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Establishment and Characterization of Molecular Clones of Porcine Endogenous Retroviruses Replicating on Human Cells

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The use of pig xenografts is being considered to alleviate the shortage of allogeneic organs for transplantation. In addition to the problems overcoming immunological and physiological barriers, the existence of numerous porcine microorganisms poses the risk of initiating a xenozoonosis. Recently, different classes of type C porcine endogenous retoviruses (PERV) which are infectious for human cells in vitro have been partially described. We therefore examined whether completely intact proviruses exist that produce infectious and replication-competent virions. Several proviral PERV sequences were cloned and characterized. One molecular PERV class B clone, PERV-B(43), generated infectious particles after transfection into human 293 cells. A second clone, PERV-B(33), which was highly homologous to PERV-B(43), showed a G-to-A mutation in the first start codon (Met to Ile) of the *env* gene, preventing this provirus from replicating. However, a genetic recombinant, PERV-B(33)/ATG, carrying a restored *env* start codon, became infectious and could be serially passaged on 293 cells similar to virus clone PERV-B(43). PERV protein expression was detected 24 to 48 h posttransfection (p.t.) using cross-reacting antiserum, and reverse transcriptase activity was found at 12 to 14 days p.t. The transcriptional start and stop sites as well as the splice donor and splice acceptor sites of PERV mRNA were mapped, yielding a subgenomic *env* transcript of 3.1 kb. PERV-B(33) and PERV-B(43) differ in the number of copies of a 39-bp segment in the U3 region of the long terminal repeat. Strategies to identify and to specifically suppress or eliminate those proviruses from the pig genome might help in the production of PERV-free animals.

Xenotransplantation, i.e., the therapeutic use of live cells, tissues, and organs from nonhuman animals, offers the chance to alleviate the shortage of human donor organs. Clinical trials for testing the general applicability of pig cells or tissues as an alternative to allogeneic source materials are ongoing. Those trials include the infusion or implantation of (encapsulated) pancreatic islet cells as a treatment for insulin-dependent diabetes mellitus (31), the implantation of fetal neuronal tissue as a therapy for Parkinson's disease (17), extracorporeal kidney perfusion (9), and perfusion through or the implantation of whole liver preparations as a treatment for hepatic failure (12, 16).

The possibility of introducing infectious agents from the animal into the individual xenograft recipient and ultimately to the public, leading to xenozoonosis, also termed xenosis, has raised numerous concerns (2, 24, 46, 62). As several pathogenic agents, including immunodeficiency viruses, have been transmitted from nonhuman primates to humans (27, 34), and as human immunodeficiency virus type 1 began as a zoonosis, probably from chimpanzees (26), nonhuman primates are considered inappropriate organ donors for xenotransplantation (10, 11; U.S. Department of Health and Human Services, Food and Drug Administration, Center for Biologics Evaluation and Research (CBER), Public health issues posed by the use of nonhuman primate xenografts in humans, April 1999, http://www.fda.gov/cber/gdlns/xenoprim.pdf), even though their tissues and organs more closely resemble those of humans than those of phylogenetically more distant animals such as pigs and are therefore less susceptible to rejection.

Pig organs are preferred for transplants (23), particularly since different strategies have been developed to overcome the major obstacle of hyperacute rejection. Enzymatic modifications of the carbohydrate surface of xenogeneic cells significantly reduce human antibody binding and complement-mediated cytolysis (4, 55, 58). In addition, affinity isolation of xenoreactive human natural antibodies of the immunoglobulin M class, of which a major fraction is directed against the terminal α-galactose determinants on porcine endothelial cells, was used to lower the natural humoral immunologic barrier to xenotransplantation (50). Inhibition of the second element responsible for hyperacute rejection, complement activation, is being pursued mainly by the use of transgenic pigs whose organs partially overcome xenograft rejection by constantly expressing human regulators of complement activation (15, 25, 42) but also by administration of complement-inhibitory therapeutics (53). As an alternative, attempts have been undertaken to establish host-specific transplantation tolerance to pig organ grafts (30). The possibility that human pathogens could be transmitted more easily by use of transgenic pigs, however, has been proposed (69).

Breeding and keeping pigs under specific-pathogen-free or qualified-pathogen-free conditions is generally assumed to reduce the potential risk of transmitting exogenous viral, bacterial, fungal, and parasitic agents by xenotransplantation. However, the remaining microbiologic obstacles include pathogens such as those which can infect fetuses congenitally or which can be stably transmitted in the germ line and other unknown or less characterized agents, e.g., porcine herpesviruses (22).

Retroviruses that reside in the pig genome (porcine endogenous retroviruses [PERV]) and show similarities to C-type viruses of other species were described almost 30 years ago (3, 7, 8, 38). Recent studies have shown that PERV released from

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porcine kidney cell lines (PK15) (52), from mitogenically activated porcine peripheral blood mononuclear cells (PBMC) (72), or from porcine aortic endothelial cells (41) are capable of infecting human cells in vitro. Approximately 50 proviral integration sites exist in the genome of different pig breeds (1, 37, 52), and at least three subtypes of PERV are known to exist (37, 63). Those PERV display very high homologies in the *gag* and *pol* genes but differ in their *env* genes; especially in the areas encoding the VRA, VRB, and PRO regions of the gp70 protein (1, 37). These regions are known to determine the host range specificities (5, 6). Although a full-length PERV cDNA has been generated from miniature swine lymphocytes (1), the designated sequence PERV-MSL is barely infectious for human cell lines in comparison with PERV class A and class B, as judged from pseudotype assays using the appropriate *env* genes (63).

Initial retrospective studies on patients who had been transplanted with porcine tissues revealed no detectable transmission of PERV (33, 51). Likewise, in a cohort of 160 patients who had been treated with various living pig tissues, no conclusive evidence of pig-to-human transmission of PERV was found, although persistent microchimerism was observed in 23 patients for up to 8.5 years (49). The treatment of the patients included short-term extracorporeal liver, kidney, and splenic perfusions, bioartificial liver perfusion, implantation with pancreatic islet cells, and transplantation of skin. The detection methods employed were based on both DNA- and RNA-dependent PCR with PERV-specific primers and on Western blot-based serological assays to detect antibody responses to PERV. As only 4 of the 23 samples showing microchimerism were PERV positive, the question of PCR contamination was raised (70). Basically, these studies suggest that PERV will not show the very high levels of transmission associated with some viruses (60). As only clinical trials will enable long-term xenograft survival and function to be tested, and as retroviruses have extended clinical latency periods following infection and therefore are difficult to identify in the absence of demonstrable disease, upcoming studies must be conducted in a well-coordinated, prospective, and stepwise manner, making use of the recently developed assays to monitor putative PERV infection and transmission to contacts.

In this communication, we report the cloning of pig-specific, polytropic proviruses isolated from the human embryonal kidney cell line 293 infected with PERV. Complete sequencing of PERV class B has been performed. Two proviral clones produced infectious and replication-competent particles after transfection into human 293 cells.

MATERIALS AND METHODS

Cell lines, transfection, and replication studies. The cell lines PK15 and 293 and a 293 cell line productively infected with PERV derived from PK15 cells, 293 PERV-PK, were kindly provided by R. Weiss (London). HeLa cells were obtained from the American Type Culture Collection (CCL-2). Plasmid DNA (1 to 10 μg) prepared with the EndoFree system (Qiagen, Hilden, Germany) was transfected into cells using lipofectamine (Life Technologies, Karlsruhe, Germany). Virus replication was detected by reverse transcription-PCR (RT-PCR), immunofluorescence microscopy with cross-reacting feline leukemia virus (FeLV) Gag antiserum, or by reverse transcriptase (RT) assays. Infectivity was confirmed by placing virions derived from cell-free supernatants of producer cells after filtration through 0.45-μm-pore-size membranes (Sartorius, Göttingen, Germany) on semiconfluent 293 or HeLa cells.

RT assay. Cell-free supernatants filtered through membranes (0.45-μm pore size) were analyzed for RT activity using the C-type RT activity assay (Cavidi Tech AB, Uppsala, Sweden) according to the instructions of the manufacturer (protocol B).

Immunofluorescence microscopy. 293 and HeLa cell lines transfected with recombinant plasmid DNA were fixed 24 to 48 h posttransfection (p.t.). Indirect immunofluorescence analysis was performed with a laser scan microscope as described previously (64). Polyclonal cross-reacting goat anti-FeLV p27 (CA) antiserum (Biodesign International) was used for detection of PERV Gag in a 1:500 dilution. Secondary goat antibodies conjugated with Cy3 (Indocarbocyanin) (Dianova, Hamburg, Germany) were employed.

