Identification of Novel Porcine Endogenous Betaretrovirus Sequences in Miniature Swine

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Received 28 August 2000/Accepted 6 December 2000

PCR amplification of genomic DNA from miniature swine peripheral blood lymphocytes, using primers corresponding to highly conserved regions of the polymerase (*pol*) gene, allowed the identification of two novel porcine endogenous retrovirus (PERV) sequences, PMSN-1 and PMSN-4. Phylogenetic analyses of the nucleotide sequences of PMSN-1 and PMSN-4 revealed them to be most closely related to betaretroviruses. The identification of PERVs belonging to the *Betaretrovirus* genus shows that endogenous retroviruses of this family are more broadly represented in mammalian species than previously appreciated. Both sequences contained inactivating mutations, implying that these particular loci are defective. However, Southern blot analysis showed additional copies of closely related proviruses in the miniature swine genome. Analyses of fetal and adult miniature swine tissues revealed a broad mRNA expression pattern of both PMSN-1 and PMSN-4. The most abundant expression was detected in whole bone marrow *c-kit*⁺ (CD117⁺) progenitor bone marrow cells, fetal liver, salivary gland, and thymus. It appears unlikely that functional loci encoding these novel PERV sequences exist, but this remains to be established. The betaretrovirus sequences described in this report will allow such investigations to be actively pursued.

The pig (*Sus scrofa*), in particular, the miniature swine, is considered to be the primary source of xenograft organs to be used in xenotransplantation (29). Because of the potential risk of zoonotic transmission of porcine endogenous retroviruses (PERVs) from pig to human recipients of xenotransplants, a major effort to characterize the potential transmission of PERV is warranted. ERVs are present in the genomes of all cells of an organism and are transmitted from parent to offspring as a provirus in the germ line DNA (6, 15, 18, 24, 33, 38). In mammals, two main types of ERVs, designated beta- and gammaretroviruses, have been identified (37). Retroviruses have acquired efficient mechanisms for survival and persistence in a wide range of host species. Some strains of retroviruses are endogenous in one species and exogenous in other species (38). Of particular importance for the potential risks associated with retroviruses and xenotransplantation is the capacity of retroviruses to change their pathogenicity following interspecies transmission, possibly resulting in emerging infections. Thus, it is possible for ERVs that are symbiotic in one host to be parasitic in another.

At least three distinct PERV gammaretrovirus sequences (PERV-A, -B, and -C) have been identified (1, 16). PERV-A and -B produced from PK-15 (porcine kidney-derived) cells (25), activated peripheral blood mononuclear cells (PBMCs) (39), or porcine aortic endothelial cells (19) are able to infect human and porcine cell lines. PERV-C has only been shown to

infect porcine cell lines (35). There has been no evidence of PERV infection in clinical samples taken from patients exposed to pig tissues or cells (12, 23, 26).

To determine whether the porcine genome harbors additional PERV loci, a PCR-based approach that utilizes the high conservation of the *pol* gene was employed (17). This approach resulted in the identification of novel betaretrovirus *pol* sequences. Genomic copy number and phylogenetic and RNA expression analyses of these novel PERV sequences are presented.

MATERIALS AND METHODS

Genomic DNA preparation. PBMCs were isolated from miniature swine (obtained from the Transplantation Biology Research Center, Massachusetts General Hospital, Charlestown) inbred for the major histocompatibility complex swine leukocyte antigen (MHC/SLA) (27–29). Three lines of inbred animals, haplotypes *SLA^{a/a}*, *SLA^{c/c}*, and *SLA^{d/d}*, were analyzed. Genomic DNA was extracted with the Genomic-tip 500/G (Qiagen, Inc.) or the PureGene DNA isolation kit (Gentra Systems, Inc.) according to the manufacturer's instructions.

PCR. PCR was performed with 500 ng of genomic DNA with standard reagents and 2.5 U of AmpliTaq Gold (Perkin-Elmer Co.) for 40 cycles (94°C for 30 s, 45°C for 30 s, and 72°C for 30 s). The oligonucleotides used were 5'-MOP-2 (5'-CCWTGGAATACTCCYRTWTT-3') and 3'-MOP-2 (5'-GTCKGAACCAATTWATATYYCC-3') (17), where R stands for A or G, Y stands for C or T, K stands for G or T, and W stands for A or T.

