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Mice Transgenic for a Human Porcine Endogenous Retrovirus Receptor Are Susceptible to Productive Viral Infection†

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Porcine endogenous retrovirus (PERV) is considered one of the major risks in xenotransplantation. No valid animal model has been established to evaluate the risks associated with PERV transmission to human patients by pig tissue xenotransplantation or to study the potential pathogenesis associated with PERV infection. In previous work we isolated two genes encoding functional human PERV receptors and proved that introduction of these into mouse fibroblasts allowed the normally nonpermissive mouse cells to become productively infected (T. A. Ericsson, Y. Takeuchi, C. Templin, G. Quinn, S. F. Farhadian, J. C. Wood, B. A. Oldmixon, K. M. Suling, J. K. Ishii, Y. Kitagawa, T. Miyazawa, D. R. Salomon, R. A. Weiss, and C. Patience, Proc. Natl. Acad. Sci. USA 100:6759–6764, 2003). In the present study we created mice transgenic for human PERV-A receptor 2 (HuPAR-2). After inoculation of transgenic animals with infectious PERV supernatants, viral DNA and RNA were detected at multiple time points, indicating productive replication. This establishes the role of HuPAR-2 in PERV infection in vivo; in addition, these transgenic mice represent a new model for determining the risk of PERV transmission and potential pathogenesis. These mice also create a unique opportunity to study the immune response to PERV infection and test potential therapeutic or preventative modalities.

Xenotransplantation has been proposed as a possible solution to the serious human donor organ shortage for transplantation. While the pig is considered a good candidate, there is a potential of a public health risk due to cross-species transmission of pathogens to immunosuppressed human patients. The development of specified-pathogen-free pig herds is a critical first step but does not eliminate the risk of transmitting an endogenous retrovirus (10, 31, 56, 59). In this context, multiple copies of porcine endogenous retrovirus (PERV) have been identified in the genome of every swine species tested (39, 43), viral mRNA is expressed in many porcine tissues (12, 29), and a number of primary and established human cell lines are permissive for PERV infection (6, 28, 30, 33, 54, 55, 60, 61, 66).

PERV belongs to the *Gammaretrovirus* genus and has been divided into three classes: PERV-A, PERV-B, and PERV-C (1, 30, 57). The first two classes productively infect human cell lines in vitro (30, 35), while PERV-C does not replicate in human cells (35). Nonetheless, the presence of PERV-C genomes in pigs has been correlated with the formation of highly infectious humantropic PERV-A/C recombinants (3, 24, 39, 40, 47, 48, 61).

While in vitro infection of human cell lines by PERV is well documented and supports concern for the potential of cross-species infection, in vivo studies by us and others with immunodeficient mice failed to demonstrate productive infection (11, 14, 58). Consistent with the lack of evidence for PERV transmission to nonhuman primates in vivo (7, 34), we demonstrated that nonhuman primate cells from a number of species and tissue types do not support productive PERV infection in vitro (44). These negative results with nonhuman primate cells reflect defects in both viral entry and assembly that raise questions about the relevance of nonhuman primate models for assessing PERV risk to humans. Finally, studies of humans transplanted or treated with porcine tissues also failed to demonstrate PERV transmission (15, 25, 27, 41, 42, 53, 63). However, interpretation of these retrospective clinical studies for the actual human risk is limited.

Partly in response to the inability of nonhuman primates to support PERV replication, researchers have tried to establish small-animal models to better evaluate the risk of PERV. Most promising were a series of studies with chimeric immunodeficient mice having human and pig cell transplants that consistently demonstrated PERV transmission to the human cells (11, 36–38, 64). However, the conclusion that transmission of PERV to human cells was directly due to xenotransplantation must be reconsidered in light of new data demonstrating that xenotropic murine leukemia virus (X-MuLV) pseudotypes PERV and is detected where human cells are positive for PERV (36, 64). These findings complicate the interpretation of results from the chimeric mouse-human-pig models, making them unsuitable for evaluation of PERV in vivo.

In the process of cloning human PERV receptors (HuPAR-1 and -2) we demonstrated that mouse cells have a nonfunctional receptor ortholog (MuPAR) (16) and that introduction of the HuPAR-2 receptor cDNA into mouse NIH 3T3 cells supports PERV infection in vitro (unpublished data). Thus, while mouse cells lack a functional PERV receptor, they have the requisite cellular factors to support PERV entry, assembly,

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and release (in contrast to what we found for nonhuman primate cells [44]). We hypothesized that creation of HuPAR-transgenic mice would create a model in which it is possible to study the risk of PERV transmission and the potential of productive infection without the confounding effect of X-MuLV pseudotyping. In the present study, we describe the introduction of the HuPAR-2 receptor to immunocompetent FVB/Nj mice, characterize these transgenic mice with respect to HuPAR-2 expression, and demonstrate that these mice represent a unique small-animal model for a productive PERV infection in vivo. We also begin to characterize the humoral immune response to PERV infection.

MATERIALS AND METHODS

Animals and cell lines. The FVB/Nj strain of mice was used in all experiments. Animal manipulations were conducted in accordance with national and institutional guidelines. Cell lines were 293T/17 (ATCC CRL-11268, fetal human kidney cell line), 14/220 293T cells (referred to as 14/220; a cell line that produces a high infectious titer of a PERV-A/C recombinant virus [24]), SIRC-A2 (ATCC CCL-60, rabbit corneal fibroblasts stably transfected with HuPAR-2), NIH 3T3-A2 (ATCC CRL-1658; mouse embryonic fibroblast cell line stably transfected with HuPAR-2), and HOS (ATCC CRL-1543; human bone osteosarcoma).

Production of transgenic mice. The HuPAR-2 cDNA was subcloned into pcDNA (Invitrogen, Carlsbad, CA) under the control of a cytomegalovirus (CMV) promoter. The construct was linearized by double digestion with BglII and Tth111l to yield a 3,876-bp DNA fragment comprising the full CMV/HuPAR-2 insert and injected into FVB/Nj fertilized oocytes according to standard techniques (26).

Southern blotting, gene expression analysis, and quantification of transgene copies. Genomic DNA from the tails of two different founder animals was subjected to restriction digestion using EcoRV or EcoRI. Digests (5 μg) were separated by gel electrophoresis (0.8% agarose), transferred to polyvinylidene difluoride membranes by Southern blotting (45), and hybridized with a ^{32}P-labeled probe covering 1,058 bp of 3′ sequence.

For expression analysis total RNA was extracted by Trizol (Invitrogen) and RNAeasy columns (QIAGEN, Valencia, CA). DNA was removed by on-column DNase I digestion. Total RNA (1 μg) was reverse transcribed using Superscript and an oligo(dT)$_{18}$ primer (Invitrogen). One-tenth volume from each reaction was used for TaqMan qPCR. To prevent detection of mRNA for the mouse ortholog of the HuPAR-2 receptor, we designed two internal probes: the first was human sequence specific (GenBank accession no. AY070775) and 3′ labeled with the fluorophore 6-carboxyfluorescein (FAM); the second was mouse sequence specific (GenBank accession no. AK008081) and unlabeled. Forward (fwd) and reverse (rev) primers were designed on the human sequence but amplify mouse sequences. The standard master mix contains TaqGold, $MgCl_2$, deoxynucleoside triphosphates, and PCR Gold buffer, with a final reaction volume of 50 μl. An unlabeled mouse receptor probe was added in a 1:10 ratio to the labeled human-specific probe. Primers were as follows: fwd primer, 5′-GGATCAGTACCCTCTGTAACCAGA-3′; rev primer, 5′-GGTGGCTCCTGCAATGGCAA-3′; mouse probe, 5′-CTCTTCCTCTTCCTCCTGTGTTGG-3′; human probe, 5′ 6-FAM TCCTCCTTCTCTCTTCCTCCTCTGCTCCTGG2, 5-di-tert-butylhydroquinone-1 3′.

