韓國家畜繁殖學會誌 21(4) : 397~403(1997)
Korean J. Animal Reprod.

Titer Amplification of GALV (Gibbon Ape Leukemia Virus) Pseudotyped Retrovirus Vectors Produced from PG13 Cells

Kim, T., Y. Y. Park, M. S. Kwon*, H-C. Yom, K. H. Kim***, Y. S. Park***, and S. P. Park*****

Dept. of Physiology, School of Medicine, Catholic University of Taegu-Hyosung

PG13 Cell로부터 생산된 GALV (Gibbon Ape Leukemia Virus)-pseudotyped Retrovirus Vector의 증폭

김태완 · 박윤엽 · 권모선* · 염행철 · 김경화*** · 박영식*** · 박세필*****

대구효성가톨릭대학교 의과대학 생리학교실

요 약

Retrovirus vector를 이용한 형질전환 동물의 생산에 있어서 시급히 선행되어야 할 가장 중요한 요소는 표적세포에 대해 높은 감염도를 가진 virus를 생산하는 system의 개발이다. 이러한 문제를 해결하기 위한 일환으로 본 연구에서는 세 가지 방법을 사용하여 세포 배양액 속의 virus의 농도를 높여줌으로써 retrovirus vector system에서 생산되는 virus의 낮은 감염도를 극복하고자 하였다. 이 실험에서 얻어진 결과는 다음과 같다.

1) Virus를 생산하는 세포 (pG13-LNβZ retrovirus producing cell)를 5 mM의 Na-butyrate로 처리했을 때 virus titer는 소의 embryonic trachea 표적세포에서 약 3배의 증가를 나타냈다.
2) Virus가 들어있는 세포 배양액을 ultrafiltration을 통해 농축한 결과, 농축액 속의 virus titer는 대조구에 비해 약 3.6배 증가하였다.
3) 초원심분리를 통해 농축된 virus stock의 titer는 소의 embryonic trachea 표적세포에서 약 1.0×10^5 $LacZ^+TU$ /ml 였는데 이 방법은 대조구에 비해 약 12.5배 만큼의 titer의 향상을 가져왔다.

따라서, 이와같이 농축된 virus stock을 이용할 경우 retrovirus vector system을 이용한 소의 수정란에의 유전자 전이율을 현저히 향상시킬 수 있으리라 사료된다.

(Key words : Retrovirus vector, Packaging cell, Sodium butyrate, $LacZ^+TU$ /ml, Concentration of virus stock)

I. INTRODUCTION

The most important features of retroviruses for their use as vectors are technical ease and effectiveness of gene transfer. This is due to their infectivity for certain target cells resulting in gene transfer. Once the cells are infected by

이 논문은 1996년도 한국학술진흥재단의 공모과제 연구비에 의하여 연구되었슴.

* 계명대 생물학과 (Dept. of Biology, Keimyung University)
** 호서대 생명과학과 (Dept. of Life Sciences, Hoseo University)
*** 경북대학교 축산학과 (Dept. of Animal Science, Kyungpook National University)
**** 마리아 불임크리닉 기초의학연구소 (Maria Infertility Medical Institute)

retroviruses, the resultant viral DNA, after reverse transcription and integration, becomes part of the host cell genome, and the stability of transgene formation maintenance (Temin, 1989) is far superior to other gene transfer systems.

Despite the high potency of the retroviral vector system in gene transfer, however, there is still no report of successful application in domestic mammals. One of the main drawbacks in the application of retrovirus vector system to the transgenic livestock production is difficulty in preparing highly concentrated virus stock. This is caused by the limited efforts expended to develop retroviral vector systems specific for domestic mammals. Most of the retroviral vector systems currently available are based on mouse leukemia virus, hence the recombinant retroviruses produced from these systems are very little infectious to target cells derived from ungulates including bovine, sheep, and pig.

Several solutions reported so far for the low titer problem include boosting virus production by treating virus-producing cells with sodium butyrate (Olsen and Sechelski, 1995), and concentration of virus stock by either centrifugal filtration (Olsen et al., 1994) or ultracentrifugation (Beddington et al., 1989). In addition, Yee et al. (1994) reported a new pseudotyped retrovirus vector system, in which the produced viruses were designed to be packaged by vesicular stomatitis virus glycoprotein G (VSV-G). Two main advantages of the VSV-G pseudotyped retrovirus vector system over other counterparts are: first, high and pantropic infectivity of the produced viruses which can infect cells derived from almost all vertebrates, and second, feasibility of ultracentrifugal concentration of virus stock without loss of virus infectivity. Tests of this new virus vector system, however, have been allowed only to the limited research groups mainly due to commercial reasons. Moreover, one report argued that high titer of the VSV-G pseudotyped retrovirus vector system was not due to real gene transfer to the target cell genome but caused by carry-over of the reporter gene product encapsidated by VSV-G (Liu et al., 1996).