Electron microscopy. Electron microscopy was performed according to standard procedures as described previously (65).

Immunoblotting. Sucrose gradient-purified PERV particles were analyzed by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS–10% PAGE) (36) and Western blotting (67) with polyvinylidene difluoride membranes (Millipore, Eschborn, Germany). Blots were incubated with a 1:5,000 dilution of goat anti-FeLV CA antiserum (Biodesign International) for 1 h or overnight, followed by a 1:10,000 dilution of protein G-conjugated horseradish peroxidase (Bio-Rad, Munich, Germany) for 1 h. Immunoreactive proteins on membranes were detected with the enhanced chemiluminescence system and exposure for 15 to 20 s on hyperfilm ECL (Amersham-Pharmacia, Freiburg, Germany).

Preparation of genomic DNA. Preparation of high-molecular-weight DNA from PBMC of different species and from cell lines was performed according to standard protocols (54).

Southern blot hybridization. Genomic cellular DNA was digested with different restriction endonucleases (10 to 40 U/μg of DNA; New England Biolabs, Frankfurt, Germany), separated in 0.7% TAE (Tris-acetate-EDTA)–agarose gels and alkali blotted onto Porablot NY Amp nylon membranes (Macherey-Nagel, Düren, Germany). Hybridization was performed with a radiolabeled *pro*/*pol* 753-bp PERV cDNA overnight at 65°C. The probe was generated by RT-PCR from mRNA of PK15 cells. Primers PK1 (TTGACTTGGGAGTGGG ACGGGTAAC, nucleotides [nt] 2927 to 2949) and PK6 (GAGGGTCACCTG AGGGTGTTGGAT, nt 3739 to 3716) in a first PCR and primers PK2 (GGTA ACCCACTCGTTTTCTGGTCA, nt 2944 to 2966) and PK5 (CTGTGTAGGG CTTCGTCAAAGATG, 3696 to 3673) in a nested PCR were derived from a published sequence (accession no. U77599). Nucleotide sequence assignments in this publication are based on the PERV-B(33) sequence (accession no. AJ133816) (see below). Filters were washed several times under stringent conditions with 2× SSC (0.15 M NaCl, 0.015 M sodium citrate)–0.1% SDS at ambient temperature and with 0.2× SSC–0.1% SDS at 65°C. Autoradiography was done for 48 to 96 h. For rehybridizations, filters were stripped and incubated with a radiolabeled 2.4-kb *Nar*I-*Bam*HI fragment of the single-copy human H1° histone gene harboring the H1° coding and 3′-flanking sequence (HSHIS10G; accession no. X03473 [20]) to reveal equal loading of DNA samples. Probes were labeled with the Multiprime DNA labeling system (Amersham-Pharmacia) with [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham-Pharmacia) as the radionucleotide.

RNA isolations. Total RNA was isolated with Trizol reagent (Life Technologies) according to the instructions of the manufacturer. Polyadenylated RNA was isolated from total cellular RNA using a PolyATract mRNA isolation kit (Promega, Mannheim, Germany).

Northern blot hybridization. For analysis of PERV mRNA expression in virus-producing cells, RNA was denatured in glyoxal according to standard techniques (54). Polyadenylated RNA (0.5 to 2.0 μg) was separated in 1% agarose–sodium phosphate (pH 6.8) gels. After capillary transfer onto nylon membranes, UV cross-linking, and additional baking of membranes for 2 h at 80°C, hybridization was done at 65°C overnight. In contrast to the Southern blot analysis, the first-stringency washing steps were performed at 42°C. A PERV long terminal repeat (LTR) U5 sequence was amplified with primers U5-for (GTGACGCACAGGCTTTGTTG, nt 489 to 508) and U5-rev (GTAAAAGA ACAATCCCCCTCGTC, nt 697 to 675). The radiolabeled U5 209-bp amplificate and the *pro*/*pol* 753-bp PERV cDNA were used as hybridization probes.

RT-PCR. Oligo(dT)-primed cDNA was synthesized employing the SuperScript preamplification system (Life Technologies) and total RNA or mRNA as the template. After RNase H treatment, 1/10 of the cDNA reaction volume was used for PCR with gene-specific primers.

Determination of splice donor and splice acceptor sites. PCR was performed with cDNA generated from 293 PERV-PK mRNA with primers PERV-PBS (GTTGGCCGGGAAATCCTGCG, nt 716 to 735) and env-R (GAGTAAGGA ACCACGCGATGGAG, nt 6291 to 6269). The PCR profile included an initial denaturation at 94°C for 10 min, 30 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C, and a final elongation for 10 min at 72°C. A 20-pmol amount of each primer and 2.5 U of AmpliTaq Gold (Perkin-Elmer Cetus, Norwalk, Conn.) were used. The elongation steps for 1 min were chosen for preferentially generating amplification products derived from subgenomic transcripts. The PCR product was cloned into pGEM-T Easy (Promega) and sequenced.

5′RACE. The Marathon cDNA amplification kit (Clontech, Heidelberg, Germany) was used for 5′ rapid amplification of cDNA ends (5′RACE) experiments according to the manufacturer's instructions with mRNA as the template. The 5′RACE products generated with anchor primer 1 in combination with PERV-specific primer PK26 (ACGCACAAGACAAAGACACACGAA, nt 1134 to 1111) were cloned into pCRII-Topo (Invitrogen, Groningen, The Netherlands) and sequenced.

Primer extension. Oligonucleotide PK-REV-PE (5′-GCAAACAGCAAGAG GATTTTTATTCCAAGCGCGCTG-3′, nt 623 to 588), which hybridizes in the LTR close to the anticipated transcriptional start site, was 5′-end labeled. In a 20-μl reaction volume containing 1× One-Phor-All buffer (Amersham-Pharmacia), 100 μCi of [γ-^{32}P]dATP, and 20 U of FPLC*pure* T4 polynucleotide kinase (Amersham-Pharmacia), 50 pmol of 5′ termini was incubated for 30 min at 37°C.

The radiolabeled primer was used in a first-strand synthesis for RT-PCR. After RNase H treatment, the RT-PCR product was separated on a 6% polyacrylamide sequencing gel and compared with the results of Sanger sequencing reactions with unlabeled oligonucleotide PK-REV-PE as the primer and the cloned 5′ LTR (pPERV-PK26/34) as the template. Sequencing reactions were carried out with the T7 sequencing kit (Amersham-Pharmacia).

PCR amplification of PERV DNA sequences. Genomic sequences containing proviral segments were initially cloned by PCR techniques. First, inverse PCR as described before (66) was performed. In brief, genomic DNA from 293 cells infected with PK15-derived PERV was digested with different restriction endonucleases (NEB), generating blunt ends. After heat inactivation and alcohol precipitation, 1 to 5 μg of DNA was self-ligated overnight in a 300-μl volume. After alcohol precipitation, circularized DNA fragments were resuspended in 50 μl of water, of which 2.5 μl served as the template for PCR with inverse-oriented oligonucleotides. PCR was carried out starting with an initial denaturation step at 94°C for 10 min, followed by 35 cycles of 45 s at 94°C, 45 s at 61°C, and 6 min at 72°C. An additional six cycles with an annealing temperature of 58°C were performed. The final extension was done for 10 min at 72°C. Each PCR mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, a 0.2 mM concentration of each deoxynucleoside triphosphate, 20 pmol of each primer, and 2.5 U of AmpliTaq Gold (Perkin Elmer Cetus). The primers PK7 (CCCACCCATCACCCAGGATTTTTT, nt 2884 to 2861) and PK9 (AGG GTTCAAGAACTCCCCGACCAT, nt 3651 to 3675) were used to generate 3-kb *Sma*I- and 2-kb *Pvu*II-religated DNA amplificates, respectively. A nested PCR with PK3 (CATCTTTGACGAAGCCCTACACAG, nt 3673 to 3696) and PK8 (TTCCTAATGGTTGTAGCAGCACTG, nt 2849 to 2826) in a second amplification generated a 3.5-kb product using self-ligated *Nae*I-digested DNA as the template. Two more inverse PCR products, a 2.2-kb *Pvu*II and a 0.6-kb *Rsa*I amplificate, were obtained using primers PK10 (AGGCGCTCACTGGGAAGT GGACTT, nt 5434 to 5457) plus PK12 (GGTGGCTTCCCCTTAGTCTCTTTC, nt 5432 to 5409) and PK20 (TGGTCGGTTATACCGATAGTCATA, nt 7559 to 7536) plus PK22 (AAAGAGAACCCGTATCCCTTACCC, nt 7561 to 7584), respectively. Amplification products were gel purified, cloned into pGEM-T Easy (Promega) or pCRII-Topo (Invitrogen), and partially sequenced.