To quantify transgene copies present in the HuPAR-2 animals, we used an approach similar to that used for the gene expression described above. Cultured primary kidney cells from HuPAR-2 transgenics and HuPAR-2-negative littermates (10^5 cells/sample) were used to extract DNA with the DNAeasy kit (QIAGEN). Six parallel extractions of DNA from each cell type were performed. Total DNA was digested with RNase and resuspended in 125 μl of H_2O. Five microliters (corresponding to about 4,000 cells) of total DNA was used in each triplicate TaqMan quantitative-PCR (qPCR) well in a final volume of 25 μl with primers, a human-specific probe, and a murine competitor probe as described above for gene expression assays. A standard curve for copy number calculation was created using serial 1:10 dilutions of a plasmid containing the HuPAR-2 coding sequence. A plasmid containing the murine ortholog was used as a negative qPCR control.

qPCR and reverse transcription-qPCR (RT-qPCR) for PERV *pol* detection. Genomic DNA or total RNA was extracted after tissue homogenization from 100 mg of each tissue (QIAGEN DNAeasy kit or Trizol). Genomic DNA was treated with RNase, and, after spectrophotometric quantification, 2 μg of DNA was used in TaqMan qPCR for detection of PERV *pol* (16). Total RNA was purified on QIAGEN RNAeasy columns, and 1 μg was reverse transcribed. The resulting cDNA was used for each qPCR with the same conditions used for genomic DNA. To check for genomic DNA contamination, controls included 1 μg of total RNA that was not reverse transcribed. All samples were normalized for input RNA using ABI primers/probe for 18S rRNA (Applied Biosystems, Foster City, CA).

Detection of PERV RNA in sera by nested RT-PCR. PERV RNA was extracted from 140 μl of serum using a QIAGEN viral RNA extraction kit. Total extracted RNA was reverse transcribed using Superscript (Invitrogen) and random hexamers. As a control, reactions without reverse transcriptase were performed. One-tenth of each reverse transcription reaction mixture was added to a PCR mixture containing 100 ng of each primer (primer sequences: fwd, 5′-AGCTCCGGGAGGCCTACTC-3′; rev, 5′-TGACAGCTTTGCTTATTTCGTAC-3′). The cycling parameters were 4 min at 95°C; 35 cycles of 95°C for 30 s, 58°C for 45 s, and 72°C for 1 min; and 10 min at 72°C (final elongation step). Nested PCR was performed with 2.5 μl (1:20 volume) of the first PCR mixture amplified for 30 cycles in 50 μl containing 100 ng of the following primers: fwd, 5′-TCCCATCATTAGAAGTTACGCAGC-3′; rev, 5′-CTTTTGACCACACCAACGGC-3′. The nested PCR product (704 bp) was detected by ethidium bromide staining. Serum samples spiked with 1 μg of total RNA from 14/220 cells and cell-free culture supernatant from 14/220 cells were used as positive controls.

Determination of $TCID_{50}$. 293T or NIH 3T3-A2 cells (10^4 cells/well) were seeded in 24-well plates. Cells were infected overnight at 37°C with 1:10 serial dilutions of different supernatants (from undiluted to 10^{-6} dilution; six wells/dilution). Genomic DNA was extracted after 72 h and analyzed by PCR for PERV-A/C *env* using the following: fwd primer, 5′-ATGTCTGCCTTCGATCAGTA-3′; rev primer, 5′-CTCAAACCACCCTTGAGTAG-3′. The 50% tissue culture infective dose ($TCID_{50}$)/ml titer was determined using the Reed and Muench formula (4) from the ratio of positive/negative wells for each dilution.

Detection of anti-PERV antibodies. Western blotting was performed as previously described (53). Briefly, 1 μg of recombinant PERV-Gag or Envelope (Env) protein was electrophoresed through a denaturing 4 to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel (Invitrogen) and transferred to polyvinylidene difluoride membranes. Mouse sera were diluted 1:50, incubated for 3 h at room temperature with the membranes followed by incubation with a 1:10,000 dilution of horseradish peroxidase–anti-mouse immunoglobulin G (Jackson Immunoresearch, West Grove, PA) for 1.5 h at room temperature, and developed using the SuperSignal West Pico kit (Pierce, Rockford, IL). For Gag controls, PERV-specific monoclonal antibodies (MAb 21 and Mab39; kind gifts from G. Byrne) (63) were used. The positive control for Env assays was an anti-PERV p15E goat polyclonal antibody (kind gift from J. Denner) (17). For detection of anti-PERV antibodies by flow cytometry, SIRC-A2 cells were exposed to PERV-containing cell culture supernatants. Naive SIRC-A2 cells and PERV-infected SIRC-A2 cells (SIRC-A2/PERV) (1×10^6) were incubated for 1 h at 4°C with 1:8 dilutions of sera. Next, washed cells were incubated with fluorescein isothiocyanate-conjugated anti-mouse antibody for 1 h at 4°C and analyzed by flow cytometry (FACScan; Becton Dickinson, San Jose, CA).

Serum infectivity assay. Human 293T cells (10^5 cells/well) were seeded in a 12-well plate 24 h before exposure to virus and then cultured in medium containing 1:5 or 1:10 dilutions of serum adjusted to a final concentration of 5 μg/ml Polybrene. Media were changed after 4 h. At 1 week qPCR detection of PERV *pol* DNA or RNA was carried out as described above.

Synthesis and purification of recombinant proteins. The coding sequences for PERV Env (GenBank accession no. AAT77168) and PERV Gag (GenBank accession no. AAT77166) were amplified using PCR of a molecular clone of 14/220 (2A; kind gift from Y. Takeuchi) (24) and cloned into the T7 IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible pET vector in frame with a C-terminal six-His tag (Stratagene, La Jolla, CA). *Escherichia coli* cells carrying extra copies of T7 RNA polymerase [BL21(DE3)pLys] were transformed with each plasmid, grown to an optical density at 600 nm of 0.6, and induced with 1 mM (final concentration) IPTG. After induction, bacteria were grown for 3 h and harvested. Gag protein was extracted using nondenaturing conditions and purified by His affinity on Talon beads (BD Bioscience, Rockville, MD). Due to insolubility, Env was extracted in 8 M urea.

Western blots and confocal microscopy for Gag proteins in tissues. Liver, brain, and kidney from transgenic animals injected with PERV were harvested at 8 weeks postinjection along with those from control transgenic animals never exposed to PERV. Total protein was extracted by Trizol (Invitrogen) according to the manufacturer's instructions, resuspended in 8 M urea, and quantified by spectrophotometry. Fifteen micrograms of total protein was loaded for each lane, and PERV Gag was detected using monoclonal anti-Gag antibody MAb 39 from G. Byrne (63), with the same conditions described above for other Western

blots. Positive (14/220 cells at 5 μg total protein/lane) and negative (293T cells at 15 μg total protein/lane) controls were loaded in each gel. An antiactin antibody (A2066; Sigma) at 1:200 dilution was used as a control for protein loading.

For confocal microscopy, kidney was fixed in 4% paraformaldehyde (Sigma) for 5 h at room temperature and then equilibrated overnight at 4°C in 10% sucrose. Tissue sections (10 μm) were permeabilized in 10% Triton X-100 for 10 min at room temperature. Gag protein was detected using MAb 39 diluted 1:200 in blocking buffer (phosphate-buffered saline, 1% donkey serum, 0.2% bovine serum albumin) for 1 h at room temperature, followed by incubation with a secondary donkey FITC-conjugated anti-mouse antibody (diluted 1:200 in blocking buffer; 1 h at room temperature). Nuclei were stained using 1:1,000-diluted TO-PRO-3 (Invitrogen) in blocking buffer for 20 min at room temperature. Finally membranes were visualized using BODIPY-phalloidin (Invitrogen) diluted 1:500 in blocking buffer for 10 min at room temperature. Serial *z* sections were acquired using a Bio-Rad (Zeiss) MRC1024 laser scanning confocal microscope and were then reassembled into a three-dimensional image and analyzed with Imaris-Autoquant software (Bitplane Inc., Saint Paul, MN).

RESULTS

Construction and characterization of mice transgenic for HuPAR-2 receptor. To characterize the risk of PERV infection associated with xenotransplantation in a more physiological context, we developed a HuPAR-2 transgenic mouse. While HuPAR-1 is also fully functional in vitro, we chose HuPAR-2 for these efforts based on infection data with the rabbit cell line SIRC suggesting that HuPAR-2 might be incrementally more efficient (data not shown). As HuPAR-2 is widely expressed in human tissues (16), we chose the ubiquitous CMV promoter to drive the transgene. Founders were generated by microinjection of oocytes from FVB/Nj mice, chosen because no replication-competent ecotropic proviruses have been detected in this strain (18, 19, 51, 52; J. Coffin, personal communication). PCR analysis of tail DNA using CMV–HuPAR-2-specific primers identified five transgenic founders. Three founders were backcrossed with wild-type FVB/Nj mice: male no. 12 was used for breeding of subsequent generations due to breeding vigor. F_2 mice derived from a cross of male no. 12 with FVB/Nj females were analyzed using EcoRI- or EcoRV-digested genomic DNA, transferred to membranes by Southern blotting, and then hybridized to a HuPAR-2-specific probe (Fig. 1). Southern analysis indicated the presence in our transgenic animals of CMV–HuPAR-2 constructs. Subsequent PCR analysis confirmed that about 50% of the F_2 progeny are transgenic for HuPAR-2, and this was consistently detected up to the current F_7 generation. The animals have been carried as heterozygotes because several attempts to make homozygotes resulted in small litter sizes.

