Identification and Promoter Analysis of PERV LTR Subtypes in NIH-Miniature Pig

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Porcine endogenous retroviruses (PERVs) are integrated into the genomes of all pigs. Since some PERVs can also infect human cells, they represent a potential risk for xenotransplantation involving pig cells or organs. The long terminal repeat (LTR) elements of PERVs show promoter activity that can affect human functional genes; therefore, we examined these elements in this study. We detected several expressed LTRs in the NIH-miniature pig liver, among which we identified 9 different subtypes. When these LTRs were compared, distinct structures that contained several insertion and deletion (INDEL) events and tandem repeats were identified in the U3 region. The transcriptional activity of the 9 LTR subtypes was analyzed in the PK15 porcine cell line and in the HepG2 and Hep3B human liver cell lines, and transcriptional regulation was found to be different in the 3 cell lines. The D LTR subtype was found to have stronger promoter activity than all other types in 4 different human cell lines (HepG2, Hep3B, U251, and 293). Using computational approaches, the D type was shown to contain 4 transcription factor-binding sites distinct from those in the U3 regions of the other subtypes. Therefore, deletion mutants were constructed and examined by a transient transfection luciferase assay. The results of this analysis indicated that the binding site for the Hand1:E47 transcription factor might play a positive role in the transcriptional regulation of PERV LTR subtype D in human liver cell lines.

INTRODUCTION

The pig has been used as an animal model of xenotransplantation, whereby solid organs, tissues, and live cells from pigs are transplanted into human patients (Louz et al., 2008). However, the risk of zoonotic infections during this process has been an important barrier to the utility of xenotransplantation. The largest problem with regard to zoonotic infections from pigs is the expression of the gene that encodes the enzyme α -1,3-galactosyl transferase, which catalyses the synthesis of a sugar

in pigs. This sugar is the major molecule recognized by human antibodies following xenotransplantation, and its production results in xenotransplant rejection (Platt, 1998; 2000). While α -1,3-galactosyl transferase-deficient pigs have been developed to address this problem, which has brought xenotransplantation closer to the clinic (Dai et al., 2002; Phelps et al., 2003), many studies have raised concerns that the presence of porcine endogenous retroviruses (PERVs) in the pig genome could be a further source of potential risk in xenotransplantation. This is because PERVs have been shown to infect human cells *in vitro* (Takeuchi et al., 1998) and have been found to be transcriptionally active and infectious in other species *in vivo* after the transplantation of pig tissues (van der Laan et al., 2000). Moreover, completely intact PERV proviruses have been identified and their replication and infection potential has been examined in human and non-human primate cells (Blusch et al., 2000; Czauderna et al., 2000). Fortunately, it has been demonstrated that human APOBEC3G proteins are capable of preventing the zoonotic transmission of PERVs (Jónsson et al., 2007), and no evidence of the transmission of pig viruses such as PERVs, porcine cytomegaloviruses (PCMV), porcine lymphotropic herpesviruses (PLHVs), or porcine circoviruses (PCVs) into primate recipients has been observed to date (Garkavenko et al., 2008). Furthermore, blood from type 1 diabetes patients has recently been transplanted into pig islets, and the DNA of white cells infected with PERVs was not observed in a long-term follow-up study (Valdes-Gonzalez et al., 2010). However, the risks that PERVs pose to xenotransplantation because of the immunorejection of glycoproteins and the virus itself are still an issue. Thus, strategies to control the expression of PERVs have been developed using antiviral drugs. Short hairpin RNAs, dolichyl-phosphate mannosyltransferase, and tetherins proteins have also been shown to inhibit the expression of PERVs (Karlas et al., 2004; Mattiuzzo et al., 2010; Yamamoto et al., 2010).

