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Investigation of Deletion Variation and Methylation Patterns in the 5' LTR of Porcine Endogenous Retroviruses

K. C. Jung, D. M. Simond[1], C. Moran[2], W. J. Hawthorne[1], J. T. Jeon[3], D. I. Jin and J. H. Lee*

Division of Animal Science and Resources, Research Center for Transgenic Cloned Pigs
Chungnam National University, Daejeon 305-764, Korea

ABSTRACT : The xenotransplantation of pig organs and cells can be related with a risk of transmission of infectious diseases to human. Previous findings indicate that the regulatory region of PERV for retroviral transcription, replication and integration into the cellular DNA is located on the 5' Long Terminal Repeat (LTR). The objective of this study is the investigation of methylation and deletion status of the PERV 5' LTR region which can be used for regulating PERV expression. We compared the sequences of genomic DNA and bisulfite-treated genomic DNA from PK-15 cells expressing PERV to observe the methylation status of the 5' LTR. Our results showed that the CpG sites of U3 were methylated and methylation was inconsistent in the R and U5 regions. Also, variable numbers of 18 bp repeats and 21 bp repeats were detected on 5' LTR by sequencing analysis. The consistent U3 methylation might be indicative of host suppression of expression of the retroviruses. (**Key Words :** Pigs, Long Terminal Repeat (LTR), Porcine Endogenous Retroviruses (PERVs), Xenotransplantation)

INTRODUCTION

Xenotransplantation using porcine organs and tissues may alleviate the shortage of organs for allotransplantation (Jung et al., 2007). However, there may be a risk of transmitting porcine endogenous retroviruses (PERVs) from pig to human in xenotransplantation because PERVs are capable of infecting human cells *in vitro* (Wilson et al., 1998) and thus have potential to cause xenozoonoses. The 5' Long Terminal Repeat (LTR) located in the promoter region has been shown to be influential in the transcription of replication-competent PERV (Scheef et al., 2001). The LTR region of PERV has sites of binding factors, such as SOX5, Ets-1, Evil, GATA, v-Myb and CEBP, which are essential for transcription (Wilson et al., 2003). CpG methylation is an important factor controlling gene expression in vertebrates (Bird and Wolffe, 1999). The study of HIV-1 has shown that methylation in the 5' LTR suppressed LTR transcription and viral expression (Bednarik et al., 1990). Also, there is evidence that transcription of HERV-K is negatively correlated with methylation levels in the 5' LTR (Lavie et al., 2005). PK-15 cells in culture show an approximately 500-fold higher level of PERV expression compared with primary fibroblasts (Dieckhoff et al., 2007). Therefore, we have analyzed CpG methylation and deletion in the U3 repeat box in a sample of 5' LTRs from pK15 cells as a first step in investigating regulation of PERV expression.

* Corresponding Author: J. H. Lee. Tel: +82-42-821-5779, Fax: +82-42-825-9754, E-mail: junheon@cnu.ac.kr

[1] Centre for Transplantation and Renal Research, Westmead Millennium Institute, Westmead Hospital, Australia.

[2] Faculty of Veterinary Science, University of Sydney, NSW 2006, Australia.

[3] Division of Applied Life Science, Gyeongsang National University, 900 Gajwa-dong, Jinju, Gyeongnam 660-701, Korea.

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MATERIALS AND METHODS

DNA extraction and sodium bisulfite treatment

The PK-15 cell line was cultured in Dulbecco's Minimal Essential Media (DMEM) with 3% fetal bovine serum. At passage, total genomic DNA was isolated from these cells using a QIAamp DNA mini Kit (Qiagen, USA) according to the manufacture's instructions. Sodium bisulfite treatment was performed according to EpiTect® Bisulfite instructions (Qiagen, USA).