Since by using Southern blots we were unable to determine the copy number of the CMV–HuPAR-2 transgene in the FVB/Nj transgenic animals, we used qPCR. DNA was extracted from 10^5 293T cells, HOS cells, NIH 3T3 cells, and primary kidney cells from both HuPAR-2 transgenics and HuPAR-2-negative littermates, and 5 μl of the final volume (about 4,000 cells) was used in TaqMan qPCR. For determination of copy number a standard curve was created using 1:10 dilutions of a HuPAR-2 plasmid. As for gene expression assays (described below), we used a competitive unlabeled mouse PAR-2-specific probe to eliminate background due to the endogenous mouse receptor (MuPAR-2). Using this technique we calculated that our transgenics contain 9.71 ± 0.8 copies of the HuPAR-2 transgene. As a comparison, the diploid HOS and partially tetraploid 293T cells contain 2.07 ± 0.34 and 3.07 ± 1.24 copies, respectively. Mouse NIH 3T3 cells as well as primary kidney cells from HuPAR-2-negative littermates were negative. Moreover no qPCR signal was detected using a MuPAR-2 plasmid at a concentration of 4×10^5 copies/μl.

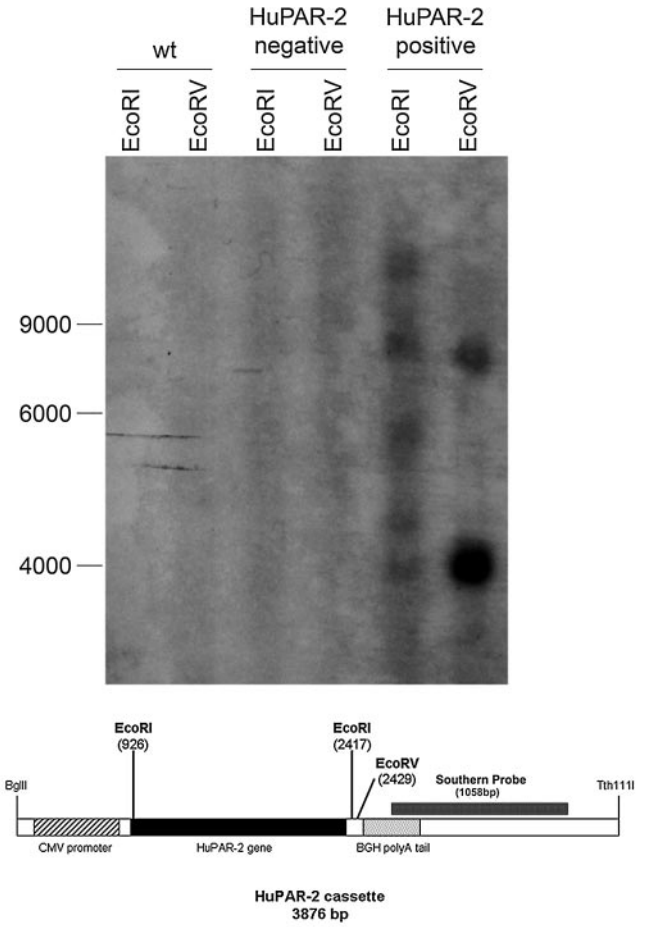

FIG. 1. Southern blot analysis of tail DNA from F_2 animals. Five micrograms of genomic DNA was digested with EcoRI or EcoRV, separated by agarose gel electrophoresis, and analyzed by Southern blot hybridization with a probe specific for sequences in the transgenic construct. Shown are wild-type FVB/Nj animals (wt), a HuPAR-2 transgene-negative F_2 littermate, and a HuPAR-2 transgene-positive F_2 animal. EcoRI cuts twice within the transgene, and EcoRV cuts once; the relationship to the probe is shown below the image. The experiment was repeated twice on different littermates with similar results.

To investigate the expression of HuPAR-2 in different mouse tissues, we designed a quantitative RT-PCR assay using Taqman technology. A competitive but unlabeled mouse PAR-2-specific probe was used in combination with a fluorochrome-labeled human-specific PAR-2 probe. This design was necessary to eliminate background due to expression of the endogenous mouse receptor (MuPAR-2) transcripts (81% sequence homology with HuPAR-2). The results were normalized relative to HuPAR-2 transcript expression in the highly PERV-permissive human 293T cells. All six mouse tissues analyzed in nine different transgene-positive F_2 animals strongly expressed HuPAR-2 (Fig. 2). Transgene-negative FVB/Nj F_2 progeny ($n = 9$), as well as wild-type FVB/Nj mice ($n = 2$), were negative for HuPAR-2 expression in all tissues analyzed

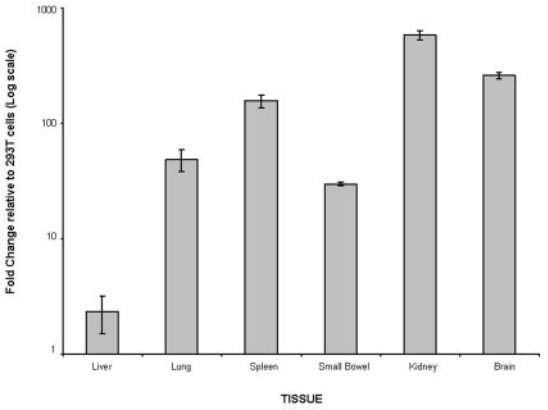

FIG. 2. Expression analysis by RT-qPCR of HuPAR-2 in transgenic mice. Values are represented as the changes in expression levels of the transgene by tissue relative to the expression of native HuPAR-2 by the highly permissive human 293T cells. Data are the averages of expression levels obtained in six tissues of nine different animals.

under conditions where the levels of the competitor for murine PAR sequences reduced the background to essentially zero (data not shown). Kidney showed the highest level of expression, about 600-fold over 293T, followed by brain and spleen (259-fold and 156-fold, respectively). Liver has considerably lower levels of expression relative to the other tissues, but still 1.5- to 2-fold greater than 293T.

Analysis of cells derived from HuPAR-2 transgenic mice for susceptibility to in vitro infection by PERV. To determine the susceptibilities of different cells from transgenic mice to PERV infection, cells from kidney and subcutaneous tissue of transgene-positive and -negative animals ($n = 3$ for each group) were explanted, collagenase digested, and cultivated in vitro for 5 days. After this, cells were trypsinized and reseeded at 50% confluence and infected with 14/220 supernatants ($TCID_{50} = 4.9 \times 10^4$/ml) in the presence of Polybrene. Infection was monitored by qPCR and RT-qPCR for PERV *pol* in both genomic DNA and total cellular RNA to distinguish between productive and nonproductive infection. Detectable levels of PERV *pol* DNA and RNA were found in kidney cells and subcutaneous fibroblasts (Fig. 3A and B) and increased between 1 and 2 weeks after infection. Note that by qPCR we consistently find low levels of PERV *pol* in murine cell lines in vitro (unpublished data), consistent with our previous study demonstrating low levels of nonproductive infection in vivo (58).

We tested the potential of infecting mitogen-activated splenocytes. Splenocytes obtained from HuPAR-2-positive and -negative F_2 animals were activated by phytohemagglutinin, and after 48 h they were exposed to 14/220 supernatants in the presence of Polybrene and maintained in culture with recombinant interleukin-2 (1 ng/ml). PERV sequences were detected in both genomic DNA and total RNA at all time points tested from activated HuPAR-2-positive splenocytes but not in any controls (Fig. 3C). To determine whether the splenocytes were productively infected, we passaged the supernatant from PERV PCR-positive splenocyte cultures onto 293T and NIH 3T3-A2 cells. By performing limiting dilutions, we determined that the titers of infectious PERV produced from the PCR-positive splenocytes were $(4.5 \pm 0.3) \times 10^3$ $TCID_{50}$/ml for 293T and $(0.6 \pm 0.1) \times 10^3$ $TCID_{50}$/ml for NIH 3T3-A2 ($P = 0.025$). As controls, the titers of 14/220 supernatants were determined to be $(4.9 \pm 1.1) \times 10^4$ $TCID_{50}$/ml on 293T cells ($n = 5$) and $(7 \pm 0.7) \times 10^3$ $TCID_{50}$/ml ($P = 0.004$) on NIH 3T3-A2 cells ($n = 5$). In sum, primary cells from the transgenic