PERVs are integrated in the pig genome and divided into 3 different replication-competent subtypes: PERV-A, PERV-B, and PERV-C (Ericsson et al., 2001; Mang et al., 2001; Patience et al., 2001). Of these subtypes, PERV-A and -B are present in the genome of all pigs and can infect human cells (Martin et al.,

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1998; Wilson et al., 1998), while PERV-C does not ubiquitously exist in the pig genome and infects only pig cells (Takeuchi et al., 1998). The PERV subtypes typically contain 3 ORFs that encode for *gag*, *pol*, and *env* between 2 long terminal repeats (LTRs) that contain regulatory elements required for transcription (Akiyoshi et al., 1998; Le Tissier et al., 1997). While *gag* and *pol* genes are highly homologous between all types of PERV, the *env* sequences differ significantly (Magre et al., 2003). Even the presence of PERV mRNA has the potential to produce infection or infectious particles, with the LTR functioning as a promoter in this process. Thus, we evaluated the sequences and structural features of PERV LTRs in this study. In particular, the promoter region of PERV LTRs containing regulatory elements for transcription was investigated, and the potential for controlling transcription through this promoter was assessed.

MATERIALS AND METHODS

Total RNA isolation and RT-PCR amplification

Total RNA was extracted from the livers of NIH-miniature pigs by using Trizol reagent (Invitrogen). The Turbo DNA-free™ kit (Ambion) was used to eliminate DNA contamination from liver total RNA. In the NO-RT experiment, which involves RT-PCR without the reverse-transcription, DNA contamination from DNase-treated total RNA samples was confirmed. M-MLV (Moloney-Murine Leukemia Virus) reverse transcriptase at an annealing temperature of 42°C was performed for the transcription reaction with the RNase inhibitor (Promega). As a standard control, the human glyceraldehyde-3-phosphate dehydrogenase (NM_002046) gene (*G3PDH*) was amplified using forward (5'-GAA ATC CCA TCA CCA TCT TCC AGG-3') and reverse primers (5'-GAG CCC CAG CCT TCT CCA TG-3'). RT-PCR amplification of the PERV LTR elements were performed using forward primer (5'-GAT GAA AAT GCA ACC TAA CCC-3') and reverse primer (5'-CCC CAA ATC ACT CAC GAG AA-3') (Huh et al., 2009). Each RT-PCR amplification step was performed as follows, after the initial denaturation step at 94°C for 4 min, cDNA was amplified for 30 cycles at 94°C for 40 s, 55°C for 30 s and 72°C for 40 s.

Molecular cloning, sequencing, and data analysis

RT-PCR products were separated on a 1.5% agarose gel, purified with a gel extraction kit (Labo Pass), and cloned into the pGL-4.11 vector (Promega). The cloned DNA was isolated using a plasmid DNA purification kit (Labo Pass). Sequencing was performed by the Macrogen company (Korea) by using primer sets corresponding to pGL-4.11 vector sequences. Sequence alignments and comparative analyses of PERV LTR elements were conducted using the BioEdit program. All nucleotide sequences reported in this paper were submitted to the DDBJ nucleotide sequences database (Supplementary Table S1). The transcription factor binding sites in the PERV LTR elements were predicted using MATCH in TRANSFAC v8.0 (<http://www.generegulation.com>).

Cell culture and transient transfection assay

PK15 (pig kidney), HepG2 (human liver), Hep3B (human liver), and 293 (human kidney) cells were grown at 37°C in a 5% (v/v) CO₂ incubator in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% (v/v) antibiotics-antimycotics. U251 (human glioma) cells were grown in RPMI1640 under the same conditions. Cells were plated in 24-well plates at 3×10^4 cells/well

and grown to 60% confluence. Cells were transfected with mixtures containing 100 ng of the pGL-4.11 LTR plasmid (9 liver subtypes, 4 liver tissue deletion mutants, and 1 subtype from heart tissue) and the pGL-4.11 basic vector linked to luciferase (Promega) by using Lipofectamine 2000 as described in the manufacturer's protocol. In addition, 100 ng plasmid of the pRL-TK vector was used to normalize for transfection efficiency. After 24 h of transfection, the cells were washed with DPBS and lysed in luciferase lysis buffer. The activities of firefly luciferase and *Renilla* luciferase in the cellular extracts were measured using the dual-luciferase reporter assay system (Promega) with a luminometer. The relative luciferase activity was obtained by normalizing the activity of the firefly luciferase with that of the *Renilla* luciferase. Each experiment was performed in triplicate. Co-transfection with siRNA was also performed as previously described. Briefly, HepG2 cells were cultured in 24-well plates and each well was transfected with 100 ng of the pGL-4.11 LTR plasmid and pRL-TK vector and 0.018 nmol of siRNA (#1066975, Bioneer) by using Lipofectamine 2000 (Invitrogen). The AccuTarget™ Negative control siRNA (Bioneer) was used as a negative control in siRNA experiments.

