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Isolation and Characterization of PERV-C *env* from Domestic Pig in Korea

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Clone PERV-C (A3) *env* was isolated from the genomic DNA of domestic pig (*Sus scrofa domesticus*) in Korea to investigate the molecular properties of PERV-C. The nucleic acid homologies between the PERV-MSL (type C) reference and the PERV-C(A3) clone was 99% for *env*, but a single base pair deletion was found in the transmembrane (TM) region of the *env* open reading frame. To examine the functional characteristics of truncated PERV-C *env*, we constructed a replication-incompetent retroviral vector by replacing the *env* gene of the pCL-Eco retrovirus vector with PERV-C *env*. A retroviral vector bearing PERV-C/A chimeric envelopes was also created to complement the TM defect. Our results indicated that truncated PERV-C *env* was not infectious in human cells as expected. Interestingly, however, the vector with the PERV-C/A envelope was able to infect 293 cells. This observation suggests that recombination within PERV-C TM could render PERV-C infectious in humans. To further characterize PERV-C/A envelopes, we constructed an infectious molecular clone by using a PCR-based technique. This infectious molecular clone will be useful to examine more specific regions that are critical for human cell tropism.

Keywords: PERV-C, pseudotype assay, xenotransplantation, envelope

Xenotransplantation is the transplantation of pig cells, tissues, and organs into human patients to relieve the shortage of human organs. Among all animal species analyzed, pigs are considered to be a suitable organ donor for xenotransplantation. The advantages of pigs as donors include similar organ donor size and physiology. However, infection associated with pig pathogens is the major concern in using porcine tissues for transplantation [3, 6, 19]. Porcine endogenous retroviruses (PERVs) are of particular concern because of their capability of infecting human cells [9]. Although there has been no evidence of PERV infection of humans *in vivo*, PERVs have been shown to infect human cells *in vitro* [21, 25]. Pig genomes have been reported to contain approximately 50 proviral copies of PERV, although many of them are defective owing to deleterious mutations. PERV integration patterns in different pig individuals showed that pigs harbor different sets of PERVs. Intact proviruses harbor genes such as *gag*, *pro/pol*, and *env* flanked by 3'- and 5'-noncoding sequences, known as long terminal repeats (LTRs). PERV is classified as an endogenous virus of the family Retroviridae, genus Gammaretrovirus, and is closely related to other mammalian type-C retroviruses such as the gibbon ape leukemia virus and murine leukemia virus [2]. PERV is divided into three classes, which differ in nucleotide sequences of the *env* gene encoding surface proteins: Two classes of infectious human-tropic replication-competent PERV (PERV-A and PERV-B), and one class of ecotropic PERV-C [16, 22]. PERV-C is capable of infecting only porcine cells. In addition to these three classes of PERVs, recombinants between ecotropic PERV-C and human-tropic PERV-A have been detected in pig cells. A recombinant PERV-A/C (PERV-A 14/220) is 500-fold more infectious than the prototype PERV-A [12], which contains the PERV-A sequence between the end of *pol* and middle of the SU (surface unit) region of *env*, with the remaining sequence derived from PERV-C. Furthermore, replication-deficient PERV may recombine with replication-competent PERV or other related endogenous retroviruses, and therefore, it is possible that new forms of infectious PERVs will emerge in the process of xenotransplantation.

The PERVs were found to be released from porcine cell lines including pig kidney cell lines PK-15 [23], MPK, and mitogenically activated peripheral blood mononuclear cells (PBMCs). PERV-A and PERV-B clones derived from PK-15 cell lines have significantly high titers, but PERV clones from primary pig cells have extremely low titers. Infection assays using pseudotype MLV harboring the retroviral MFGnls*LacZ* vector and different PERV envelopes

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indicated that PERV-A, -B, and -C each use different receptors. Recently, two genes encoding functional human PERV-A receptors have been isolated [8].

The PERV envelope proteins are located on the virion surface and synthesized as polyproteins. Based on homology to murine leukemia viruses, the PERV envelope consists of a surface unit (SU) containing the receptor binding domain (RBD) followed by a proline-rich region (PRR) and transmembrane (TM) glycoproteins containing membrane anchor determinants [15]. The RBD of MLV consists of two variable regions (VRA and VRB) and plays a critical role for receptor specificity, whereas the PRR is a critical determinant for the envelope's conformation and fusogenicity. On the other hand, the study of the region of PERV envelope protein required for receptor binding has not been determined.

