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# Study of Full-Length Porcine Endogenous Retrovirus Genomes with Envelope Gene Polymorphism in a Specific-Pathogen-Free Large White Swine Herd

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**Specific-pathogen-free (SPF) swine appear to be the most appropriate candidate for pig to human xenotransplantation. Still, the risk of endogenous retrovirus transmission represents a major obstacle, since two human-tropic porcine endogenous retroviruses (PERVs) had been characterized in vitro (P. Le Tissier, J. P. Stoye, Y. Takeuchi, C. Patience, and R. A. Weiss, Nature 389:681–682, 1997). Here we addressed the question of PERV distribution in a French Large White SPF pig herd in vivo. First, PCR screening for previously described PERV envelope genes *envA*, *envB*, and *envC* (D. E. Akiyoshi, M. Denaro, H. Zhu, J. L. Greenstein, P. Banerjee, and J. A. Fishman, J. Virol. 72:4503–4507, 1998; Le Tissier et al., op. cit.). demonstrated ubiquity of *envA* and *envB* sequences, whereas *envC* genes were absent in some animals. On this basis, selective outbreeding of pigs of remote origin might be a means to reduce proviral load in organ donors. Second, we investigated PERV genome carriage in *envC* negative swine. Eleven distinct full-length PERV transcripts were isolated. The sequence of the complete envelope open reading frame was determined. The deduced amino acid sequences revealed the existence of four clones with functional and five clones with defective PERV PK-15 A- and B-like envelope sequences. The occurrence of easily detectable levels of PERV variants in different pig tissues in vivo heightens the need to assess PERV transmission in xenotransplantation animal models.**

For a number of anatomical, physiological, and ethical reasons, pigs are considered the most adequate organ source for xenotransplantation, an alternative therapy to alleviate the chronic human transplant shortage (15, 30, 39). To overcome immunological barriers, transgenic pigs bearing human complement-inhibiting proteins have been developed, leading to increased control of hyperacute rejection in primate models (8, 13, 19, 24, 31, 41, 47; M. Winkler, M. Loss, M. Przemeck, J. Schmidtko, H. Arends, R. Kunz, A. Jalali, J. Klempnauer, E. Cozzi, and D. J. G. White, Abstr. 5th Int. Congr. Xenotransplant. abstr. 184, p. 64). However, xenotransplantation circumvents the natural barriers against infection, raising the risk of cross-species transmission of pathogens after intimate and prolonged contact of living pig and human cells (2, 4, 5, 27, 32). Whereas most pathogens liable to be transmitted from a pig graft to a human recipient can be ruled out by specific-pathogen-free (SPF) rearing conditions, this might not apply to new (14, 17, 23, 40) or hitherto unknown organisms and definitively does not apply to pathogens that are inherited as part of the germ line, i.e., porcine endogenous retroviruses (PERVs) (42). In theory, PERVs share the pathogenic potential of retroviruses in general, which includes insertional mutagenesis and immunosuppression by themselves or after recombination with human retroviruses (12, 34).

Recent findings showed that human-tropic type C PERVs are released in vitro from porcine pig kidney cell line PK-15 (PERV-PK15), from stimulated miniature swine primary peripheral blood mononuclear cells (PERV-MSL), and spontaneously from porcine aortic endothelial cells (PERV-PK15-like) (1, 18, 22, 26, 37, 45). Analysis of these PERVs revealed genomes of about 8.1 kb with close amino acid sequence similarities (>95%) for the *gag* and *pol* open reading frames (ORFs). In contrast, sequence discrepancies occurring in envelope ORFs led to the distinction of three envelope genes, termed *envA*, *envB*, and *envC* (1, 18). *gag*, *pol*, and *env* transcripts and close variants of the last have been discovered in many different pig herds (9; D. Cunningham et al., Abstr. 5th Int. Congr. Xenotranspl., abstr. 1154, 1999; C. Herring et al., Abstr. 5th Int. Congr. Xenotranspl., abstr. 1153, 1999; J. H. Lee et al., Abstr. 5th Int. Congr. Xenotranspl., abstr. 0164, 1999). In spite of these observations, until now, no case of PERV infection has been reported for patients and primates who had been treated with living pig tissues (16, 21, 25, 28). Albeit promising, the apparent absence of PERV transmission in these assays needs to be relativized with respect to the short period of exposure to pig cells before xenograft rejection, the number of cells introduced, the degree of cell contact (extracorporal versus in situ), and the genetically unmodified donor material used (35–37, 44). Compared to future xenotransplantation settings, these conditions might have minimized the viral load, a factor which determines successful PERV transmission in vitro (C. Patience, B. Oldmixon, T. Ericsson, and G. Andersson, Abstr. 5th Int. Congr. Xenotranspl., abstr. 1109, 1999). For all these reasons, it will be important to learn more about the incidence of PERV particles in vivo. Aside from work done by Akiyoshi et al. on PERV-MSL (*envC*) genomes in miniature swine (1), many unknowns remain concerning the type and level of transcription of replication-competent PERVs in vivo. Indeed, general observations made for endogenous retroviruses as for other nonessential genes show that multiple mutations accumulate during evolution (20). Among the 50 proviral loci estimated in the pig genome using a protease probe (26), only a small subset are expected to be infectious. Characterization of active full-length PERVs is of special interest