Real-time RT-PCR

Real-time RT-PCR was performed to detect the expression of *Hand1* and assess the knockdown efficiency following siRNA transfection. The amplification efficiencies and correlation coefficients of the *Hand1* gene were estimated following real-time RT-PCR amplification by using the slopes of the standard curves obtained from a 10-fold dilution series. Amplification efficiency was calculated using the following formula: efficiency (%) = $(10^{(-1/\text{slope})} - 1) \times 100$. Real-time RT-PCR amplification was carried out on a Rotor Gene 3000 (Corbett Research). In each reaction, 1 µl of cDNA was used as a template for amplification, while an amplification reaction without template was performed to establish non-specific background amplification. Each real-time RT-PCR template was added to 14 µl of reaction mixture containing 7 µl of H₂O, 5 µl of QuantiTect® SYBR® Green PCR Master Mix (Qiagen), and 1 µl of each primer (10 pmol). Real-time RT-PCR to detect *Hand1* or housekeeping gene transcripts was carried out over 50 cycles of 94°C for 10 s, 60°C for 15 s, and 72°C for 15 s. Melting curve analysis was conducted for 30 s at 55-99°C. All samples were amplified in triplicate. For the standard control, the human *G3PDH* gene was amplified by real-time PCR with forward (5'-GAA ATC CCA TCA CCA TCT TCC AGG-3') and reverse (5'-GAG CCC CAG CCT TCT CCA TG-3') primers designed using the human *G3PDH* sequence (GenBank accession no. NM_002046). Real-time PCR amplification of *Hand1* was conducted using forward (5'-CCT AGC CAC CAG CTA CAT CG-3') and reverse primers (5'-TTT AAT CCT CCT CTC GAC TGG G-3').

RESULTS

Identification and structural analysis of PERV LTR elements in the liver tissue from NIH-miniature pig

RT-PCR was performed to amplify the PERV LTR elements from the liver tissue of the NIH-miniature pig (Fig. 1), and the products were randomly inserted into the pGL-4.11 vector for sequence analysis. Of the PERV LTR elements transcribed, 25 were identified (Supplementary Table S1) and divided into 9 subtypes on the basis of the structural features (Fig. 2). The sequences of the PERV LTR elements were compared using multiple alignments of the PERV-A and -B family LTR consensus sequences (Fig. 2), and several deletions, insertions, and

Fig. 1. RT-PCR amplification of PERV LTR elements from liver and heart tissues of the NIH-miniature pig. Different sized products were detected ranging from 500 bp to 700 bp. As a standard control, *GAPDH* (120 bp) was used.

tandem duplication sequences were identified in the U3 region but not in the conserved R and U5 regions of the LTRs. The B and G type LTR elements distinctive sequences with PERV-A and -B family LTR element sequences, with the B type LTR element containing 50 bp and the G type LTR element 17 bp. While the F, H, and I type LTR elements were found to have completely deleted internal sequences in the U3 region of the PERV LTR elements, the A, B, C, D, and G type LTR elements contained sequences generally conserved with those of the PERV-A and -B family LTR elements. We also observed differences in the number of tandem repeats and sequences. These tandem repeats were close to the PERV-A and -B family LTR

element tandem sequences. Finally, the E type LTR element sequences were quite different from the other LTR element sequences, with the U3 region showing only a 22% similarity with the PERV-A family LTR sequences, and tandem repeat sequences distinct from those of the other LTR elements. For instance, the S9 tandem repeat containing only the E type element was found in the PERV-C family LTR sequences, and the E type element showed high similarity (70%) to the PERV-C family LTR sequences. In all, we identified 9 types of PERV LTR elements that distinguish the U3 region, including specific tandem repeats and INDEL events (Fig. 2).