Cloning and sorting of PCR products. The approximately 640-bp PCR products were purified and cloned into the pCR2.1-TOPO vector (Invitrogen). Bacterial colony PCRs with 5'-MOP-2 and 3'-MOP-2 followed by restriction enzyme digestion were used to screen a total of 84 colonies. From the restriction enzyme digestion profile, colonies were assigned to one of seven different groups.

Nucleotide sequence and phylogenetic analyses. Representative clones were sequenced by Lark Technologies, Inc., with a model 373 automated sequencer (Applied Biosystems-Perkin-Elmer). A neighbor-joining phylogenetic tree (30) was constructed from translated N-terminal sequences. The sequences used and their GenBank accession numbers are listed in Table 1. Distances were calculated by using a PAM250 similarity matrix. The programs PROTDIST and NEIGHBOR were used to deduce a tree that was then drawn by DRAWTREE. The last three programs are part of the PHYLIP package (37). Numbers denote

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TABLE 1. ERV *pol* sequences used for phylogenetic tree construction

Abbreviation	Full name	Host species	Accession no.
ASLV	Avian sarcoma/leukosis virus	Chicken	AF052428
BaEV m7	Baboon endogenous virus strain M7	Baboon	M16550
ERVfrd	Human endogenous retrovirus clone ERV FRD	Human	U27240
ERVftd	Human endogenous retrovirus clone ERV FTD	Human	U27241
HERV-I	Human endogenous retrovirus-I	Human	AC007276
HERV-E	Human endogenous retrovirus-E	Human	M10976
HERV-F2	Human endogenous retrovirus-F2	Human	AC000378
HERV-H	Human endogenous retrovirus-H	Human	AC005386
HERV-L	Human endogenous retrovirus-L	Human	M89211
HERV-K10	Human endogenous retrovirus-K10	Human	M14123
HERV-KC4	Human endogenous retrovirus-KC4	Human	X80240
HERV-R	Human endogenous retrovirus-R	Human	AC04054
HERV-T	Human endogenous retrovirus-T	Human	Z70664
HERV-W	Human endogenous retrovirus-W	Human	AF135487
HIV-1	Human immunodeficiency virus type 1	Human	K03455
HML-1	Human MMTV-like element-1	Human	AF030038
HML-3	Human MMTV-like element-3	Human	HS453A3
HML-4	Human MMTV-like element-4	Human	AF020092
HML-5	Human MMTV-like element-5	Human	HSA1
HML-6	Human MMTV-like element-6	Human	AF079797
HML-7	Human MMTV-like element-7	Human	U91321
HRV-5	Human retrovirus-5	Human	U46939
HSRV	Human spumaretrovirus	Human	U21247
HTLV-1	Human T-cell leukemia virus type 1	Human	D13784
IAPha	Syrian hamster intracisternal A particle	Hamster	M10134
IAPm	Mouse intracisternal A particle	Mouse	M17551
JSRV	Jaagsiekte sheep retrovirus	Sheep/goat	M80216
MLV	AKV murine leukemia virus	Mouse	J01998
MMTV	Mouse mammary tumor virus	Mouse	M15122
MPMV	Simian Mason-Pfizer D-type retrovirus	Monkey	M12349
MuERV-L	Mouse endogenous retroviral sequence-L	Mouse	Y12713
PERV-A	Porcine endogenous retrovirus-A	Pig	AF38601
PERV-B	Porcine endogenous retrovirus-B	Pig	Y17013
PERV-C	Porcine endogenous retrovirus-C	Pig	AF38600
PERV-Ts	PERV-Tsukuba-1	Pig	AF038599
PERV-L	Porcine endogenous retrovirus-L	Pig	AJ233661
PERV-B3	Porcine endogenous retrovirus-B3	Pig	AF274712
PMSN-1	PERV miniature swine new-1	Pig	AF277320
PMSN-4	PERV miniature swine new-4	Pig	AF277322
WDSV	Walleye dermal sarcoma virus	Walleyed perch	AF033822

the percentage of 600 bootstraps at which a certain branch occurred. Values above 60% are not shown. A few branches in the HML cluster had values as low as 22% but are not shown because of space limitations.