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with regard to localization of intact proviruses, a prerequisite for eliminatory cross-breeding or gene knock-out technology.

This study focused on proviral PERV distribution and characterization of transcriptionally active full-length type C PERVs in a specific-pathogen-free (SPF) Large White pig herd. We recorded absence of envelope *envC* genes in the genome of some animals of the herd. Results for long reverse transcriptase (RT)-PCR conducted on *envC*-negative swine revealed persistent high levels of full-length type C PERV genomes in seven pig organs in vivo. There is compelling evidence for at least 11 distinct biologically active proviral loci in three animals analyzed from this herd. Envelope data analysis showed distinct and intact ORFs for four PERVs with close homologies with PERV PK-15 A- and B-like sequences. On the contrary, five other PERVs with truncated envelope ORFs will constitute no infectious risk in xenotransplantation.

## MATERIALS AND METHODS

**Viruses and cell cultures.** The production of PERV PK-15 and Tsukuba-1 particles by, respectively, pig kidney PK-15 and pig spleen Schimozuma cell lines has been described previously (39). Schimozuma substrain G2 was a gift from B. Kaeffer (INRA, Nantes, France). Porcine PK-15, human K562, and green monkey kidney MARC cell lines were kindly provided by E. Albina (AFSSA, Ploufragan, France). These cells, except for K562, were grown in minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS), 100 U of penicillin per ml, and 100 mg of streptomycin per ml K562 cells were kept in RPMI medium, chicken hepatocellular carcinoma LMH cells (ATCC CRL-2117) were kept in Williams medium, and equine fibroblast ED cells (ATCC CCL-57) were kept in MEM with FCS and antibiotics as above.

**SPF pigs.** All samples were from Large White pigs raised under SPF conditions (6). Periodic controls ensure the maintenance of well-defined health status for this herd (C. Cariolet, personal communication). For the maintenance of the Ploufragan SPF pig out-bred herd, females are selected among the descendants. In order to avoid consanguinity, two boars delivered by hysterectomy and raised under sterile conditions until weaning are introduced each year. Hence, pig families were defined as the descendants of one boar. Tissue samples were harvested shortly after slaughter and stored in liquid nitrogen. Genomic full-length transcripts were cloned from pigs 6309, 6282, and 6407, weighing about 100 kg and 132 to 140 days of age at sacrifice. In order to avoid RNA degradation, a sample of pancreas was snap-frozen in liquid nitrogen, and RNA was extracted immediately after resection. Whole blood samples were collected from three females (sows 1 to 3 [$S_1$ to $S_3$]) and 17 offspring of four boars ($O_1$ to $O_4$). For practical reasons, no blood could be obtained from boars.

**Isolation of DNA.** DNA was extracted from whole blood samples using the Qiagen tissue kit (Qiagen).

**Isolation of mRNAs.** From 20 to 30 mg of frozen tissue sample was ground in liquid nitrogen. The frozen powder was lysed (LiDS), homogenized (Qiashredder; Qiagen) and fixed for 4 min on 1.25 mg of paramagnetic oligo$(dT)_{25}$ beads (Dynal) according to the manufacturer's instructions. mRNA was eluted in Tris-HCl (10 mM [pH 7.5]) and stored at −80°C until use. For the extraction of mRNA from cell lines, cells were rinsed in phosphate-buffered saline (PBS) and directly subjected to lysis as stated here above.

