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 (Jönsson et al., 2007), and no evidence of the transmission of pig viruses such as PERVs, porcine cytomegaloviruses (PCMV), porcine lymphotropic herpesviruses (PLHV), or porcine circoviruses (PCV) 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.,...
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 glyceraldehydes-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 was 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 45 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 pG-L.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 pG-L.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 × 10⁴ 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 (%) = [(1-(-1/slope)-1)×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′-AGG CAC CAG CTA CAG GAG AA-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 pG-L.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