Porcine Endogenous Retrovirus Transmission Characteristics of an Inbred Herd of Miniature Swine

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Here we report the identification of inbred miniature swine that failed to produce human-tropic replication-competent porcine endogenous retroviruses (HTRC PERVs), using in vitro coculture assays. When HTRC PERVs were isolated from transmitting animals, all were recombinant viruses, with the receptor-binding domain of PERV-A combining with PERV-C-related sequences.

The number of patients awaiting transplantation increases every year due to a lack of suitable donors. Although the xenotransplantation of porcine tissues has the potential to alleviate this shortage (5), there remain certain risks associated with the use of pigs as donor animals. In particular, the inadvertent transmission of porcine microorganisms to the recipient of a xenograft is a concern (1, 7, 14, 17, 20). Because porcine endogenous retroviruses (PERVs) are unaffected by barrier derivation technologies and can infect human cells in vitro (13), these viruses are perceived as a major safety concern.

Three replication-competent classes of PERVs (PERV-A, -B, and -C) have been identified in the genomic DNA of pigs and porcine cell lines (9, 18). The PERV-A and PERV-B classes can infect human and pig cells in vitro. PERV-C is not able to infect human cells but can replicate in porcine cell lines (13, 18). Although the majority of PERV loci in porcine DNA are most likely noninfectious (3, 4, 8, 9), human-tropic viruses have been isolated from porcine cell lines as well as primary tissues (2, 11–13, 16, 18, 21). Interestingly, all clones of replication-competent PERV-A and PERV-B classes with significant infectious titers have been isolated from PK15 cells or cell lines infected by viruses released from PK15 cells (6, 8, 19). Conversely, all PERV clones derived from primary pig cells have had extremely low titers and limited replication competence (6, 8, 19).

We investigated the PERV transmission characteristics of a unique inbred herd of miniature swine by using in vitro coculture assays. Three independent swine leukocyte antigen (SLA) haplotypes (a, c, and d) and three classes of recombinant SLA haplotypes (g, h, and k) are maintained within this herd. Due to its proven sensitivity, the coculture of porcine peripheral blood mononuclear cells (PBMC) with the human 293 cell line (6, 8, 10, 21, 22) and the porcine cell line ST-IOWA (10, 13, 21)

has been used to compare the relative infectivity of PERVs produced from different pigs and the replication competence of PERV. In addition to these systems, we varied both the producer cells and target cell lines used in the coculture assays in an attempt to identify conditions under which additional human-tropic replication-competent (HTRC) PERVs could be identified. Pig aortic endothelial cells were investigated as an alternative primary cell source of PERV because single polynucleotide polymorphisms in PERV long terminal repeats (LTRs) have been reported that might confer differential locus responsiveness to activation by phytohemagglutinin and phorbol myristate acetate or tumor necrosis factor alpha (15). Production of PERVs from pig aortic endothelial cells was lower than that from PBMC and resulted in less-reliable coculture assays (data not shown). Therefore, only those assays using PBMC as the primary producer cells are reported below.

Because of the presence of PERV-C in miniature swine, we used the porcine cell line ST-IOWA and the human cell line HT1080 as target cell lines for the transmission assays. The

TABLE 1. Transmission of PERV from lines of inbred miniature swine[a]

Miniature swine line haplotype	No. of positive transmission assays/no. of animals tested with indicated cell line		
	ST-IOWA	HT1080	293
a/a	7/7	NT	2/7
c/c	8/8	0/1	4/8
d/d	28/28	0/6	3/28
g/g	10/10	0/1	1/10
h/h	4/4	NT	1/4
k/k	4/4	NT	1/4

[a] Infection of porcine and human cell lines following exposure to miniature swine PBMC. Prior to coculture, PBMC were stimulated with phytohemagglutinin P (2.5 μg/ml) and phorbol 12-myristate-13-acetate (1 ng/ml). Infection of cells was measured by the presence of RT activity in the culture supernatants (HS-kit Mn^{2+} RT; Cavidi Tech AB) and, for transmitting animals, was typically detectable after 10 to 14 or 20 to 30 days in ST-IOWA and 293 cultures, respectively. 293 and HT1080 cell cocultures were maintained for a minimum of approximately 60 days before being classified as negative for PERV replication. PERV-C RNA expression was detected in ST-IOWA cells concurrently with the development of RT activity in the culture supernatants. NT, not tested.