Based on this sequence information, the entire PERV *gag-pol-env* gene cassette was directly amplified using the Expand long-template PCR system (Boehringer, Mannheim, Germany). PCR was performed in a 50-μl volume containing 50 mM Tris-HCl (pH 9.2), 16 mM $(NH_4)_2SO_4$, 1.75 mM $MgCl_2$, a 0.2 mM concentration of each deoxynucleoside triphosphate, 20 pmol each of primers PK29 (5′-ATCAGCAGACGTGCTAGGAGGATC-3′, nt 895 to 918) and PK28 (5′-CCACGCAGGGGTAGAGGACTGCGG-3′, nt 8781 to 8758), 100 ng of genomic DNA from PERV-infected 293 cells, and 2.5 U of enzyme mix, according to the instructions of the manufacturer. Cycle conditions included an initial denaturation at 94°C for 2 min and 10 cycles of 30 s at 94°C, 45 s at 65°C, and 9 min at 68°C. The following 25 cycles had an increment of 10 s in the elongation step. The final extension was done for 30 min at 72°C.

Likewise, primers PK34 (AAAGGATGAAAATGCAACCTAACC, nt 3 to 26) and PK26 (see above) were used for direct amplification of a PERV 5′ LTR sequence. Amplification products were gel purified and cloned into pGEM-T Easy (Promega). The resulting clones, pPERV-PK28/PK29 and pPERV-PK26/PK34, containing 7.8 and 1.0 kb of PERV sequences, respectively, were further characterized.

Detection of integrated PERV by PCR. PERV *pro-pol* sequences were PCR amplified from cellular DNA after transfection with primers PK44 (CTGGAA TTGTCTGACCTAG, nt 3815 to 3833) and PK46 (GCCATCCTCTTACCTTC CAC, nt 4689 to 4670) in a first PCR and primers PK45 (GCTAAGAAGGCC CAGATTTGC, nt 3848 to 3868) and PK47 (CTTCCGTCAGTGAACCAGGT TAG, nt 4659 to 4637) for nested PCR. PERV *env* sequences were amplified with primers PK17 (GCTAATCTTCCAGAATACCTCCAG, nt 5384 to 5408) and PK19 (CCCTAATCCGAGCATTACAGCTAG, nt 7607 to 7584).

Generation of a human λ bacteriophage library. For the construction of a genomic DNA library, high-molecular-weight DNA from 293 PERV-PK cells was partially digested with *Sau*3AI and cloned into the lambda Fix II/*Xho*I partial fill-in vector (Stratagene, Amsterdam, The Netherlands). Phage particles were generated according to the manufacturer's instructions using Gigapack XL packaging extract (Stratagene). The bacteriophage library, after one round of amplification, was screened with the ^{32}P-labeled *pro-pol* probe (see above). Recombinant λ clones were purified to homogeneity, and bacteriophage DNA was prepared using the Nucleobond AX ready-to-use system (Macherey-Nagel).

Subcloning. DNA from λ bacteriophage clones was digested with *Not*I (10 U/μg of DNA; NEB) and separated on standard 0.7% TAE–agarose gels. Inserts were gel purified using the JETsorb DNA extraction kit (Genomed, Bad Oeynhausen, Germany), ligated into the *Not*I site of pBS-KS (Stratagene), and transformed into *Escherichia coli* DH10B.

Plasmid pPERV-B(33)/ATG is a derivative of pPERV-B(33) which showed a G-to-A mutation at nt 6191 in the first start codon of the *env* open reading frame (ORF). Basically, pPERV-B(33)/ATG was generated by replacing a *Bsr*GI restriction fragment (nt 5920 to 7245) from pPERV-B(33) with the cognate fragment from pPERV-PK28/PK29. The replacement of the 1,326-bp restriction fragment was performed in a plasmid containing an *Eco*RV-*Sph*I fragment (nt 2321 to 7410) of pPERV-B(33) subcloned into pGEM-5 (Promega). Finally, an *Nde*I-*Sph*I fragment (nt 5199 to 7410) of the resulting plasmid, pGEM-5-*Eco*RV-PERV-B(33)-*Sph*I-ATG, containing the ATG triplet was ligated into appropriately digested pPERV-B(33).

Sequence analysis. The DNA sequences of both strands were determined by primer walking with the double-stranded dideoxy chain termination method using Thermo Sequenase fluorescent dye terminator cycle sequencing and precipitation kits according to the instructions of the manufacturer (Amersham-Pharmacia). Sequencing reactions were performed in a Vistra DNA Labstation 625 (Molecular Dynamics, Krefeld, Germany) and cycle sequenced on an ABI 373A or 377 DNA sequencing system (Applied Biosystems, Weiterstadt, Germany) at the Paul Ehrlich Institute or by Agowa GmbH, Berlin, Germany. All primers were commercially purchased (Eurogentec, Seraing, Belgium; ARK Scientific, Damnstadt, Germany).

Nucleotide sequence accession numbers. The complete nucleotide sequences of pPERV-PK28/29 (Y17013), pPERV-PK26/34 (Y17012), PERV-B (33) (AJ133816), and PERV-B(43) (AJ133818) have been deposited in GenBank.

The sequences used for homology studies are PERV-MSL (AF038600) (1), Tsukuba-1 (AF038599) (1), PK-15 ERV (AF038601) (1), Moloney murine leukemia virus (MoMLV) (J02255, J02256, and J02257) (59), gibbon ape leukemia virus (GaLV) (M26927) (18), baboon endogenous virus (BaEV) (D10032, D00088, and N00088) (35), FeLV (M18247 and M19392) (21), and human endogenous retrovirus ERV3 *pol-env* 3′ LTR (M12140) (14).

RESULTS

Porcine type C retroviruses are released from productively infected human 293 cells. The human kidney cell line 293, which had been originally infected with PERV virions derived from porcine kidney cell line PK15 (293 PERV-PK [52]) and which permanently releases particles, was used in our studies. Virions produced by 293 PERV-PK cells were serially passaged on 293 cells using RT assays to monitor retroviral replication (data not shown). As revealed by ultrastructural analysis (Fig. 1), the morphology of the viruses produced by freshly infected human 293 cells is typical of a type C retrovirus, as reported for pig kidney cell lines (3). These infected human cells were employed for subsequent molecular characterization and cloning of PERV sequences.

PERV sequences are specific to pigs. A PERV 753-bp *pro-pol* probe was generated by RT-PCR with mRNA from 293 PERV-PK cells. A variety of animal DNA samples were investigated in a genomic Southern blot analysis, demonstrating that PERV sequences could only be detected in DNA of porcine origin, such as PBMC and PK15 cells (Fig. 2, lanes 3 and 7), but not in mouse, dog, sheep, goat, bovine, or human DNA (Fig. 2) even though (endogenous) murine leukemia virus (MLV) *pol* sequences show up to 64% homology to PERV sequences (see below). This result indicated that proviral PERV sequences exist in several integration sites in the cell line 293 PERV-PK. The intensity of a ~2.1-kb *Eco*RI-hybridizing marker fragment in 293 PERV-PK DNA (Fig. 2, lane 9) was as strong as in porcine PBMC or PK15 DNA (Fig. 2, lanes 3 and 7). At least two more hybridizing fragments were detected in 293 PERV-PK DNA but at lower intensities (Fig. 2, lane 9). A rehybridization analysis with a probe derived from the single-copy histone $H1^o$ gene, which is well conserved throughout evolution (20), demonstrated equal loading of DNA (data not shown), suggesting that at least five integrated proviral copies of PERV exist in the cell line 293 PERV-PK. This estimation was confirmed by a Southern blot analysis with different restriction enzymes for 293 PERV-PK DNA. *Eco*RV, *Hpa*I, and *Sca*I were identified as single cutters in the PERV sequence by computer analysis in relation to the *pro-pol* probe, which served as a proviral landmark and revealed numerous more or less distinct hybridizing bands (data not shown). Distinct single bands generated by *Dra*I, *Nae*I, *Pvu*II, and *Sma*I indicated the existence of at least two internal recognition sites in the proviral PERV sequence (data not shown). Some of those fragments formed the basis for inverse PCR-mediated cloning techniques after religation.