**Generation of genomic full-length PERV clones.** mRNA derived from 125 μg (375 μg for pancreas) of oligo$(dT)_{25}$ beads was reverse transcribed in 50 μl of Thermoscript RT-PCR system reaction mixture with oligo(dT) primers as specified by the vendor (Gibco BRL). Negative controls without reverse transcriptase were prepared for all samples. RT conditions consisted of 5 min at 50°C, 60 min at 55°C, and 10 min at 60°C. Template mRNA was removed by addition of 5 U of *Escherichia coli* RNase H (Gibco BRL) at 37°C for 20 min. Then 5 μl of cDNAs or 2 ng of plasmid Tsukuba-1 DNA (kindly provided by J. P. Stoye) was subjected to PCR amplification with primers conserved in all three type C PERVs. The forward primer was designed from the leader region (L-fov, 5′-ACGTGCTAGGAGGATCACAGGCTGC-3′, nucleotides [nt] 342 to 356 in Tsukuba-1 or 347 to 372 in PK-15) and backward primer from the untranslated region downstream of the envelope gene (L-rev, 5′-GTTGTCTAAGTACCATGATCTGGACTGCAC-3′, nt 7476 to 7506 in Tsukuba-1 or 6665 to 6684 in PK-15). PCR with these primers should generate a 7.2-kb product for genomic porcine PERV type C RNA and products in the ~3-kb range for spliced viral mRNAs. Amplification was carried out in 50 μl of Platinum *Taq* High Fidelity reaction mixture (Gibco BRL) with dimethyl sulfoxide added immediately prior to cycling to a final concentration of 10%. The initial denaturation step was 1 min at 94°C, followed by 10 cycles of 10 s at 94°C, 30 s at 66°C, ramp for 1 min to 68°C, 8 min at 68°C, and 25 cycles of 10 s at 94°C, 30 s at 66°C, ramp for 1 min to 68°C, 9 min at 68°C, and 20 min of final extension at 68°C. Full-length (approximately 7.2 kb) PCR products were gel purified (QIAEXII; Qiagen), ligated into the Topo XL cloning vector, and introduced into Top10 electrocompetent cells (Invitrogen). Eighty colonies with the 7.2-kb insert were isolated. Plasmid DNA was prepared with the Qiaprep 8 miniprep kit (Qiagen).

**PCR.** PCR with primers specific for *gag* (1), *pol* (45), and envelope genes *envA*, *envB*, and *envC* (18, 38) was carried out using the primers described elsewhere but adapted here to higher melting temperatures: gag-fov (5′-CCCGATCAGGAGCCCTATATCCTTACGTG-3′), gag-rev (5′-CGCAGCGGTAATATCGCGATCTCGT-3′), pol-fov (5′-GACGGGTAACCCACTCGTTTCTGGT-3′), pol-rev (5′-ACGTACTGGAGGAGGGTCACCTAG-3′), envA-fov (5′-GAGATGGAAAGATTGGCAACAGCG-3′), envA-rev (5′-AGTGATGTTAGGCTCAGTGGGGAC-3′), envB-fov (5′-AATTCTCCTTTGTCAATTCCGGCCC-3′), envB-rev (5′-CCAGTACTTTATCGGGTCCCACTG-3′), envC-fov (5′-CTGACCTGGATTAGAACTGGAAGC-3′), and envC-rev (5′-GTTATGTTAGAGGATGGTCCTGGTC-3′). Reaction mixture (20 μl) containing 3 U of recombinant *Tetrahymenis thermus* (rTth) polymerase (Roche) bound to rTth antibody (Ozyme) and 1 U of uracil desoxynucleotide glycosylase (Gibco BRL) was subjected to 15 min at 37°C, 10 min at 94°C, followed by 30 rounds of thermocycling of 30 s at 94°C, 45 s at 65°C, 40 s at 72°C, and 10 min of final extension at 72°C.

**RFLP.** For the restriction fragment length polymorphism (RFLP) assay, 80 full-length clones were digested with *Eco*RI (NEB).

**Sequence analysis of PERV elements.** Clones were cycle sequenced on a 373 DNA sequencing system (Applied Biosystems) with the dye terminator cycle sequencing kit and Ampli Taq DNA polymerase FS (Applied Biosystems).

**Nucleotide sequence accession numbers.** The complete envelope coding region was determined for one clone out of each profile group and deposited in the EMBL database (AJ288584 to AJ288592). Infobiogen's software was used to deduce the putative amino acid sequence. All sequence alignments were carried out using the INRA multialignment software (11), and sequence comparisons were carried out with NCI's BLAST (3) and LFASTA (7) software.

## RESULTS

**Distribution of proviral elements in SPF pigs in Ploufragan.** DNA extracted from blood samples of three sows and 17 offspring derived from four boars were screened by PCR for the distribution of *gag*, *pol* (data not shown), and envelope *envA*, *envB*, and *envC* gene sequences (Fig. 1). *gag*, *pol*, and envelope *envA* and *envB* genes were ubiquitously present in all animals. In contrast, our herd was heterogeneous for *envC*, which was absent in all mother sows tested, in offspring of boar $B_4$, and in three of five offspring of boar $B_3$. In all subsequent studies on transcriptionally active PERVs, only *envC*-negative swine, presumably cleaner in terms of proviral load, were used.