In this study, we isolated PERV-C *env*, using a PCR-based approach, from the genomic DNA of domestic pig (*Sus scrofa domesticus*) in Korea to study the property of PERV-C *env*. In addition, we employed a retroviral vector bearing PERV-C envelope and full-length molecular clones containing PERV-C/A envelopes to examine more specific regions that are critical for human cell tropism.

High-molecular-weight liver DNA (Seegene, Korea) was obtained from domestic pig (*Sus scrofa domesticus*). TELCeB6 (kindly provided by J. J. Battini), 293 human embryonic kidney (ATCC 1573), PK-15 pig kidney (ATCC CCL-33), and ST swine testis (ATCC CRL-1746) cells were maintained in DMEM supplemented with 10% fetal calf serum and antibiotics. To determine the nucleotide sequence of the *env* of the PERV, genomic DNA was prepared from the domestic pig and used as PCR substrate.

The primers to amplify the viral *env* were as follows:

PERVenv-1: 5'-ACCTCGAGACTCGGTGGAAG-3'
PERVenv-2: 5'-CTTTCATTCCCCACTTCTTCTCT-3'

PERVenv-1 (sense, nucleotides 124 to 144, GenBank Accession No. Y12238) and PERVenv-2 (antisense, nucleotides 2,282 to 2,259, GenBank Accession No. Y12238) primers were designed to amplify the 2–2.5 kb PERV *env* fragment containing the intact ORF, as previously described [20, 24]. The PCR reactions were carried out in a MJ Research PTC-150 Thermal Cycler (PE Applied Biosystems, Foster City, CA, U.S.A.). The reactions were performed for 30 cycles with a 1 min denaturation step at 94°C, a 1 min annealing step, and a 1 min extension step at 72°C. The 2.1 kb PCR products were cloned into the pCR2.1-TOPO vector and sequenced (SolGent, Korea). KasI-EcoRI fragments of the amplified PERV-C *env* PCR product and a synthetic double-strand linker with BamHI and KasI sticky ends were cloned into the pBluescript II SK vector at the BamHI-EcoRI sites, as previously described [22]. To construct recombinant pBluescript-PERV-C (containing a ClaI-XbaI fragment of MoMLV), the ClaI-XbaI fragment was added at the N-terminal of the PERV-C *env* coding sequences that are already cloned into the pBluescript II SK vector. Therefore, the ClaI-XbaI fragment of MoMLV containing the PERV-C *env* was prepared to replace retroviral vector Moloney MLV *env*. To complement the TM defects in pBluecript-PERV-C, clone pBluecript-PERV-C/A was constructed by replacing the BsmI/EcoRI fragment of pBluecript-PERV-C with the corresponding fragment of the PERV-A *env* PCR product (Fig. 2). Clone pBluecript-PERV-C/A was digested with SacI and EcoRI, and then cloned into the SacI/EcoRI sites of pIRES2-EGFP plasmid. pIRES2-EGFP-C/A contains residues 1-605 of PERV-C *env* in frame with the residues 606-660 of PERV-A *env*. LacZ pseudotype virus was generated by cotransfection of 293 cells with the ecotropic PERV envelope expression vector, pCL-PERV-C and pCLMFG-*lacZ* (Imgenex Co., San Diego, CA, U.S.A.). To prepare the pCL-PERV-C, the ClaI-XbaI *env* fragment of pBluecript-PERV-C was ligated into the ClaI-XbaI cloning site of the pCL-Eco retrovirus packaging vector (Imgenex Co., San Diego, CA, U.S.A.).