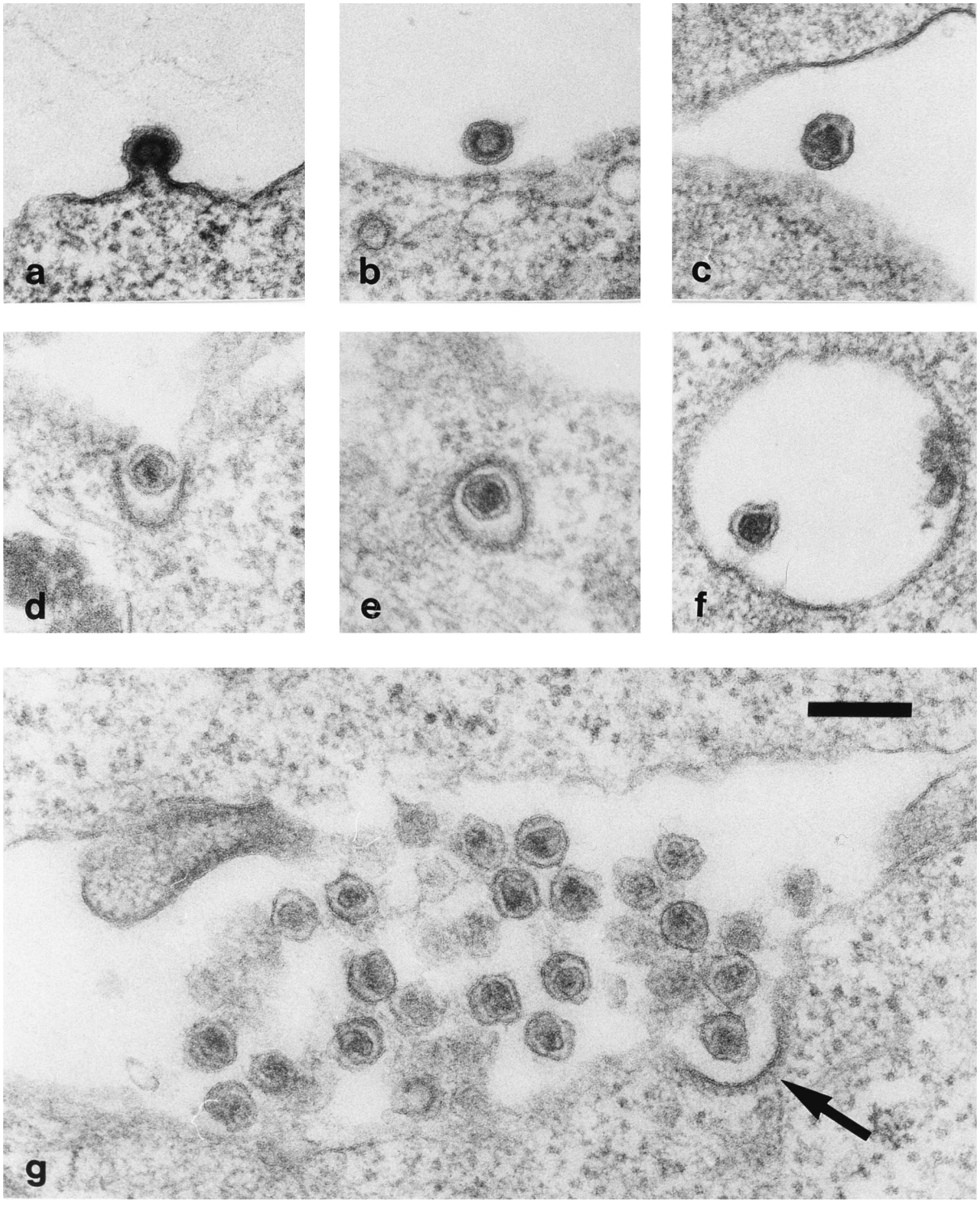

FIG. 1. Morphology of PERV. Ultrathin sections showing PERV particles budding from (a to c) and infecting (d to f) human kidney cells (293 line). Budding proceeds in a typical C-type manner, while virus entry follows the pathway of receptor-mediated endocytosis via coated pits (d and arrow in g) and coated vesicles (e) towards endosomes (f). The diameter of the particles is approximately 110 nm. The arrow points to a virus particle during cell entry. The bar in panel g represents 200 nm. 293 cells were infected with originally PK15-derived PERV particles, which can be passaged on 293 cells.

Cloning of PERV sequences by inverse and long PCR techniques. Partial sequences were obtained after cloning of *Sma*I (nt 943 to 4024), *Pvu*II (nt 1925 to 3999), *Nae*I (nt 804 to 5470), *Pvu*II (5372 to 7633), and *Rsa*I (nt 7502 to 8109) DNA fragments, which were amplified by inverse PCR using DNA from 293 cells infected with PERV after restriction enzyme digestion and religation. With oligonucleotides PK28 and PK29, derived from these sequences, the PERV coding region comprising a 7.8-kb *gag-pol-env* gene cassette was amplified, producing clone pPERV-PK28/29. In addition, a 5′ LTR was

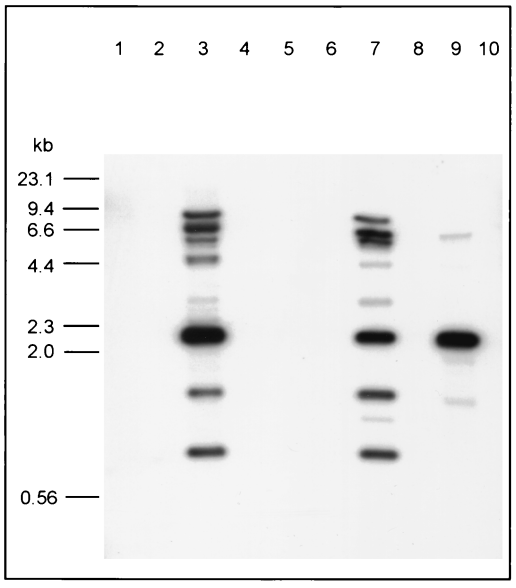

FIG. 2. Presence of PERV sequences in different genomes and restriction enzyme analysis of PERV-infected 293 cell line. Southern blot analyses of *Eco*RI-digested genomic DNA samples from different species using a PERV *pro-pol* probe. Lane 1, mouse; lane 2, dog; lane 3, pig PBMC; lane 4, sheep; lane 5, goat; lane 6, bovine; lane 7, pig (PK15 cell line); lane 8, human 293 cell line; lane 9, human cells infected with PERV (293 cell line infected with PK15-derived PERV); lane 10, human teratocarcinoma cell line GH. A 753-bp *pro-pol* fragment served as the hybridization probe. The sizes of *Hin*dIII-digested λ are given.

amplified using primers PK 26 and PK34, generating pPERV-PK26/34. Both retroviral clones were fully sequenced, and pPERV-PK28/29 was characterized as PERV-B. This sequence, ranging from nt 895 to 8781 based on the PERV-B(33) standard sequence (see below), exhibits *gag* (nt 1154 to 2728) and *env* (nt 6189 to 8162) ORFs and demonstrates homologies of 94.0 to 99.2% to the *gag* and *pol* genes of PERV-MSL, Tsukuba-1, and PK-15 ERV (1), respectively, using the BLASTN program (National Center of Biotechnology Information genetic databases). PK-15 ERV is a partially (in *pol*) deleted PERV cDNA sequence isolated from PK15 cells. The *env* gene of PERV-PK28/29 is almost identical (99.8%) to a PERV-B sequence described previously (accession no. Y12239) (37). Thus, pPERV-PK28/29 belongs to class B of PERV sequences. A single-nucleotide deletion (A, nt 5573) leads to a truncated *pol* ORF (nt 2729 to 6316) in pPERV-PK28/29.

As this PCR strategy was not optimal to fully clone and characterize different complete proviral PERV sequences which potentially could be replication competent, particularly as several copies exist in the infected human cell line 293 PERV-PK, genomic cloning was performed.

Generation and screening of a gene library. For the cloning of complete proviral PERV genomes, including both LTRs, a λ bacteriophage library of 293 PERV-PK DNA was generated. A total of 1.5×10^6 independent primary clones were obtained and screened with the PERV *pro-pol* probe. After three rounds of screening, six clones were purified to homogeneity. Restriction enzyme analyses of subcloned *Not*I inserts into pBS-KS revealed two identical clones [pPERV-B(34) and pPERV-B(41)]. A third and unclassified clone, pPERV-(46), was excluded from further analysis because of a truncated *pol* gene detected by PCR (not shown). Two of the four remaining clones, pPERV-B(33) and pPERV-B(43), were characterized in detail. Clone PERV-B(34) demonstrated a truncation in the *env* gene, and clone PERV-A(42), encompassing an *env* class A gene, has not yet been fully characterized.