**Isolation of genome-length viral RNAs in pig tissues.** The specificity of long RT-PCR for PERV versus other ERV genomes was tested on RNA derived from human, simian, equine, avian, and porcine cell lines. Only porcine RNA yielded amplification products (data not shown). Long RT-PCR efficiently amplified full-length and additional shorter PERV type C transcripts from all pig tissues studied (Fig. 2). Faint bands larger than 8 kb likely correspond to either PCR artifacts or readthrough transcription of viral into cellular genes (20). Subgenomic fragments, derived from either truncated proviruses or spliced viral mRNAs, were observed in the 2.3-kb to 6-kb range. The intensity of these bands varied in a tissue-specific manner, but three times showed reproducibly consistent patterns in four different pigs: ~2.3-kb, 2.8-kb, 3-kb, and 3.8-kb transcripts in the lung; ~2.3-kb, 2.8-kb, 3-kb, 4-kb, and 5-kb transcripts in the thymus; a strong 3-kb transcript band in the liver; ~3-kb and 3.3-kb transcripts in the heart; ~3-kb, 4-kb, and 6-kb transcripts in the kidney; ~3-kb and strong 3.3-kb bands in the spleen; and a ~3.3-kb band in the pancreas. These patterns are more complex than those observed by Akiyoshi et al. for transcripts in miniature swine hybridized with an *envC* (PERV-MSL*env*) probe (1). The multitude of products found here underscores the great number of PERV type C proviruses fixed in the germ line of our and probably most other pig herds.

Subsequent PCR screening of 80 cloned full-length transcripts derived from heart, liver, lung, spleen, thymus, and kidney from two pigs (6309 and 6282) and pancreas from one pig (6407) ascertained retroviral origin: all clones presented

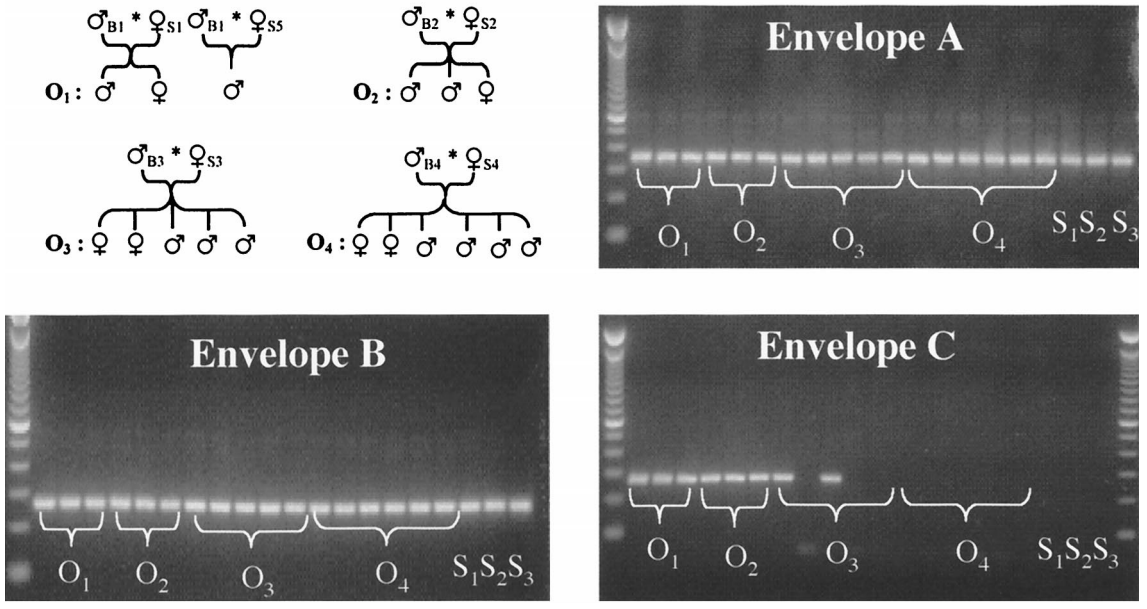


FIG. 1. Distribution of proviral elements in SPF pigs in Ploufragan. PCR with primer pairs specific for *envA*, *envB*, and *envC* resulted in amplification products of 359 bp, 263 bp, and 281 bp, respectively. Here, a pig family was defined as the descendants of one boar. $S_1$ to $S_3$, mother sows; $O_1$ to $O_4$, piglets sired by boars 1 to 4, respectively.

