PCR-based cloning and immunocytological titration of infectious porcine endogenous retrovirus subgroup A and B

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Two pig endogenous retroviruses (PERV), PERV-A and -B, productively infect human cells and are therefore considered to constitute a potential risk in pig-to-human xenotransplantation. A PCR-based cloning technique to isolate infectious PERV proviruses was established. Overlapping 3′ and 5′ halves of PERV proviral genomes were amplified using DNA extracted from human 293 cells infected with PERV-A or -B. These clones were fused at a unique restriction site in the overlapping region and tested for their infectivity. Representative constructs possessed the same infectious properties as their parent isolates. We also developed a polyclonal anti-PERV serum by using recombinant PERV capsid protein derived from one of the infectious constructs as immunogen and established an immunocytological method for detection and titration of PERV infection. This detection method proved to be more sensitive than the current method of choice (transfer of MLV-lacZ vectors) for infectivity assessment of PERV. These findings should be considered for future characterization of PERV isolates.

Introduction

Xenotransplantation is considered a potential solution to overcome the increasing shortage of organs and tissues available for transplantation into humans and overall pigs are the most suitable source species (Cozzi et al., 2000; Takeuchi & Weiss, 2000). Clinical trials using porcine tissues are currently ongoing for a number of different therapeutic indications. These procedures, however, bring with them the possibility of introducing infectious agents from the pig into the xenograft recipient and ultimately the community at large (Birmingham, 1999; Platt, 2000; Takeuchi & Weiss, 2000). The risk of zoonosis may be reduced by extensive screening to provide source animals free of exogenous pathogens. However, endogenous agents and agents that current detection strategies do not identify are difficult or impossible to eliminate by current barrier derivation technologies. For this reason there has been extensive interest in studying the biology of pig endogenous retroviruses (PERVs) carried in the chromosomes of every pig (Patience et al., 1997; Le Tissier et al., 1997).

Among several PERV families within the pig genome (Ericsson et al., 2001; Patience et al., 2001), one family produces infectious viruses whose closest relatives are the gammaretroviruses gibbon ape leukaemia virus (GALV) and mouse leukaemia virus (MLV) (Le Tissier et al., 1997; Patience et al., 1997; Akiyoshi et al., 1998). Since the discovery of PERVs in the 1970s (Breese, 1970; Armstrong et al., 1971; Todaro et al., 1974), it has been shown that several pig cell lines, including the pig kidney cell line PK-15, as well as primary pig cells such as endothelial and mitogenically activated peripheral blood mononuclear cells release PERV particles which are infectious for human cells (Patience et al., 1997; Martin et al., 1998a, 2000a, b; Wilson et al., 1998, 2000). Molecular isolation and analysis of partial and full-length proviruses confirmed the presence of at least three different classes of PERV genomes, termed PERV-A, -B and -C, with high homology in the gag and pol genes but a significant divergence within sequences encoding the outer envelope glycoprotein (Le Tissier et al., 1997; Patience et al., 1997; Akiyoshi et al., 1998; Czauderna et al., 2000; Wilson et al., 2000; Krach et al., 2001). Pseudotyping experiments with the PERV envelope glycoproteins indicated that PERV-A, -B and -C use different receptors from each other for cell entry (Takeuchi et al., 1998) and that a significant number of cell lines of human and animal origin are permissive to entry of PERV-A and -B. While it was shown that the host range of PERV-C is mostly restricted to porcine cells (Takeuchi...
et al., 1998), a range of different cell lines derived from human, primates, mink, mouse, rat, rabbit, bat, hamster and dog is permissive to entry of PERV-A and/or -B (Takeuchi et al., 1998; Blusch et al., 2000; Martin et al., 2000a, b; Wilson et al., 2000). PERV-A and -B can productively infect human primary cells of endothelial and vascular origin (Martin et al., 2000a, b), cell types which would become extensively exposed to potential xenografts.

PERVs have thus raised major concern regarding the safety of xenotransplantation, and the potential of cross-species infection by PERV has been reported by recent findings of PERV infection in non-obese diabetic severe combined immunodeficiency (NOD-SCID) mice after pig islet cell transplantation (Deng et al., 2000; van der Laan et al., 2000). However, studies of immunosuppressed non-human primates (Martin et al., 1998b; Switzer et al., 1999; Templin et al., 2000; Winkler et al., 2000) and extensive retrospective studies of patients treated with living pig tissues or cells mostly for a short period of time did not result in any evidence of PERV infection (Patience et al., 1998; Heneine et al., 1998, 2001; Paradis et al., 1999; Pitkin & Mullen, 1999; Dinsmore et al., 2000; Herring et al., 2001; Tacke et al., 2001). While the lack of evidence of PERV infection in primates and humans has been reassuring, these observations do not rule out that a risk exists.

