Absence of Replication-Competent Human-Tropic Porcine Endogenous Retroviruses in the Germ Line DNA of Inbred Miniature Swine

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The potential transmission of porcine endogenous retroviruses (PERVs) has raised concern in the development of porcine xenotransplantation products. Our previous studies have resulted in the identification of animals within a research herd of inbred miniature swine that lack the capacity to transmit PERV to human cells in vitro. In contrast, other animals were capable of PERV transmission. The PERVs that were transmitted to human cells are recombinants between PERV-A and PERV-C in the post-VRA region of the envelope (B. A. Oldmixon, J. C. Wood, T. A. Ericsson, C. A. Wilson, M. E. White-Scharf, G. Andersson, J. L. Greenstein, H. J. Schuurman, and C. Patience, *J. Virol.* 76:3045–3048, 2002); these viruses we term PERV-A/C. This observation prompted us to determine whether these human-tropic replication-competent (HTRC) PERV-A/C recombinants were present in the genomic DNA of these miniature swine. Genomic DNA libraries were generated from one miniature swine that transmitted HTRC PERV as well as from one miniature swine that did not transmit HTRC PERV. HTRC PERV-A/C proviruses were not identified in the germ line DNAs of these pigs by using genomic mapping. Similarly, although PERV-A loci were identified in both libraries that possessed long *env* open reading frames, the *Env* proteins encoded by these loci were nonfunctional according to pseudotype assays. In the absence of a germ line source for HTRC PERV, further studies are warranted to assess the mechanisms by which HTRC PERV can be generated. Once identified, it may prove possible to generate animals with further reduced potential to produce HTRC PERV.

Xenotransplantation carries the concerns of cross-species transmission of infectious pathogens present in the donor species (7, 17, 18, 23). Studies indicate that many exogenous microorganisms can be eliminated from the donor herd by using various barrier methods and specialized qualified-pathogen-free rearing techniques (3, 25, 26). However, porcine endogenous retroviruses (PERVs) are an exception to this rule, as proviral copies are inherited through the germ line DNA. Three subgroups of PERV, PERV-A, -B, and -C, have been identified (24). Either PERV-A, PERV-B, or viruses derived from recombination events between PERV-A and PERV-C, are able to result in productive infection of human cells in vitro, such as the kidney epithelial 293 cell line (1, 10, 12–14, 16, 21, 24, 27). All three PERV subgroups can replicate in porcine cell lines (21, 24). Although in vitro transmission characteristics indicate a potential for in vivo transmission, infection by PERV of humans exposed to living porcine tissue has not been reported thus far (5, 6, 8, 19, 20, 22).

Genomic mapping studies have been used to investigate the distribution of the three subgroups of PERV in the genomic DNA of pigs. PERV-A and PERV-B loci have been identified in the germ line DNA of all pigs tested to date (11). Generally, PERV-A loci are present at a higher copy number than the

PERV-B subgroup. In most pig breeds, the PERV-C subgroup either is absent or is represented by only a limited number of elements. In contrast, the copy number of PERV-C can be elevated in miniature swine (MS) (2, 21). Previous mapping data have shown that the majority of PERVs in the genome are defective due to mutations in their coding sequences (9, 15). Interestingly, those PERVs that are clearly replication competent have been isolated from the genomic DNAs of immortalized pig cell lines (10). In contrast, those clones isolated from primary pig cells have been of weak competence (15). However, because the transmission phenotype of the cells from which these mapping studies were produced is unknown, it is not possible to determine which, if any, loci are expressed and, moreover, whether these viruses can be transmitted.

In vitro transmission studies of an inbred herd of MS have identified animals that reproducibly do not transmit PERV to human cells (16). The identification of animals within the highly inbred MS herd that lack human-tropic replication-competent (HTRC) PERV (16) may circumvent the need for strategies such as specific breeding or gene targeting to remove HTRC PERV from the herd, which might be required in other outbred breeds of pig. On all occasions when HTRC PERV was transmitted to human cells, the viruses were recombinants between the PERV-A and PERV-C subgroups with recombination in the post-VRA region of the *env* gene (16), indicating that recombination may play a vital role in the generation of HTRC PERV. Accordingly, for analysis of the PERV burden of the genomic DNA of pigs to be complete, genomic mapping

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must identify (i) all HTRC PERVs in the genome that possess independent replication competence; (ii) long replication-defective PERV-A loci, i.e., those loci that contain an *env* open reading frame (ORF) beyond the VRA region, which might contribute toward the production of HTRC PERV via recombination; and (iii) transcriptionally silent but potentially replication-competent proviruses that might become activated and infect human cells. Genomic mapping of PERV loci can be used to achieve these goals.

