Journal of Microbiology (2015) Vol. 53, No. 5, pp. 348–353
DOI 10.1007/s12275-015-5134-0

Identification of Porcine Endogenous Retrovirus (PERV) packaging sequence and development of PERV packaging viral vector system

Jiwon Choi[1], Hoon-mi Kim[1], Jong Kwang Yoon[1], Yeondong Cho[1], Hee-Jung Lee[1], Kang Chang Kim[1], Chang-Kyu Kim[2], Gye-Woong Kim[3], and Young Bong Kim[1]*

[1]*Department of Bioindustrial Technologies, Konkuk University, Seoul 143-701, Republic of Korea*
[2]*Division of Animal Resources and Life Science, Sangji University, Wonju 220-702, Republic of Korea*
[3]*Department of Animal Resources Community, KongJu National University, Kong-ju 314-701, Republic of Korea*

(Received Mar 11, 2015 / Revised Apr 9, 2015 / Accepted Apr 13, 2015)

Studies of the retroviruses have focused on the specific interaction of the nucleocapsid protein with a packaging signal in the viral RNA as important for this selectivity, but the packaging signal in porcine endogenous retrovirus (PERV) has not been defined. Herein, we identified and analyzed this packaging signal in PERV and found hairpin structures with conserved tetranucleotides in their loops and nucleocapsid recognition sequences; both of which are key elements in the viral packaging signal of MLV. We evaluated packaging efficiency of sequence variants isolated from viral and proviral integrated genomes. All viral packaging sequences (Ψ) were identical, while five distinct packaging sequences were identified from proviral sources. One proviral sequence (Ψ1) was identical to that of the viral Ψ and had the highest packaging efficiency. Three variants (Ψ2, Ψ3, Ψ4) maintained key elements of the viral packaging signal, but had nucleotide replacements and consequently demonstrated reduced packaging efficiency. Despite of the same overall hairpin structure, the proviral variant (Ψ5) had only one GACG sequence in the hairpin loop and showed the lowest packaging efficiency other than ΔΨ, in which the essential packaging sequence was removed. This result, thus, defined the packaging sequences in PERV and emphasized the importance of nucleotide sequence and RNA structure in the determination of packaging efficiency. In addition, we demonstrate efficient infection and gene expression from the PERV-based viral vector, which may serve as a novel alternative to current retroviral expression systems.

Keywords: Porcine endogenous retrovirus, packaging signal, viral vector

*For correspondence. E-mail: kimera@konkuk.ac.kr; Tel.: +82-2-450-4208; Fax: +82-2-455-1044

Introduction

Porcine endogenous retrovirus (PERV) is a member of the family *retroviridae* and perpetually resides within the porcine genome. PERV is classified into subfamilies, PERV-A, -B, and -C by host tropism; PERV-A and PERV-B infect cross-species, whereas PERV-C only infects pig cells. All of these three subfamilies of PERV are found in pigs, primary cultured porcine cells, and porcine cell lines (Klymiuk and Aigner, 2005; Kimsa *et al.*, 2014). In addition, PERVs are transmitted vertically across generations from host germ line cells infected by retrovirus (Gifford and Tristem, 2003; Bae and Jung, 2014).

Endogenous retroviruses commonly persist within their host without apparent detriment; however, the assembly and egress of the virus is still possible which could initiate further rounds of infection and induce an immune response. Subfamilies PERV-A and PERV-B have demonstrated the ability to infect human cells *in vitro* (Martin *et al.*, 1998; Wilson *et al.*, 2000; Abe *et al.*, 2014). This infectious potential is especially concerning from the standpoint of xenotransplantation of porcine tissues. Pigs currently represent the best animal candidate for organ donation, but the presence of PERVs within their genome is a barrier to their use.

The life cycle of PERV is generally like that of other retroviruses, consisting of viral entry through receptor binding, uncoating, reverse transcription, replication, integration within the host genome, viral gene expression, particle assembly, and budding. However, little is known regarding the mechanistic details of the PERV packaging system. PERVs are type-C retroviruses and, as such, share some sequence and structural features with MLV, which is well-studied for virus packaging. In MLV, the virally encoded gag polyprotein undergoes proteolytic cleavage into three component proteins: matrix protein (MA), capsid (CA), and nucleocapsid (NC) (D'Souza *et al.*, 2001). Of these, NC participates in the selective packaging of two copies of the unspliced full-length genome through its interaction with the viral RNA psi (Ψ) sequence (Sun *et al.*, 2014). The Ψ site is located within the 5'-UTR of the viral genome and contains four stem loop structures, each of which participates in encapsidation, dimerization and transportation. NC contains a single zinc finger motif (CCHC), which has the ability to recognize and bind the psi sequence (Basyuk *et al.*, 2005; Seif *et al.*, 2013).