Southern blot analysis of porcine genomic DNA for PERV-MSN1 and PERV-MSN4 sequences. Genomic DNA (5 µg) from miniature swine and domestic pigs (Clontech) was digested with *EcoRI* (New England Biolabs), fractionated on a 0.8% agarose gel, and transferred to nylon membranes (Schleicher and Schuell). A PCR product from a PMSN-1 or PMSN-4 clone with 5'-MOP-2 and 3'-MOP-2 primers was used as a template for [α - 32 P]dCTP random-primed probes (Amersham Pharmacia Biotech). The hybridizations were performed at 60°C for PMSN-1 and at 57°C for PMSN-4 for 1.5 h with ExpressHyb solution (Clontech) and then washed under high-stringency conditions (0.03 M NaCl, 0.03 M sodium citrate, and 0.1% sodium dodecyl sulfate [SDS] at 60°C for PMSN-1 and at 57°C for PMSN-4). The membranes were exposed for autoradiography at -70°C for 10 days before development. A single membrane was used and initially hybridized with the PMSN-1 probe as described previously. After development, the PMSN-1 probe was removed by boiling for 15 min in a mixture of 15 mM NaCl, 15 mM sodium citrate, and 0.5% SDS. The membrane was then probed for PMSN-4 sequences.

RNA preparation and cDNA synthesis. Total RNA was extracted from tissues or cells by using TRIzol reagent (Gibco Life Technologies) followed by cDNA synthesis with random hexamers and 0.5 to 1 µg of RNA with the Superscript preamplification system (Gibco Life Technologies) according to the manufacturer's instructions. The quality of the cDNA was tested by 18S rRNA PCR (data not shown).

PMSN-1- and PMSN-4-specific RT-PCR. One-tenth of the total cDNA reaction mixture was amplified with standard reagents and 2.5 U of AmpliTaq Gold

(Perkin-Elmer) for 35 cycles (96°C for 10 s, 59°C for 30 s, and 72°C for 30 s). PMSN-1 reverse transcriptase (RT)-PCR was performed with the forward oligonucleotide PMSN1F (5'-GCATGGAACCTACGGGG-3') and reverse oligonucleotide PMSN1R (5'-GAAAGGCTCAGCATCTGTG-3'). PMSN-4 RT-PCR was performed with the forward oligonucleotide PMSN4F (5'-TGCAATCCCC TTAGACTGGG-3') and reverse oligonucleotide PMSN4R (5'-CGGCAACAC TTCCACTGA-3'). PCRs were electrophoresed on 2% agarose gels stained with ethidium bromide and analyzed for the presence of the expected products.

Nucleotide sequence accession number. The nucleotide sequences of PMSN-1.1 and -1.2 have been submitted to GenBank under accession no. AF277320 and AF277321, respectively. The nucleotide sequence of PMSN-4 has been submitted to GenBank under accession no. AF277322.

RESULTS

Identification of PERV *pol* genes. Pan-*pol* PCR with degenerate primers corresponding to conserved regions of known retrovirus *pol* genes was performed and yielded products of the expected size of approximately 640 bp. The PCR products were grouped by restriction enzyme digestion, which identified seven different groups of amplicons (Materials and Methods). The majority of the clones fell into two groups of 61 and 14 clones out of a total of 84. The first group was designated PERV miniature swine new-1 (PMSN-1), and the sec-

ond group was designated PMSN-4. Partial nucleotide sequence analysis established that these two main groups contained sequences that were of retroviral origin. The remaining five groups contained sequences corresponding to noninfectious, nonretroviral porcine retroelements (e.g., porcine LINEs) and will not be discussed further. Representative clones from each main group were subjected to complete nucleotide sequence analyses (described below).

The nucleotide sequences of a total of three PMSN-1 clones obtained from different miniature swine were determined. Alignment of nucleotide sequences derived from these PMSN-1 clones showed that two were identical (PMSN-1.1) and that the third clone (PMSN-1.2) displayed differences at 11 positions (seven nucleotide substitutions and two 2-bp insertions). Restriction analysis of a small number of PMSN-1 clones showed approximately equal numbers of PMSN-1.1 and PMSN-1.2 varieties (data not shown). The nucleotide sequences of PMSN-1.1 clones were used for phylogenetic analysis. Both varieties of PMSN-1 have single frameshift mutations and four premature stop codons. The nucleotide sequences of three PMSN-4 clones obtained from different miniature swine were determined. The clones were derived from three different miniature swine. The nucleotide sequences of all three clones were identical and possessed three frameshift mutations and four premature stop codons.

