CD8 was produced at small amount.

Surface staining of stable transfectant

We also generate stable virus-producing cell lines containing each recombinant. To test conveniently the ability of virus production, we checked the production of virus with surface staining followed by flow cytometric analysis. Expressions of recombinant on cell surfaces are largely much higher in CD8 than CD4 (Figure 2). Furthermore recombinant with LNCX in both CD4 and CD8 were smallest expression among tested vectors. The marked differences between LNSX and LXSN were not found from surface staining.

DISCUSSION

We have tested a set of retroviral vectors which facilitate cDNA transfer and expression in eukaryotic cells. We have inserted human CD4 or CD8 cDNAs into each of these vectors. In general, SV40 promoter has been proven the weakest promoter for cDNA expression, the LTR is the best and the CMV promoter is intermediate. From our data, we have found that transfection efficiency is dependent on the length of construction. However, virus titers obtained following transient transfection are quite high in LNSX vector. This is consistant with the data of vector alone [7]. In both of transient transfection assay and viral titer, LNCX showed lowest data in either constructs inserted with CD4 or CD8. This might be in part due to the infected cell type and particular cDNA inserted into the vectors.

On the basis of our datas, it is suggested that viral infection can be monitored by using cell surface molecules such as CD4 or CD8.

SUMMARY

We have tested the ability of vectors to generate high titer-virus. LN, LNSX, LNCX and

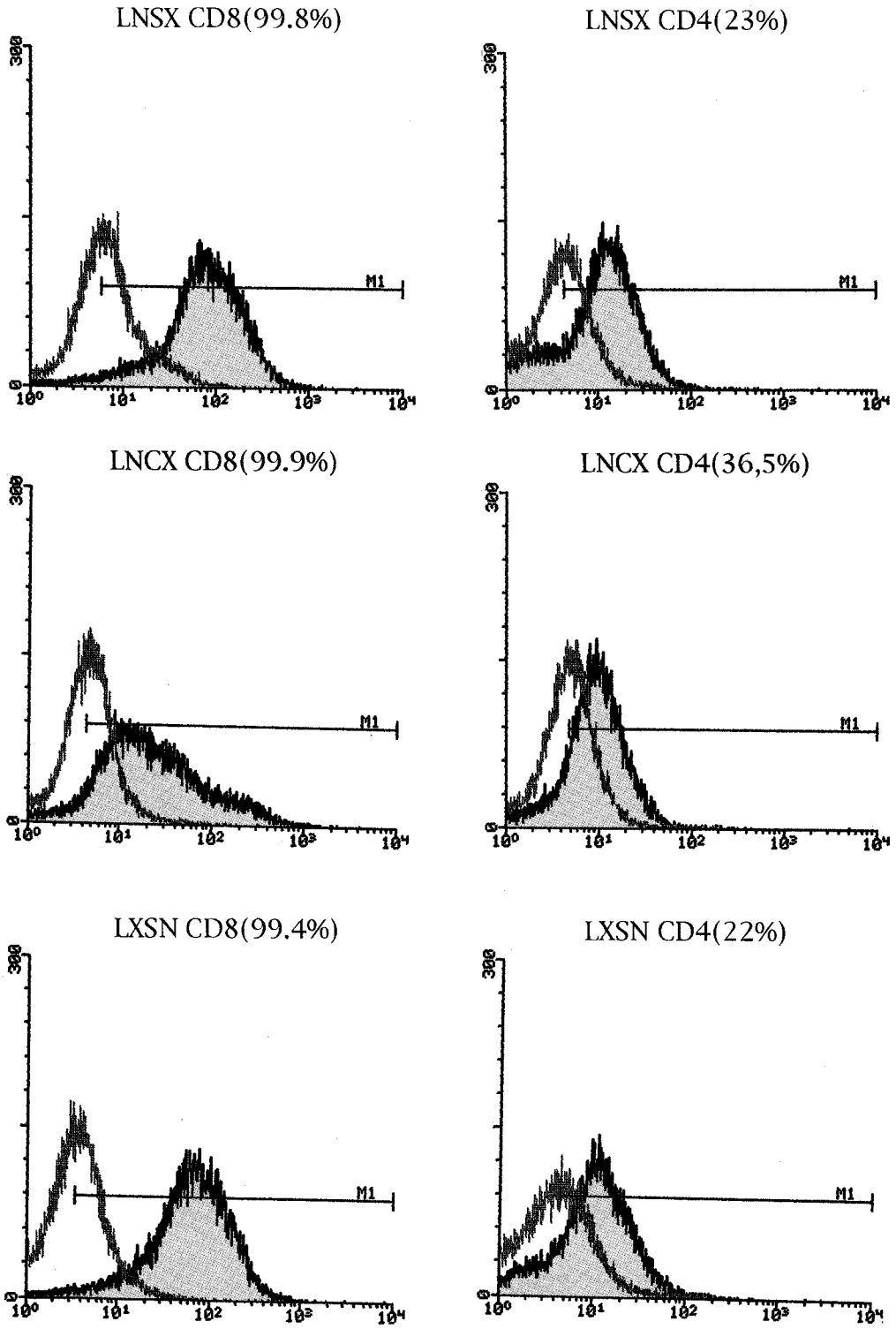

Figure 2. Surface staining of stable transfectant. Stable transfectants were surface stained with anti mouse CD4 or CD8 antibody (filled). Surface expression of these CD4 or CD8 were compared to the negative control stained with only second antibody (open).

LXSN were tested, and human CD4 and CD8 were inserted into these vectors to test easily the viral titer. Among these construction, LXSN which contain SV40 early promoter in front of *neo* gene exhibited best viral titer. Recombinant containing CD8 which has half length of CD4 generally showed much higher viral titration and surface expression than CD4.

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