Previous studies have shown that MLV and PERV share some features in their 5'-UTRs including the tRNA primer binding site (PBS), the splice donor (SD) site, two identical GACG tetra-loops, and a UCUG sequence (Konings *et al.*, 1992), but the psi motif in PERV has not previously been identified. Thus, we characterized the psi sequences of PERV

to determine how variations impact the efficiency of viral packaging and egress.

Materials and Method

Bioinformatics analysis

The nucleotide and protein sequences of PERV and MLV were retrieved from GeneBank in NCBI. Multiple alignments of genomic sequences in this study were carried out using the BioEdit program. The secondary structures of virus and provirus RNA sequences were predicted by using M. Zukers' mfold program (Jaeger *et al*., 1989).

Cell culture and isolation of viral RNA/genomic DNA

Porcine kidney cells (PK-15 cell, ATCC CCL33) were grown in Dulbecco's modified eagle medium (DMEM, Gibco BRL) supplemented with 10% fetal bovine serum (FBS, Gibco BRL), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL). The culture medium was collected, centrifuged at 1,500 rpm for 5 min to remove cell debris, and filtered through 0.45 μm pore filters. Viral RNAs were obtained from PK-15 cell culture supernatant using Trizol reagent (Invitrogen) followed by DNase digestion (TaKaRa) treatment for 30 min at 37°C. The genomic DNA was isolated from PK15 cell lysate using QIAmp DNA mini kit (Qiagen).

Construction of plasmids: pCL MFG-LacZ/PERV-Ψ and pcDNA3.1-PERVgag/pol

Viral RNA was incubated with 10 μl of oligo-dT primers at 65°C for 10 min and mixed with 5 x reverse transcriptase buffer [50 mM Tris HCl, 250 mM KCl, 10 mM $MgCl_2$, (pH 8.1)], 10 mM dNTPs, 100 mM DTT, and M-MLV reverse transcriptase (Bioneer) at 42°C for 1 h. Then, PERV Ψ was amplified by primers: PERV psi_F 5'-ACTAGTTGATCAG CAGACGTGCTAGG-3' and PERV psi_R 5'-AGATCTCGC GGAGGGGGAAGCTTTC-3' for cloning viral Ψ. The same primers were also used for the amplification of Ψ for the integrated form of PERV in genomic DNA. The PCR products were cloned into pGEM-T easy vector (Promega) for sequence alignment. These fragments containing viral/genomic Ψ sequences were subcloned into pCLMFC-LacZ using *Spe*I and *Bgl*II site to replace MLV Ψ with PERV Ψ.

The DNA fragment corresponding to PERV gag/pol, which was in pGEM-T Easy vector (Kim *et al*., 2009) was excised out by Not I, was transferred to the pcDNA3.1(+) expressing vector to result in gag/pol expression under CMV promoter.

Production of pseudotyped PERV and comparison of viral packaging efficiency

To produce PERV pseudotype with MLV glycoprotein and PERV pseudotyped with vesicular stomatitis virus (VSV) envelope glycoprotein, pCL-Eco, pCLMGFLacZ/PERV Ψ and pCLMGFLacZ/PERV Ψ, pcDNA3.1/PERVgag-pol, pHCMVG were transfected to 293T cells in a 6-well culture plate using calcium phosphate method. Two days after transfection, supernatant was collected, centrifuged at 1,200 rpm for 5 min and filtered through 0.45 μm filter units (Millipore). The filtrate was considered as inoculum containing VSV-G pseudotyped PERV and infected Crandell Rees feline kidney (CRFK, ATCC CCL-94) cells. 2 days postinfection, cells were fixed with 2% paraformaldehyde followed by washing with PBS twice and stained with X-gal by applying the staining solution [5 mM $K_4Fe(CN)_6$-$3H_2O$, 5 mM $K_3Fe(CN)_6$, 1 mg/ml X-gal in 0.1 M phosphate buffer supplemented with 2 mM $MgCl_2$] for 3 h and washed with PBS three times. Numbers of X-gal stained cell were counted manually.