Promoter activity analysis and prediction of TF binding sites in PERV LTR subtypes

To investigate the promoter activity of the 9 PERV LTR subtypes, we performed luciferase assays following the transient transfection of the PK15 pig cell line and HepG2 and Hep3B human liver cell lines (Fig. 3). The A, B, C, and G type LTR elements, which have a conserved structure, showed high promoter activity in the PK15 cell line, while the other LTR elements showed low promoter activity. In contrast, the promoter activity of almost all of the LTR element subtypes was very low in the human liver cell lines, with the exception of the D type LTR element, which showed strong promoter activity (Fig. 3). Therefore, we expect that differences in the sequences and structural features of each LTR could affect its ability to function as a promoter.

In order to verify the relationship between sequence variation and promoter activity, we analyzed transcription factor (TF) binding sites in the 9 PERV LTR element subtypes by using TRANSFAC v8.0. As was observed with regard to their structures, large differences were observed in the TF binding sites contained in the U3 regions of each LTR, which include several

Fig. 2. Structural illustration of 9 identified PERV LTR elements and their sequences. The 9 PERV LTR elements were divided on the basis of their structures into U3, R, and U5 regions. White boxes indicate conserved sequences between the 9 types of PERV LTR elements and the PERV-A, and -B family LTR element sequences, while gray and black boxes indicate sequence differences compared with PERV-A and -B family LTR elements and other subtype sequences. Different box numbers indicate different tandem repeat sequences. Inverted triangles indicate the TATA box.

Fig. 3. Luciferase reporter assay for analysis of PERV LTR element promoter activity. Nine types of PERV LTR elements were transiently transfected into PK15, HepG2, and Hep3B cell lines, and luciferase assays were performed. Relative luciferase activity is shown in the schematic diagram. Results are expressed as the ratio of the luciferase activity to that of the promoterless pGL4.11 reporter plasmid. Relative luciferase activity was obtained by normalizing the activity of the firefly luciferase with the *Renilla* activity. Each experiment was performed in triplicate, and data is presented as the mean with error bars representing the standard error.

Fig. 4. Analysis of transcription factor binding sites predicted to be in the PERV LTR elements by using TRANSFAC v8.0. Closed circles represent each transcription factor binding site, with upper circles indicating the sense direction and lower circles indicating the antisense direction.

INDEL events (Fig. 4). For instance, D type LTR elements were found to contain several different TF-binding sites (Hand1:E47, MEF-2, TFIIA, SOX-9, Muscle TATA box, Gfi-1, and Whn), of which the SOX-9, Muscle TATA box, and Whn TF binding sites were unique to the D-type LTR elements (Fig. 4).

Regulation of promoter activity in TF binding site deletion mutants

To assess the effects of the TF binding sites present in the D type LTR element on its promoter activity, we manufactured 4 deletion mutants (Del1, Del2, Del3, and Del4) in which the Hand1:E47, Sox9, Muscle TATA box, Whn, and MEF2 TF binding sites were removed sequentially. We then performed luciferase assays in transiently transfected HepG2 and Hep3B human liver cell lines (Fig. 5). The Del1 mutant showed remarkably low promoter activity in comparison to the original D type LTR element, while the other deletion mutants also showed negligible promoter activities (Fig. 5). These results indicate that Hand1:E47 might play a positive role in the regulation of the promoter activity of PERV LTR in human liver cell lines.

To investigate whether *Hand1* expression affects the promoter activity of the D type PERV LTR, we transfected cells with siRNAs targeting *Hand1* before performing luciferase reporter assays using the D type PERV LTR. Figure 6 shows that silencing *Hand1* expression down regulated the promoter activity of the D type PERV LTR, supporting the findings from our deletion mutant study.

DISCUSSION

Organs harvested from miniature pigs have the potential to be widely used in patients waiting for transplantation. Thus, numerous studies have evaluated the risks associated with such xenotransplantations. Among a meaningful arguments, the LTR elements is suggested an alternative. The LTR elements have various sequences that affect promoter activity. Thus, the presence of LTR elements could have the potential to promote expression of downstream coding genes. Various studies using luciferase reporter genes have shown that the tandem repeat sequences of LTR elements have strong promoter activity

Fig. 5. Comparative luciferase reporter assay for the promoter activity of 4 deletion mutants (Del1, Del2, Del3, and Del4) in HepG2 and Hep3B human liver cell lines. Promoter activity decreased remarkably in the Del1 mutant in both HepG2 and Hep3B. Each experiment was performed in triplicate, and data is presented as the mean with error bars representing the standard error. The white box indicates the U3 region, the gray box indicates the R region, and black box indicates the U5 region.