In this study, we tried to amplify titers of the viruses produced from PG13 packaging cells (Miller et al., 1991) by three methods: (i), boosting of virus productivity from the sodium butyrate-treated virus-producing cells; (ii) and (iii), concentrations of virus stock by either filtration or ultracentrifugation. The major difference of PG13 cell line compared to other general mouse leukemia virus (MLV)-based packaging cell line is encapsidation of the produced viruses with GALV (Gibbon ape leukemia virus) envelope. We have previously reported that viruses encapsidated with GALV envelope protein is more infectious to bovine cells than viruses packaged with either xenotropic or amphotropic MLV envelope (Kim et al., 1992a, b).

Ⅱ. MATERIALS AND METHODS

1. Retrovirus vector-producing cell

Construction of GALV pseudotyped retrovirus vector-producing cells (designated as PG13-LNβZ) was made by infecting PG13 packaging cells (Miller et al., 1991) with the LNβZ retrovirus vector produced from amphotropic PA317 (Miller and Buttimore, 1986) virus-producing cells (designated as PA317-pLNβZ), followed by G418 (800 μg/ml) selection for 2 weeks. PA317-pLNβZ cells were made by transient transfection of PA317 cells with the plasmid pLNβZ (Kim et al., 1993b) whose structure is shown at Fig. 1.

The virus-producing cells (PG13-LNβZ) and EBTr (bovine embryonic trachea) target cell line (American Type Culture Collection, CCL44) were maintained in Dulbecco's Modified Eagle

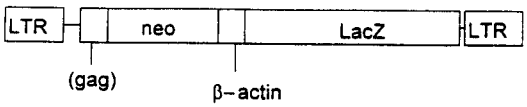

Fig. 1. Structure of pLNβZ retrovirus vector. LTR, long terminal repeat; (gag), 5' portion of of Molony murine leukemia virus; neo, G418 resistant gene; β-actin, rat β-actin promoter; LacZ, *E coli* β-galactosidase gene.

Medium (DMEM) with 4.5 g/l of glucose (Sigma Chemical Co., St. Louis, MO) supplemented with FCS (10%), gentamycin (50 mg/l; Gibco). All cells were grown in a 37℃, 5% CO_2 incubator. To maximize virus-production, the PG13-LNβZ cells were maintained in the medium containing G418 (600 μg/ml) (Emerman and Temin, 1984b).

2. Transfection and infection

Polybrene/DMSO (dimethyl sulfoxide) mediated DNA transfection (Kawai and Nishizawa, 1984) was performed following the established protocols of our laboratory. Briefly, 10 μg of plasmid DNA in 1 ml of medium supplemented with polybrene (30 μg/ml) was added to NIH3T3-based packaging cells plated on the previous day (5×10^5 cells/60 mm dish). After 6 hours of incubation at 37℃ with 5% CO_2 in air, the DNA-medium mixture was aspirated, and DMSO shock was applied by adding 2 ml of 25% DMSO in medium to a 60 mm dish for 1 min. Following 3 washes with medium, cells were fed with 4 or 5 ml of medium and incubated overnight before trypsinization to split the transfected cells. Selection medium was added on the next day of splitting. Fresh selection medium was provided every 2 or 3 days.

Infection of target cells was performed following the procedure of Miller and Rosman (1989). Briefly, 4 ml of the mixture of non-selection medium, various amounts of virus-containing medium (filtered through a 0.22 μm pore-size filter), and polybrene (5 μg/ml of final concentration) was added to target cells which were plated on the previous day. The virus-containing medium was harvested from the virus-producing cells which had been fed with non-selection medium on the previous day. Following one day of culture, infected cells were trypsinized and split in non-selection medium. Addition of selection medium (G418) or X-gal staining of the cells was done on the next day of splitting. Counting of $LacZ^+$ cells was done with the aid of dissecting microscope at 80X magnification. Two or more adjacent $LacZ^+$ cells were counted as one $LacZ^+$ cell under the assumption that a cell can replicate during the time between infection and staining. Virus titer in $LacZ^+$ TU/ml (transforming unit per ml) was calculated by the dilution factor (one ml divided by the volume of virus-containing medium) and splitting factor.