To generate the PERV-C/A pseudotype, the pIRES2-EGFP-C/A plasmid was transfected into TELCeB6 cells, which express a high amount of MLV-base vector core encoding a *lacZ* gene [16]. Viral supernatants were collected, filtered, and used to infect 293, PK-15, and ST that had been plated in 6-well culture dishes at a density of 1.5×10^5 per well. The cells were infected with 1 ml of virus in the presence of 8 μg/ml polybrene for 3 h before 2 ml of fresh medium was added to each well. Two days after infection, cells were fixed with 0.5% glutaraldehyde and stained to reveal the presence of β-galactosidase activity. Infectious titers were expressed as the number of blue CFU per milliliter of virus supernatant. To construct infectious PERV-C/A clones using a PCR-based method, 5'halves spanning 5,071 nt from the 5'LTR to integrase in *pol* and 3'halves spanning 3,926 nt from *pol* to U5 in the 3'LTR were amplified from human 293 cells infected with PK-15-derived PERV, as previously described [4]. The PCR reactions were carried out in a MJ Research PTC-150 Thermal Cycler (PE Applied Biosystems, Foster City, CA, U.S.A.). The reactions were performed at 94°C for 4 min followed by 30 cycles with a 1 min denaturation step at 94°C, a 1 min annealing step at 58°C, and a 12 min extension step at 72°C. The unique restriction site, NheI, was used to fuse the 5' and 3'halves to full-length molecular clones (pBluecrpit KSII - PERV-A). PERV-C/A *env* coding sequences were excised from pBluecript-PERV-C/A by KasI and EcoRI, and cloned into the PERV-A type full-length molecular clone from which most of PERV-A *env* had been removed by digestion with KasI and EcoRI. To test the infectivity of the recombinant PERV-C/A full-length molecular clone in 293 cells, the product-enhanced reverse

transcription (PERT) assay was performed, as previously described [18]. PERT assays involve converting an RNA template to cDNA and then amplifying the cDNA using product-specific primers. Briefly, genomic RNA of bacteriophage MS2 template was used. The supernatants from full-length molecular clone transfected 293 cells were used to reverse transcribe an MS2 substrate RNA. For reverse transcription, 3.2 μg of MS2 RNA template/RT-1 primer (5'-CATAGGTCAAACCTCCTAGGAATG-3') in 1.4 μl of H_2O was added to 23.6 μl of buffer consisting of 56 mM Tris·HCl (pH 8.3), 56 mM KCl, 9 mM $MgCl_2$, 11.2 mM dithiothreitol, 1 unit of RNase inhibitor, 0.13 μg of BSA, 0.4% Triton X-100, 1 mM dNTP, and 3 μl of disrupted samples. For RT-PCR, primer RT-1 and primer RT-2 (5'-TCCTGCTCAACTTCCTGTCGAG-3') were used. Amplification of the MS2 cDNA was performed for 30 cycles with the following cycle; a 30 sec DNA denaturation step at 94°C, 100 sec annealing step at 55°C, and 110 sec extension step at 72°C. The GenBank accession number of the sequence reported in this paper is EU090250.

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PERV-MSL      MHPTLNRRHLPIRGGKPKRLKIPLSFASIAWFLTLSITSQTNGMRIGDSLNSHKPLSL
PERV-C(1312)  ------------------------------------------------------------
PERV-C(A3)    -----S-----T------------------------------------------------
PERV-MSL      TWLITDSGTGININNTQGEAPLGTWWPDLYVCLRSVIPSLTSPPDILHAHGFYVCPGP  VRA
PERV-C(1312)  ------------------------------------------------------------
PERV-C(A3)    --S---------------------------------------------------------
PERV-MSL      PNNGKHCGNPRDFFCKQWNCVTSNDGYWKWPTSQQDRVSFSYVNTYTSSGQFNYLTWI  VRB
PERV-C(1312)  ------------------------------------------------------------
PERV-C(A3)    ------------------------------------------------------------
PERV-MSI      RTGSPKCSPSDLDYLKISFTEKGKQENILKWVNGMSWGMVYYGGSGKQPGSILTIRLK
PERV-C(1312)  ------------------------------------------------------------
PERV-C(A3)    ------------------------------------------------------------
PERV-MSL      INQLEPPMAIGPNTVLTGQRPPTQGPGPSSNITSGSDPTESSSTTKMGAKLFSLIQGA  PRR
PERV-C(1312)  ------------------------------------------N-----------------
PERV-C(A3)    ------------------------------------------------------------
PERV-MSL      FQALNSTTPEATSSCWLCLASGPPYYEGMARRGKFNVTKEHRDQCTWGSQNKLTLTEV
PERV-C(1312)  ------------------------------------------------------------
PERV-C(A3)    --------------------L---------------------------------------
PERV-MSL      SGKGTCIGKVPPSHQHLCNHTEAFNQTSESQYLVPGYDRWWACNTGLTPCVSTLVFNQ
PERV-C(1312)  ------------------------------------------------------------
PERV-C(A3)    ------------------------------------------------------------
PERV-MSL      TKDFCIMVQIVPRVYYYPEKAILDEYDYRNHRQKREPISLTLAVMLGLGVAAGVGTGT
PERV-C(1312)  -------------------------K----------------------------------
PERV-C(A3)    ------------------------------------------------------------
PERV-MSL      AALVTGPQQLETGLSNLHRIVTEDLQALEKSVSNLEESLTSLSEVVLQNRRGLDLLFL
PERV-C(1312)  ------------------------------------------------------------
PERV-C(A3)    ------------------------------------------------------------
PERV-MSL      KEGGLCVALKEECCFYVDHSGAIRDSMNKLRERLEKRRREKETTQGWFEGWFNRSLWL
PERV-C(1312)  --------------------------------K------------------------P--
PERV-C(A3)    ---------------------------------------------------------P--
PERV-MSL      ATLLSALTGPLIVLLLLLTVGPCIINKLIAFIRERISAVQIMVLRQQYQSPSSREAGR  R
PERV-C(1312)  ------------------------------------------------------------
PERV-C(A3)    --------****************************************************
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Fig. 1. Comparison of amino acid sequences of envelope proteins of PERV-MSL, PERV-C(1312), and PERV-C(A3).