Results

Multiple alignment and predicted secondary RNA structure of PERV packaging sequences

The genomic structure of PERV is similar to that of MLV, in which the packaging signal is located in the 5'UTR. To define the PERV packaging region, we analyzed the sequence of the 5' UTR region of PERV-B, which was reported to be produced and viral genome was integrated in PK-15 cells (Chung *et al*., 2014). Previous studies have shown that type-C retroviruses, which PERV and MLV belong to, share features including a primer binding site (PBS), a splicing donor (SD), and two identical GACG hairpin loop motifs as indicated by arrows in Fig. 1A (Konings *et al*, 1992). Further studies have demonstrated that GACG-containing hairpin loops are key factors of retrovirus packaging and have additionally identified GGNG sequences which help nucleocapsid protein (NC) bind to the hairpin loop structures (D'Souza *et al*., 2001; Hagan and Febris, 2003).

Most porcine cells are contaminated by PERV or include the PERV genome in the integrated form in the host genome. To clone the packaging signal of PERV, PK-15 cells were used to analyze the PERV Ψ variants. Using primers designed to amplify the PERV Ψ sequence, PCR products were cloned from virus isolated from PK-15 cell culture supernatant or genomic DNA from PK-15 cells. 12 viral Ψ and 11 genomic Ψ sequences were cloned into pGEM-T easy vector and analyzed. All 12 viral Ψ sequences were identical, whereas five distinct genomic Ψ sequences were observed. One isolate out of 11 proviral Ψs had the same sequence as the viral Ψ (Ψ1) and the rest of the 10 isolates had single nucleotide polymorphisms (SNP) in 7 sites. Two out of the 11 isolates (Ψ2) grouped together with three nucleotide replacements, three (Ψ3) had a different five replacements and another three (Ψ4) contained two nucleotide replacements (Fig. 1A). We predicted the secondary structure of the packaging sequences of each isolate using M. Zukers' mfold program (Fig. 1B). Isolates 1, 3, 4, and 5 had similar secondary RNA structures while isolate 2 had one hairpin structure less than the rest. Isolates 1, 2, 3, and 4 had two conserved hairpin structures containing the important GACG sequences, whereas isolate 5 had the same overall hairpin structure but one GACG was changed to TACG. Finally, all five isolates had conserved GGNG sequences between the two hairpin structures (Fig. 1C). Thus, we delineated a 146 bp region (Fig. 1A) within PERV as the putative packaging signal region containing two GACG hairpin loops with GGNG sequences between the two hairpin loops (Fig. 1C).