*gag* elements and all except two clones showed *pol* elements. The latter, assumed to contain deletions or rearrangements which might render them noninfectious, were sorted out. The remaining clones were divided into 11 categories according to PCR results obtained for envelope genes (*envA* plus *envB*, or double positives, noted *envAB*) and *Eco*RI digestion profile patterns (1 to 8) (Table 1). These categories indicate the existence of at least 11 distinct full-length PERV genome vari-

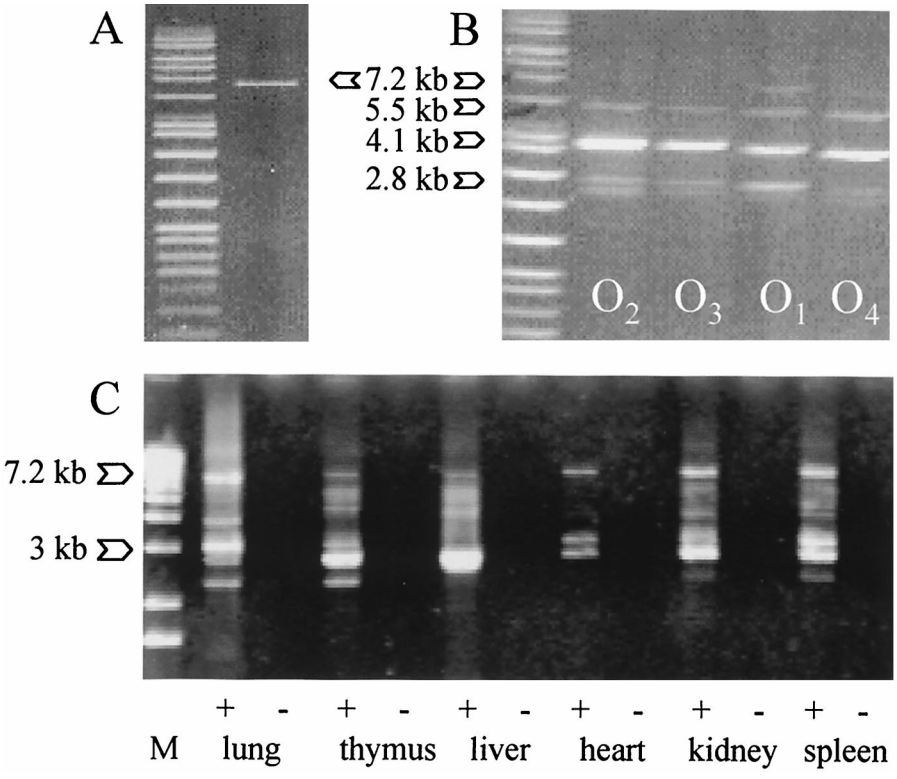


FIG. 2. Characterization of full-length PERV RNA and DNA. Long PCR performed on (A) plasmid Tsukuba-1 DNA, (B) porcine DNA extracted from whole blood of one offspring in each pig family, and (C) cDNA derived from major pig organs (lanes +) and negative controls without reverse transcriptase (lanes −). A specific amplification product was obtained for cloned PERV sequence, whereas porcine DNA revealed multiple products of viral origin. RT-PCR demonstrated the existence of full-length and spliced viral RNA in all pig tissues studied.

TABLE 1. Characterization of full-length PERV genomes transcribed in three SPF pigs in vivo[a]

| Group | No. of clones | No. of pigs | Occurrence in organs | Envelope sequence | Envelope (amino acids) |
|---|---|---|---|---|---|
| $envA_1$ | 27 | 3 | Heart, kidney, lung, pancreas, spleen, thymus | PERV-$A_1$ | 661 |
| $envA_2$ | 2 | 2 | Heart, thymus | PERV-$A_2$ | 661 |
| $envB_1$ | 25 | 3 | Kidney, liver, pancreas, spleen | PERV-$B_1$ | 655 |
| $envB_4$ | 1 | 1 | Spleen | PERV-$B_4$ | 404 |
| $envB_5$ | 1 | 1 | Kidney | ND[b] | |
| $envB_6$ | 9 | 3 | Heart, kidney, liver, pancreas, thymus | PERV-$B_6$ | 288 |
| $envB_7$ | 1 | 1 | Pancreas | PERV-$B_7$ | 632 |
| $envB_8$ | 2 | 2 | Liver, kidney | ND | |
| $envAB_3$ | 5 | 1 | Heart, liver, thymus | PERV-$AB_3$ | 280 |
| $envAB_4$ | 2 | 1 | Liver, thymus | PERV-$AB_4$ | No protein |
| $envAB_7$ | 3 | 2 | Heart, kidney | PERV-$AB_7$ | 202 |

[a] Each group includes clones with identical envelope PCR and RFLP profile (I to VIII) results.
[b] ND, not determined.

ants. In consequence, there are at least 11 different transcriptionally active proviral insertion sites in the genome of these pigs. All tissues except lung transcribed three or more different variants. In particular, members of group $envB_1$, which present a profile pattern identical to PERV PK-15 B (GenBank Y17013), and of group $envA_1$ were prominent, i.e., all three pigs presented one or both types. Clones belonging to group $envA_1$ were observed in all tissues except liver. However, we only isolated seven clones from liver and lung. This small sample size might not fully reflect the retroviral diversity in these tissues.