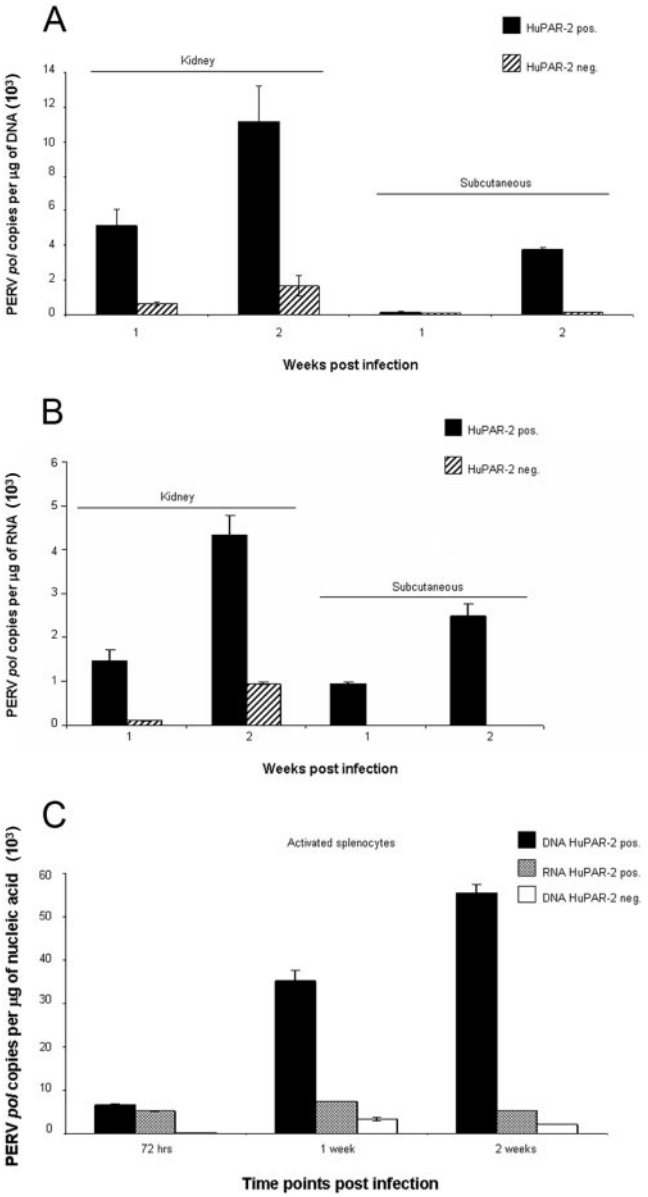

FIG. 3. PERV infection analysis of three primary cell lines from HuPAR-2 transgenic animals in vitro. PERV infection of kidney cells and subcutaneous fibroblasts was monitored as PERV *pol* copies per µg of genomic DNA (A) or total RNA (B) in HuPAR-2 transgene-positive animals (black bars) and transgene-negative animals (striped bars). Data were collected at two time points for each cell type, 1 and 2 weeks postinfection, to monitor productive infection. (C) PERV infection was monitored in mitogen-activated splenocytes 72 h after activation with phytohemagglutinin and then up to 2 weeks in culture with recombinant IL-2. PERV *pol* copies per µg of genomic DNA (black bars) and total RNA (gray bars) are shown for HuPAR-2 transgene-positive and -negative animals (white bars). The negative RNA data for the transgene-negative animals are not shown. Data represent the averages of four experiments.

TABLE 1. Analysis of PERV *pol* copy numbers in genomic DNA and total RNA from mice after PERV inoculation

Animal	Copy no. in:												Wk
	DNA[a] from:						RNA[b] from:						
	Liver	Lung	Spleen	Small bowel	Kidney	Brain	Liver	Lung	Spleen	Small bowel	Kidney	Brain	
1(−)[c]	Neg[d]	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	4
655	255	344	Neg	Neg	37	1,670	64	48	94	55	774	Neg	4
656	Neg	152	Neg	Neg	86	74	26	52	14	127	45	207	4
676	Neg	365	Neg	Neg	22	318	Neg	Neg	Neg	Neg	185	103	4
679	23	421	Neg	Neg	56	1,700	11	161	18	358	222	181	4
532	Neg	Neg	Neg	Neg	28	280	225	3,480	257	Neg	3,980	3,800	4
533	Neg	Neg	Neg	Neg	94	1,390	264	206	Neg	54	592	450	4
536	Neg	Neg	Neg	Neg	16	246	254	Neg	98	129	663	111	4
2(−)	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	8
658	175	304	Neg	656	2,480	520	1,200	840	70	Neg	708	163	8
671	96	351	Neg	12	2,800	986	1,380	132	45	22	611	955	8
672	115	818	Neg	21	1,460	1,420	1,410	246	24	51	Neg	942	8
675	138	436	Neg	13	987	1,320	5,410	331	21	Neg	645	814	8
526	176	359	Neg	94	2,980	959	1,760	505	327	110	902	590	8
527	56	1,330	Neg	28	286	1,800	1,490	263	65	107	300	321	8
535	23	32	Neg	26	189	959	2,200	473	317	96	415	710	8
3(−)	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	12
667	1,969	Neg	Neg	32,000	4,470	1,340	127	111	21	222	73	459	12
668	17,789	37	Neg	9,550	1,600	2,550	Neg	Neg	22	Neg	Neg	20	12
686	14,589	16	Neg	5,490	1,420	3,580	Neg	Neg	19	71	Neg	24	12
687	2,309	Neg	Neg	7,810	1,160	1,500	114	Neg	16	65	Neg	Neg	12
525	42,600	Neg	Neg	19,600	5,730	42,700	Neg	Neg	Neg	Neg	Neg	Neg	12
530	16,000	18	Neg	44,000	600	7,360	Neg	54	Neg	Neg	Neg	17	12
531	52,100	32	Neg	2,350	3,350	4,240	Neg	38	Neg	Neg	Neg	24	12

[a] Data are expressed as copy numbers per 2 μg of genomic DNA (18S rRNA sequences used as control); background from nontransgenic littermates has been subtracted.
[b] Data are expressed as copy numbers per 1 μg of total RNA (18S rRNA sequences used as control); background from nontransgenic littermates has been subtracted.
[c] (−) indicates HuPAR-2-negative animals injected in the same way as positive ones.
[d] Neg, any result <10 copies in 2 μg of DNA or <10 copies in 1 μg of total RNA after background subtraction.

animals are capable of supporting productive replication of PERV.

Analysis of in vivo infection of HuPAR-2 transgenic mice. The susceptibility of HuPAR-2 transgenic mice to PERV infection was evaluated by inoculating them at between 8 and 10 weeks of age with cell-free supernatants from 14/220 cells. Alternating intraperitoneal or intravenous injections were done at 3-day intervals for a total of three or four injections in each of 21 mice so that the total exposure to virus was about 3×10^5 to 5×10^5 infectious units/animal by $TCID_{50}$. Animals were sacrificed at different time points after the first injection, and both genomic DNA and total RNA from multiple tissues were analyzed for the presence and copy numbers of PERV *pol* nucleic acid sequences by TaqMan qPCR and RT-qPCR (Table 1).

Genomic PERV *pol* sequences became detectable by qPCR as early as 4 weeks in all the HuPAR-2 transgenic mice tested ($n = 7$). All transgenic mice had at least one tissue with >20 copies PERV DNA, and by 8 weeks most mice had up to four or five tissues with >1,000 copies of PERV DNA per 2 μg genomic DNA (equivalent to approximately 3×10^5 cells). Moreover, PERV *pol* copies in genomic DNA increased as a function of time from 4 to 12 weeks. In total, all 21 HuPAR-2 transgenic animals exposed to PERV were positive for PERV *pol* sequences in genomic DNA. Likewise, 20/21 HuPAR-2 transgenic mice were positive for PERV *pol* gene expression by RT-qPCR analysis. PERV *pol* expression was detected in multiple tissues as early as 4 weeks, peaked at 8 weeks, and then decreased at 12 weeks. Control HuPAR-2-negative F_2 littermates injected with the same amount of virus were negative for both PERV *pol* DNA and RNA at all time points tested and all tissues examined. The sensitivity of this assay was defined experimentally as 10 copies of PERV *pol* in a background of 3.3 μg DNA (approximately 5×10^5 cells) (58). To validate the negative results, PERV *pol*-negative tissues were also analyzed for DNA quality by PCR using primers for MuPAR-2 and 18S rRNA sequences.