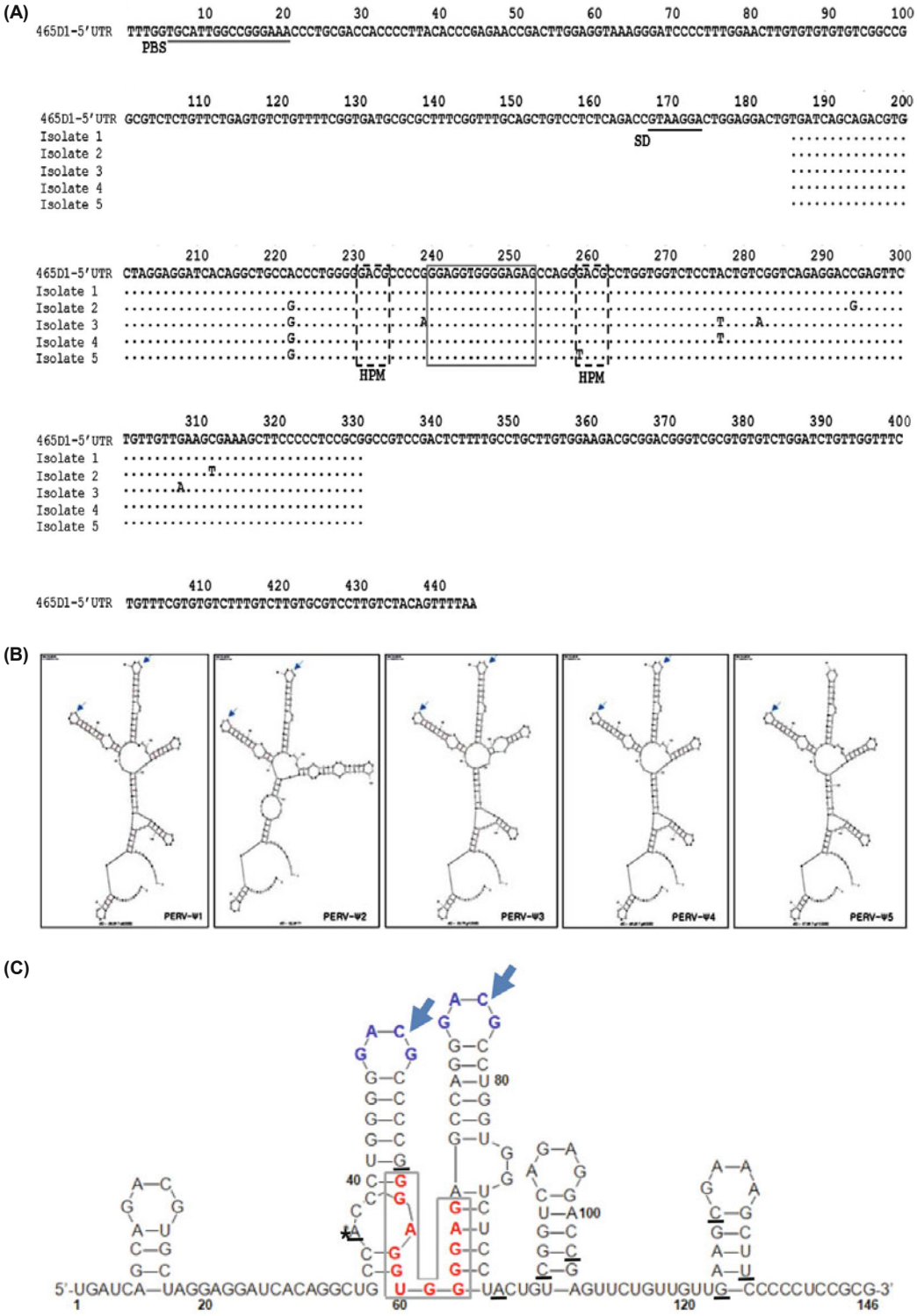

Fig. 1. Multiple alignment and RNA structure of the packaging sequences of isolate 1 to 5. (A) The packaging sequences for PERV isolates 1 to 5 are aligned to a 5'-UTR region in PERV-B strain 465D1 corresponding to nucleotides 630 through 1074. The packaging sequences from 12 viral RNA isolates are all the same as those in 465D1 and named isolate 1. One of the proviral RNA packaging sequences is identical to that of isolate 1 while the other 10 proviral RNA sequences are grouped as isolates 2, 3, 4, and 5 depending on the nucleotide differences. The nucleotides that differ from the 5'-UTR of 465D1 are indicated, while nucleotides that are unchanged from the 465D1 sequence are shown as a dot. GACG tetranucleotides located in two hairpin regions are indicated in the dashed boxes. The gray outlined box marks the GGNG sequence. (B) The secondary RNA structure of PERV packaging sequences in isolates 1 to 5 predicted by M. Zukers' mfold program. (C) The predicted RNA secondary structures of the PERV packaging sequences isolated from the proviral genome. Arrows in (B) and (C) indicate the double hairpin motif containing GACG tetranucleotides in the loop; the black boxed region between them indicates the GGNG sequence. Changed nucleotides are underlined and an asterisk indicates a common point mutation observed in isolates 2 to 5. PBS, primer binding site; SD, splice donor; HPM, hairpin motif.

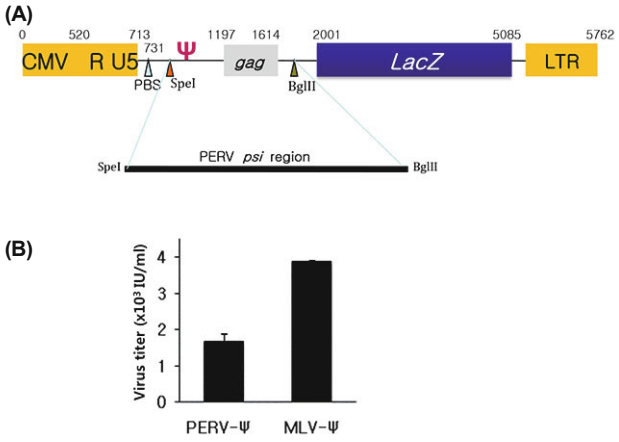

Fig. 2. Schematic representation of pCL MFG-LacZ/PERV-Ψ and titer of viruses. (A) pCLMFG-LacZ-containing MLV packaging signal sequence and partial *gag* gene was replaced with PERV packaging signal with *Spe*I and *Bgl*II digestion. (B) The titer of virus was measured by counting of X-gal stained CRFK cells, to which supernatant from 293T cells, which had been co-transfected with pCL-Eco and each viral DNAs, PERV-Ψ or MLV- Ψ containing the viral packaging sequence, and thus contained virus, was applied. The error bar indicates the standard deviation from three experiments.