PCR with genomic DNA

The PERV 5' LTRs for sequence analysis were

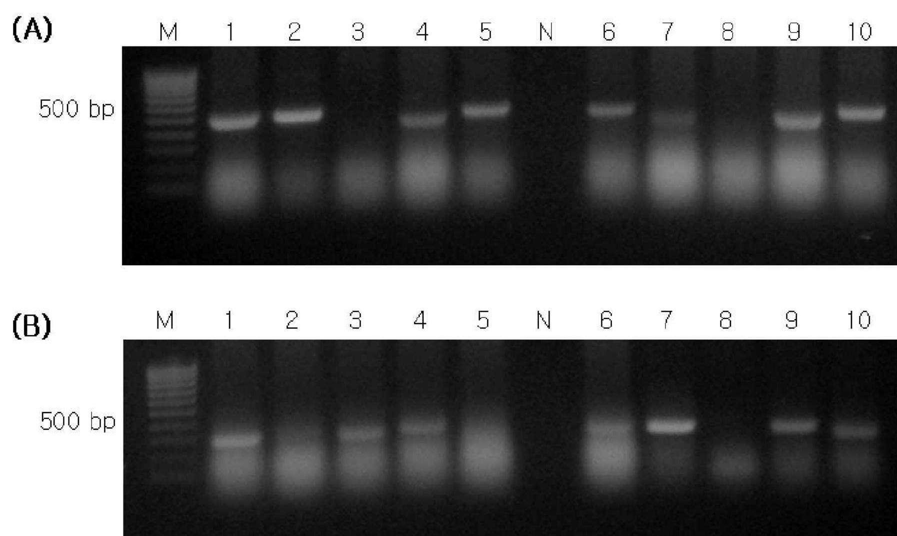

Figure 1. The PCR based screening of clones containing PERV 5' LTR. (A) Eight positive clones containing inserts amplified from untreated genomic DNA. (B) Seven positive clones containing inserts amplified from bisulfite-treated genomic DNA.

amplified under the following conditions: 95°C for 5 min, follow by 42 cycles of 30 sec at 95°C, 40 sec at 60°C, 60 sec at 72°C and final extension for 10 min at 72°C, using the following primers: PERV-G-F (5' GCTATCCTGGC CTAAGTAAG 3') and PERV-G-R (5' CCAACGCACCAA ATGAAGG 3').

PCR with bisulfite-treated genomic DNA

PCR was performed using bisulfite-treated genomic DNA as template with primers PERV-M-F (5' GGAGTTATTTTAAAATGATTGGTTT 3') and PERV-M-R (5' AAACCAATAAAAAAACAATCCC 3'), designed by Methyl Primer Express® Software v1.0 (Applied Biosystems, USA). Amplification was performed by a modified touchdown PCR, with denaturation at 95°C for 2 min, followed by 15 cycles of 30 sec at 95°C, 60 sec at 55°C, extension for 60 sec at 72°C, followed by 25 cycles of 30 sec at 95°C, 60 sec at 52°C and 60 sec at 72°C and a final extension at 72°C for 10 min. The bisulfite treatment converts unmethylated cytosine to thymine in the template DNA so that the sequence of the amplicon will differ (C→T) at all sites of methylation compared with the reference sequence from an untreated template.

Cloning and sequencing of the PERV 5' LTR

PCR amplicons were gel purified using UltraClean™ GelSpin™ DNA Extraction Kit (MOBIO, USA) and subcloned into plasmid of pCR® 2.1-TOPO TA Cloning Kit (Invitrogen, Australia) and sequenced using the facility at AGRF (Australian Genome Research Facility) in Brisbane, Australia.

RESULTS AND DISCUSSION

Multiple copies of PERV are present in pK15 and amplification products can arise from about 50 different PERV integration sites (Le Tissier et al., 1997). Unsurprisingly, several different sized amplicons resulted from both the untreated and bisulfite-treated genomic DNAs (Figure 1). Therefore, three PERV LTR clones from untreated genomic DNA (clone numbers 2, 4, 5 in Figure 1A) and two clones from bisulfite-treated genomic DNA (clone numbers 1, 9 in Figure 1B) were selected and sequenced. Figure 2 shows LTR variants differing in the copy numbers of interleaved 18 bp repeats and 21 bp repeats located in the U3 region of the PERV 5' LTR, as well as deletions with respect to the reference sequence (Scheef et al., 2001).

These 18 and 21 bp subrepeats are located in the enhancer element of the U3 region (Wilson et al., 2003), containing binding sites for transcription factors which contribute to the activation of transcription (Wilson et al., 2003). Variation in promoter activity has been suggested to result from the presence or absence of combinations of the 18 and 21 bp subrepeats in the LTR region (Scheef et al., 2001). Vectors used to infect human cells showed stronger promoter activity when possessing five 39 bp repeats (18 and 21 bp subrepeats) in comparison to low activity in vectors with the repeat deleted (Scheef et al., 2001). It has also been shown that the deletion of enhancer segments of the Moloney Murine Leukemia Virus (M-MuLV) LTR reduced infectivity 20- to 100-fold in NIH-3T3 mouse cells (Hanecak et al., 1986).