The PERV-C(A3) *env* sequence was compared with that of PERV-MSL (AF038600 as a reference strain). Dashes mean the same amino acid as top. Asterisks indicate the position of amino acid deletion relative to PERV-MSL. The VRA, VRB, PRR, and R regions are shaded.

In our previous study, we analyzed the PERV *env* from the genomic DNA of domestic pig (*Sus scorfa domesticus*) in Korea for the existence of additional PERV envelopes. Thus, a 2.1 kb product was amplified, and 5 clones were classified into PERV-A, B, and C subtypes by using subtype-specific restriction enzyme KpnI, Bsu36I, and AvaI. In the present study, PERV-C *env* was cloned and sequenced. Except for the deletion of the R region and part of the membrane-spanning domain, the present PERV-C(A3) *env* gene was remarkably similar and most closely related to previously described PERV-C (Fig. 1), such as the PERV-MSL (GenBank Accession No. AF038600) and the replication-competent molecular clone PERV-C(1312) [17]. PERV-MSL was identified from miniature pig lymphocyte cDNA libraries, and therefore, the infectivity was unknown. The nucleic acid homology between PERV-C (A3) and clone PERV-MSL was 99% for *env*, whereas the amino acid homology was 99.2% for *env*. There were no amino acid changes within the VRA and VRB regions, but the region encoding the R peptide was truncated. To examine the expression of PERV-C(A3) *env*, PERV-C *env* genes were subcloned into a pCL-Eco retroviral vector and transfected into 293 cells with pCLMFG-*lacZ*. Supernatants containing pseudotype viruses were collected from transfected 293 cells, filtered, and used to infect 293, PK-15, and ST cells. We found that the PERV-C pseudotype efficiently infected PK-15 and ST porcine cells, but was not infectious for 293 cells as expected. Interestingly, the PERV-C/A pseudotype vector was able to infect 293 cells, although at a very low titer (Table 1, Fig. 3). To investigate the molecular properties of PERV-C/A, we isolated replication-competent molecular clones from biological PERV isolates using a PCR-based technique. Genomic DNA of PERV-infected 293 cells and PK-15 cells were isolated and used as templates in PCR reactions to amplify PERV proviruses in two overlapping halves. 5' and 3' fragments of PERV were isolated, subcloned, and fused to generate full-length proviruses using a unique NheI restriction site located within the overlap. The PERV-B 5' half clone and PERV-A 3' half clone were fused to generate a PERV-B/A chimera provirus. The KasI-EcoRI fragments of PERV-C/A *env* was ligated to the PERV-B/A chimera from

Table 1. Host range and cell tropism of PERV-C(A3) envelope.

Target cell origin	Titer of LacZ pseudotype[a]	
	LacZ(PERV-C)	LacZ(PERV-C/A)
Swine testis (ST)	480	250
Pig kidney (PK-15)	100	60
Human embryonic kidney (293)	<2	160

[a]Titers are given as number of CFU per ml.

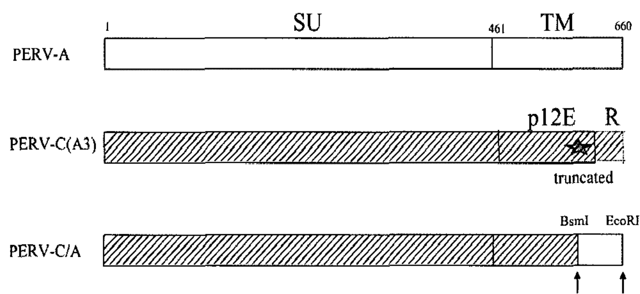


Fig. 2. Schematic representation of PERV-A (open), PERV-C (A3) (hatched), and PERV-C/A.