In this study we investigated MS with known in vitro transmission phenotypes. Genomic libraries were constructed from peripheral blood mononuclear cells (PBMC) of two SLA^{d/d} inbred MS (16); one of these (MS 13519) was capable of transmitting HTRC PERV-A/C recombinants to 293 cells in vitro, whereas MS 11852 lacked transmissible HTRC PERV and infected only the porcine cell line ST-IOWA. Study of these animals facilitates direct comparison of in vitro transmission data with genomic mapping data. We did not detect HTRC PERV in either of these libraries, which is indicative of the absence of HTRC PERV in the germ line DNA of MS. In addition, taken together with the data presented by Wood et al. (28), our data challenge the assumption that the most significant source of HTRC PERV is directly from intact germ line proviruses.

MATERIALS AND METHODS

Genomic library screening. Lambda libraries were constructed from the PBMC of SLA^{d/d} MS that had been classified as either a nontransmitter of HTRC PERV (MS 11852) (designated HTRC-NT) or a transmitter of HTRC PERV (MS 13519) (designated HTRC-T) (16). The library for MS 11852 was constructed from DNA partially digested with *Sau3A*I and ligated into *Xho*I-digested lambda GEM-12 (Promega, Southampton, United Kingdom) that had been partially filled with Klenow and T and C deoxynucleotides to create an *Sau3A*I-compatible end. Titer of the library were determined in KW251 cells (Promega). For MS 13519, the library was prepared from DNA partially digested with *Mbo*I and ligated to *Bam*HI-digested lambda DASH II vector (CMT Inc., Phillipsburg, N.J.), and titers were determined in P2 (MRA) cells (CMT Inc.). Neither library was amplified prior to use. For each library, a total of 1.5×10^6 PFU was plated on NZY agar plates. Duplicate filters were lifted by using Hybond NX (Amersham Pharmacia Biotech, Little Chalfont, Bucks, United Kingdom) and denatured and neutralized as described previously (9). Filters were UV-cross-linked and prehybridized for 30 min in Quickhyb solution (Stratagene, Amsterdam, The Netherlands). For *gag* screening, the probes and reagents used were as described by us previously (9). For *env* screening, a PERV-A-specific *env* probe was derived by using a nested set of primers specific to the variable region of the PERV-A envelope sequence. Primary PCR was performed on PK-15 cDNA by using conserved primers pCENV1 (5'-ACCAACGGCTGTGAAAGTCGAAG-3') and pCENV2 (5'-AAGTACCATGATCTGGACTGCA C-3'). Secondary amplifications were carried out with specific primers PV4USU (5'-GGAGATGGAAAGATTGGCAACAG-3') and PV4DSU (5'-CAGAGGTGTATTGTGAATCAGAG-3'). Products were purified, cloned into the vector pGEM-2T (Promega), and verified by restriction digestion and sequencing. An internal 334-bp insert was released by a *Spe*I-*Sac*II digest and used as the PERV-A specific probe. Probes were labeled with [α -³²P]dCTP by using Prime-it II (Stratagene). The library filters were hybridized and washed as described previously (9), exposed to X-ray film at -70°C for up to a week, and developed by using a Compact $\times 4$ automatic film processor (X-ograph Imaging Systems, Gloucester, United Kingdom).

Clones that gave positive hybridizations on both the *gag* and *env* filters were then purified to homogeneity through multiple rounds of rescreening. Lambda DNA was prepared from liquid lysates of these clones by standard methodologies.

Analysis of proviral clones for ORFs. To determine the lengths of the *env* genes of the lambda clones, genomic DNA was prepared from all *gag*- and *env*-positive lambda clones and analyzed by using PCR primer pairs external to the original *env* probe region. These primers (cenv-3 [5'-GGTTATAACAGGTGGTGGGCATG-3'] and cenv-4 [5'-CCAAGGAGACCTGTTGAACCGTC-

3']), which are conserved between all three subtypes of PERV, amplify a portion of the *env* gene and span the junction between the surface envelope (SU) and transmembrane (TM) protein-coding regions of *env*. PCRs were initiated by using the PCR core kit (Qiagen, Sussex, United Kingdom) with 50- μ l mixtures containing 1.5 mM MgCl₂, 1 U of *Taq* polymerase (Qiagen), and 200 ng of lambda DNA; reactions were cycled in an ABI9700 instrument (Perkin-Elmer Biosystems, Warrington, United Kingdom) at 94°C for 1 min 30 s followed by 35 cycles of 94°C for 15 s, 65°C for 30 s, and 72°C for 1 min and then followed by 72°C for 7 min, to yield a product of approximately 588 bp. Primers were used at a concentration of 10 pmol per reaction mixture.

Protein truncation test (PTT) PCR screening for *gag* and *env* ORFs was carried out as described previously on all clones that possessed long *env* genes (9). PCR products from clones that tested positive for full-length *gag* and *env* ORFs were cloned into the pCR2.1-Topo vector (Invitrogen, Paisley, United Kingdom). A minimum of two products for each clone were sequenced by ABI PRISM Dye terminator cycle sequencing on an ABI373 automated sequencer (Applied Biosystems, Warrington, United Kingdom). DNAs from all lambda clones that maintained full-length *gag* and *env* genes were digested with either *Sma*I or *Eco*RI in order to determine whether the clones isolated were unique.