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TABLE 2. Summary of 293 cell transmission assays from repeat blood draws of individual miniature swine

No. of negative assays/ no. of positive assays	No. of animals tested
1/0	42
2/0	4
3/0	1
4/0	2
0/1	6
0/2	2
0/3	1
1/1	2
1/2	1

HT1080 cell line is of particular interest because it is the only human cell line that has been reported to express a receptor for PERV-C (18). Although all ST-IOWA coculture assays became infected by replication-competent PERV-C released from the PBMC, infection was not detected with the HT1080 cell line (Table 1). Although it cannot be excluded that

PERV-C may have required an extended adaptation period for its replication to become productive in the HT1080 cells, tissue culture-adapted classes of PERV-A and PERV-B, but not PERV-C, were capable of replication in this cell line, albeit at low levels (data not shown). In addition, it has recently been observed that retrovirus pseudotype particles lacking all *env* proteins can also infect HT1080 cells (Y. Takeuchi and F.-L. Cosset, personal communication). This suggests that the PERV-C infectivity reported for this human cell line is the result of nonspecific uptake mechanisms and therefore that no independent human-tropic capacity can currently be associated with PERV-C.

Animals representing all lines of miniature swine were identified whose PBMC transmitted HTRC PERVs to 293 cells (Table 1). However, the frequency of transmission within the haplotype lines varied markedly (Table 1). In particular, two lines of miniature swine ($SLA^{d/d}$ and $SLA^{g/g}$) showed low incidences of transmission. It is noteworthy that the three $SLA^{d/d}$ animals showing transmission are closely related (one sow and

A

Sequence	Pre VRA region																										
	14	15	20	21	22	31	32	36	38	40	41	42	47	49	85	98	113	118	137	140	143	158	159	164	167	173	174
PERV-A	T	C	A	G	T	A	A	C	T	T	G	G	C	C	C	T	T	G	C	G	G	A	T	T	C	T	C
PERV-C	C	T	G	A	C	T	G	A	A	G	A	G	C	T	A	C	C	A	T	A	A	T	C	C	T	C	A
T2A3	C	T	G	A	C	T	G	A	A	G	A	G	C	T	A	T	T	G	C	G	G	A	T	T	C	T	C
T2A5	C	T	G	A	T	A	A	C	T	T	G	G	C	C	C	T	T	G	C	G	G	A	T	T	C	T	C
T6E5	T	C	A	G	T	A	A	C	T	T	G	G	C	C	C	T	T	G	C	G	G	A	T	T	C	T	C

B

Sequence	VRA region																					
	182	185	189	191	194	195	196	197	198	199	200	201	202	204	207	215	219	221	226	231	236	242
PERV-A	A	C	G	T	C	A	A	T	G	A	C	C	A	G	A	C	G	A	G	T	G	C
PERV-C	T	T	A	T	-	-	-	-	-	-	-	-	-	A	T	A	A	C	A	C	A	T
T2A3	A	C	G	T	C	A	A	T	G	A	C	C	A	G	A	C	G	A	G	T	G	C
T2A5	A	C	G	T	C	A	A	T	G	A	C	C	A	G	A	C	G	A	G	T	G	C
T6E5	A	C	G	**C**	C	A	A	T	G	A	C	C	A	G	A	C	G	A	G	T	G	C

Sequence	VRA region continued																					
	257	268	270	273	278	287	288	289	290	302	305	313	317	318	223	335	336	351	352	362	365	368
PERV-A	C	A	G	T	T	T	C	A	G	C	G	G	C	A	T	G	A	G	T	A	C	A
PERV-C	A	G	A	C	C	C	A	G	A	T	A	A	T	G	C	A	T	A	C	G	T	G
T2A3	C	A	G	T	T	T	C	A	G	C	G	G	C	A	T	G	A	G	T	A	C	A
T2A5	C	A	G	T	T	T	C	A	G	C	G	G	C	A	T	G	A	G	T	A	C	A
T6E5	C	A	G	T	T	T	C	A	G	C	G	G	C	A	T	G	A	G	T	A	C	A

FIG. 1. Alignment of pre-VRA (A) and VRA (B) regions of the *env* genes of HTRC PERV. Only those nucleotides that vary between the PERV-A (shaded boxes) and PERV-C (unshaded boxes) reference sequences are indicated. HTRC PERV loci were PCR amplified from PERV-infected 293 cells using oligonucleotides designed to match LTR sequences conserved between the three infectious classes of PERV (5′-CCTGGTGGTCTCCTACTGTCG-3′ and 5′-GCTTTTATGGGGTTCACAACAAA-3′) with TaKaRa DNA polymerase (Intergen). The sequences were divided into three families as defined by the position at which their nucleotide sequence underwent transition between PERV-C and PERV-A. Representative members of each family are presented. A nucleotide substitution that did not correlate with either reference sequence was identified at base 192 in sequence T6E5 (black box).
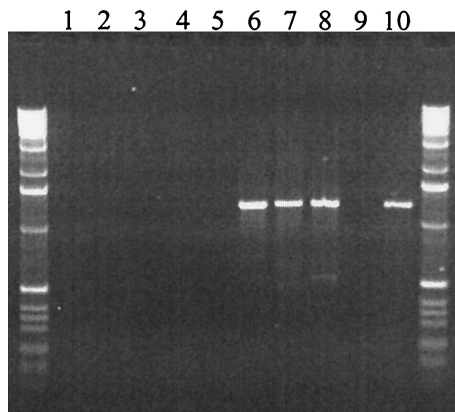
FIG. 2. PCR analysis of miniature swine genomic DNA indicating that PERV-A–PERV-C TM recombinants are not present in miniature swine genomic DNA but develop during tissue culture. The sense primer specific for PERV-A is located in the VRA region, and the PERV-C specific antisense primer is located in the TM region. Samples are as follows: water control (lane 1), DNA from miniature swine (nontransmitting animals, lanes 2 and 3; transmitting animals, lanes 4 and 5), 293 cells infected with HTRC-PERV (lanes 6 to 8), 293 cells (lane 9), and a plasmid-positive control of a cloned HTRC-PERV *env* gene (lane 10). Control amplifications of cytochrome *b* sequences were successful and comparable from each sample (data not shown).