**Envelope sequence data.** Alignment of the deduced amino acid sequence (Fig. 3) revealed intact envelope ORFs for four restriction groups: variants PERV-$A_1$ and PERV-$A_2$ shared homologies greater than 95 and 96%, respectively, with sequence PERV PK-15A, and variants PERV-$B_1$ and PERV-$B_7$ showed homologies greater than 97% to sequence PERV PK-15 B. The main sequence discrepancies occurred in a small stretch of 14 amino acid residues in the C-terminal regions of PERV-$A_1$, PERV-$A_2$, and PERV-$B_1$, whereas 24 amino acid residues were deleted at the C-terminal region of PERV-$B_7$. Five other clones, PERV-$B_4$, PERV-$B_6$, PERV-$AB_3$, PERV-$AB_4$, and PERV-$AB_7$, presented insertions or deletions causing frameshifts and consecutive disruption of the translation process.

## DISCUSSION

We used Large White outbred SPF pigs to assess PERV carriage in vivo and suitability as an organ source for xenotransplantation. Consistent with observations made for other pig herds (37), PERV *envC* genes were absent in some of the animals, i.e., in all three mother sows tested, in the offspring of boar $B_4$, and in three of five offspring of boar $B_3$. Matched to the genealogical tree, these results sustain the hypothesis that our envelope *envC* primers are complementary to a single proviral insertion site on a somatic chromosome in the heterozygous boar $B_3$ and its positive offspring. Hence, donor screening will permit us to preclude infectious risk for this subtype of PERVs in xenotransplantation. However, we cannot rule out that distantly related PERV *envC* viruses have not been recognized by the PCR primers used here. In the future, determination of chromosomal locations of PERVs in herds of remote origin might prove to be an efficient means of reducing PERV carriage by selective outbreeding.

Long PCR with primer pairs conserved in all three PERV sequences (PERV PK-15 A and B and PERV-MSL) confirmed predictions of whole and partial inserts in the genome of our pig herd (Fig. 2). Since proviral DNA load does not necessarily correlate with viral RNA load, we investigated whether the intact proviruses are dormant or biologically active. In contrast to other endogenous retroviruses which are transcribed at extremely low levels and require nested RT-PCR for their detection (43), simple RT-PCR was sufficient to detect constitutive transcription of full-length PERV genomes in all tissues tested. There are several implications to these findings. (i) Obviously, these viral genomic RNAs stem from long terminal repeats acting as strong promoters in porcine cells, which invalidates earlier assumptions that PERV expression might be triggered by in vitro culture conditions. (ii) Among the 11 different PERV genomes recognized here, a high percentage were of the $envB_1$ type, which displays the restriction pattern expected for PERV PK-15 B (GenBank Y17013). This apparent similarity indicates replication competence for this group. (iii) Finally, the sample size and experimental conditions used might not account for minority or distantly related PERV genomes, thereby understating the viral diversity. A conceivable consequence of the presence of multiple variants is that earlier in vitro studies on the host range and interference of PERVs (37, 46) might not fully reflect the viral population encountered in different pig herds in vivo.

To assess infectious genomes, we sequenced the complete envelope coding region for one clone in each profile group. The deduced amino acid sequence revealed four variants with intact envelope ORFs and close homologies with sequences PERV PK-15 A and B. The main sequence discrepancies were located in a small stretch of amino acids at the C terminus. While amino acid residues 632 to 657 were missing in PERV-$B_7$, the three other ORF variants showed C-terminal sequences markedly different from those of PERV-PK-15 but similar to each other. A likely reason for this phenomenon is that a predecessor PERV, after having acquired the C-terminal sequence, reintegrated into the pig genome. Indeed, ERVs (20) are prone to amplify in the genome in a retrotransposable fashion, thereby introducing genetic diversity and rapid genetic drift between separate strains. As a 16-amino-acid C-terminal fragment is removed from the end of the transmembrane envelope protein in murine leukemia virus-related viruses before budding (10), we suppose that this variation from PERV PK-15 A and B sequences does not impede replication competence in these clones. Nonetheless, downstream infectious