3. Boosting titer by sodium butyrate treatment

To boost virus production, confluent PG13-LNβZ cells were cultured in the medium supplemented with various concentrations of sodium butyrate (1~30 mM). After 24 hours of sodium butyrate treatment, the medium was harvested and applied to the EBTr target cells after filtration through a 0.22 μm pore size filter to remove cells and cell debris.

4. Concentration of the virus stock by centrifugal filtration

15 ml of the virus (0.2-μm filtered) was added to a NanoSpin filter (100,000 MW cut-off, Gelman Sciences) prerinsed by 15 ml of H_2O-through filtration device for 20 min., then centrifuged

in a fixed angle rotor at 500g at 4℃. Every 30 min., centrifugation was stopped to discard the filtrate until the volume of retentate containing the virus reaches the desired reduction in volume. The harvested retentate was filtered through 0.2-μm syringe filter before applying to the EBTr target cells.

5. Concentration of the virus stock by ultracentrifugation

Using swinging bucket rotor (Beckman SW 41 Ti), 0.2-μm filtered virus filled in a polyallomer tube was centrifuged at 100,000g for 2 hours at 4℃. The resulting pellet was resuspended in a small volume of Hank's Balanced Salt Solution (HBSS: 0.4 g KCl, 0.06 g KH_2PO_4, 8 g NaCl, 0.048 g Na_2HPO_4, and 1 g D-glucose /liter), and then filtered through 0.2-μm syringe filter before applying to the EBTr target cells.

Ⅲ. RESULTS

1. Boosting titer by sodium butyrate treatment

To boost virus production from the PG13-LNβZ virus-producing cells, various concentrations of the sodium butyrate were added to the culture medium. As shown in Table 1, 5 mM concentration of sodium butyrate in the medium resulted in the best virus productivity. Compared to the titer of the control (0 mM sodium butyrate), treatment of virus-producing cells with 5mM sodium butyrate increased virus production more than three folds.

2. Amplication of virus titer by centrifugal filtration

Initial 15 ml of virus was concentrated to 1.5 ml after centrifugal filtration. The titer of the 10X concentrated virus stock after 10X reduction in volume was 3.2×10^4 Lac^+TU /ml resulting in 3.6 folds of titer increase, while recovery of total infectivity was 36% $\{3.2\times10^4/(8.8\times10^3)\times10)\}$ (Table 2).

3. Boosting titer by ultracentrifugation

The pellet resulting from centrifugation of virus-containing medium (30 ml), was resuspen-

Table 1. Boosting virus production by sodium butyrate treatment

Sodium butyrate (mM)	Titer ([a]$LacZ^+$ TU /ml) ([b]SE)	Fold(s) of increase
0	5.5×10^3 (6.6×10^2)	1
1	1.0×10^4 (1.7×10^3)	1.8
5	1.7×10^4 (8.2×10^2)	3.1
10	7.9×10^3 (9.9×10^2)	1.4
20	6.0×10^3 ($.2\times10^2$)	1.1
30	5.6×10^3 (5.7×10^2)	1.0

[a]$LacZ^+$ TU /ml, $LacZ^+$ transforming unit /ml; [b]SE; standard error

Table 2. Boosting titer by centrifugal filtration

Treatment	Titer ([a]$LacZ^+$ TU /ml) ([b]SE)	Fold(s) of increase	Rate of recovery
no filtration	8.8×10^3 (6.5×10^2)	1	1
filtration	3.2×10^4(3.6×10^3)	3.6	0.36

[a]$LacZ^+$ TU /ml, $LacZ^+$ transforming unit /ml; [b]SE; standard error

Table 3. Boosting titer by ultracentrifugation

Treatment	Titer ([a]LacZ$^+$ TU /ml) ([b]SE)	Fold(s) of increase	Rate of recovery
no centrifugation	8.0×10^3 (7.5×10^2)	1	1
centrifugation	1.0×10^5 (3.6×10^3)	12.5	0.083

[a]LacZ$^+$ TU /ml, LacZ$^+$ transforming unit /ml; [b]SE; standard error

Table 4. Ultracentrifugal concentration of the viruses harvested from PG13-LNβZ cells treated with sodium butyrate boosting titer by ultracentrifugation