Fig. 6. The D type PERV LTR promoter activity requires *Hand1* expression. (A) After transfection with siRNA, the knockdown efficiency of *Hand1* was confirmed using real-time RT-PCR. *Hand1* expression was shown to decrease more than 50% compared with negative controls. (B) The promoter activity of the D type PERV LTR was examined in HepG2 cells transfected with control or *Hand1* siRNA by using the luciferase reporter assay. Relative luciferase activity

was applied by normalizing the activity of the firefly luciferase with the *Renilla* activity. The promoter activity of the D type PERV LTR was shown to decrease in cells in which *Hand1* expression was knocked down. Each experiment was performed in triplicate, and data is presented as the mean with standard error represented by error bars.

Fig. 7. Luciferase reporter assay for analysis of the promoter activity of a PERV LTR element identified in heart tissues from NIH-miniature pig. The PERV LTR element that was identified in the heart tissue was transiently transfected into human cell lines (U251 and 293), and the luciferase activity was measured. Relative luciferase activity is shown in the schematic diagram. Results are expressed as the ratios of the luciferase activity to that of the promoterless pGL4.11 reporter plasmid. Relative luciferase activity was obtained by normalizing the activity of the firefly luciferase with the *Renilla* activity. Each experiment was performed in triplicate, and data is presented as the mean with standard error represented by error bars.

(Denner et al., 2003; Maksakova and Mager, 2005; Scheef et al., 2001). Thus, the central question addressed in our study was how the promoter activity of PERV LTR elements can be effectively controlled in human cell lines. To that end, we analyzed the transcribed PERV LTR elements in liver tissues from the NIH-miniature pig. These PERV LTR element sequences were found to have U3, R, and U5 regions. Several PERV LTR element sequences containing U3 region have been previously identified in various pig tissues by RT-PCR amplification (Huh et al., 2009; Park et al., 2010). Likewise, liver tissues from the NIH-miniature pig showed U3, R, and U5 regions in the present study. Surely we confirmed that DNA contamination in RNA samples was absent by NO-RT experiment. These data indicating that the transcribed PERV LTR elements containing U3, R, and U5 regions could result from another promoter. As shown in Fig. 2, 9 types of PERV LTRs containing different structural features were identified in the NIH-miniature pig liver. While the A, B, C, and G types of PERV LTR showed high promoter activity in the pig cell line, their activities were markedly decreased in the human liver cell lines (Fig. 3). In contrast, the D type PERV LTR showed stronger promoter activity in the human liver cell lines than the pig cell line. Furthermore, the D type LTR was found to have similar sequences with PERV-A, and -B family LTR sequences, which are known to infect human cell lines. The U3 region has a highly conserved structure, and although INDEL (insertion and deletion) events were not detected (Fig. 2), the tandem repeat sequences in this region

were highly conserved. The D type LTR also contained 2 of the original 18 bp repeats and 1 of the original 21 bp repeat sequences. The other tandem repeat sequences of subtype were not significantly different from the original sequences-only transitions burst within C and T. Well-conserved sequences are expected to result in strong promoter activity in human liver cells (Fig. 3), and the other LTR subtypes, which contained significantly different sequences and enormous deletions in the U3 region and tandem repeat compared to the PERV-C family LTR, had low promoter activity in both human and pig cell lines (Fig. 2). Recombinant PERV-A/C has been reported in various studies. Some recombinant PERV-A/C was detected in normal tissues, but not in the germline tissues (Bittmann et al., 2012). However, the most important fact is that the recombinant PERV-A/C could infect human cell (293) (Wood et al., 2004). Furthermore, some reports suggest that they have adapted to human cells (293) (Karlas et al., 2010). Therefore, recombinant PERV-A/C could be a potential risk factor in human to pig xenotransplantation (Denner, 2008). In this study, the E type LTR showed high sequence similarity with PERV-C family, but had low promoter activity in both human and pig cell lines. However, recombinant PERV-A/C may be discovered in the future, and therefore, further research will be needed.