Analysis of replication-defective PERV loci. Lambda clones that tested negative by *env* PTT PCR but positive by *gag* PTT PCR were designated defective. To determine whether the negative *env* PCR was due to disrupted ORFs and not due to the presence of recombinant sequence not being amplified because of our primer selection, clones testing positive for *env* sequences according to PCR with the primers cenv3 and cenv4 but negative by *env* PTT PCR were amplified by long terminal repeat-long terminal repeat PCR as described previously (16), and the *env* region was sequenced.

Detection of PERV-A/C *env* recombinants by PCR. Genomic DNAs were prepared from the PBMC of MS 11852 and MS 13519, and 293 cells were infected by a PERV-A/C recombinant and control plasmids containing PERV-A, -B, or -C *env* sequences. The DNAs were analyzed by using PCR primers specific to PERV-A/C *env* recombinant sequences. The primers A-VRA (forward) (5'-CCTACCAGTTATAATCAATTAAATTATGGC-3') and C-TM (reverse) (5'-CTCAAACCAACCTTGAGTAGTTTCC-3') were used to amplify sequences from 100 ng of genomic DNA in 50- μ l reaction mixtures containing 25 pmol of primers in PCR master mix (Qiagen) with the following cycling parameters: 1 cycle of 94°C for 5 min; 35 cycles of 94°C for 10 s, 60°C for 30 s, and 72°C for 90 s; and 1 cycle of 72°C for 10 min.

Analysis of competence of PERV clones and Env proteins in vitro. Transfection of lambda DNA was used to assess the replication competence of PERV loci, using techniques described by others (15). Briefly, 10 μ g of DNA was prepared from clones by the liquid lysate method and transfected into the 293 human and the ST-IOWA porcine cell lines by using Lipofectamine Plus in accordance with the manufacturer's instructions (Invitrogen). As an internal control for transfection efficiency, each transfection also contained a 10-ng spike of the expression plasmid pEGFP-N1 (BD Biosciences Clontech, Palo Alto, Calif.), and transfected cells were examined after 48 h of culture by fluorescence microscopy. Cultures were maintained for up to 60 days and monitored for reverse transcriptase (RT) activity by using a sensitive enzyme-linked immunosorbent assay-based system (HS-Mn²⁺ kit; Cavid Tech AB, Uppsala, Sweden) as described previously (15, 16). PERV-A60 DNA (1) was used as a positive control in the transfection assays.

Expression vectors were used to assess the competence of the Env proteins putatively encoded by the PERV-A proviral loci. Amplification of the *env* sequences from library clone DNA was carried out in 50- μ l reaction mixtures containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, a 150 nM concentration of each primer, a 200 nM concentration of each deoxynucleoside triphosphate, 2.5 U of Amplitaq (Perkin-Elmer Biosystems), 200 ng of lambda DNA, and the primers *env* F (5'-GGATCCTAATACGACTCACTATAGGAA CAGACCACCATGCATCCACGTTAAGCCG-3') and *env* R (5'-GCTCTAG ACTAAGCGTAGTCTGGGACGTCGTTAGGTTAGAGTGGGAAGGGT AGAGGTCAGT-3') and were cycled as follows: 1 cycle of 95°C for 3 min; 30 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 2 min 10 s; and 10 min at 72°C. The primers used were previously described by us (9); the T7 RNA polymerase promoter sequence is incorporated in the sense primer, and the antisense primer is modified to contain an in-frame hemagglutinin epitope tag as utilized in the PTT assay (9). The PCR products were cloned into the pCR2.1-Topo cloning vector (Invitrogen) according to the manufacturer's instructions. The PERV-A envelope sequences were excised from the pCR2.1-Topo vector by digestion with *Spe*I and *Xba*I and subcloned into the expression vectors pCR3.1 (Invitrogen) and pCIneo at the *Xba*I site. The correct orientation of the *env* sequences was confirmed by digestion of the constructs with *Xho*I, and the constructs were sequenced on an ABI373 automated sequencer (Applied Bio-

A

B

FIG. 1. Genomic library screening results for transmitting and nontransmitting MS. (A) HTRC-NT MS11852; (B) HTRC-T MS13519. Lambda clones were identified by radioactive hybridization screening prior to PCR and sequencing.