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PERV-A   MHPTLSRRHL PIRGGKPKRL KIPLSFASIA WFLTLSITPQ VNGKRLVDSP NSHKPLSLTW LLTDSGTGIN   70
PERV-A1  .......... .....E..K. .......... .......... ......A... .F........ .....S...T   70
PERV-A2  .......... .......... .......... .......... .......... .......... ..........   70
PERV-B   MHPTLSWRHL PTRGGEPKRL RIPLSFASIA WFLTLTITPQ ASSKRLIDSS NPHRPLSLTW LIIDPDTGVT   70
PERV-B1  .........P .......... G.....G... .......... .......... .......... ..........   70
PERV-B7  .......... ......R... ........F. .......I.. .......... .......... ..........   70

PERV-A   INSTQGEAPL GTWWPELYVC LRSVIPGLND QATPPDVLRA YGFYVCPGPP NNEEYCGNPQ DFFCKQWSCI  140
PERV-A1  .......... .......... ........S. .......... ...N...... .......... .........V  140
PERV-A2  .......... .......... .......... .......... .......... .......... .......R.V  140
PERV-B   VNSTRGVAPR GTWWPELHFC LRLINPAVKS TPPNLVRSYG FYCCPGTEKE KYCGGSGESF CRRWSCVTSN  140
PERV-B1  .......... .......... .......... .......... .......... .......... ..........  140
PERV-B7  .......... .......... .......... .......... .......... .......... ..........  140

PERV-A   TSNDGNWKWP VSQQDRVSYS FVNNPTSYNQ FNYGHGRWKD WQQRVQKDVR NKQISCHSLD LDYLKISFTE  210
PERV-A1  .......... ......A... ...S...... .......... .......... ......N... ..........  210
PERV-A2  .......... .......... .......... .......... .......... .......... ..........  210
PERV-B   DGDWKWPISL QDRVKFSFVN SGPGKYKVMK LYKDKSCSPS DLDYLKISFT EKGKQENIQK WINGMSWGIV  210
PERV-B1  .......... .......... .......... .......... .......... .......... .M........  210
PERV-B7  ........F. .......... ......R... .......... .......... .......... ..........  210

PERV-A   KGKQENIQKW VNGISWGIVY YGGSGRKKGS VLTIRLRIET QMEPPVAIGP NKGLAEQGPP IQEQRPSPNP  280
PERV-A1  .......... ...M....M. ...T..R... .......... R......... .......... ..........  280
PERV-A2  .......... ...M...... .......... .......... .......... .......... ........D.  280
PERV-B   FYKYGGGAGS TLTIRLRIET GTEPPVAVGP DKVLAEQGPP ALEPPHNLPV PQLTSLRPDI TQPPSNGTTG  280
PERV-B1  .......... ....---... .......... .......... .......... .......... ..........  277
PERV-B7  .......... .V........ .......... .......... .......... .......... ..........  280

PERV-A   SDYNTTSGSV PTEPNITIKT GAKLFSLIQG AFQALNSTTP EATSSCWLCL ASGPPYYEGM ARGGKFNVTK  350
PERV-A1  .V......L. .P...F.... .......... .......... .......... .L........ ..........  350
PERV-A2  .......... .......... .....N.... .......... .......... .......... .T........  350
PERV-B   LIPTNTPRNS PGVPVKTGQR LFSLIQGAFQ AINSTDPDAT SSCWLCLSSG PPYYEGMAKE GKFNVTKEHR  350
PERV-B1  ........H. .......... .......... .......... .......... .......... ..........  347
PERV-B7  .....M.... ........K. .........K ..K....E.N .......... .......... ..........  347

PERV-A   EHRDQCTWGS QNKLTLTEVS GKGTCIGMVP PSHQHLCNHT EAFNRTSESQ YLVPGYDRWW ACNTGLTPCV  420
PERV-A1  ..T....... .......... .......R.. .......... .......... .......... ..........  420
PERV-A2  .......... .......... .......... .......... .P.......P .......... ..........  420
PERV-B   NQCTWGSRNK LTLTEVSGKG TCIGKAPPSH QHLCYSTVVY EQASENQYLV PGYNRWWACN TGLTPCVSTS  420
PERV-B1  .......... .......... .......... ........X. .......... .......... ..........  417
PERV-B7  .......... .......... .......... ..P....... .......... .......... ..........  420

PERV-A   STLVFNQTKD FCVMVQIVPR VYYYPEKAVL DEYDYRYNRP KREPISLTLA VMLGLGVAAG VGTGTAALIT  490
PERV-A1  .......... .......... .......... .......... .........V .......... .E........  490
PERV-A2  .......... .......... ..H....T.. .......... .....F.... .......... ..........  490
PERV-B   VFNQSKDFCV MVQIVPRVYY HPEEVVLDEY DYRYNRPKRE PVSLTLAVML GLGTAVGVGT GTAALITGPQ  490
PERV-B1  .......... .......... ....M..... E......... .....I.... .......... ..........  487
PERV-B7  .......... .......... .......... ........G. .......... .......... ..........  490