The *pol* genes of PMSN-1 and PMSN-4 are related to betaretroviruses. Phylogenetic alignments were performed based on translated sequences from the amino terminus of available *pol* sequences in the databases, as well as a recently identified pig betaretrovirus sequence denoted PERV-B3 (26a), by using a stretch from the motif AINA and its analogs to two amino acids before the superconserved motif YVDD. The alignment had 129 columns. The complete alignments are available at <http://www.kvir.uu.se> at the Uppsala University subdirectory. The phylogenetic tree obtained is presented in Fig. 1. The abbreviations and identities of all viruses included in the tree are listed in Table 1. In this analysis, PMSN-1 clustered between PMSN-4 and HML-6, while PMSN-4 was between JSRV and PMSN-1, and PERV-B3 was most similar to HML-1 (Fig. 1). These three novel PERV sequences branched together with the HML sequences, with only HRV-5 and the intracisternal A particles (IAPs) being clearly more ancestral relative to the rest of the family *Retroviridae* (Fig. 1). We conclude that the three novel PERV sequences belong to genus *Betaretrovirus*, and although they are unique, they are related to several human MMTV-like (HML) sequences.

Southern blot analysis of PMSN-1 and PMSN-4 in the miniature swine genome. To estimate the number of PMSN-1 and PMSN-4 loci in the miniature swine genome, Southern blot analysis of *Eco*RI-digested genomic DNA prepared from 11 different miniature swine and from a single domestic pig was performed (Fig. 2). Initially the filter was hybridized with a PMSN-1 probe. In one animal, the apparent lack of hybridizing bands was due to the small amount of DNA loaded in each lane, because weak signals were evident upon prolonged exposure (not shown). This blot indicates that at least two copies of PMSN-1 are present within the miniature swine as well as domestic swine genomes. Some variation was evident, with some animals having singly or doubly hybridizing bands of 4, 6, and/or 7 kb.

FIG. 1. Phylogenetic tree analysis. The tree is based on an alignment and calculations of N-terminal protein sequences from ERV *pol* sequences listed in Table 1. Numbers denote the percentage of 600 bootstraps in which a certain branch occurred. Values above 60% are not shown. A few branches in the HML cluster had values as low as 22% but are not shown because of limited space. Four sequences in the betaretrovirus cluster are indicated to the right of the tree due to space limitation. Exogenous retroviruses, some of which are also known to be endogenous, are shown in italics.

The same filter was used for hybridization with the PMSN-4 probe after the PMSN-1 probe was removed (Materials and Methods). One major hybridizing band of 11 kb was found in all swine (Fig. 2B, lanes 2 to 9, 11, and 12) along with additional weak bands, indicating at least a single copy of PMSN-4. The fainter bands may represent divergent copies of PMSN-4 loci or cross-hybridization to related PERV sequences.

Expression of PMSN-1 and PMSN-4 mRNA. The mRNA expression of PMSN-1 and PMSN-4 *pol* was analyzed by RT-PCR with RNA prepared from several porcine tissues. The 13 different cell types and tissues that were used for RNA preparation and the MHC haplotype and animal designations are listed together with the results obtained (Table 2). Two MHC homozygous animals (*SLA^{a/a}*), one MHC heterozygous animal (*SLA^{a/d}*), one *SLA^{g/g}* homozygous animal, and fetal tissues from one *SLA^{d/d}* homozygous animal were analyzed.

Representative results are shown in Fig. 3. The PMSN-1 PCR produces a 422-bp product for PMSN-1.1 and a 426-bp product for PMSN-1.2 that could not be separated by the agarose gel electrophoresis employed (Fig. 3A). The PMSN-4 PCR produced a 155-bp product (Fig. 3B). The mRNA expression of both PMSN-1 and PMSN-4 showed individual variation between different miniature swine, with PMSN-1 showing a broader range of tissues and cell types. The highest mRNA expression of PMSN-1 was detected in salivary glands, in one fetal liver sample, and in *c-kit⁺* (CD117⁺) hematopoietic progenitor cells (Table 2 and Fig. 3). All seven fetal tissues were found to be positive for expression. PMSN-4 expression appeared to be greatest in *c-kit⁺* enriched bone marrow cells.