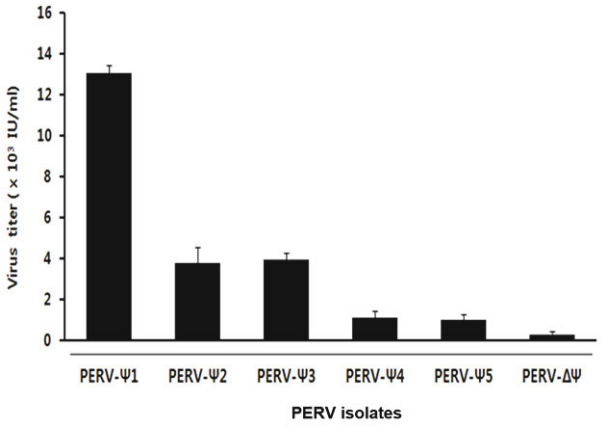

Fig. 3. Infectivity of PERV variants, containing the Ψ sequences from viral RNAs or proviral genomic DNAs. Supernatant containing virus was collected from 293T cells two days post-transfection with the PERV three plasmid system (pPERV-Ψ, pPERVgag/pol, and pHCMVG) and applied to CRFK cells. Three days after infection, X-gal staining was performed to measure the infectivity of the virus produced from the transfected cells. Data were derived from 4 independent experiments and standard deviations are indicated by error bars.

Viral production from a plasmid containing the PERV packaging sequence

To determine the efficacy of this putative PERV packaging sequence, we used it as a replacement for the MLV packaging sequence of the retroviral reporter plasmid, pCLMFG-LacZ (Novus Biologicals) (Fig. 2A). We generated this new reporter plasmid using the Ψ1 sequence isolated as described above from viral and pro-viral sources. We co-transfected this new reporter plasmid with the retrovirus packaging vector, pCL-Eco, which provides gag and pol, and measured viral titer in comparison to that produced with the unaltered reporter plasmid. As shown in Fig. 2B, the virus titer using pCLMGFLacZ/PERV Ψ plasmid was two-fold lower than that using MLV Ψ. Although less efficient, this vector was packaged, resulting in an MLV-pseudotyped virus, which thus establishes the PERV sequence we identified as a functional packaging sequence. Furthermore, because all of the viral components in the system except for the packaging sequence in the new pCLMGFLacZ/PERV Ψ are MLV-based, this result demonstrated that the MLV gag and pol proteins are capable of recognizing the PERV packaging sequence and cross-packaging virus.

Three-plasmid system to produce VSV-G pseudotyped PERV

The PERV Ψ sequence was further characterized by assessing efficiency in the presence of PERV-derived gag and pol rather than those from well-established retroviral vector. The pCLMGFLacZ/PERV Ψ (pPERV-Ψ1) vector was co-transfected with pcDNA3.1/PERVgagpol and pHCMVG, such that PERV gag and pol were expressed and the glycoprotein was replaced by VSV-G. The supernatant from 293T cells, which had been transfected with pPERV-Ψ1 was able to produce $1.4x10^4$ IU/ml of virus resulting in LacZ expression following infection of CRFK cells (Fig. 3). The titer of the virus was about 8 times higher than that of MLV-pseu-