			↓	↓	↓	↓
NTr1	(600)	VTLLSALTGPLVILLLLLTGPGCLINRFVAFVREQVSAVRIMVLRQQYQ				
NTr2	(601)	VTLLSALTGPLVILLLLLTGPGCLINRFVAFVREQVSAVRIMVLRQQYQ				
NTr3	(601)	VTLLSALTGPLVILLLLLTGPGCLINRFVAFVREQVSAVRIMVLRQQYQ				
NTr4	(601)	VTLLSALTGPLVILLLLLTGPGCLINRFVAFVREQVSAVRIMVLRQQYQ				
Tr1	(600)	MTLLSALTGPPVLLLLLTIGPGCLINRFVAFVRE*VSAVQIMVLRQQYQ				
Tr2	(601)	VTLLSALTGPLVILLLLLTGPGCLINRFVAFVREQVSAVRIMVLRQQYQ				
Tr3	(601)	VTLLSALTGPLVILLLLLTGPGCLINRFVAFVREQVSAVRIMVLRQQYQ				
Tr4	(601)	VTLLSALTGPLVILLLLLTGPGCLINRFVAFVREQVSAVRIMVLRQQYQ				
Tr5	(601)	VTLLSALTGPLVILLLLLTGPGCLINRFVAFVREQVSAVRIMVLRQQYQ				
T142.1	(601)	LATLLSALTGPLVILLLLLTGPGCIINKLIAFIRERISAVQIMVLRQQYQ				
T142.8	(601)	LATLLSALTGPLVILLLLLTGPGCIINKLIAFIRERISAVQIMVLRQQYQ				
t1.2c9	(583)	LATLLSALTGPLVILLLLLTGPGCIINKLIAFIRERISAVQIMVLRQQYQ				
t2a3	(601)	LATLLSALTGPLVILLLLLTGPGCIINKLIAFIRERISAVQIMVLRQQYQ				
t2a5	(601)	LATLLSALTGPLVILLLLLTGPGCIINKLIAFIRERISAVQIMVLRQQYQ				
t6e5	(601)	LATLLSALTGPLVILLLLLTGPGCIINKLIAFIRERISAVQIMVLRQQYQ				
			↓			
NTr1	(650)	GLPS*GETDL				
NTr2	(651)	GLPS*GETDL				
NTr3	(650)	GLPS*GETDL				
NTr4	(651)	GLPS*GETDL				
Tr1	(649)	GLLSQGETDL				
Tr2	(651)	GLPS*GETDL				
Tr3	(651)	GLPS*GETDL				
Tr4	(651)	GLPS*GETDL				
Tr5	(651)	GLPS*GETDL				
T142.1	(651)	SPSS-REAGR				
T142.8	(651)	SPSS-REAGR				
t1.2c9	(633)	SPSS-REAGR				
t2a3	(651)	SPSS-REAGR				
t2a5	(651)	SPSS-REAGR				
t6e5	(651)	SPSS-REAGR				

FIG. 2. Amino acid sequence comparison of the 3' ends of the *env* regions of PERV-A clones isolated from both the HTRC-T 13519 (Tr1 to -5) and HTRC-NT 11852 (NTr1 to -4) libraries and PERV-A/C recombinants isolated from 293 cells infected with MS PBMC (t1.2c9 [accession number AF417229], t2a3 [AF417230], t2a5 [AF417231], and t6e5 [AF417232]). T142-derived PERV A/C recombinant sequences are those from the coculture of PBMC from animal 13519 (T142.1 [AY364234] and T142.8 [AY364236]). *, stop codon. Arrows highlight amino acid differences.

systems). Plasmid DNAs were transfected into the retroviral packaging cell line TELCeB by using Lipofectamine (Invitrogen) according to the manufacturer's instructions. Stable clones were selected by the addition of 800 μ g of G418 per ml 48 h after transfection. The infectious titers of the pseudotypes were determined by plating on 293 and ST-IOWA cells in the presence of 8 μ g of Polybrene per ml and β -galactosidase staining as described previously (24).

Nucleotide sequence accession numbers. Primers were derived from sequences taken from the GenBank database, for PERV-A, GenBank accession codes Y12238; for PERV B, Y17013/Y12239; and for PERV-C, AF038600. Sequences for the clones NTr1 to -4 are as follows: *gag* accession AY368583 to -6 and *env* genes accession numbers AY368587 to -9 and AY288779. For Tr1 to -4 *env* genes accession codes are AY368579 to -82; for Tr5, AY371067.

RESULTS AND DISCUSSION

To identify PERV clones that may be important in the formation of human-tropic viruses, we analyzed genomic DNA libraries made from MS PBMC genomic DNA. Each library contained an average insert size of approximately 18.2 kb and three to five genome equivalents. A primary screen to identify lambda clones that contained PERV-A proviral sequences was performed with (i) a PERV-A-specific *env* probe that is located within the VRA region and (ii) a *gag* probe, the sequence of which is conserved across the PERV subgroups (9). Double-positive clones were plaque purified prior to further analysis. A PCR-based secondary screen that analyzed the lengths of the

gag and *env* genes of the clones was performed. In order for a clone to be included in third-round analysis, we required that the clone should produce near-full-length *gag* and *env* PCR products. Third-round analysis consisted of PTT and sequencing analysis of *gag* and *env* PCR products to detect loci that had full-length *gag* and *env* ORFs. In addition, those *gag*-positive clones that possessed *env* ORFs that extended beyond the VRA region were also included in final analysis, because these "long" defective PERV-A loci could be the genetic substrate for the formation of human-tropic PERV. Finally, restriction fragment length polymorphism analysis was performed on the qualifying clones to identify unique loci.