Analysis of full-length PERV sequences and ORFs. The retroviral portions of pPERV-B(33) and pPERV-B(43) were sequenced and compared. PERV-B(33) and PERV-B(43) display proviral sequences of 8,918 and 8,750 bp, respectively (Fig. 3A). Only minor differences are evident throughout the retroviral genes, which show nucleotide and amino acid homologies of 99.8% (three nucleotide exchanges) and 99.8% (one amino acid exchange) for *gag*, 99.9% (five nucleotide exchanges) and 99.6% (five amino acid exchanges) for *pro-pol*, and 99.7% (five nucleotide exchanges) and 99.2% (five amino acid exchanges) for *env* (Table 1). According to their *env* sequences, both proviruses belong to PERV class B (37). PERV-B(33) and PERV-B(43) sequences show ORFs for all retroviral proteins in a type C-specific manner except for *env* in the case of PERV-B(33), in which the first putative start codon is mutated at nt 6191 from G to A (Fig. 3A). The nucleotide and amino acid homologies of PCR clone pPERV-PK28/29 and of genomic clone pPERV-B(33) are 99.6% (six nucleotide exchanges) and 99.0% (five amino acid exchanges) for *gag*, 99.8% (six nucleotide exchanges, one nucleotide deletion) and 99.7% (two amino acid exchanges, one frameshift) for *pro-pol*, and 99.7% (five nucleotide exchanges) and 99.2% (five amino acid exchanges) for *env* (Table 1). The *env* ORFs of pPERV-B(43) and pPERV-PK28/29 differ in only one codon (nt 8091, A to G, Ser versus Pro).

The existence of short ORFs preceding the *gag* gene, such as the one at nt 839 to 895, is known for several retroviruses (13). The ATG of the PERV-B(33) *gag* ORF is located at position 1154 (Fig. 3A). The entire encoded Gag sequence shows closest amino acid homology to the Gag of PERV-MSL (1) and of GaLV (18), 94.5 and 63.0%, respectively (Table 1). The sequence Asn_1-Met_1-Gly_2-Gln_3-Thr_4 is identical to that of MoMLV (59) and PERV-MSL (1). Some highly conserved amino acid motifs for mammalian type C retroviruses are present in the Gag-Pol polyproteins of PERV-B(33) and PERV-B(43). Identical to the situation in PERV-MSL (1) and similar to other type C retroviruses, including GaLV (18) and BaEV (35), the consensus sequence for the N terminus of p30 starts at nt 1736 with a proline (48), and the C-terminal end is located at nt 2501 to 2512 (Thr-Lys-Ile-Leu). Amino acid comparisons with the p30 and p27 capsid proteins of other retroviruses revealed scores of 96.1% (PERV-MSL), 75.7% (GaLV), 68.2% (BaEV), 61.7% (FeLV), and 61.1% (MoMLV). A zinc finger motif consisting of the Cys-His box (Cys-Xaa2-Cys-Xaa4-His-Xaa4-Cys) located at nt 2630 to 2671 is a hallmark of the nucleic acid-binding protein (nucleocapsid, p10) of other type C retroviruses (29, 45).

The *pro* region encodes the protease, which cleaves the *gag* and *pol* polyproteins. The stop codon at position 2728 that separates the *gag-pro-pol* precursor is suppressed by a tRNA accepting glutamine and accounting for a readthrough (32). A highly conserved amino acid motif characteristic of aspartyl proteases is located at nt 2801 to 2824 (Leu-Val-Asp-Thr-Gly-Ala-Glu-His), demonstrating a six-of-eight amino acid match to the consensus sequence (13).

The tetrapeptide motif located at positions 3746 to 3757, YVDD, is highly conserved in many retroviruses, and the two aspartate residues of the common YXDD motif are invariant among all the RNA-dependent DNA and RNA polymerases (74). The PERV-B(33) Pro-Pol polyprotein shows closest homology with PERV-MSL (96.9%) and GaLV (68%), whereas MLV displays scores of 64% at the nucleotide and 62% at the amino acid level (Table 1). The 3′ end of the *pol* ORF coding

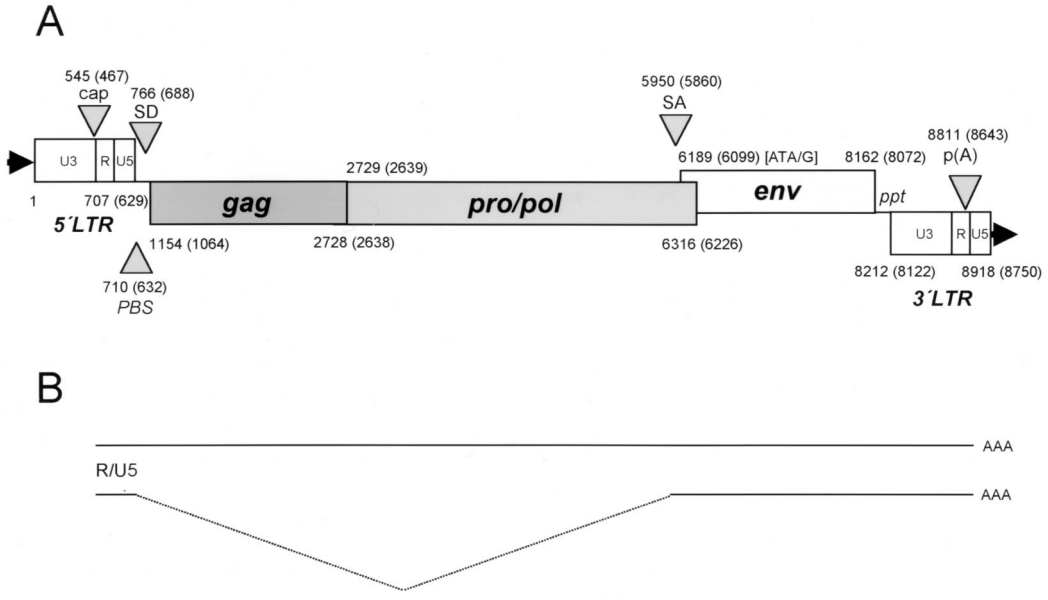

FIG. 3. Proviral organization of clones PERV-B(33) and PERV-B(43). (A) Proviral sequences are 8,918 and 8,760 bp long for PERV-B(33) and PERV-B(43), respectively. Genes and ORFs are shown as open boxes; the numbers indicate the first and last nucleotide in the PERV-B(33) and PERV-B(43) (in parentheses) sequences. A 39-bp repeat is found four times in the U3 element of the PERV-B(33) LTRs, whereas the PERV-B(43) LTRs harbor two 39-bp repeat elements. Cap, transcriptional start site; PBS, primer binding site ($tRNA_{Gly4}$); SD, splice donor; SA, splice acceptor; ppt, polypurine tract; p(A), poly(A) addition site. (B) Two mRNA species are expressed from proviral PERV. A full-length (8.3 kb) and a subgenomic (3.1 kb) *env* mRNA are indicated.

for RT and integrase (nt 6316) overlaps the 5′ coding region of the *env* gene starting at nt 6189.

The cleavage site between the surface protein gp70 and the transmembrane protein p15E predicted by Le Tissier et al. (37) is located downstream of the Arg-Pro-Lys-Arg sequence at nt 7565 in pPERV-B(33). The *env* gene is followed by a polypurine tract (nt 8199 to 8211) upstream of the 3′ LTR (Fig. 3A). The results of comparisons of PERV-B(33) sequences with the genes of other retroviruses are summarized in Table 1.

No significant homologies of PERV with human endogenous retroviral (HERV) type C-like sequences were found. With regard to putative recombination events, however, partial homologies of PERV-B(33) *env* (nt 6157 to 6196) with ERV-3 (HERV-R) *pol-env* sequences (nt 293 to 332) (accession no. M12140) (14) are noteworthy.