The numbers represent the amino acid position for the envelope surface glycoprotein (1 to 460) and transmembrane (461 to 660) regions of the PERV-A virion (GenBank Accession No. Y12238). Asterisks represent deletions. The arrow indicates the restriction sites for BsmI and EcoRI. SU, surface; TM, transmembrane; p12E, R peptide removed from TM; R, the C-terminal 16 amino acids.

which the corresponding fragment had been deleted. To test for their ability to produce a virus stock, the PERV-B/A chimera containing PERV-C/A *env* was transfected into 293 cells. Progeny virus released into the supernatant medium was detected by PERT assay (Fig. 4). Reverse transcriptase (RT) activity was detectable in 293 cell-free supernatants from 30 days posttransfection (lanes 3, 4, and 5). The positive control showed RT activity (lane 1), whereas untransfected 293 cells showed no RT activity (lane 2).

The screening of pigs harboring PERV-C is important because a few point mutations in PERV-C SU Env enable PERV-C infections in human recipients [11]. By selecting animals lacking PERV-C expression, one will minimize the risk of recombination events in xenotransplantation. Here, we isolated PERV-C *env* from domestic pig (*Sus scrofa domesticus*), and experiments to amplify the full-length PERV-C are currently under way.

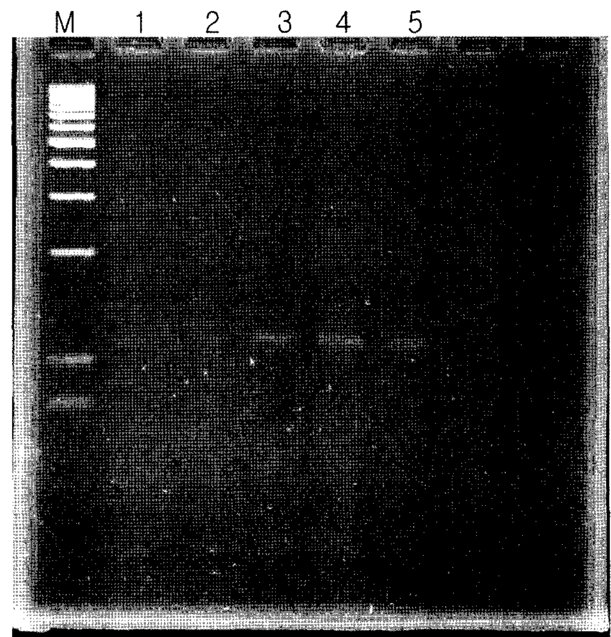
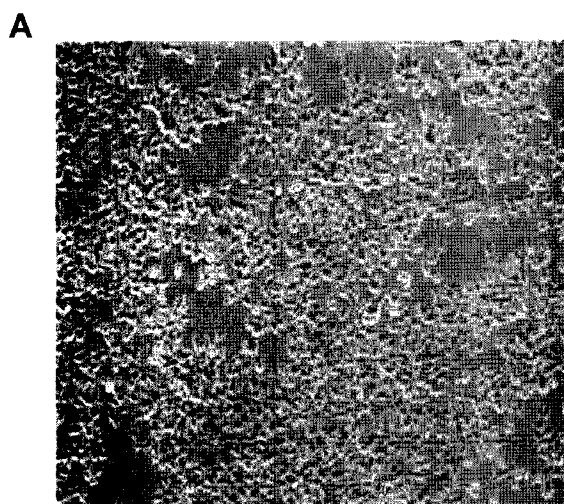


Fig. 4. Construction of recombinant PERV-C/A full-length molecular clones and screening for PERV-C/A proviruses by PERT assay.

To identify infectious proviruses, the 3 clones were initially tested for their ability to express reverse transcriptase by transfection into 293 cells (lanes 3–5). Lanes 1 and 2 are supernatants of PERV-infected 293 cells and untransfected 293 cells, respectively.