Treatment	Titer ([a]LacZ$^+$ TU /ml) ([b]SE)	Fold(s) of increase	Rate of recovery
no treatment	1.1×10^4 (1.5×10^3)	1	1
sodium butyrate (5 mM)	2.9×10^4 (2.4×10^3)	2.6	
sodium butyrate (5 mM)+ ultracentrifugation	2.1×10^5 (2.7×10^4)	19	0.13

[a]LacZ$^+$ TU /ml, LacZ$^+$ transforming unit /ml; [b]SE; standard error

ded in 200 μl of HBSS. The titer of the concentrated virus stock after 150X reduction in volume was 1.0×10^5 LacZ$^+$TU /ml on EBTr target cells, resulting in 12.5 fold increase in titer. However, recovery rate of total infectivity was only 8.3% $\{1.0\times10^5/(8.0\times10^3)\times150)\}$ (Table 3).

4. Ultracentrifugal concentration of the viruses produced from the PG13-LNβZ cells treated with sodium butyrate

To maximize virus titer, viruses harvested from the PG13-LNβZ treated with 5 mM of sodium butyrate were centrifuged. The titer of the concentrated virus stock after 150X reduction in volume was 2.1×10^5 LacZ$^+$TU /ml on EBTr target cells (Table 4). Two fold increase in titer compared to centrifugal concentration alone (Table 3), seems to be due to increased virus productivity by sodium butyrate treatment which contributed about three fold increase in virus titer (Table 1). Recovery rate of total infectivity was very low also (13 %, Table 4).

Ⅳ. DISCUSSION

Considering the small size of bovine blastocyst (~180 μm in diameter), we estimated that the medium taken from virus-producing cells should contain at least 106 LacZ TU /ml of viruses to infect bovine embryos efficiently. To meet this requirement, we tried to amplify titers of the viruses produced from PG13 packaging cells (Miller et al., 1991) by three methods: (i), boosting of virus productivity from the sodium butyrate-treated virus-producing cells; (ii) and (iii), concentration of virus stock by either filtration or ultracentrifugation. All three methods we tested contributed real increase in virus titer, but even the highest titer resulting from ultracentrifugal concentration was only one tenth of our expectation (1.0×10^5 LacZ$^+$ TU /ml). Possible causes for this low titer are: (i) Addition of LacZ gene in the retroviral vector might contribute decrease in titer (Soriano et al., 1991; Adam et al., 1991); (ii) EBTr cells might be inadequate for target cells because growth and morphological characteristics of the cells tend to decline as the passage increases (ATCC cell line manual); (iii) Inhibitory interaction between LTR and β-actin promoter in a retrovirus

vector (Emerman and Temin, 1984a,b, 1986); (iv) Subcloning of PG13-LNβZ virus-producing cells might increase virus productivity.

In terms of recovery rate, centrifugal filtration method was better than ultracentrifuged one (36% vs 8.3%). However, application of this method to transgenic animal production seems to be inadequate. The titer of the virus stock harvested after centrifugal filtration is far lower than that of the virus stock prepared by ultracentrifugation (3.2×10^4 Lac^+TU/ml vs 1.0×10^5 $LacZ^+TU$/ml). Moreover, preparation of concentrated virus stock by centrifugal filtration was too laborious and sticky (data not shown). In retrovirus vector-mediated transgenic animal production, stickiness of virus-containing medium might be serious problem because high osmolarity of the sticky virus stock in which the embryo to be cultured during infection is detrimental to the embryo viability.

Application of the retroviruses harvested from the sodium butyrate-treated virus-producing cells in transgenic animal production seems to be problematic because sodium butyrate treatment contributed only two or three folds of increase in titer (Tables of 1 and 4) and butyrate in the virus stock might affect embryo viability.

Combined with observations in this study, ultracentrifugation-mediated boosting of virus titer seems to be the best choice in retrovirus vector-mediated transgenic animal production. Of course, mainly due to inherent instability of the retrovirus envelope protein, ultracentrifugation method resulted in very low row recovery rate in total infectivy, substitution of the retrovirus envelope protein with other appropriate protein such as VSV-G will solve this problem (Yee et al., 1994). Based on the experiences accumulated in this study, we are developing VSV-G pseudotyped retrovirus vector system.