Pig genomes have been reported to contain up to 50 copies of PERV, although many of them are likely to be defective (Patience et al., 1997; Le Tissier et al., 1997; Akiyoshi et al., 1998; Bosch et al., 2000; Krach et al., 2001). PERV integration patterns in different pig individuals showed only limited conservation (Le Tissier et al., 1997), suggesting that individual pigs harbour different sets of PERV with significant heterogeneity. Because retroviruses can evolve fast, it is possible that new forms of infectious PERV will emerge in the process of xenotransplantation. Replication competent, molecular clones of PERV will be useful to study PERV biology by genetic approaches. Infectious clones have recently been isolated by screening a phage genomic library derived from human 293 cells infected with PK15-derived PERV (Czauderna et al., 2000; Krach et al., 2001). In this study, we isolated infectious PERV clones using a PCR-based method, which is less labour intensive and more widely applicable than the classical method of phage library construction followed by hybridization screening. In addition, a titration method for PERV infection was established using polyclonal antibodies raised against PERV capsid protein, which allowed us to quantitatively compare infectivity of PERV-A and -B clones and their parental isolates as well as other standard gammaretrovirus strains.

Methods

- **PCR based cloning.** PERV proviruses were amplified in two overlapping halves. Primers (+) GGACTAGTGGGGTTGTTACCTGAGGGAGG and (−) CCACTAGTCCGGCTCGGAAAGGCGACACCCC were designed to amplify a 5071 nt long 5′ half spanning the region from the 5′ LTR to integrate in pol. These primer sequences correspond to nucleotide positions 67 to 93 and 5165 to 5189, respectively, in PERV clone 293-PERV-A(42). 3′ halves spanning 3926 nt from pol to US in the 3′ LTR were amplified with primers (+) GGACTAGTGGGGTTGTTACCTGAGGGAGG and (−) CCACTAGTCCGGCTCGGAAAGGCGACACCCC, whose sequences correspond to nucleotide positions 4845 to 4871 and 8793 to 8819, respectively, in PERV clone 293-PERV-A(42). PCR reactions were performed in 100 µl volumes using Pfu polymerase (Stratagene) according to the manufacturer’s instructions with 100 ng genomic DNA derived from PERV infected or uninfected 293 cells as template. Genomic DNA was extracted with the QiAamp DNA Blood Mini Kit from Qiangen according to the manufacturer’s instructions. PCR reactions were run on a PTC-225 Feltier Thermal Cycler (MJ Research) at 94°C for 4 min followed by 30 cycles of 1 min denaturation at 94°C, 1 min annealing at 58°C and a 12 min extension step at 72°C. PCR products were analysed by gel-electrophoresis and purified using a Qiagen Gel Extraction Kit according to the manufacturer’s instructions. PCR products of the 5′ half were subcloned using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen) according to the manufacturer’s instructions. PCR products of the 5′ and 3′ halves were ligated into the Zero Blunt TOPO vector (Invitrogen) using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen) according to the manufacturer’s instructions. PCR products of the 5′ and 3′ halves were used to fuse 5′ and 3′ halves to full-length proviruses. Sequences of the clones were obtained by dye terminator cycle sequencing using a CEQ 2000 and accessories (Beckman). Homology scores of sequences were revealed using sequence analysis software Geneworks (Aladdin Systems Inc.).

- **PCR screening.** PCR reactions were performed in 50 µl volumes using Taq polymerase (Promega) according to the manufacturer’s instructions with 50 to 100 ng genomic DNA as template. Reactions were run at standard conditions using an extension time of 1 min per kb target sequence. Primer sequences for different amplicons were previously described (Patience et al., 1997).