The TM(p15E) protein is known to serve to anchor the envelope complex to the virion membrane and is also a vital component of the viral entry process. At the time of viral budding, p15E is processed further by a viral protease to produce a 12-kDa protein (p12E) and R peptide (16 amino acids oligopeptide), and the R peptide inhibits membrane fusion [5]. In this study, sequence data analysis



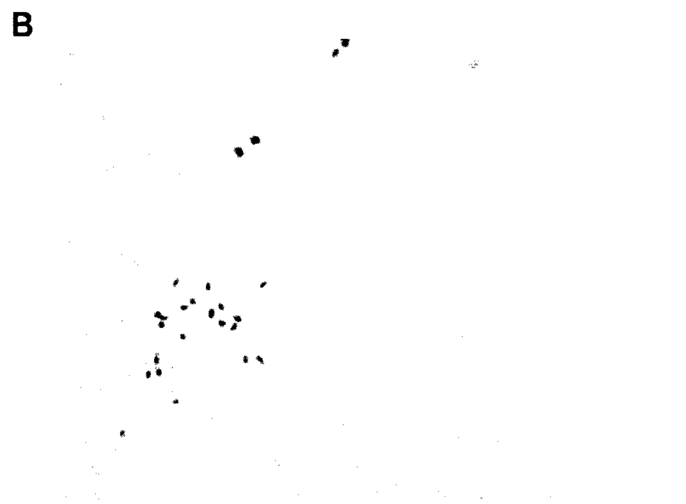


Fig. 3. Susceptibility of 293 cells to PERV-C pseudotype infection.

293 cells were inoculated with 1 ml of virus in the presence of 8 μg/ml polybrene. Two days after infection with PERV-C *env* pseudotype (**A**) and PERV-C/A *env* pseudotype (**B**), cells were fixed and stained with X-Gal.

showed a truncated envelope ORF for PERV-C *env* with close homologies with PERV-MSL and PERV-C(1312). Single base-pair deletion was found in the transmembrane region of the *env* ORF, and this deletion represented truncation of R peptide. The infectivity of retroviral vectors carrying the truncated PERV-C Env proteins to transduce ST cells, 293 cells, and PK-15 cells was measured by β-galactosidase staining (Table 1). The results showed that the envelope cytoplasmic tail was not required for viral entry. However, a question remains as to how the R peptide and the removal of membrane-spanning regions of PERV-C *env* could lead to the conformational alterations of the ectodomain of the protein and the strength of the SU-TM interaction. It may be that the residual amino acids of the membrane-spanning domain are sufficient to achieve membrane anchoring of the PERV-C envelope. To address whether this envelope was defective in the membrane fusion, an XC cell fusion assay would be necessary [13]. To complement the TM defect, we constructed a PERV-C/A recombinant vector. Interestingly, vectors bearing a chimeric envelope PERV-C/A TM could render PERV-C infectious in humans (Table 1, Fig. 3). One hundred amino acid residues at the C-terminus of the PERV-A TM could lead to the infection of 293 cells. This finding led us to propose that recombinant PERV-C/A *env* alters the strength of the SU-TM interaction, as previously described in Moloney *murine leukemia virus* [1]. Furthermore, we constructed an infectious molecular clone by using a PCR-based technique to examine regions that are critical for human tropism. Although the first description of a replication-competent PERV-C provirus was recently reported [17], the novel active full-length PERV-C would be an important tool for further characterization. We constructed infectious recombinant PERV clones using a PCR-based method for the first time to investigate the molecular properties of PERV-C. Up to now, very few infectious clones of PERV have been analyzed and assessed for their ability to evolve by recombination. Although our recombinant clone replaced the *env* region from the previously cloned infectious PERV provirus, these clones would be useful to understand PERV-C replication. As shown in Fig. 4, 293 cells infected with cell-free supernatants of PK-15 cells (lane 1) as positive control and our recombinant PERV-C/A-transfected 293 cells (lanes 3, 4, and 5) showed RT activity, evidenced by PERT assays. For PERT assays, cell-free supernatants were harvested 30 days after transfection, because most porcine endogenous retroviruses studied to date grew poorly. Viruses of the PERV-C class are capable of infecting only porcine cells. However, our result showed that the PERV-C/A envelope was able to infect 293 cells, demonstrated by pseudotype assay. Hence, mutations in PERV-C *env* eventually enable PERV-C infections in human recipients. It is likely that selecting animals lacking PERV-C expression would minimize the risks for xenotransplantation. Until now, the basic properties of PERV-C envelope proteins has not been defined. Only a few studies on PERV recombination in terms of its antigenic determinants have just been reported [7, 11]. For further study to examine specific regions that are critical for human cell tropism, we plan to employ retroviral vectors bearing several PERV-C/PERV-A recombinant envelopes.

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