The first-round screen of the genomic library from MS 11852, an HTRC PERV-nontransmitting (HTRC-NT) pig, identified a total of 106 PERV-A-positive clones (Fig. 1A). PCR analysis of purified clones indicated that 62 of the clones possessed *gag* and long *env* genes. The remaining 44 clones were excluded due to truncations of either their *env* (33 of 44 cases) or *gag* (2 of 44 cases) genes or of both genes (9 of 44 cases). Within the 62 *gag*- and *env*-positive clones, 22 were found to be positive by both *gag* and *env* PTT PCR, indicating that these loci were potentially replication competent. The remaining 40 clones were excluded due to the absence of the *env* ORF (30 of 40 cases), the *gag* ORF (5 of 40 cases), or both

TABLE 1. Nucleotide sequence and replication competence analysis of PERV-A loci

Clone ^a	<i>gag</i> ORF	<i>env</i> ORF ^b	Replication competence ^c in:	
			293 cells	ST-IOWA cells
NTr1	Full length	Premature stop	Negative	Negative
NTr2	Full length	Long <i>env</i>	Negative	Negative
NTr3	Full length	Long <i>env</i>	Negative	Negative
NTr4	Full length	Long <i>env</i>	Negative	Negative
Tr1	Full length	Premature stop	Negative	Negative
Tr2	Full length	Long <i>env</i>	Negative	Negative

^a The NTr and Tr designations refer to loci identified from nontransmitting and transmitting MS, respectively.

^b Long *env* refers to *env* sequences terminated approximately 6 amino acids short of full length. Premature stop refers to a significantly truncated *env* ORF at nt 247 for NTr1 and nt 1183 for Tr1.

^c The PK15-derived control PERV transfection resulted in detectable replication within 10 days of transfection.

ORFs (5 of 40 cases). RFLP analysis of the 22 double-positive loci indicated that 9 were unique, and of these, sequence analysis indicated that only 4 (NTr1 to -4) had full-length *gag* genes, i.e., genes of similar size to the *gag* genes of known replication-competent PERV. Analysis of the *env* genes of the four associated PERV-A sequences indicated that they possessed greater than 97% homology to each other and to the previously characterized germ line PERV-A *env* sequence (GenBank accession number AF435967) (data not shown) (11). Interestingly, none of these clones possessed full-length *env* ORFs due to two common point mutations that introduced stop codons at either nucleotide (nt) 1906 (CGA to TGA) or nt 1963 (CAA to TAA). The stop codon at nt 1963 terminates the putative Env proteins six amino acid residues before the termination site observed with PK15-derived PERV-A, which can grow to high titers in human cells in vitro (Fig. 2) (for complete sequence comparison, see <http://www.vet.gla.ac.uk/retrovirus/PERV.html>).

The genotypes of the PERV clones identified during analysis of the genomic DNA library made from MS 13519, an HTRC PERV-transmitting (HTRC-T) animal, are summarized in Fig. 1B. A total of 120 PERV-A-positive clones were identified during the primary screen of the library. PCR analysis of purified clones indicated that 72 of the clones initially possessed *gag* and *env* sequences. Of these 72 clones, 29 were positive for long *gag* and *env* genes as determined by PTT PCR. Of the

TABLE 2. Infectivity of PERV pseudotypes for human and porcine cells

PERV clone ^a	Infectivity (IU/ml) on the following target cell line:	
	293	ST-IOWA
PERV-A	10 ⁴	10 ⁴
PERV-B	10 ³	10 ³
PERV-C	<4	10 ²
NTr1	<4	<4
NTr4	<4	<4
Tr1	<4	<4
Tr2	<4	<4

^a The NTr and Tr designations refer to loci identified from nontransmitting and transmitting MS, respectively.

FIG. 3. Genomic DNAs isolated from transmitting and nontransmitting MS and analyzed by PCR for the presence of PERV-A/C recombinant *env* sequences (top) and pan-PERV *env* sequences (bottom), using conserved primers. Spiking experiments with recombinant PERV-A/C DNA confirmed the ability of the PCR to detect a single copy of PERV-A/C provirus (not shown). Lanes: 1, PERV-A control (PCR-A60); 2, PERV-B control (PCR-B17); 3, PERV-C *env* control (PoEV3); 4, MS13519; 5, MS11852; 6, 293 PERV-A/C genomic DNA; 7, 293 cell genomic DNA; M, size marker.

remainder, 14 were defective in *env*, 8 were defective in *gag*, and the final 21 clones were negative for *gag* and *env* PTT PCR products. Within the 29 *gag*- and *env* positive clones, nucleotide sequencing revealed that five PERV-A clones (Tr1 to -5) possessed long *gag* and *env* genes. The remaining 24 clones were either defective PERV-A or PERV-B; the latter were probably identified due to cross-hybridization of the PERV-A probe during the lower-stringency hybridization employed in the primary screen.