PERV-A   GPQQLEKGLS NLHRIVTEDL QALEKSVSNL EESLTSLSEV VLQNRRGLDL LFLKEGGLCV ALKEECCFYV  560
PERV-A1  .......... D......... .......... ...V...... .......... .......... ..........  560
PERV-A2  .......... .......... .......... .......... .......... .......... ..........  560
PERV-B   QLEKGLGELH AAMTEDLRAL EESVSNLEES LTSLSEVVLQ NRRGLDLLFL REGGLCAALK EECCFYVDHS  560
PERV-B1  .......... .......... .......... .......... .......... .......... ..........  557
PERV-B7  .......... .......... .......... .....G.... .......... .......... ..........  560

PERV-A   DHSGAIRDSM SKLRERLERR RREREADQGW FEGWFNRSPW MTTLLSALTG PLVVLLLLLT VGPCLINRFV  630
PERV-A1  .......... .......... .......... ....L..... .......... .......... I......G..  630
PERV-A2  .......... ........K. HK.K..G... ......K... V......... ...I...... .....T....  630
PERV-B   GAIRDSMSKL RERLERRRRE READQGWFEG WFNRSPWMTT LLSALTGPLV VLLLLLTVGP CLINRFVAFV  630
PERV-B1  .......... .......... .......... .......... .......... .......... ..........  627
PERV-B7  .......... .......... .......... .......... .......... .......... ..........  630

PERV-A   AFVRERVSAV QIMVLRQQYQ GLLSQGETDL .  660
PERV-A1  .......... .......RML KGEF.HTGGR Y  661
PERV-A2  .....Q.... .......RML KGEF.HTGGR Y  661
PERV-B   RERVSAVQIM VLRQQYQGLL SQGETDL.     657
PERV-B1  .......... ....RMLKGE F.HTGGRY     655
PERV-B7  ..-------- ---------- --------     632
```

FIG. 3. Amino acid sequences of untruncated envelope ORFs deduced from PERV transcripts. Dashes indicate gaps, and dots indicate identical amino acids.

particle formation remains to be demonstrated. Sequence analysis of PERV variants $B_4$, $B_5$, $B_6$, $AB_4$, and $AB_7$ revealed multiple frameshifts with introduction of stop codons leading to truncated envelope proteins. Taken together with possible changes in other regions of the genome, the apparent envelope ORF alterations will not support replication in these genomes. The presence of multiple defective genomes in pig cells in vivo which are susceptible of virion assembly through complementation by helper viruses and interfere with replication-competent viruses is in agreement with the observation that a single in vitro passage of PERVs through human cells selects virus populations with increased infectious titers (46).

PCR analysis of envelope genes revealed double positive *envAB* in three of eight RFLP profile groups, all of which proved to code for defective envelope proteins. Since *envA*- and *envB*-specific primers bind to analogous but disparate regions of envelope gene sequences of two PK-15 PERVs (18), these findings suggest the presence of mutations or crossovers yielding novel mosaic genotypes. Evidence for *envAB* recombinant PERVs had been reported for two of sixty-four proviral envelope sequences derived from Australian Westran pigs (J. H. Lee et al., Abstr. 5th Int. Congr. Xenotranspl., abstr. 0164, 1999), and more recently, *envAC* recombinant viral RNAs were observed in U.S. minipig peripheral blood mononuclear cell cultures (46). Paradoxically, envelope sequence analysis of our *envAB* clones did not show recombination between *envA* and *envB* sequences but numerous point mutations. In spite of these alterations, we were unable to identify primer binding sites for *envA* amplification. As all PCR experiments were repeated twice at 3 month intervals on freshly prepared plasmid material in a PERV-free atmosphere, we suppose that *envAB* double-positive PCR results are due to crossovers of PERV PK-15 A- and B-like sequences upstream of the envelope ORF sequenced here rather than to PCR contamination.

This work demonstrates that full-length PERV genomes actively replicate to easily detectable levels in French Large White SPF pigs in vivo. Envelope analysis revealed the existence of five PERV variants coding for truncated envelope proteins, indicating that these genomes are devoid of infectious risk in a xenotransplantation setting. On the contrary, all tissues examined presented at least one of four PERVs with functional PERV PK-15-like envelope ORFs. Experiments trying to map potentially replication-competent variants to a Large White bacterial chromosome library (29) are currently under way. In the light of our findings, it seems illusory to control expression of PERVs in transplanted tissues. We therefore hope that the sequence data presented here will be valuable for the comparison of PERV distribution in different pig herds in an endeavor to rule out potentially infectious proviruses.

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