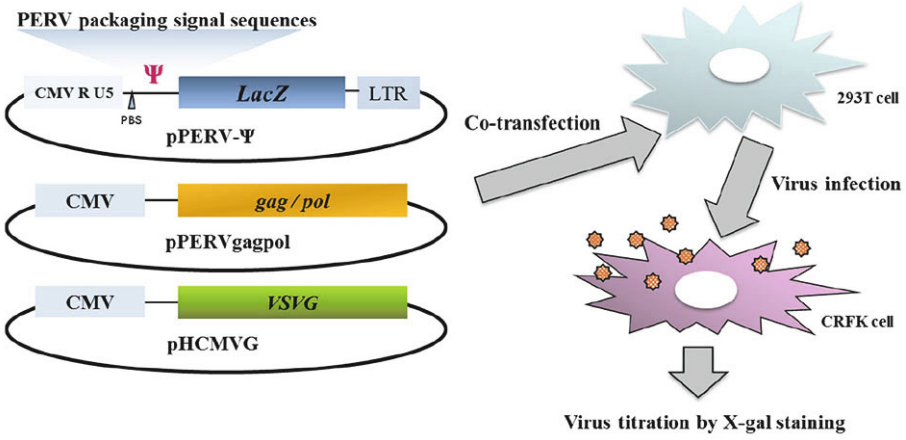

Fig. 4. Schematic diagram of transfection with the three plasmid PERV system and viral infection. On the left, the three plasmid system is depicted: pPERV-Ψ, in which the PERV packaging sequence from the virus has replaced the corresponding MLV sequence; pPERV gag/pol, in which PERV gag/pol was cloned into pcDNA3.1; and pHCMVG, in which VSV glycoprotein has been placed under the control of the CMV promoter. Following transfection into 293T cells, the three-plasmid system produces virus which is collected in the supernatant and applied to infect CRFK cells. Viral packaging efficiency is measured by X-gal staining, which represents the virus titer.

dotyed PERV produced using the two-plasmid system. Thus, as might be expected, the PERV pol and gag are more efficient than their MLV counterparts when packaging sequence containing the PERV Ψ. These findings demonstrate that the three-plasmid system illustrated in Fig. 4 provides the necessary components to safely and efficiently produce infectious PERV.

Comparison of virus packaging efficiency among isolates

To compare the packaging efficiency of PERV Ψ sequences, we replaced the MLV Ψ in pCLMFG-LacZ with that of PERV from each of the five isolates. The supernatant that was taken two days post- transfection was applied to CRFK cells to estimate the packaging efficiency of each pseudotyped PERV. Infected cells were identified by X-gal staining and counted, thus representing the packaging efficiency of each packaging sequence. Variants of Ψ did not alter infectivity of CRFK cells, and viral titer was thus measured for each of the five packaging sequences, Ψ1 through Ψ5, or ΔΨ, in which the packaging signal sequence was deleted (Fig. 3). PERV-Ψ1, which is identical to the viral Ψ, showed the highest packaging efficiency among the five isolates. Isolates PERV-Ψ2 and -Ψ3 had 3.4 times lower efficiency than PERV-Ψ1, and PERV-Ψ4 and -Ψ5 were even less efficient at 11.9 and 13.3 times lower compared to -Ψ1, respectively. PERV-ΔΨ still demonstrated packaging function although it was very low (Fig. 3).

Discussion

For the purpose of xenotransplantation, the use of pig tissues has a variety of potential benefits, including the availability of organs and cell lines for genetic modification; however, their use has potential risks as well, including the risk of infection by virus residing within the pig genome, which possesses the ability to infect human cells. Although the expression of the PERV genome in hosts is down-regulated by DNA methylation and histone deacetylation, more than 50 copies of PERV have been reported to be integrated into the pig genome and porcine cell lines have been shown to produce PERV (Patience *et al.*, 2001; Lee *et al.*, 2011; Wolf *et al.*, 2013). Thus, before pig tissues can be safely used for xenotransplantation purposes more must be learned of this risk and methods which may be used to circumvent it.

The process by which PERV RNA is packaged to produce virus from the integrated genome in the host has not previously been reported. Thus, we defined, characterized, and compared variants of the PERV packaging signal to find the relationship between the sequence variation and the packaging efficiency. The packaging sequence was identified in the 5'UTR region and was composed of several stem-loop structures with GACGs in the loop and NC recognition sequences. These sequences, which were comprised of 146 bp, were cloned from virus in the media of PK-15 cells or from provirus in the genome of PK-15 cells. All 12 clones isolated from viruses had the same viral sequence, whereas only one out of 11 clones from proviral genomic sequences matched the non-integrated viral clones. Despite the unique viral packaging sequence found in the virus, the nucleotide polymorphism identified in the integrated proviral genome suggests that mismatch nucleotide errors occur frequently by retrovirus reverse transcriptase in the process of conversion from viral RNA to proviral genomic DNA (Menéndez-Arias, 2009). Because the viral reverse transcriptase lacks the proof reading function derived from 3' to 5' exonuclease, low fidelity of the reverse transcriptase likely generated at least 5 kinds of proviral genomic sequences. On the other hand, finding just one unique viral sequence despite of the detection of 5 proviral sequences suggests the importance of the nucleotide sequence and its resulting secondary or tertiary RNA structure on packaging efficiency.