Amino acid sequence analysis revealed no remarkable differences between the nucleotide sequences of the long *env* genes of the PERV-A loci present in the HTRC-T pig and HTRC-NT pigs (Fig. 2) (<http://www.vet.gla.ac.uk/retrovirus/PERV.html>), which is a reflection of the highly inbred nature of the MS herd (inbreeding coefficient, >0.9).

Studies of replication competence were performed with the PERV-A clones that may have possessed replication competence, i.e., the four isolated from the HTRC-NT library and two representative clones of the five isolated from HTRC-T library identified as possessing full-length *gag* ORFs as well as long *env* ORFs (i.e., truncated at nt 1906 or 1963). Purified lambda clone DNA was transfected into human (293) and pig (ST-IOWA) target cells, along with an enhanced green fluorescent protein reporter vector. Following transfection, approximately 10 to 30% of target cells expressed the enhanced green fluorescent protein reporter vector, indicating that the transfection was successful (data not shown). The cultures were monitored for supernatant RT activity for approximately 60 days. No production of RT activity was detected in either of the cell lines, indicating that the clones were not replication competent (Table 1). In contrast, RT activity was detectable with the control PERV-A clone (PERV-A60) after approximately 10 days in culture (data not shown).

In order to further analyze the competence of the clones, representative *env* genes of long PERV-A loci were cloned into mammalian expression vectors and transfected into the murine