To confirm the PCR evidence of PERV infection, we determined the expression of PERV Gag proteins in putatively infected tissues using two approaches, Western immunoblots and confocal microscopy. First, we harvested liver, kidney, and brain tissue from HuPAR-2 transgenics 8 weeks after the injection of PERV. HuPAR-2-negative littermates that were injected with PERV in parallel served as the receptor-negative controls. HuPAR-2 transgenics that were never injected with PERV served as the PERV-negative controls. Finally, 293T and 14/220 cells served as the negative and positive controls for the Gag Western blots. As shown in Fig. 4, the results clearly demonstrate positive expression of Gag protein in all the HuPAR-2 transgenic tissues tested, and these were confirmed by DNA/RNA PCR to be PERV positive (data not shown). Interestingly, a very weak positive Gag signal is consistently found in the kidney and brain tissues of the HuPAR-2-negative littermates exposed to PERV; this signal is not seen in the

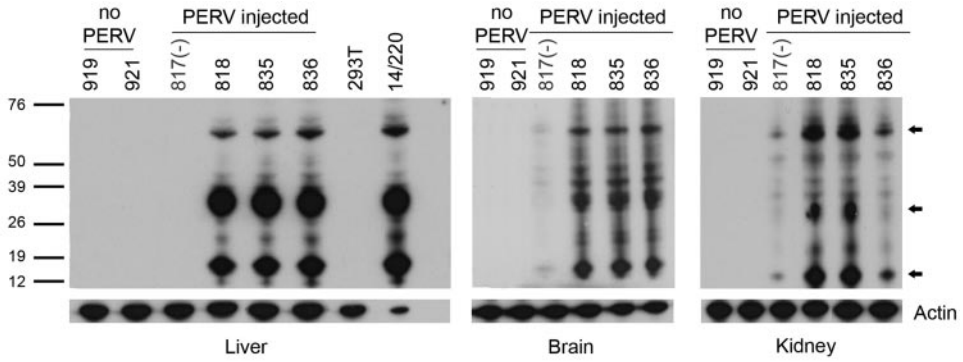

FIG. 4. Detection of PERV Gag protein in tissue samples from PERV-infected animals. Western blots were done on liver, brain, and kidney tissues harvested from three HuPAR-2 transgenic animals 8 weeks after injection with PERV (mice 818, 835, and 836) and one HuPAR-2-negative littermate as a receptor-negative control [817(−)]. Two HuPAR-2 transgenic animals (919 and 921) were never exposed to PERV and serve here as PERV-negative controls. Negative and positive controls for detection of PERV Gag were human 293T cells and human 14/220 cells. The latter were done for all gels with similar results but only shown for the liver samples to save space. On the left molecular mass in kilodaltons is indicated, and on the right the arrows indicate the three different forms of PERV Gag detected by MAb 39 antibody. The results were confirmed in a second experiment of the same design (not shown).

HuPAR-2-positive animals not exposed to PERV. These results correlate with the in vitro PCR data for both DNA and RNA in receptor-negative kidney cells shown in Fig. 3 and are also consistent with our original publication demonstrating the nonproductive infection of mouse cells in immunodeficient mice after pig islet xenotransplants (58). Second, we examined the same tissues using confocal microscopy and staining for PERV Gag protein using the same antibody used in the Western blots, MAb 39. For example, staining for Gag is clearly identified in renal tubular epithelial cells (Fig. 5A), hepatocytes, and endothelial cells (Fig. 5C).

We tested the possibility of PERV transmission from HuPAR-2 transgenic cells that were infected in vivo. Primary kidney cells were isolated from three different PERV-infected animals. Culture supernatants were then titered on human 293T cells or stably transfected murine NIH 3T3-A2 cells. The resulting infectious titers were $(1.2 \pm 0.9) \times 10^3$ $TCID_{50}$/ml with 293T and $(1.5 \pm 0.5) \times 10^2$ $TCID_{50}$/ml with NIH 3T3-A2.

To confirm productive infection in vivo, we tested mouse sera for evidence of viremia by (i) RT-PCR analysis for PERV RNA, (ii) exposure of 293T cells to mouse sera to detect infectious virus, and (iii) flow cytometry and Western blot analysis to detect the presence of PERV-specific antibodies. RNA was extracted from serum samples, reverse transcribed to cDNA, and analyzed by nested PCR for the presence of PERV genomic RNA by using primers that amplified sequences spanning from *gag* to *env*. PERV RNA was detected in the serum from 12 of 21 HuPAR-2-positive animals (Table 2). All the PERV-exposed negative littermates and a wild-type FVB/Nj control were negative for PERV RNA.

To prove that PERV RNA present in the serum at 4, 8, or 12 weeks is associated with infectious virus, 293T cells were cultured in 1:5 dilutions of mouse serum for 1 week and then genomic DNA was isolated and tested by qPCR. Of the 12 transgenic animals with evidence of PERV RNA in their serum, 10 transmitted PERV to human cells in vitro, indicating the RNA detected correlates with infectious virus (Fig. 6).

As further evidence of productive infection, we tested whether infected transgenic mice developed an immune response to PERV. Sera from infected mice were used to stain SIRC-A2 rabbit cells infected with virus from 14/220 cells, and these cells were compared to uninfected SIRC-A2 cells as negative controls. The cell staining data in Table 2 were analyzed by measuring the change in mean fluorescence (Δ mean fluorescence). We averaged the Δ mean fluorescence from the HuPAR-2 transgenics at each time point and compared it to the average of the matching HuPAR-2 transgenic negative littermates (measured in triplicate). Resulting Δ mean fluorescence values were as follows: HuPAR-2 transgenics at 4 weeks, 22.79 versus 3.21 for the negative littermate ($P = 0.008$); HuPAR-2 transgenics at 8 weeks, 28.68 versus 0.105 for the negative littermate ($P = 0.01$); HuPAR-2 transgenics at 12 weeks, 25.33 versus −0.24 for the negative littermate ($P = 0.04$). Thus, by this analysis only HuPAR-2 transgenic animals develop an antibody-mediated immune response to PERV, as evidenced by significantly higher staining of PERV-infected SIRC-A2 cells.

Immune response to PERV in HuPAR-2 transgenic mice. We cloned full-length PERV Gag and Env proteins with His tags and tested the purified proteins in Western blots using 1:50 dilutions of mouse sera. All PERV-infected transgenic animals show a band at the expected molecular mass (59 to 60 kDa) for the full-length recombinant Gag-His protein (Fig. 7A). Faint bands were also observed for PERV-injected receptor-negative littermates at all time points. However, transgenic animals consistently demonstrated an increase in band densities as a function of time, confirmed by densitometry to be about 3-fold (standard deviation, ±0.5-fold) brighter at 12 weeks than at 4 weeks ($P = 0.042$). In contrast, PERV Env antibodies were detected only in two transgenic animals, both at 12 weeks (Fig. 7B, mice 686 and 525).

To determine whether any of the detected PERV-specific antisera had neutralizing activity, we incubated four antibody-positive sera with serial dilutions of 14/220 supernatant (from 1:10 to 1:10,000) and then tested these for infection on fresh 293T cells (Fig. 8). Neither preimmune serum nor serum reactive to PERV Gag that was heat denatured at 95°C for 15 min to destroy antibody structure (Fig. 7) inhibited PERV