In order for the proviral genomic sequence to be packaged into virus, transcribed RNAs from the proviral DNA need to be transported from the nucleus, dimerize, and interact with NC (Konings *et al.*, 1992; Mougel and Barklis, 1997; Basyuk *et al.*, 2005). In HIV-1, the GGNG tetranucleotide has been identified as essential for the interaction with the zinc fingers of the NC protein (Kim *et al.*, 2000). Herein, we have reported the identification of GGNG sequences between two hairpin-containing GACGs in the 5'-UTR of PERV. The hairpin structures with GACGs are important to form the dimerization of the stable kissing-loop complex (De Tapia *et al.*, 1998; Mundigala *et al.*, 2014). In addition, the hairpin structure in Ψ is involved in the cytoplasmic localization of viral RNAs (Basyuk *et al.*, 2005). Although the nucleotide polymorphisms observed in Ψ-3, -4, and -5 does not affect the predicted secondary hairpin loop structure, they may affect RNA dimerization and transport to the cytoplasm, thus resulting in reduced packaging efficiency. The lowest packaging efficiency was found in the Ψ-5 isolate and was likely due to the mutation in one of the GACG tetranucleotides, which would result in the disruption of NC binding and a low rate of PERV production.

In addition to the identification of the PERV Ψ sequence and characterization of its efficiency, this research also demonstrated that the three plasmid system used herein, including the retroviral expression plasmid with the PERV packaging signal, the PERV-gag-pol-expressing plasmid and the VSV-glycoprotein-expressing plasmid, produced replication-incompetent viruses and showed efficient gene expression. The use of virus produced from this system is thus different from an infectious replication-competent clone of PERV, such as has been designed for the study of its pathogenicity (Xiang *et al.*, 2013). Furthermore, since PERV has been reported to not cross-package with human endogeneous retrovirus (HERV) (Suling *et al.*, 2003), we propose that this PERV-based three plasmid system might be a safe and effective viral expression alternative to MLV- and HIV-based systems.

Acknowledgements

This research was supported by a grant of the KIPET (Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries) from Ministry of Agriculture, Food and Rural Affairs (314003-02-1-SB010 and 112157-03-2-SB020).

References

Abe, M., Fukuma, A., Yoshikawa, R., Miyazawa, T., and Yasuda, J. 2014. Inhibition of budding/release of porcine endogenous retrovirus. *Microbiol. Immunol.* **58**, 432–438.

Bae, E.H. and Jung, Y.T. 2014. Tetherins of various species inhibit the release of porcine endogenous retrovirus from human cells. *Acta Virol.* **58**, 53–60.

Basyuk, E., Boulon, S., Skou Pedersen, F., Bertrand, E., and Vestergaard Rasmussen, S. 2005. The packaging signal of MLV is an integrated module that mediates intracellular transport of genomic RNAs. *J. Mol. Biol.* **354**, 330–339.

Chung, H.C., Nguyen, V., Moon, H.J., Kim, H.K., Park, S.J., Lee, J.H., Choi, M.G., Kim, A.R., and Park, B.K. 2014. Inhibition of porcine endogenous retrovirus in PK15 cell line by efficient multi-targeting RNA interference. *Transpl. Int.* **27**, 96–105.

D'Souza, V., Melamed, J., Habib, D., Pullen, K., Wallace, K., and Summers, M.F. 2001. Identification of a high affinity nucleocapsid protein binding element within the Moloney murine leukemia virus Psi-RNA packaging signal: implications for genome recognition. *J. Mol. Biol.* **314**, 217–232.

De Tapia, M., Metzier, V., Mougel, M., Ehresmann, B., and Ehresmann, C. 1998. Dimerization of MoMuLV genomic RNA: redefinition of the role of the palindromic stem-loop H1 (278-303) and new roles for stem-loops H2 (310-352) and H3 (355-374). *Biochemistry* **37**, 6077–6085.

Gifford, R. and Tristem, M. 2003. The evolution, distribution and diversity of endogenous retroviruses. *Virus Genes* **26**, 291–315.

Hagan, N. and Fabris, D. 2003. Direct mass spectrometric determination of the stoichiometry and binding affinity of the complexes between nucleocapsid protein and RNA stem-loop hairpins of the HIV-1 Psi-recognition element. *Biochemistry* **42**, 10736–10745.