Yoder et al. (1997) suggested that the cytosine methylation of integrated retroviral genomes is a result of

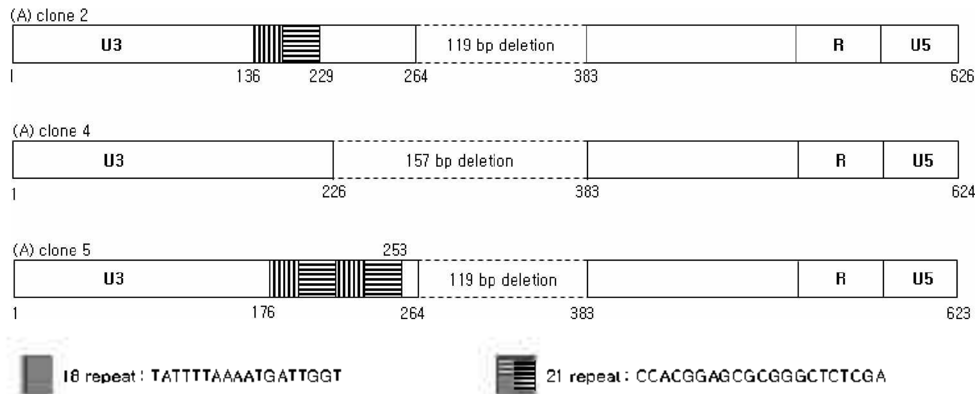

Figure 2. The structure of PERV 5' LTR and identified variable deletions and different repeats.

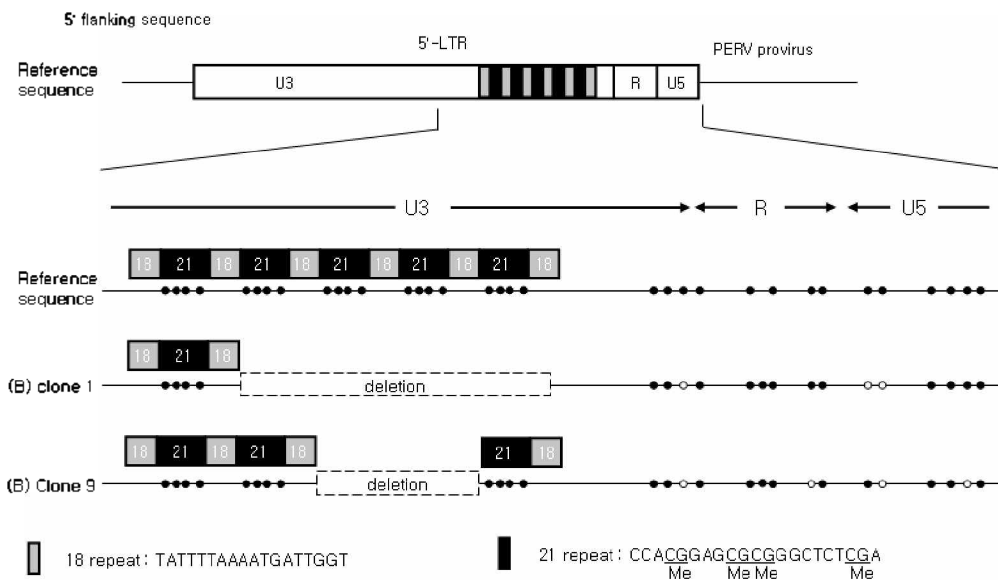

Figure 3. DNA methylation status and variable deletion within the PERV 5' LTR from Figure 1(B). Methylated CpG sites are indicated by solid circles and unmethylated CpG sites are indicated by open circles. Methylation sites in the 21 bp repeat are denoted as Me. Reference sequence is from 293-PERV-B (43)-746 LTR (Scheef et al., 2001).

the host's suppression of expression of the provirus. CpG methylation of the LTRs of human endogenous retrovirus (HERV-K) (Lavie et al., 2005) and proviral HIV have been shown to suppress transcription (Bednarik et al., 1987). Demethylation of the CpG sites in the 5' LTR is expected to allow the binding of transcription factors to increase proviral transcription levels of PERV (Dieckhoff et al., 2007). We found one unmethylated CpG site in the U3 and another in the U5 region (Figure 3). In summary, variability in numbers of repeats was detected on the 5' PERV LTR region and unmethylated CpG sites were detected in U3 and U5 regions. However the consistency of the methylation status at these sites needs to be verified for more of the numerous PERV integrations in the pK15 cell line before their role in regulation of PERV expression can be confidently evaluated.

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