PERV LTRs. The LTRs of pPERV-B(33) and pPERV-B (43) are limited by the inverted repeat sequence TGAAAGG/CCTTTCA. The LTRs differ in the length of the U3 element. A 39-bp sequence motif exists four times in the PERV-B(33) LTR (nt 331 to 369, 370 to 408, 409 to 447, and 448 to 486) and two times in the PERV-B(43) LTR (Fig. 3A). Part of this repeated sequence consists of an 18-bp segment which precedes the quadruplex/duplex stretch at nt 313 to 330. A comparison with the PERV-MSL LTR (1) reveals, after reconstitution of the appropriate cDNA portions, an overall homology of 70%. However, in the PERV-MSL LTR, only one copy of the 39-bp motif (five nt exchanges) exists, which is flanked on each side by a single 18-bp segment. Extensive homology is also found downstream of this sequence in the PERV-MSL U3 element. A retroviral TATA box demonstrating a sequence context homologous to other promoters (28) is present at nt 517. The R region is located at nt 546 to 624, followed by the U5 element (nt 625 to 707) in the PERV-B(33) LTR. These elements show extended or partial homologies with the corresponding sequences of the PERV-MSL and GaLV LTRs. A MatInspector analysis (Genomatix, Munich, Germany) revealed a primer binding site at nt 710 to 727 that is complementary to the last 18 nt at the 3′ end of a $tRNA_{Gly4}$. By contrast, PERV-MSL encompasses a $tRNA_{Pro}$ sequence (1). A polyadenylation signal consensus sequence (AATAAA) is located at nt 8811 to 8816 in PERV-B(33) (Fig. 3A).

RNA expression pattern of PERV. Total RNA and mRNA from uninfected 293, 293 PERV-PK, PK15, and GH (a human teratocarcinoma cell line) cells were hybridized in a Northern blot analysis with different probes. Full-length transcripts of ~8.3 kb were found with the *pro-pol* probe for the 293 PERV-PK and the PK15 cell lines (Fig. 4A). According to the β-actin control hybridization, mRNA steady-state levels of (polytropic) PERV expressed in 293 cells are higher than those in PK15 cells (Fig. 4A). With a U5-specific probe, a genomic RNA of ~8.3 kb and a subgenomic transcript of ~3.1 kb were found in 293 PERV-PK and at lower steady-state levels in

TABLE 1. Comparison of nucleotide and amino acid sequences of PERV-B(33) and PERV-B(43) *gag*, *pro-pol*, and *env* ORFs with other viral genomes[a]

Virus	% Nucleotide identity (% amino acid identity) with PERV-B(33) gene (protein)		
	gag	*pro-pol*	*env*
PERV-B(43)	99.8 (99.8)	99.9 (99.6)	99.7 (99.2)
PERV-PK28/29	99.6 (99.0)	99.8 (99.7)	99.7 (99.2)
PERV-MSL	94.3 (94.5)	96.7 (96.9)	74.0 (69.0)
BaEV	62.0 (54.0)	63.2 (63.0)	53.0 (33.0)
FeLV	58.0 (51.1)	61.3 (61.0)	57.0 (48.0)
GaLV	66.0 (63.0)	66.0 (68.0)	59.0 (51.9)
MoMLV	61.0 (53.0)	64.0 (62.0)	58.0 (49.3)

[a] One frameshift in PERV-PK28/29 is required to maintain colinearity with the *pol* ORFs. The start codon of PERV-B(33) *env* (nt 6189 to 6191) was restored to create colinear ORFs. Homology scores were revealed using sequence analysis software DNASIS (Hitachi).

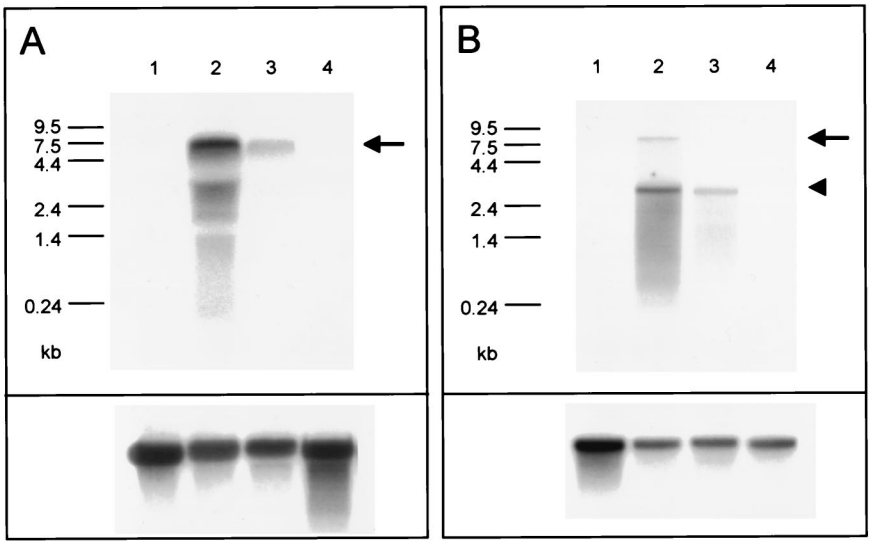

FIG. 4. PERV RNA expression pattern. Northern blot analysis of total RNA (A) and oligo(dT)-selected RNA (B) from different cell lines. Equal amounts of RNA were separated on sodium phosphate-agarose gels, transferred to nylon membranes, and hybridized with a radiolabeled *pro-pol* probe (A) and with a U5 probe (B). A β-actin probe served as a control (lower panels). Lane 1, human kidney cell line 293; lane 2, 293 cells infected with PERV; lane 3, porcine kidney cell line PK15; lane 4, human teratocarcinoma cell line GH. A full-length transcript of about 8.3 kb is indicated (arrows). A subgenomic *env* transcript of ~3.1 kb hybridizes with the U5 probe (arrowhead in panel B). RNA prepared from uninfected 293 cells and the human teratocarcinoma cell line GH served as negative controls.

PK15 cells (Fig. 4B). The specificity of the probes used was confirmed by the absence of hybridizing RNAs in uninfected 293 cells and GH cells (Fig. 4).

Identification of the transcription start site. To map the cap site of PERV mRNA, a combination of two RT-PCR-based methods was performed. The 5′RACE products generated with an anchor primer in combination with the gene-specific primer PK26-R were cloned and sequenced. The 5′RACE product ended at position 569 (not shown). For the exact determination of the transcriptional start site, a primer extension analysis was carried out. An RT-PCR was performed with the oligonucleotide PK-REV-PE, derived from the 5′RACE sequence. The same primer was used in a regular Sanger sequencing reaction. As shown in Fig. 5, the RT-PCR product identified the cap site at nt 545 (see also Fig. 3A).

Identification of splice donor and acceptor sites. The splice donor and splice acceptor sites which trigger the expression of the subgenomic PERV *env* mRNA were experimentally determined. A PCR with an elongation step of 60 s preferentially amplified subgenomic transcripts using 293 PERV-PK cDNA with the primers PERV-PBS and env-R. After sequencing a ~390-bp product, a junction of CTTGGAGCCTCT was revealed, demonstrating that the splice donor and the corresponding splice acceptor sites are located at nt 766 (A) and at nt 5950 (G), respectively (data not shown). All mapping results are summarized in Fig. 3A. Based on these analyses, the theoretical size of the *env* transcript of 3.1 kb corresponded to the experimentally defined subgenomic mRNA (Fig. 3 and 4B).

Transfection and infection studies. To functionally characterize proviral sequences, plasmids pPERV-B(33), pPERV-B (33)/ATG, and pPERV-B(43) were repeatedly transfected in triplicate into 1×10^5 to 3×10^5 293 cells. Plasmid pPERV-B (33)/ATG, harboring a restored *env* start codon at nt 6189, was constructed to investigate potential differences in retroviral replication, since the first ATG in the *env* gene of pPERV-B (33) is located at position 6693, whereas the *env* start codon in PERV-PK28/29 and in the previously published PERV-B *env* sequence (37) is located 504 bp upstream at nt 6189, similar to PERV-B(43).

The replacement of the 1,325-bp *Bsr*GI fragment (nt 5920 to 7245) derived from pPERV-PK28/29 introduced four nucleotide and amino acid exchanges in the *env* ORF of PERV-B (33), at nt 6191 (Ile to Met), nt 6562 (Asp to Gly), nt 6568 (Gly to Glu), and nt 7005 (Ser to Pro).