V. SUMMARY

For the ultimate goal of efficient retrovirus vector-mediated transgenic animal production, we tried to increase virus titer by employing three methods; boosting virus production by treating virus-producing cells with sodium butyrate, concentration of virus stock by either filtration or ultracentrifugation. Compared to the control, applications of sodium butyrate (5 mM) treatment and filtration resulted in only 3 and 3.6 folds of titer increases on bovine EBTr target cells, respectively. However, concentration of virus-containing medium by ultracentrifugation showed 12.5 folds of titer increase compared to the control (1.0×10^5 $LacZ^+$ TU/ml), indicating the best method which can enhance retrovirus vector-mediated transgenic animal production.

VI. REFERENCES

1. Adam, M. A., N. Ramesh, A. D. Miller, and W. R. A. Osborne. 1991. Internal initiation of translation in retroviral vectors carrying picornavirus 5' nontranslated regions. J. Virology, 65:4985-4990.
2. Beddington, R. S. P., J. L. Morgernstern, and A. Hogan. 1989. An *in situ* transgenic enzyme marker for the midgestation mouse embryo and the visualization of inner cell mass clones during early organogenesis. Development, 106:37-46.
3. Emerman, M. and H. M. Temin. 1984a. High-frequency deletion in recovered retrovirus vectors containing exogenous DNA with promoters. J. Virology, 50:42-49.
4. Emerman, M. and H. M. Temin. 1984b. Genees with promoters in retrovirus vectors can be independently suppressed by an epigenetic mechanism. Cell, 39:459-467.
5. Emerman, M. and H. M. Temin. 1986. Quan-

titative analysis of gene suppression in integrated retrovirus vector. Mol. Cell. Biol., 6:792-800.

6. Kawai, S., and M. Nishizawa. 1984. New procedure for DNA transfection with polycation and dimethyl sulfoxide. Molecular and Cellular Biology, $:1172-1174.
7. Kim, T., M. L. Leibfried-Rutledge and N. L. First 1993a. Gene transfer into bovine cells and embryos using replication-defective retroviral vectors encapsidated with xenotropic murine leukemia virus envelopes. Animal Biotechnology, 4:53-69.
8. Kim, T., M. L. Leibfried-Rutledge, and N. L. First. 1993b. Gene transfer in bovine blastocysts using replication-defective retroviral vectors packaged with Gibbon ape leukemia virus envelopes. Mol. Reprod. Develop., 35:105-113.
9. Liu, M-L., B. L. Winther, and M. A. Kay. 1996. Pseudotransduction of hepatocytes by using concentrated pseudotyped vesicular stomatitis virus G glycoprotein (VSV-G) -Moloney leukemia virus-derived retrovirus vectors: Comparison of VSV-G and amphotropic vectors for hepatic gene transfer. J. Virology, 70:2497-2502.
10. Miller, A. D., and C. Buttimore. 1986. Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. Mol. Cell. Biol., 6:2895-2902.
11. Miller, A. D., and G. J. Rosman. 1989. Improved retroviral vectors for gene transfer and expression. BioTechniques, 7:980-990.
12. Miller, A. D., J. V. Garcia, N. von Suhr, C. M. Lynch, C. Wilson, and M. V. Eiden. 1991. Construction and properties of retrovirus packaging cells based on Gibbon ape leukemia virus. J. Virol., 65:2220-2224.
13. Olsen, J. C., L. G. Johnson, and J. R. Yankaskas. 1994. Methods for the use of retroviral vectors for transfer of the CFTR gene to airway epithelium. "In Gene Therapy Protocols" edited by P. D. Robbins, 153-168. Humana Press, Totowa, New Jersey, USA.
14. Olsen, J. C., and J. Sechelski. 1995. Use of sodium butyrate to enhance production of retroviral vectors expressing CFTR cDNA. Hum. Gen. Ther., 6:1195-1202.
15. Soriano, P., G. Friedrich, and P. Lawinger. 1991. Promoter interactions in retrovirus vectors introduced into fibroblasts and embryonic stem cells. J. Virology, 65:2314-2319.
16. Temin, H. M. 1989. Retrovirus variation and evolution. Genome, 31: 17-22.
17. Yee, J. K., A. Miyanohara, P. LaPorte, K. Bouic, J. C. Burns, and T. Friedmann. 1994. A general method for the generation of high-titer, pantropic retroviral vectors: highly efficient infection of primary hepatocytes. Proc. Natl. Acad. Sci. USA, 91:9564-9568.

(접수일자 : 1997. 11. 16. /채택일자 : 1997. 12. 5)