AY288779	A	(384)	QHLCNHTAEAFNRTSESQYLVPGYDRWWACNTGLTPCVSTLVFNQTKDFCV
AF435967	A	(384)	QHLCNHTAEAFNRTSESQVSGTWLCRWWACNTGLTPCVSTLVFNQTKDFCV
AF435966	A	(384)	QHLCNHTAEAFNRTSESQYLVPGYDRWWACNTGLTPCVSTLVFNQTKDFCV
Y12238	A	(384)	QHLCNHTAEAFNRTSESQYLVPGYDRWWACNTGLTPCVSTLVFNQTKDFCV
AJ133817	A	(384)	QHLCNHTAEAFNRTSESQYLVPGYDRWWACNTGLTPCVSTLVFNQTKDFCV
AY099323	A	(384)	QHLCNHTAEAFNRTSESQYLVPGYDRWWACNTGLTPCVSTLVFNQTKDFCV
AJ279057	B	(381)	QHLCCSTVVYEQASENQYLVPGYNRWWACNTGLTPCVSTSVFNQSKDFCV
AJ133816	B	(381)	QHLCYSTVVYEQASENQYLVPGYNRWWACNTGLTPCVSTSVFNQSKDFCV
AY099324	B	(381)	QHLCYSTVVYEQASENQYLVPGYNRWWACNTGLTPCVSTSVFNQSKDFCV
Y12239	B	(381)	QHLCYSTVVYEQASENQYLVPGYNRWWACNTGLTPCVSTSVFNQSKDFCV
AY288779	A	(434)	MVQIVPRVYYYPEKAVLDEYDYYRNPKEPISLTLAVMLGLGVAAGVGT
AF435967	A	(434)	MVQIVPRVYYYPEKAVLDEYDYYRNPKEPISLTLAVMLGLGVAAGVGT
AF435966	A	(434)	MVQIVPRVYYYPEKAVLDKYDYYRNPKEPISLTLAVMLGLGVAAGVGT
Y12238	A	(434)	MVQIVPRVYYYPEKAVLDEYDYYRNPKEPISLTLAVMLGLGVAAGVGT
AJ133817	A	(434)	MVQIVPRVYYYPEKAVLDKYDYYRNPKEPISLTLAVMLGLGVAAGVGT
AY099323	A	(434)	MVQIVPRVYYYPEKAVLDEYDYYRNPKEPISLTLAVMLGLGVAAGVGT
AJ279057	B	(431)	MVQIVPRVYYHPEEVVLDEYDYYRNPKEPVS LTLAVMLGLGTAVGIGT
AJ133816	B	(431)	MVQIVPRVYYHPEEVVLDEYDYYRNPKEPVS LTLAVMLGLGTAVGVGT
AY099324	B	(431)	MVQIIIPRVYYHPEEVVLDEYDYYRNPKEPVS LTLAVMLGLGTAVGVGT
Y12239	B	(431)	MVQIVPRVYYHPEEVVLDEYDYYRNPKEPVS LTLAVMLGLGTAVGVGT
AY288779	A	(484)	GTAALITGPQQLEKGLSNLHRIVTEDLQALEKSVSNLEESLTSLEVV LQ
AF435967	A	(484)	GTAALITGPQQLEKGLSNLHRIVTEDLQALEKSVSNLEESLTSLEVV LQ
AF435966	A	(484)	GTAALITGPQQLEKGLSNLHRIVTEDLQALEKSVSNLEESLTSLEVV LQ
Y12238	A	(484)	GTAALITGPQQLEKGLSNLHRIVTEDLQALEKSVSNLEESLTSLEVV LQ
AJ133817	A	(484)	GTAALITGPQQLEKGLSNLHRIVTEDLQALEKSVSNLEESLTSLEVV LQ
AY099323	A	(484)	GTAALITGPQQLEKGLSNLHQIVTEDLQALEKSVSNLEESLTSLEVV LQ
AJ279057	B	(481)	GTAALITGPQQLEKGLGELHAAMTEDLRALEESVSNLEESLTSLEVV LQ
AJ133816	B	(481)	GTAALITGPQQLEKGLGELHAAMTEDLRALEESVSNLEESLTSLEVV LQ
AY099324	B	(481)	GTAALITGPQQLEKGLGELHAAMTEDLRALEESVSNLEESLTSLEVV LQ
Y12239	B	(481)	GTAALITGPQQLEKGLGELHAAMTEDLRALEESVSNLEESLTSLEVV LQ
AY288779	A	(534)	NRRGLDLLFLKEGGLCVALKEECCFYVDHSGAIRDMSKLRERLEKRHKE
AF435967	A	(534)	NRRGLDLLFLKEGGLCVALKEECCFYVDHSGAIRDMSKLRERLEKRHKE
AF435966	A	(534)	NRRGLDLLFLKEGGLCVALKEECCFYVDHSGAIRDMSKLRERLERRRRE
Y12238	A	(534)	NRRGLDLLFLKEGGLCVALKEECCFYVDHSGAIRDMSKLRERLERRRRE
AJ133817	A	(534)	NRRGLDLLFLKEGGLCVALKEECCFYVDHSGAIRDMSKLRERLERRRRE
AY099323	A	(534)	NRRGLDLLFLKEGGLCVALKEECCFYVDHSGAIRDMSKLRERLERRRRE
AJ279057	B	(531)	NRRGLDLLFLREGGLCAALKEECCFYVDHSGAIRDMSKLRERLERRRRE
AJ133816	B	(531)	NRRGLDLLFLREGGLCAALKEECCFYVDHSGAIRDMSKLRERLERRRRE
AY099324	B	(531)	NRRGLDLLFLREGGLCAALKEECCFYVDHSGAIRDMSKLRERLERRRRE
Y12239	B	(531)	NRRGLDLLFLREGGLCAALKEECCFYVDHSGAIRDMSKLRERLERRRRE
AY288779	A	(584)	KEAGQGWFEQWGNKSPWVTLLSALTGPLVLLLLLLTVGPCLINRFVAFV
AF435967	A	(584)	KEAGQGWFEQWGNKSPWVTLLSALTGPLLILLLLLLLTVGPCLINRFVAFV
AF435966	A	(584)	READQGWFEQWGNRSPWMTLLSALTGPLVLLLLLLTVGPCLINRFVAFV
Y12238	A	(584)	READQGWFEQWGNRSPWMTLLSALTGPLVLLLLLLTVGPCLINRFVAFV
AJ133817	A	(584)	READQGWFEQWGNRSPWMTLLSALTGPLVLLLLLLTVGPCLINRFVAFV
AY099323	A	(584)	READQGWFEQWGNRSPWMTLLSALTGPLVLLLLLLTVGPCLINRFVAFV
AJ279057	B	(581)	READQGWFEQWGNRSPWMTLLSALTGPLVLLLLLLTVGPCLINRFVAFV
AJ133816	B	(581)	READQGWFEQWGNRSPWMTLLSALTGPLVLLLLLLTVGPCLINRFVAFV
AY099324	B	(581)	READQGWFEQWGNRSPWMTLLSALTGPLVLLLLLLTVGPCLINRFVAFV
Y12239	B	(581)	READQGWFEQWGNRSPWMTLLSALTGPLVLLLLLLTVGPCLINRFVAFV
AY288779	A	(634)	REQVSAVRIMVLRQQYQGLPS*GETDL
AF435967	A	(634)	REQVSAVRIMVLRQQYQGLPS*GETDL
AF435966	A	(634)	RERSAVQIMVLRQQYQGLLSQGETDL
Y12238	A	(634)	RERSAVQIMVLRQQYQGLLSQGETDL
AJ133817	A	(634)	RERSAVQIMVLRQQYQGLLSQGETDL
AY099323	A	(634)	RERSAVQIMVLRQQYQGLLSQGETDL
AJ279057	B	(631)	RERSAVQIMVLRQQYQGLLSQGETDL
AJ133816	B	(631)	RERSAVQIMVLRQQYQGLLSQGETDL
AY099324	B	(631)	RERSAVQIMVLRQQYQGLLSQGETDL
Y12239	B	(631)	RERSAVQIMVLRQQYQGLLSQGETDL