FIG. 2. Genomic DNA prepared from miniature swine was analyzed by Southern blotting of PMSN-1 (A) and PMSN-4 (B). Lane 1 contains partially *Eco*RI-digested *SLA^{d/d}* miniature swine genomic DNA, followed by nine distinct *SLA^{d/d}* miniature swine genomic DNA samples (lanes 2 and 3 and 5 to 11; lane 10 is extremely faint). In lane 4, genomic DNA from an *SLA^{c/c}* haplotype miniature swine was analyzed. Lane 12 contains domestic pig genomic DNA. All samples were digested with *Eco*RI. Sizes in kilobase pairs of the hybridizing bands are indicated to the right. The filter was exposed for 10 days in both cases.

DISCUSSION

Transmission of human-tropic gammaretrovirus PERVs constitutes a potential risk in pig-to-human xenotransplantation (9, 34). To better understand such risks, further molecular and functional characterization of PERVs is warranted. Therefore, we employed a pan-*pol* PCR approach using degenerate retroviral *pol* primers to identify PERVs that belong to other retroviral genera. In this study, two novel PERV *pol* elements denoted PMSN-1 and PMSN-4 were identified.

Phylogenetic analyses revealed that both PMSN-1 and PMSN-4 cluster with betaretroviruses. Together with PERV-B3 (26a), these sequences represent the first described PERVs belonging to this genus. Furthermore, these sequences diverged close to the *Betaretrovirus* root, suggesting a relatively distant relationship to previously known mammalian betaretroviruses. This is also reflected by the overall low nucleotide sequence similarities of approximately 60% between PMSN-1 and other betaretrovirus sequences (not shown). PMSN-1 was found to be most similar to HML-6 (21). A description of the HML groups was recently published (3). PMSN-4 appeared more distantly related to betaretrovirus sequences, branched

TABLE 2. Expression of PMSN-1 and PMSN-4 mRNA in miniature swine tissues

Tissue designation	Haplotype	Expression of:	
		PMSN-1	PMSN-4
13463, heart	<i>a/a</i>	Not done	+
13432, bone marrow	<i>a/d</i>	+	+
13432, <i>c-kit</i> ⁺	<i>a/d</i>	++	++
12694, spleen	<i>a/a</i>	—	—
12694, liver	<i>a/a</i>	+	—
12694, thymus	<i>a/a</i>	—	—
12694, PBL	<i>a/a</i>	—	—
12694, lung	<i>a/a</i>	—	—
13590, bone marrow	<i>g/g</i>	+	—
13590, adrenal	<i>g/g</i>	+	—
13590, thyroid	<i>g/g</i>	+	—
13590, lung	<i>g/g</i>	—	—
13590, liver	<i>g/g</i>	+	—
13590, kidney	<i>g/g</i>	+	—
13590, spleen	<i>g/g</i>	+	—
13590, lymph node	<i>g/g</i>	—	+
13590, salivary gland	<i>g/g</i>	+++	—
13590, thymus	<i>g/g</i>	++	+
13590, <i>c-kit</i> ⁺	<i>g/g</i>	+	—
13590, <i>c-kit</i> [—]	<i>g/g</i>	—	—
13363, bone marrow	<i>h/h</i>	+	—
Fetal liver		+++	—
Fetal liver	<i>d/d</i>	+	—
Fetal lung	<i>d/d</i>	+	—
Fetal kidney	<i>d/d</i>	+	+
Fetal salivary gland	<i>d/d</i>	+	+
Fetal heart	<i>d/d</i>	+	—
Fetal spleen	<i>d/d</i>	+	—
Fetal thymus	<i>d/d</i>	+	+

off closer to the root, and was closely related to Jaagsiekte sheep retrovirus (JSRV), MMTV, and Mason-Pfizer monkey virus (MPMV). PERV-B3 was more similar to PMSN-1 than to PMSN-4 and clustered with HML-5. As seen in Fig. 1, PERVs span a sequence spectrum similar to that of human ERVs (HERVs). This may indicate a pool of common ERVs in vertebrates created by interspecies transfers during vertebrate evolution. An alternative explanation is that the *pol* sequences originated from a common ancestor of modern host species.