Jaeger, J.A., Turner, D.H., and Zuker, M. 1989. Improved predictions of secondary structures for RNA. *Proc. Natl. Acad. Sci. USA* **86**, 7706–7710.

Kim, C.H., Kao, C.C., and Tinoco, I. Jr. 2000. RNA motifs that determine specificity between a viral replicase and its promoter. *Nat. Struct. Biol.* **7**, 415–423.

Kim, N.Y., Lee, D., Lee, J., Park, E.W., Jung, W.W., Yang, J.M., and Kim, Y.B. 2009. Characterization of the replication-competent porcine endogenous retrovirus class B molecular clone originated from Korean domestic pig. *Virus Genes* **39**, 210–216.

Kimsa, M.C., Strzalka-Mrozik, B., Kimsa, M.W., Gola, J., Nicholson, P., Lopata, K., and Mazurek, U. 2014. Porcine endogenous retroviruses in xenotransplantation--molecular aspects. *Viruses* **6**, 2062–2083.

Klymiuk, N. and Aigner, B. 2005. Reliable classification and recombination analysis of porcine endogenous retroviruses. *Virus Genes* **30**, 357–362.

Konings, D.A., Nash, M.A., Maizel, J.V., and Arlinghaus, R.B. 1992. Novel GACG-hairpin pair motif in the 5' untranslated region of type C retroviruses related to murine leukemia virus. *J. Virol.* **66**, 632–640.

Lee, D., Lee, J., Yoon, J.K., Kim, N.Y., Kim, G.W., Park, C., Oh, Y.K., and Kim, Y.B. 2011. Rapid determination of perv copy number from porcine genomic DNA by real-time polymerase chain reaction. *Anim. Biotechnol.* **22**, 175–180.

Martin, U., Kiessig, V., Blusch, J.H., Haverich, A., von der Helm, K., Herden, T., and Steinhoff, G. 1998. Expression of pig endogenous retrovirus by primary porcine endothelial cells and infection of human cells. *Lancet* **352**, 692–694.

Menéndez-Arias, L. 2009. Mutation rates and intrinsic fidelity of retroviral reverse transcriptases. *Viruses* **1**, 1137–1165.

Mougel, M. and Barklis, E. 1997. A role for two hairpin structures as a core RNA encapsidation signal in murine leukemia virus virions. *J. Virol.* **71**, 8061–8065.

Mundigala, H., Michaux, J., Feig, A.L., Ennifar, E., and Rueda, D. 2014. HIV-1 DIS stem loop forms an obligatory bent kissing intermediate in the dimerization pathway. *Nucleic Acids Res.* **42**, 7281–7289.

Patience, C., Switzer, W., Takeuchi, Y., Griffiths, D.J., Goward, M.E., Heneine, W., Stoye, J.P., and Weiss, R.A. 2001. Multiple groups of novel retroviral genomes in pigs and related species. *J. Virol.* **75**, 2771–2775.

Seif, E., Niu, M.J., and Kleiman, L. 2013. Annealing to sequences within the primer binding site loop promotes an HIV-1 RNA conformation favoring RNA dimerization and packaging. *RNA* **19**, 1384–1393.

Suling, K., Quinn, G., Wood, J., and Patience, C. 2003. Packaging of human endogenous retrovirus sequences is undetectable in porcine endogenous retrovirus particles produced from human cells. *Virology* **312**, 330–336.

Sun, M., Grigsby, I., Gorelick, R.J., Mansky, L.M., and Musier-Forsyth, K. 2014. Retrovirus-specific differences in matrix and nucleocapsid protein-nucleic acid interactions: implications for genomic RNA packaging. *J. Virol.* **88**, 1271–1280.

Wilson, C.A., Wong, S., VanBrocklin, M., and Federspiel, M.J. 2000. Extended analysis of the *in vitro* tropism of porcine endogenous retrovirus. *J. Virol.* **74**, 49–56.

Wolf, G., Nielsne, A.L., Mikkelsen, J.G., and Pedersen, F.S. 2013. Epigenetic marking and repression of porcine endogenous retroviruses. *J. Gen. Virol.* **94**, 960–970.

Xiang, S., Ma, Y., Yan, Q., Lv, M., Zhao, X., Yin, H., Zhang, N., Jia, J., Yu, R., and Zhang, J. 2013. Construction and characterization of an infectious replication competent clone of porcine endogenous retrovirus from Chinese miniature pigs. *Virol. J.* **10**, 228.