As an initial marker of PERV expression, retroviral Gag

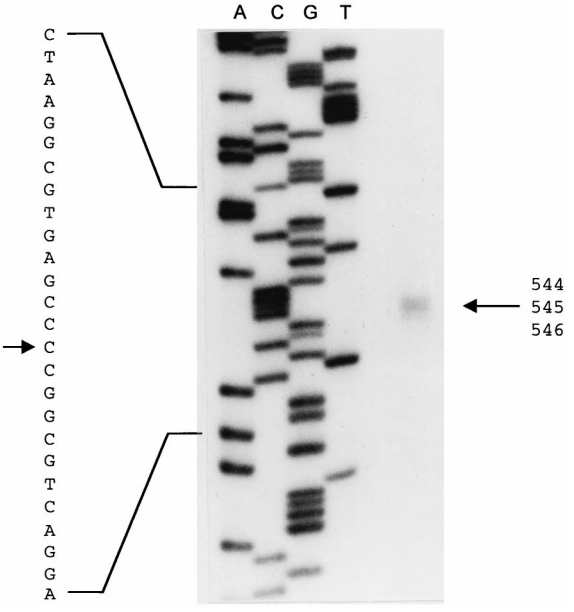

FIG. 5. Identification of the transcription initiation site of PERV mRNA. Polyadenylated RNA prepared from the cell line 293 PERV-PK was reverse transcribed using the ^{32}P-end-labeled oligonucleotide PERV-PK-REV. The cDNA was separated by SDS–6% PAGE together with a four-lane Sanger sequencing reaction performed with the same unlabeled primer and the cloned 5′ LTR (pPERV-PK26/34) as the template. Two to three end-labeled transcripts were detected on the autroradiograph. The cap site was assigned to nt 545 (C) in the PERV-B(33) LTR (arrows). The lower-strand sequence is given on the left side.

proteins were detected by indirect immunofluorescence with cross-reacting FeLV CA antiserum. At 24 to 48 h p.t., 20 to 50% of cells transfected with pPERV-B(33), pPERV-B(33)/ATG, and pPERV-B(43) demonstrated Gag staining (not shown). In parallel cultures, 293 cells transfected with pPERV-B(43) and pPERV-B(33)/ATG showed RT activities in cell-free supernatants after 12 to 14 days (not shown). Those cultures were maintained and analyzed at regular time points. At 6 to 12 weeks p.t., all cells transfected with pPERV-B(43) and pPERV-B(33)/ATG stained positive for Gag (Fig. 6A). These PERV-expressing cells released virion-bound RT at levels of 600 to 900 mU/ml (Fig. 6B). The 293 PERV-PK cells used as a positive control gave RT activities of 1,800 mU/ml in cell-free supernatants (Fig. 6B). Purified virus particles from those cultures were positive for the capsid protein (p30) by immunoblotting with FeLV CA antiserum (Fig. 6C). PCR analyses revealed integration of proviral DNA as demonstrated for *pol* (Fig. 6D) and *env* sequences (not shown). Cell-free supernatants from those particle-producing cultures were used to transfer PERV to fresh 293 cells. Similar to the transfection experiments, indirect immunofluorescence analysis showed Gag expression 24 to 48 h postinfection, and RT assays became positive at 10 to 14 days postinfection (not shown), demonstrating that pPERV-B(43) and pPERV-B(33)/ATG encode infectious retroviruses. Those virus transfers were continued serially on 293 cells every 4 weeks for up to 6 months (not shown). On the other hand, weak RT activities in pPERV-B(33) Gag-expressing cultures were found only transiently after transfection (not shown), and virions could not be transmitted to fresh 293 cells.

In addition, HeLa cells were transfected with the three recombinant PERV plasmids. Retroviral Gag expression was seen in 10 to 20% of cells 24 to 48 h p.t. However, even over longer periods of cultivation, no RT activity was found, and the number of Gag-expressing cells decreased even though integration of proviral PERV sequences was found (not shown).

DISCUSSION

As demonstrated by the almost complete cloning of a PERV-C proviral sequence (PERV-MSL [1]), the pig genome harbors intact copies of PERV. Those elements are not merely pig endogenous retroviral sequences, as suggested earlier (57), for some of those endogenous retroviral sequences encode replication-competent retroviruses similar to those present in normal chickens, cats, baboons, and several strains of mice (71).

PERV-B sequences encode infectious particles. This report presents the first structural and functional description of complete PERV-B sequences with replicative capacities. Both proviruses [PERV-B(33) and PERV-B(43)] were isolated from a human 293 cell line productively infected with PERV-PK which harbors at least four additional PERV sequences isolated by genomic and PCR cloning.

The capacity of PERV-B(43) to produce infectious viral particles was directly shown after transfection of cloned proviral sequences into human cells and subsequent serial passaging on human 293 cells (Fig. 6). The second proviral genome, PERV-B(33), was presumed to be defective due to a point mutation in the *env* start codon, generating a Met-to-Ile mutation. For this reason, the codon was genetically restored by replacement with a homologous sequence derived from the PCR clone PERV-PK28/29. After transfection of this proviral construct, PERV-B(33)/ATG was able to produce infectious particles which were serially passaged on human 293 cells. On the other hand, no infectious and replication-competent virus was found after transfection of PERV-B(33). It should be mentioned, however, that in addition to the introduction of ATG, there are four predicted amino acid differences between the two molecular clones, and the contribution of any or all of these changes to the defective nature of PERV-B(33) and rescue by exchange of the fragment from pPERV-PK28/29 was not evaluated.

For endogenous MLVs, four classes of sequences have been described (61). The MLV *env* sequence of the prototype polytropic proviral clone MX27 exhibits the same methionine-to-isoleucine mutation of the start codon as PERV-B(33). Those sequences and members of the modified polytropic endogenous MLV class, however, can contribute their *env* genes to the observed recombinant viruses derived from distinct parental MLV (61). Therefore, given the fact that PERV-B(33) preexisted in this form in the pig genome, it is conceivable that this sequence is involved in recombination events of PERV sequences and/or that gene mutations can lead to expression of replication-competent pig retroviruses. It seems likely, however, that PERV-B(33), in addition to the defective clones PERV-PK28/29, PERV-B(34), and PERV-(46), which was not further classified, was integrated into the 293 host genome via cross-packaging of viral RNA derived from PK-15 cells and subsequent RT after infection of 293 cells by PERV particles. In this context, it cannot be completely excluded that the single-nucleotide deletion in *pol* of the amplified sequence of clone PERV-PK28/29 was generated by a PCR artifact, even though we used polymerases with proofreading activity.

PERV host range. The host range and cell tropism of retroviruses mainly depend on the *env* gene. Two of three distinct PERV can infect some human cells in culture (37, 52, 63, 72). However, these reports did not address the entire genetic context of proviral PERV, and the main conclusions were drawn from pseudotype experiments with PERV type A, B, and C *env* genes cloned into MLV-based retroviral backbones (63, 73). These data suggest that different receptors exist on susceptible cells (37, 63), although the receptors have not yet been identified on cells from nonhuman primates, suggesting that primate xenotransplantation models may not be appropriate for the study of PERV zoonosis (60, 63). In this study, we have shown that two full-length molecular clones of the PERV-B class can replicate on human cells. So far, we do not know whether PERV-A viruses which demonstrate the same functional properties exist. Our preliminary investigations of one isolated clone [PERV-A(42)], based on sequence analysis, however, suggest a similar potential (not shown).

In addition, the promoter activity of LTRs and other regulatory factors is important for the host range. The use of defined LTRs from functional PERV in reporter gene studies will allow analysis of cell and tissue specificities in addition to the host range and interference studies (63). The U3 regions of the PERV-B(33) and PERV-B(43) LTRs differ in the numbers of a 39-bp repeat that are present. Our first studies indicate significantly different promoter activities of these LTRs in human cells. As no steroid hormone-responsive element or NF-κB sites could be found in the PERV-B(33) or PERV-B(43) LTRs, it remains to be shown whether immunosuppressive drugs such prednisolone and cyclosporin A have a direct or indirect effect on LTR activities.

Putative impact of replication-competent PERV in xenotransplantation. The different pig breeds studied so far harbor different sets and distinct numbers of PERV copies in their genomes (1, 37). For miniature swine inbred for major histocompatibility complex loci, it was shown that PERV-MSL exists in multiple copies but that some individuals apparently harbor additional proviral loci (1), a phenomenon which could be explained by new infections, genetic recombination, or retrotransposition of transcriptionally active PERV in the minipig