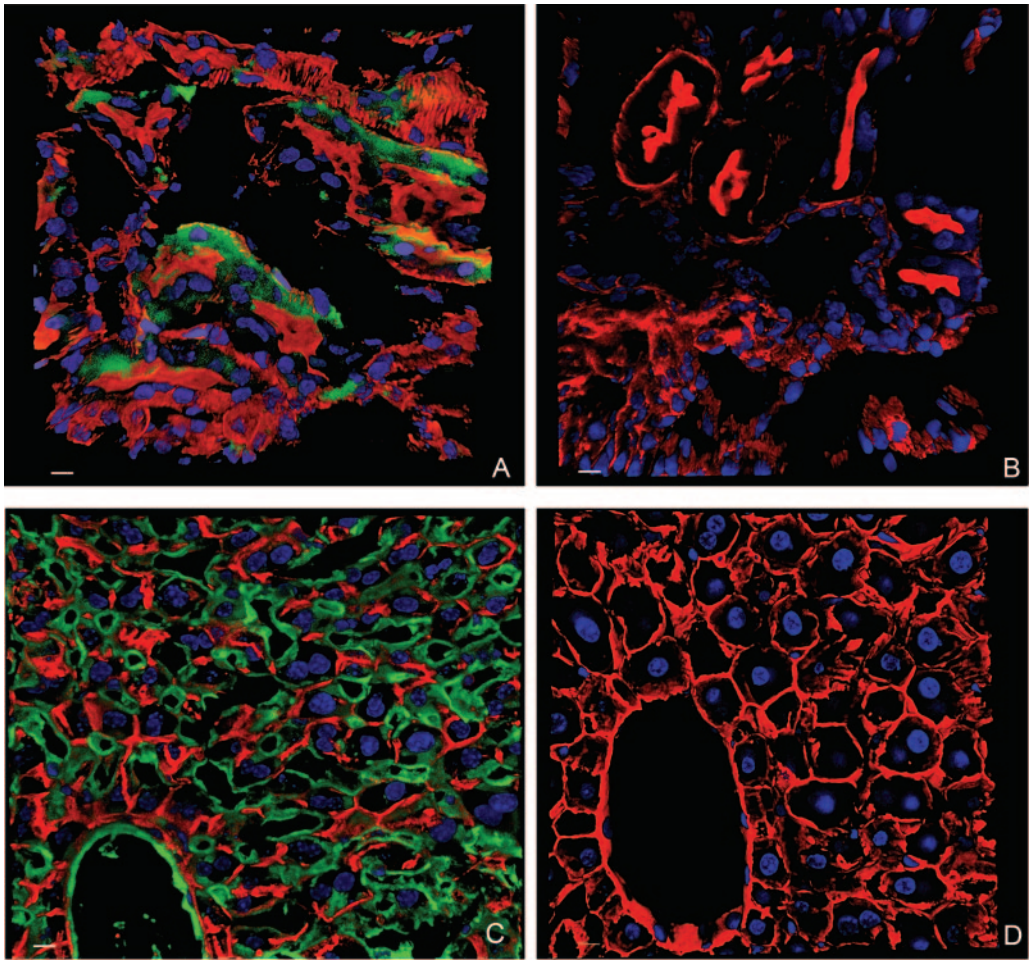

FIG. 5. Detection of PERV Gag protein in kidney and liver samples from PERV-infected animals by confocal microscopy. PERV Gag is visualized in green using MAb 39. Cellular nuclei and membranes are stained, respectively, in blue (TO-PRO-3) and red (BODIPY-phalloidin). (A) Kidney from HuPAR-2 transgenic animal 8 weeks after PERV injection; (B) kidney from HuPAR-2 transgenic animal never exposed to PERV; (C) liver from HuPAR-2 transgenic animal 8 weeks after PERV injection; (D) liver from HuPAR-2 transgenic animal never exposed to PERV. Bars, 10 μm for all four images.

infection. In contrast, nondenatured sera from four animals shown to be reactive to PERV Gag were all able to reduce the infectivity of PERV in this assay (Fig. 8). These results demonstrate that at least some animals with anti-PERV Gag antibodies also have PERV-specific neutralizing antibodies.

Analysis of neonatal HuPAR-2 transgenic mice for susceptibility to PERV. We tested the potential of viral infection in neonatal transgenic animals where early exposure to PERV should generate a form of functional immune tolerance (5, 22, 46). Newborns from transgenic mothers crossed to wild-type FVB/Nj mice were injected intraperitoneally with 50 μl of 14/220 supernatant within 1 day after birth. At 8 weeks the animals were classified as HuPAR-2 positive or -negative by tail genotyping.

Exposure to PERV resulted in a decreased rate of weight gain compared to nontransgenic littermates. Twenty-four weeks after PERV exposure the average weight for HuPAR-2-positive animals was 27.75 ± 4.4 g, while that for their negative littermates was significantly higher, 34.95 ± 4.2 g ($P = 0.02$). The average weight for age-matched animals not injected with PERV was 34.54 ± 4.8 g for HuPAR-2-positive mice and 35.31 ± 3.9 g for negative littermates ($P = 0.61$; $n = 8$ for each group). Thus, the 21% lower weight of the transgenic animals appears to be due to the neonatal exposure to PERV, not the transgene itself.

We sacrificed animals at 9, 24, 28, and 32 weeks, extracted genomic DNA and total RNA, and tested for the presence of PERV *pol* nucleotide sequences by qPCR and RT-qPCR (Table 3). Animal 771, sacrificed at 9 weeks after injection due to very poor weight gain, was positive for PERV *pol* DNA in all the analyzed tissues at levels similar to or higher than those of adult animals at 8 weeks postinjection. Animals at 24 and 28 weeks showed evidence of PERV DNA in multiple tissues. However, of the tissues examined, only liver and brain were positive for PERV *pol* RNA, but not consistently in all animals, and only at very low levels (Table 3).

To test the original hypothesis that neonatal exposure to PERV prevents an immune response, we tested serum samples for reactivity to PERV-infected SIRC-A2 cells by flow cytometry and for reactivity to Env and Gag recombinant proteins in Western blots. None of the sera showed specific reactivity to

TABLE 2. Analysis of sera from PERV-infected mice by flow cytometry and RT-PCR

Animal	Mean fluorescence		Δ mean fluorescence	SD	PERV RNA RT-PCR result[c]	Wk
	SIRC-A2[a]	SIRC-A2/PERV[b]				
CTR[d]	6.62	5.93	0.32	1.16	−	0
1(−)[e]	21.40	24.61	3.21	0.22	−	4
655	86.25	123.63	37.38	23.91	−	
656	53.17	82.32	29.15	15.07	+	
676	33.34	48.6	15.26	14.27	+	
679	41.72	58.67	16.95	8.63	+	
533	17.3	22.68	5.38	4.37	−	
532	16.69	47.69	31	1.15	+	
536	33.49	57.89	24.4	1.41	+	
2(−)	9.46	9.57	0.105	1.29	−	8
658	43.33	89.41	46.08	29.45	−	
671	23.54	52.22	28.68	0.23	+	
672	14.78	20.21	5.43	8.68	+	
675	11.93	25.76	13.83	4.19	−	
527	105.16	163.42	58.26	2.7	−	
535	34.82	60.46	25.64	11.2	+	
526	35.27	58.1	22.83	5.51	+	
3(−)	5.96	5.72	−0.24	0.52	−	12
667	29.92	73.03	43.11	20.3	+	
668	34.73	53.25	18.52	13.06	−	
686	35.28	36.51	1.23	10.05	−	
687	28.68	35.57	6.89	4.45	−	
525	68.82	134.69	65.87	49.28	−	
530	55.42	70.92	15.5	51.39	+	
531	67.92	94.09	26.17	9.27	+	

[a] SIRC-A2 cells are rabbit cells stably transfected with HuPAR-2 receptor.
[b] SIRC-A2/PERV cells are SIRC-A2 cells infected with cell-free supernatants from 14/220 cells.
[c] Nested RT-PCR was performed on reverse-transcribed PERV genomes after isolation from serum with a QIAGEN blood kit. + or −, positive or negative signal, respectively, by PCR amplicon on agarose gel stained by ethidium bromide.
[d] CTR indicates a control serum from different FVB/Nj transgenic and wild-type animals.
[e] (−) indicates negative transgenic animals injected in the same way as positive ones.

SIRC-A2/PERV-infected cells (Table 4) or purified PERV proteins (data not shown).

To check for evidence of viremia, we tested fresh sera for PERV RNA by nested RT-PCR and for infectivity on 293T cells. With the exception of serum from animal 771, sacrificed 9 weeks after PERV exposure, no viral RNA or infectious virus was detected in sera tested from mice sacrificed at 24 weeks or later. Interestingly, only serum for mouse 771 was positive for both PERV RNA and release of infectious virus (data not shown).

DISCUSSION

We introduced one of the two human receptors for PERV (HuPAR-2) under the control of a CMV promoter into the genomes of fully immunocompetent FVB/Nj mice. The CMV promoter was chosen for two reasons: (i) the strong promoter activity in mammalian cells provides high levels of transgene expression and (ii) previous studies indicate that mRNA for HuPAR-2 is expressed in almost all human tissues (16). Analysis of receptor expression by RT-qPCR on the tissues from transgenic mice indicated that HuPAR-2 was highly expressed (Fig. 2). Transgenic tissues express between 2 times (liver) and 600 times (kidney and brain) the amount of HuPAR-2 present in the highly permissive human 293T cell line.

First, we demonstrated the functionality of HuPAR-2 expressed by primary cells of the transgenic mice as a PERV receptor in vitro. Primary cells derived from transgenic mice were exposed to PERV from 14/220 cells, a naturally occurring recombinant between PERV-A and PERV-C, with the tropism and receptor specificity of PERV-A. Detection of both PERV *pol* DNA and RNA demonstrated that the expression of HuPAR-2 allowed virus infection (Fig. 3A to C). Moreover, the ability to pass virus from infected transgenic mouse cells to 293T or NIH 3T3-A2 cells indicated production of infectious virions, although infection appears to be less efficient in murine cells than human cells. Similar levels of PERV *pol* DNA detected in the transgenic primary cells were reached only after 2 to 3 weeks compared to approximately 1 week in human 293T cells. As another point of reference, RNA/DNA ratios for infected transgenic kidney cells 2 weeks after infection in vitro are approximately 0.4 while the ratios for the human cells, HOS and the adenovirus-transformed 293T, at 2 weeks are approximately 40 and 420, respectively. While the data here and in our original paper identifying the receptors (16) support the basic premise that the lack of a functional PERV receptor is the main block to infection in mice, these results suggest that other cellular factors establishing high-titer productive infections may be necessary. In support of this conclusion are data for nonreceptor mechanisms limiting human immunodeficiency virus type 1 (HIV-1) infection in mouse models (8, 32). We have also shown that nonhuman primate cells have postentry blocks for PERV infection (44), and others have described postentry blocks for HIV (9, 23, 65).