two offspring) and, similarly, that the single transmission-positive animal identified within the SLA$^{g/g}$ subline is from a line of animals distinct from the nontransmitting SLA$^{g/g}$ animals. To assess the reliability of the coculture assays, and therefore the significance of negative transmission results with 293 cells, repeat cocultures for individual animals were initiated using PBMC isolated from additional blood samples. Although a low false-negative rate in the assay system was identified, animals were identified that consistently produced transmitting or nontransmitting phenotypes, following repeat testing on up to four occasions (Table 2). The identification of transmitting animals within all of the lines of SLA-defined miniature swine indicates that critical PERV proviruses are unlikely to be closely linked to the SLA on chromosome 7.

To gain information regarding the genomic organization of the HTRC PERVs produced by transmitting miniature swine, we examined the PERV proviruses present in eight independently infected 293 cultures using LTR-to-LTR PCR. In total, 127 PERV clones were analyzed by sequencing regions of their *gag*, *pol*, and *env* genes. All clones possessed *gag*, *pol*, and *env* transmembrane (TM) sequences most closely related to PERV-C and the *env* receptor-binding domain of PERV-A. To determine whether recombination events were required to generate the PERV-A–PERV-C hybrid sequences detected in the HTRC PERVs, we analyzed the sequence of PERV loci present in the genomic DNA of miniature swine. PERV-A clones were generated using LTR-to-LTR PCR, and regions of the *gag*, *pol*, and *env* genes of nominally full-length clones were sequenced. Analysis indicated that many of these PERV-A clones possessed *gag* and *pol* genes most closely related to PERV-C, suggesting that the PERV-C *gag-pol* and PERV-A *env* hybrid sequences were derived directly from genomic sequences. Alignment of the *pol- env* gene sequences of approximately 30 HTRC-PERV clones indicated that three distinct

families of PERV-C *pol* and PERV-A *env* sequences were present and is suggestive of only a limited number of PERV-A loci having contributed toward the formation of HTRC PERVs in the samples tested (Fig 1). In contrast, PERV-A variable region A (VRA)–PERV-C TM hybrid sequences were not detected in the genomic DNA of miniature swine by PCR (Fig. 2), indicating that they were being generated by recombination during the in vitro coculture period. Interestingly, although PERV-A loci with long *env* open reading frames (ORFs) were identified in miniature swine DNA, their *env* TM ORFs were disrupted within a few residues of the carboxy terminus. In HTRC PERVs, this region was replaced by in-frame PERV-C sequences that repaired the TM ORF, suggesting a growth advantage of the recombinant viruses over the truncated PERV-A viruses or rescue of defective sequences during the in vitro coculture period. Further experiments are required to identify the interactions driving these recombination events. In this regard, it is noteworthy that an *env* TM truncation has also been identified in a PERV-A clone derived from PK15 cells (8). Cells infected by this virus produced only transient reverse transcriptase (RT) activities at levels approximately 1,000-fold below those infected by tissue culture-adapted PERVs (6, 8, 19) and lower than those identified with porcine cells that behave as if uninfected by PERVs (13, 18, 21, 22). While such minimal replication competence can be demonstrated using cloned viral DNA in combination with highly sensitive RT detection systems, it remains to be determined if such clones can be isolated directly from primary pig cells using coculture systems.

In summary, we report the in vitro PERV transmission characteristics of a unique herd of inbred miniature swine and demonstrate that animals can be identified that consistently do not transmit HTRC PERVs. When HTRC PERVs were isolated from transmitting miniature swine, they were shown to be recombinant viruses derived from PERV-A and PERV-C. It will be important to determine whether a transmitting phenotype is inherited in a strictly Mendelian manner or whether additional factors such as exogenous PERV transmission between animals affects the production of HTRC PERV by pigs.

Nucleotide sequence accession numbers. Representative members of full-length HTRC PERV *env* sequences (accession no. AF417227 through AF417232), HTRC PERV-A and genomic PERV locus *gag* sequences (accession no. AF417210 through AF417221), and near-full-length genomic PERV loci *env* sequences (accession no. AF417222 through AF417226) have been deposited at GenBank.

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