FIG. 4. Comparison of PERV-A and -B sequences from the GenBank database in the transmembrane region of the envelope. The GenBank accession number for each sequence is indicated at the start of the sequence. The nonrecombinant PERV-A sequence NTr3 (AY28879), which was isolated from MS11852, is included for comparison. Arrow, recombination site; *, stop codon.

leukemia virus-based pseudotype producer cell line TELCeB, which encodes β -galactosidase activity. As shown in Table 2, Env function was not detected for any of the clones when tested on 293 or ST-IOWA cells.

To date, it has been assumed that (i) the production of human-tropic PERV is governed by germ line proviral loci and (ii) the HTRC PERV-A/C recombinants detected by using in vitro transmission assays are tissue culture phenomena. However, the results reported by Wood et al. (28), indicating that PERV-A/C recombinants exist in vivo, raise the possibility that the viruses might not be present in the genomes of transmitting pigs. Therefore, we extended the analysis of the genomic DNAs and the genomic library DNAs (data not shown) from both animals in an attempt to identify the presence of recombinant viruses. By PCR, PERV-A/C recombinants were not detected in either the genomic DNA (Fig. 3) or the genomic library DNA from the transmitter animal. It is noteworthy that PERV-A/C recombinants were also not detected during the screening of the HTRC-NT and HTRC-T genomic libraries. These results are consistent with the hypothesis proposed by Wood et al. (28), indicating that these viruses are not present in the germ line DNA of MS and that recombination may be critical for the formation of human-tropic PERV.

To investigate the potential importance of PERV recombination in more detail, we analyzed the functional *env* sequences that have been deposited in the GenBank database. We compared the *env* sequences of five functional PERV-A and four functional PERV-B GenBank sequences (isolated either from the PK-15 cell line [1, 4, 11] or from various pig breeds [4, 15], along with a representative PERV-A *env* sequence isolated from the MS genomic library. Alignment of these sequences indicated that all but one of the PERV-A *env* genes are recombinants with PERV-B within the TM region (Fig. 4), with the exception being an *env* sequence (accession number AF435967) which was derived from a weakly replication-competent PERV-A cloned from the genomic DNA of Large White pigs (15). Interestingly, in contrast to the apparent PERV-A/B recombinants identifiable in cell lines producing high titers of PERV, the proviral PERV-A sequences identified in the MS libraries appear to be nonrecombinant and most similar to the PERV-A sequence under accession number AF435967 (Fig. 4). The Env protein products encoded by the MS-derived sequences were nonfunctional in pseudotype assays (Table 2). Given the reproducible nature of the recombination with PERV-B, it is tempting to speculate that the generation of HTRC PERV-A *env* genes that can infect human cells at a significant titer, such as the PK-15 cell line-derived envelope described by Le Tissier et al. (11), may be the result of recombination between PERV-A and PERV-B elements in vitro.

In summary, in this study we were unable to identify PERV proviral loci derived from either an HTRC-T or an HTRC-NT MS library that possess replication competence in human 293 cells. Current data indicate that HTRC PERV possesses *env* genes that are recombinants between PERV-A and -C *env* sequences. Our PCR and genomic mapping studies indicate that these recombinant viruses are not present in the germ line DNA of MS, which, taken together with our observation that recombinant PERVs can be identified in the PBMC of MS (28), indicates that recombination is an important mechanism

in the generation of PERVs that are able to infect human cells. The identification of human-tropic recombinant PERV in vitro but not in the germ line suggests that the recombination event may occur de novo in each pig. A number of mechanisms potentially underlie the generation of replication-competent PERV. First, because PERV loci were not identified in transmitting pigs that correlated with the potential to produce HTRC PERV, it is possible that these elements are being generated in pig cells from a number of defective germ line PERV elements. Thus, in a worst-case scenario, despite an organ or tissue being free of replication-competent retroviruses at the time of transplant, replication-competent retroviruses might be generated in the organ while it is in the xenograft recipient, thus exposing them to risk of infection. Second, the PERV-A/C recombinant may exist as an exogenous agent or may be generated as a result of infection of the pig with an infectious PERV. If this proves to be correct, it may be possible to dramatically reduce the rate of recombinant PERV formation via established specific-pathogen-free pig derivation procedures. Further studies are required to define the mechanism by which PERV-A/C recombinant viruses are formed so that appropriate strategies can be developed to remove these viruses from the herd and improve the safety of xenotransplantation.

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