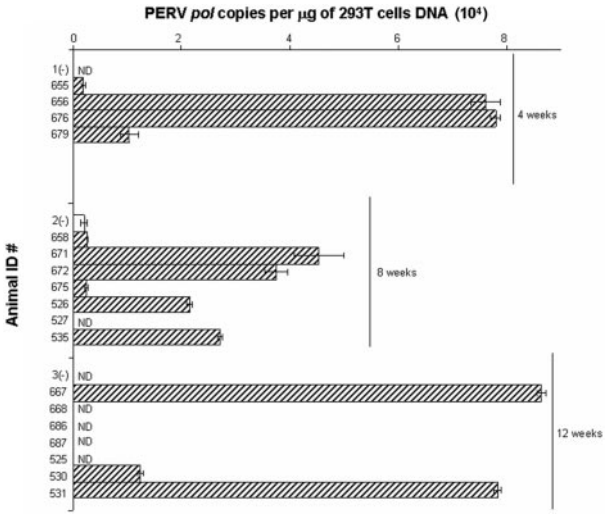

FIG. 6. Analysis of sera from PERV-infected animals tested for infection on 293T cells. Sera from three groups of infected animals (4, 8, and 12 weeks after exposure to PERV) were diluted 1:5 by volume and used to infect triplicate cultures of 293T cells in vitro. 293T cells were harvested 1 week later, and results are expressed as PERV *pol* copies per μg of genomic DNA. Standard deviations are shown. ND, infection not detected in 293T cells.

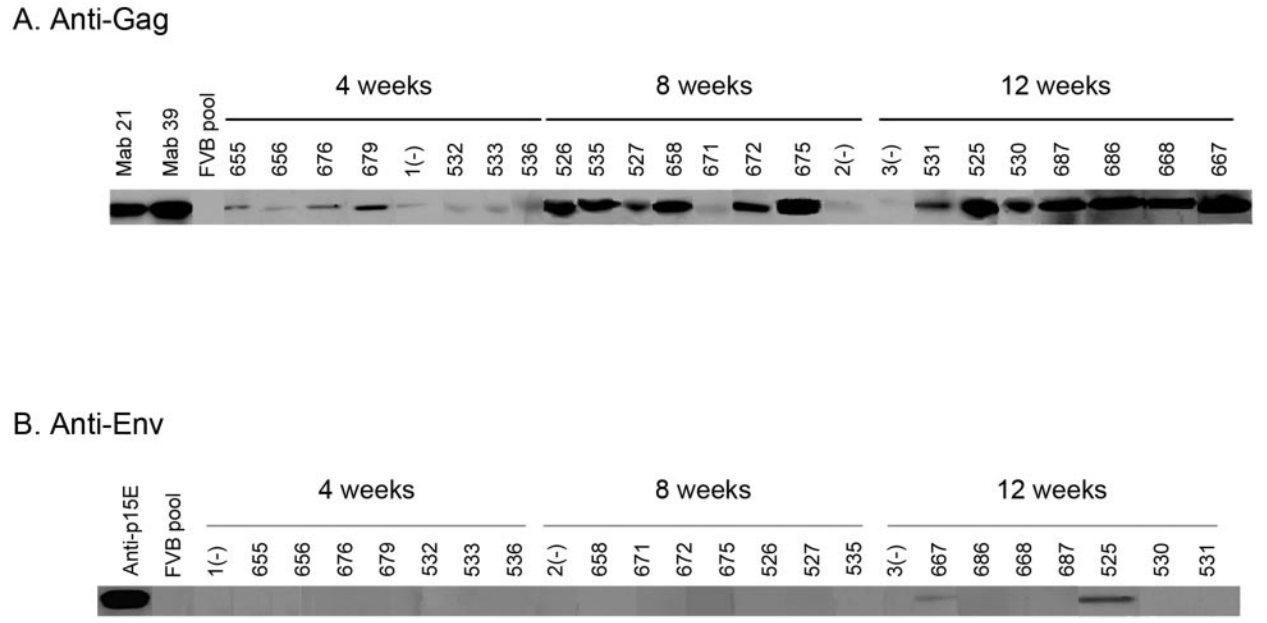

FIG. 7. Anti-Gag and anti-Env antibody detection in the sera of infected HuPAR-2 transgenic mice. Western blots were done with 1 μg of recombinant PERV Gag or Env proteins and stained with 1:50 dilutions of sera from HuPAR-2 transgene-positive and -negative littermates that were inoculated in vivo with high-titer infectious PERV 14/220. The numbers given for each lane represent individual animals, and the negative littermate controls are designated with (−). In addition we made a pool of preimmune sera from 12 animals denoted as "FVB pool." Sera were tested at 4, 8, and 12 weeks after PERV inoculation. As positive controls two monoclonal anti-PERV Gag antibodies (Mab21 and Mab39) and a goat polyclonal anti-PERV Env (anti-p15E) were used.

We next tested the permissiveness of HuPAR-2 transgenic mice in vivo by injecting animals with PERV at the equivalent of 3×10^5 to 5×10^5 $TCID_{50}$ total. All animals were positive for PERV *pol* DNA at different time points in multiple tissues (Table 1). Additionally, we demonstrate here that we could detect increasing copy numbers for PERV *pol* during the observation period from 4 to 12 weeks, consistent with a productive infection. However, it is important to acknowledge the poor correlations between DNA and RNA detections in the different tissues and different animals. One explanation for a DNA-negative tissue to be RNA positive is the presence of PERV RNA in the blood, as we have demonstrated. That could also alter the relative number of copies of PERV RNA detected. Another potential factor impacting these correlations is the issue of sampling errors, as DNA and RNA were prepared from separate sections in tissues that may not be uniformly infected. Finally, it is possible that the transcriptional control of PERV RNA or the half-lives of PERV transcripts are different in different cell types or tissues.

The key difference in our study using HuPAR-2 transgenic mice from previously published studies with NOD/SCID mice is the use of cell-free, filtered supernatants containing infectious PERV instead of cellular transplants. Thus, no cellular microchimerism is possible in this model, eliminating the issue of distinguishing PERV present in pig cells that are chimeric in the mouse tissues posttransplant (11, 20, 58). Therefore, any

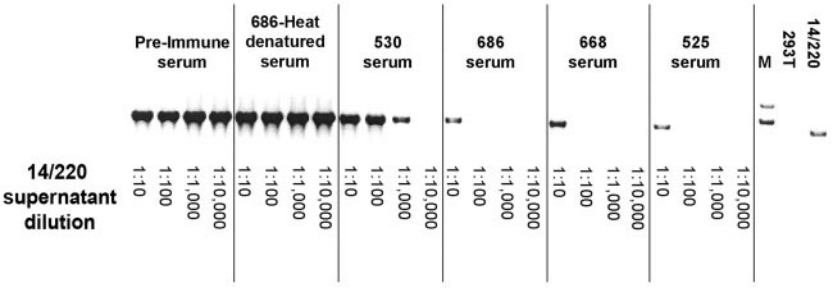

FIG. 8. Demonstration of in vitro PERV-neutralizing antibodies in sera from four animals that were Western blot positive for anti-PERV antibodies. Serial dilutions from 1:10 to 1:10,000 of cell-free supernatants from 14/220 cells were incubated for 1 h at room temperature with a 1:10 dilution of animal sera. Then 5 μg/ml Polybrene was added, and the supernatants were transferred to wells with 50% confluent 293T cells. The cells were washed free of virus and Polybrene after 4 h, and at 72 h the cells were harvested for PCR of PERV *env*. The positive control for infection was 14/220 supernatant without exposure to mouse sera. The negative control was mouse sera heat denatured at 95°C for 15 min. As a positive control for detecting PERV *env* DNA, PCR was done on 14/220 cells (lane 14/220). M, DNA marker, 1-kb ladder (Invitrogen); 293T, negative PCR control using DNA from human 293T cells.