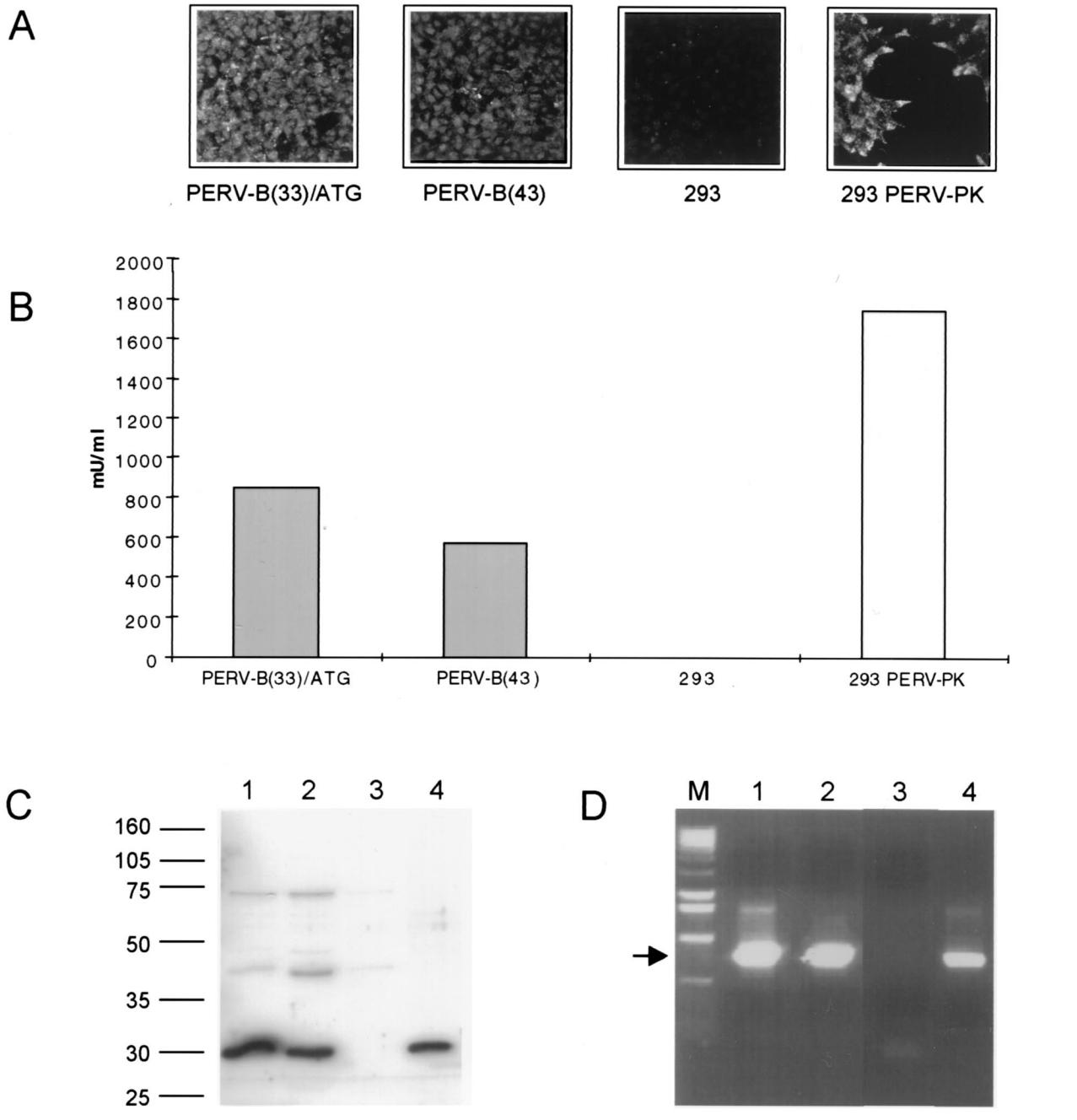

FIG. 6. Analysis of retrovirus expression after transfection of molecular clones PERV-B(33) and PERV-B(43). (A) Indirect immunofluorescence analysis of PERV Gag expression 12 weeks p.t. using cross-reacting FeLV CA antiserum. Molecular clones PERV-B(33)/ATG and PERV-B(43) in 293 cells were used at 12 weeks p.t.; uninfected 293 cells and 293 cells productively infected with PERV derived from PK15 (293 PERV-PK) served as controls. Expression of Gag from PERV-B(33)/ATG and PERV-B(43) was detected as early as 24 h p.t. in 20 to 50% of cells (not shown). (B) RT activities in cell-free supernatants 12 weeks p.t. RT produced by PERV-B (33)/ATG and PERV-B(43) was detected as early as 12 to 14 days p.t. (not shown). The controls were the same as in A. (C) Immunoblot analysis of PERV Gag proteins. Sucrose gradient-purified particles from PERV-producing cells [PERV-B(33)/ATG and PERV-B(43) at 12 weeks p.t.] were separated by SDS-PAGE. The p30 capsid protein (arrow) cross-reacts with FeLV CA antiserum. Lane 1, PERV-B(33)/ATG; lane 2, PERV-B(43); lane 3, 293 cell line; lane 4, 293 PERV-PK cell line. The positions of molecular mass markers are indicated (in kilodaltons). (D) DNA PCR of PERV *pro-pol* sequences demonstrating integration of proviral PERV sequences. Lane 1, PERV-B(33)/ATG; lane 2, PERV-B(43); lane 3, 293 cell line; lane 4, 293 PERV-PK cell line. The arrow marks an 812-bp amplification product obtained with primers PK45 and PK47. Lane M, molecular size markers (1-kb ladder; Life Technologies).

genome. Furthermore, mRNA expression of PERV in different porcine tissues has been reported (1, 52). Significant steady-state levels have been shown for PBMC, spleen, thymus, lymph node, and lung, where full-length and subgenomic RNAs were found (1). These patterns of expression are identical to those observed in PK15 and in 293 PERV-PK cells (1) (Fig. 4).

In recent retrospective analyses of xenotransplanted human patients, no evidence was found for expression, infection, and replication of PERV in the human host (33, 49, 51). Even though these reports are somewhat reassuring with regard to safety, particularly as PERV replicates even in permissive tissue culture cells to relatively low titers (63), it has to be stated

that (i) no long-term xenotransplantation of physiologically functional pig tissues has been achieved so far and (ii) no vascularized pig organs have been successfully used in human patients. Therefore, although these studies found no evidence of a risk, they do not rule out that a risk exists (70). An additional level of risk has been pointed out which arises from the presence of HERV in human transplant recipients and the subsequent chance of recombination with PERV yielding viruses with new properties (70). It has to be stated that no HERV with replicative capacities has been identified so far (39). For instance, in the HERV-K family, which comes closest of all known HERV to encoding infectious virus, no corresponding replication-competent virus could be found in the human genome (66). Nevertheless, expression of types B and D HERV-K full-length mRNAs was observed in numerous tissues (40). As PERV belong to the C-type retroviruses, related HERV sequences might offer targets for recombination. ERV-3 (HERV-R) is a defective C-type provirus that is selectively expressed during differentiation of placental syncytiotrophoblasts and elicits mass production of a nonglycosylated and unprocessed 65-kDa ENV protein in this tissue (68), a phenotype which seems to be dispensable in humans (19). Thus, even if genetic recombination occurred, based on the limited sequence homologies in *env*, a resulting recombinant PERV-HERV might not gain any advantageous functions.

The aim of establishing long-term xenotransplantation and the presence of infectious PERV and other unknown agents make it necessary to closely monitor animal donors, human recipients, and their contacts in prospective xenotransplantation trials. This will enable a comprehensive evaluation of the risks of cross-species transmission of microorganisms, as suggested previously (47, 60). Even though PERV do not replicate as efficiently as polytropic murine and feline ERV in human cells (37, 52), the possibility remains that PERV or recombined viruses could infect xenograft recipients (70) and, like their closest viral relatives MLV, FeLV, and GaLV, cause leukemic diseases. Besides PERV, other new horizontally transmissible viruses of swine, including hepatitis viruses and herpesviruses, have recently been discovered (22, 43, 44), adding to the list of putative human pathogens. Therefore, regulatory frameworks similar to those set up by the U.S. Food and Drug Administration (U.S. Department of Health and Human Services, Public Health Service guideline on infectious disease issues in xenotransplantation, Fed. Register vol. 61, no. 185, 23 September 1996, p. 49920–49949, http://www.fda.gov/cber/xeno.pdf) and the United Kingdom Xenotransplantation Interim Regulatory Authority (U.K. Xenotransplantation Interim Regulatory Authority, Draft report of the infection surveillance steering group, 7 July 1999, http://www.open.gov.uk/doh/ukxira.htm) are needed to help to provide pathogen-free pigs for xenotransplantation.

Specific tools for detection of PERV expression and replication have been established, including nucleic acid and immunologic assays (33, 49, 51). We have generated PERV-specific antisera and have expressed recombinant PERV proteins in order to unequivocally distinguish those viruses from other agents (U. Krach, N. Fischer, F. Czauderna, R. Kurth, and R. R. Tönjes, submitted for publication).

Should methods such as knock-out technology become available for pigs in the future, inherited genes, including vertically transmitted PERV, could be eliminated. Functional characterization and genetic mapping of replication-competent endogenous retroviruses, including those PERV presented here, will help eliminate functional proviruses from transplant donor animals or at least allow the expression of these PERV classes to be controlled.

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