Endogenous betaretroviruses may be biologically active, and some HERV-K loci show conservation of complete and intact retroviral genes with open reading frames (ORFs) encoding Gag, Pol, and Env proteins (4, 20, 22). Enzymatic activities for the HERV-K dUTPase, protease, and endonuclease have been reported (10, 14, 31). Recently, it was shown that several HERV-K members also encode functional RT polymerase and an RNase H domain (4).

As discussed above, while the *pol* genes of betaretroviruses are highly similar (22, 42), the viruses diverge into two categories, with *env* genes resembling either betaretroviruses or the murine leukemia virus (MLV)-like group of gammaretroviruses. This feature implies that a recombination event involving the *env* gene from an MLV-like virus into one resembling MMTV at some point occurred during evolution (7). MMTVs exist in both endogenous and infectious exogenous forms. Moreover, the role of MMTV integration and the development of mammary carcinoma are well-established features

FIG. 3. RT-PCR analysis of PMSN-1 and PMSN-4. Agarose gel electrophoresis was performed with PMSN-1 (A) and PMSN-4 (B) RT-PCR products obtained from RNA prepared from the following cell and tissue types: lanes 1 and 8, *c-kit*⁺ cells; lanes 2 and 9, whole bone marrow (RNAs were prepared from an *SLA*^{alid} heterozygous miniature swine). The remaining samples (lanes 3 to 7 and 10 to 14) were prepared from cells obtained from an *SLA*^{alid} homozygous miniature swine. Lanes 3 and 10, spleen; lanes 4 and 11, liver; lanes 5 and 12, thymus; lanes 6 and 13, peripheral blood lymphocytes; lanes 7 and 14, lung. Lanes 8 to 14 represent negative controls without the presence of RT. In panel A, control amplifications with a PMSN-1 PCR product (lane 15), a negative control (lane 16), and miniature swine genomic DNA (lane 17) are shown. In panel B, heart +/- RT (lanes 15 and 16), control amplifications with a PMSN-4 PCR product (lane 17), miniature swine genomic DNA (lane 18), and a negative control (lane 19) are shown. The sizes of the products obtained are indicated to the left.

(36). Betaretrovirus particles have been identified from several mammalian species (11, 13, 32). Their established role in pathogenesis in other species is an additional cause of concern for pig-to-human xenotransplantation. However, based on our results, a role for pig betaretroviruses in transmitted disease appears unlikely. Southern blot analysis showed that only a relatively few copies of these PERVs are present in the porcine genome. In both the PMSN-1 and -4 *pol* sequences described here, as well as in the PERV-B3 *pol* sequence, mutations disrupting the ORFs were present, which suggests that the few copies present are most likely defective.

Transcription of these novel *pol* sequences was shown in several different cell types by RT-PCR. Individual variation of PMSN-1 expression between different animals was evident. Differences in expression of human betaretrovirus sequences between individuals and between different tissues were reported earlier (2, 41). The expression of PMSN-1 mimics the expression pattern of HERV-K (HML-2), to which PMSN-1 is relatively closely related (Fig. 1) (4). In contrast, PMSN-4 mRNA expression had a more limited tissue distribution. Among the different cell types analyzed, the highest PMSN-4 mRNA expression was detected in hematopoietically derived cells, like bone marrow cells enriched for the expression of *c-kit*⁺ (CD117⁺), and in fetal liver. However, due to the highly mutated and defective nature of the clones sequenced, it appears unlikely that these PERVs will pose risks in xenotransplantation.

In vitro studies have shown transmission of gammaretrovirus PERVs to human cells (25, 39, 40). However, no evidence of PERV transmission to human recipients of xenografted cells has been documented, and productive in vivo PERV transmission to human recipients of pig cells appears to be unlikely (5,

12, 23, 26). Recombination between defective porcine and human retroviral sequences that would generate a pathogenic retrovirus seems only a remote possibility. The identification of the betaretrovirus PERV sequences described here will allow further assessment of the potential risks associated with PERV transmission following xenotransplantation of miniature swine organs.

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