TABLE 3. Analysis of PERV *pol* copy numbers in genomic DNA and total RNA from tissues of mice inoculated with PERV neonatally

Animal	Copy no. in:												Wk[c]
	DNA[a] from:						RNA[b] from:						
	Liver	Lung	Spleen	Small bowel	Kidney	Brain	Liver	Lung	Spleen	Small bowel	Kidney	Brain	
771	2,620	2,947	20,000	515	1,727	18,601	758	ND[d]	Neg	Neg	Neg	457	9
772(−)[e]	Neg	Neg	Neg	Neg	Neg	Neg	ND	ND	Neg	ND	ND	Neg	24
783(−)	Neg	Neg	Neg	Neg	Neg	Neg	ND	ND	Neg	ND	ND	Neg	24
773	Neg	Neg	183	Neg	Neg	84	ND	ND	Neg	ND	ND	Neg	24
786	Neg	Neg	128	Neg	Neg	1,650	ND	ND	Neg	ND	ND	Neg	24
777(−)	Neg	Neg	Neg	Neg	Neg	Neg	Neg	ND	Neg	ND	Neg	Neg	28
774	80	Neg	Neg	Neg	1,098	431	Neg	ND	Neg	ND	Neg	32	28
775	515	Neg	186	Neg	3,191	630	Neg	ND	Neg	ND	Neg	43	28
776	291	Neg	874	Neg	1,171	793	Neg	ND	Neg	ND	Neg	52	28
782	180	120	12	Neg	976	103	Neg	ND	Neg	ND	Neg	Neg	32

[a] Data are expressed as copy number per 2 μg of genomic DNA (18S rRNA sequences used as control).
[b] Data are expressed as copy number per 1 μg of total RNA (18S rRNA sequences used as control).
[c] Animals were injected at day 1 with about 50 μl of cell-free supernatant from 14/220 cells.
[d] ND, not done.
[e] (−) indicates negative transgenic animals injected in the same way as positive ones.
[f] Neg, any result <10 copies in 2 μg of DNA or <10 copies in 1 μg of total RNA after background subtraction.

positive result for PERV DNA is directly attributable to infection of murine cells.

Moreover, in this context, another major advantage of our HuPAR-2 transgenics is that X-MuLV pseudotyping is not a factor in our experimental designs, since there are no nonmurine cells present for X-MuLV to infect. Thus, our results provide the first proof of productive in vivo infection by PERV in any animal.

Total RNA extracted from the tissues of PERV-infected transgenic mice were positive for PERV *pol* sequences by RT-qPCR in 20/21 animals tested (Table 1). Interestingly, the fact that a number of DNA-negative tissues were found to be RNA positive suggests an ongoing viremia, and this was confirmed by analyzing serum samples in two ways: (i) for the presence of viral RNA genomes in whole blood and (ii) for the ability of serum to transmit virus to fresh 293T cells in culture. All serum samples positive for PERV RNA were also able to transmit the virus to human cells, confirming the presence of infectious virus in the circulation. Unlike previous reports for mice and small rodents including our own (11, 20, 49, 50, 58), these data suggest a productive infection.

TABLE 4. Analysis of sera from infected neonatal mice by flow cytometry and RT-PCR

Animal	Mean fluorescence		Δ mean fluorescence	SD	PERV RNA RT-PCR result[c]	Wk
	SIRC-A2[a]	SIRC-A2/PERV[b]				
771	5.76	7.31	1.55	2.72	+	9
772(−)[d]	3.03	2.86	−0.17	2.2	−	24
783(−)	3.02	2.91	−0.11	1.91	−	
773	2.96	5.45	2.49	5.68	−	
786	2.13	1.61	−0.52	1.04	−	
777(−)	2.76	2.42	−0.34	3.75	−	28
774	2.58	1.71	−0.87	2.86	−	
775	2.67	4.85	2.18	4.06	−	
776	2.78	3.05	0.27	2.1	−	
782	1.69	2.47	0.78	3.13	−	32

[a] SIRC-A2 cells are rabbit cells stably transfected with HuPAR-2 receptor.
[b] SIRC-A2/PERV cells are SIRC-A2 cells infected with cell-free supernatants from 14/220 cells.
[c] Nested RT-PCR was performed on reverso-transcribed PERV genomes after isolation from serum with a QIAGEN blood kit. + or −, positive or negative signal respectively, by PCR amplicon on agarose gel stained by ethidium bromide.
[d] (−) indicates negative transgenic animals injected in the same way as positive ones.

Surprisingly, spleen tissue was negative for proviral DNA in all 21 animals tested (Table 1), although low levels of PERV RNA were detected in multiple spleen samples, perhaps reflecting the viremia already discussed. These results were not predicted given the close relationship of PERV to gibbon ape, feline, and murine leukemia viruses that are associated with hematopoietic cell infections (13, 62). In contrast to our in vivo results, mitogen-activated splenocytes from HuPAR-2 transgenic animals can be infected productively with PERV in vitro (Fig. 3C). Further work will be necessary to understand the reason why in vivo infection of splenocytes is not observed, but we speculate that cell activation might be one of the factors influencing the ability of transgenic lymphocytes to be infected.

We tested sera from infected adult HuPAR-2 transgenic animals for anti-Gag and anti-Env antibodies. All animals showed anti-Gag reactivity (Fig. 7A). Transgenic animals showing productive infection at 12 weeks had consistently higher levels of anti-PERV antibody (threefold by densitometry) than animals at 4 or 8 weeks. In contrast, anti-Env reactivity was detected in only 2 animals out of 21 at 12 weeks (Fig. 7B). Interestingly, these two animals did not transmit PERV from their sera to fresh 293T cells, and no PERV genomes were detected by nested PCR in their blood (Fig. 6; Table 2). Moreover, we demonstrate using an in vitro neutralization

assay that PERV-neutralizing antibodies are present in these two animals. Further investigation is required to answer the question if this reflects PERV-neutralizing anti-Env antibodies, as previously suggested by Fiebig et al. (17). However since the sera from other mice without anti-Env antibodies neutralized PERV infection of 293T cells, it is also possible that some anti-Gag antibodies can block virus infection. We are currently testing the impact on PERV infection of preimmunization with Gag and Env recombinant proteins. These studies together with more detailed investigations of the antibody epitopes and the cellular immune response to PERV infection will be facilitated by this new transgenic receptor model.

Neonatal animals were inoculated with PERV to test the possibility that circumventing antiviral immunity by inducing tolerance in animals in this early postnatal period might result in a dramatic increase in levels of viral replication. We observed that the PERV-infected transgenic neonates had on average 21% lower body weights than their transgene-negative littermates injected at the same time, suggesting that an early viral infection altered their normal development. Comparison with transgenic animals not exposed to PERV demonstrated that the failure to gain weight was not the effect of the transgene alone. Interestingly, unlike those from the infected adults, six spleens of a total of seven analyzed were proviral DNA positive. In this context, we could not find any evidence of anti-PERV antibodies in the sera of these animals by flow cytometry and Western blotting at 9, 24, 28, and 32 weeks, consistent with the conclusion that we did induce tolerance for antibody production. In spite of this lack of anti-PERV antibody response the neonatal animals did not develop a sustained productive infection, as we would have predicted. There are two possible explanations. (i) Neonatal animals were injected only once with about 100-fold less virus than a single adult injection; thus, it is possible that initial viral load determines the extent of productive PERV infection. (ii) If the primary target of PERV in vivo is a relatively fixed cell population that is only turning over slowly (for example, endothelial cells), it is possible that viral titers would decrease as a function of time due to saturation of the target and development of viral interference. Alternatively, we hypothesize that newborn mice inhibit a productive PERV infection by mounting a cellular immune response (a hypothesis that has not been tested). Indeed, mice exposed as neonates to poorly replicating murine leukemia virus, or very low infectious titers, developed $CD8^+$ cytotoxic T cells and cleared infection (2, 21).

In conclusion, we have created a new small-animal model that supports productive PERV replication. In fact, HuPAR-2 transgenic mice are the only animals ever documented to have a productive PERV infection, as this has not been observed in pigs, NOD/SCID mice, guinea pigs, and nonhuman primates. Thus, our model provides a unique opportunity to study the nature of PERV infection from the context of pathogenicity, tissue tropism, and humoral and cellular immunity. One obvious hypothesis is that immunosuppression of these transgenic mice with drugs commonly used in clinical transplantation, such as the calcineurin inhibitors FK506 and cyclosporine, will dramatically alter the course of the productive viral infection. Though we acknowledge that a mouse model for PERV infection is still only a model for what might happen in human xenotransplant recipients, it is evident that many parallels exist for human and mouse immunity and response to viral infections that underline the value of this new model for future studies.

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