Taken together, we have confirmed in this study that variations in the U3 region can more strongly affect promoter activity than in the well-conserved R and U5 regions. This is in accord with the findings of many previous studies that have examined the relationship between the U3 region and LTRs transcriptional regulatory sequences (Della et al., 2011; Mukerjee et al., 2010).

Several transcription factor binding sites present within the U3 region of PERV LTRs could affect promoter activity. In this study, we identified potential transcription factor binding sites present in the U3 regions of 9 LTR subtypes by performing a search using TRANSFAC v8.0 (Fig. 4). The D type PERV LTR was found to contain transcription factor binding sites such as those for Hand1:E47, SOX-9, Muscle TATA box, and Whn (Fig. 4). We also showed that the D type PERV LTRs have strong promoter activity in human cell lines, and we expect that only the transcription factors with binding sites within the D type PERV LTR can affect its promoter activity. Figure 5 shows that the Hand1:E47 binding site may be crucial for strong promoter activity in human cell lines, which was confirmed using siRNA transfection (Fig. 6). Transcription factor Hand1 (class B basic helix-loop-helix; bHLH) forms heterodimers with class A bHLH proteins, including E12/E47, HEB, or ITF-2 (Hu et al., 1992), that function as transcriptional activators (Quong et al., 1993) after binding sites such as the Hand1:E47 heterodimer binding site found in the D type PERV LTR. While A, B, C, D, and G type PERV LTRs also have binding sites for these factors and were shown to display a high promoter activity in the pig cell line (Fig. 3), the A, B, C, and G type PERV LTRs were found to have drastically lower promoter activity in human liver cells. The Hand1:E47 binding site in the D type PERV LTR is therefore an essential factor that promotes the transcription of this LTR in human cell lines. While the F type PERV LTR also has a Hand1:E47 binding site (Fig. 4), this LTR did not show promoter activity in either human or pig cell lines (Fig. 3). Generally, the Hand1 transcription factor has been reported to be a MEF2 cofactor (Morin et al., 2005). Thus, this may be due to the lack of the MEF-2 transcription factor, which does not exist in the F type PERV LTR. Therefore, the correlation between the Hand1:E47 and MEF-2 transcription factors may be the main factor dictating the promoter activity of the D type PERV LTR in human liver cell lines.

In heart tissues, we only identified one PERV LTR subtype (Fig. 1), which contained the same sequences as the D type PERV LTR identified in the liver tissues. This LTR subtype also showed strong promoter activity in the human brain (U251), kidney (293), and liver cell lines (HepG2 and HepB3) (Fig. 7). In addition, a strikingly similar LTR subtype has previously been detected in the kidney tissues of NIH-miniature pig (Park et al., 2010), which also showed stronger promoter activity in a human kidney cell line than in a pig kidney cell line. Multiple alignments of these PERV LTR sequences from NIH-miniature pigs are shown in Supplementary Fig. S1. The 2 subtypes of LTR (D and G) detected in the kidney tissues of NIH-miniature pig were similar to those found in the liver and heart. Totally, the 11 subtypes LTR were identified in the NIH-miniature pig. Since the D type PERV LTRs identified in the liver, heart, and kidney tissue of NIH-miniature pig have stronger promoter activity in human cell lines (Figs. 3 and 7), the promoter activity of D type PERV LTRs may have a species-specific feature. Thus, if we can regulate the transcription factors involved in its expression, we can reduce the risks associated with xenotransplantation due to PERV.

In conclusion, we have identified novel and commonly transcribed PERV LTR elements in the liver and heart tissue of the NIH-miniature pig and confirmed their promoter activity via a luciferase reporter assay. We also analyzed the *cis*-element regulation of the identified PERV LTR elements by using deletion mutants. In this analysis, the Hand1:E47 binding site was shown to play a potential positive role in the transcriptional regulation of LTR subtype in human cell lines. These results may contribute to the development of safe xenotransplantation methods via the control of PERV expression.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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