- **Bacterial expression of capsid protein.** A His-tagged capsid (His6-capsid) expression vector was constructed by PCR with Pfu polymerase (Stratagene) and the primers (+) GATCGCCATGGTACC-GCTGCGCACCTATGG and (−) CCACTAGTCCGGCTCGGAAAGGCGACACCCC. PCR products were digested with the restriction enzymes SpeI (SpeI was included in the primers), ligated into SpeI-digested pBluescript SKII and transformed into the bacterial strain HB101. Mini-preparations of DNA of positive transformants of 5′ and 3′ halves were checked for orientation of insertion. The unique restriction site NheI, located within the overlap between the 5′ and 3′ halves of PERV-A and -B, as well as the vector-specific restriction site NotI, which was present in both pBluescript KSII and pCR TOPO but not in the PERV sequence, were used to fuse 5′ and 3′ halves to full-length proviruses. Sequences of the clones were obtained by dye terminator cycle sequencing using a CEQ 2000 and accessories (Beckman). Homology scores of sequences were revealed using sequence analysis software Geneworks (Aladdin Systems Inc.).
of 50 mM Tris, 600 mM NaCl, 5 mM imidazole pH 8, 0.1% Triton X-100, 0.1% Tween, 0.1% Triton X-100, 150 mM NaCl, 5 mM imidazole pH 8, 0.1% Tween, 0.1% Triton X-100 and (c) 50 ml of 50 mM Tris, 150 mM NaCl, 10 mM imidazole pH 8, 0.1% Tween, 0.1% Triton X-100. The His6-capsid protein was eluted in a buffer consisting of 50 mM Tris, 150 mM NaCl, 100 mM imidazole pH 8, 1 mM PMSF and protease inhibitor cocktail. His6-capsid fractions were pooled and dialysed against 50 mM Tris, 150 mM NaCl, 0.5 mM DTT at 4 °C overnight.

Antibody production. His6-capsid protein (440 μg) was adjusted to a volume of 2 ml with PBS and added to one vial of the adjuvant MPL + TDM + CWS (M6601, Sigma), according to the manufacturer’s instructions, and injected into a rabbit. Total serum was recovered after four further boosts with the same antigen/adjuvant preparation.

Immunoblotting and in situ staining. Cell-lysates were analysed by SDS–PAGE, transferred onto nitrocellulose membrane using a semi-dry blotting system (Hoefer) and probed with rabbit antibodies (dilution was 1/500 for the antibody produced in this study as well as that obtained from Q-One Biotech) using chemiluminescence-based detection (Amersham) according to the manufacturer’s instructions. The detection of lacZ pseudotypes was performed as previously described (Takeuchi et al., 1994). For in situ staining with anti-PERV capsid antibody 293 cells were fixed for 15 min with an ice-cold 1:1 mixture of methanol and acetone and then left to dry. Fixed cells were stored at −20 °C if not processed immediately. Cells were washed twice in PBS and then blocked for 10 min at room temperature with PBS containing 10% foetal calf serum (FCS) followed by a 1 h incubation with PBS containing 1% FCS and anti-PERV capsid antibody at a dilution of 1:250. This was followed by two washes in PBS, 1% FCS and a 1 h incubation with PBS, 1% FCS containing secondary goat anti-rabbit antibody conjugated to alkaline phosphatase (Jackson). After two washes in PBS, 1% FCS and two washes in PBS, alkaline phosphatase was detected using NBT/BCIP ready-to-use tablets (Roche) according to the manufacturer’s instructions.

Viruses and cell culture. The origins of the MLV-A and GALV SF virus strains, the lacZ pseudotypes and the human embryonic kidney fibroblasts 293 used here have been described previously (Takeuchi et al., 1998). The cell-free infection was carried out essentially as described previously (Takeuchi et al., 1994). Briefly, 105 cells per well of 293 cells were seeded in a 96-multiwell plate; 24 h later virus dilutions were added in the presence of 4 μg/ml polybrene and then incubated for 3 days before fixation and staining. Transfections into 293 cells were performed using lipofectamine from Gibco according to the manufacturer’s instructions.

Nucleotide sequence accession numbers. The complete nucleotide sequences of PERV-17 (AY099324) and PERV-60 (AY099323) have been deposited at GenBank. Sequences used for sequence comparison are PK15-ERV (AF038601) (Akiyoshi et al., 1998), 293-PERV-A(42) (A1133817) (Kraich et al., 2001) and 293-PERV-B(43) (A1133818) (Czauderna et al., 2000).

Results and Discussion

Isolation of infectious PERV-A and PERV-B clones.

To investigate the relationship between biological and molecular properties of PERVs we have isolated replication competent molecular genomes from biological PERV isolates using a PCR-based technique. Viral isolates of PERV-A and -B were previously obtained by a limiting dilution of PK-15 cell supernatant on human 293 cells (Takeuchi et al., 1998). Clonal PERV-A or -B introduced by initial, single infection have spread to be saturated by many rounds of infection in these cell populations. These cultures are therefore likely to be mixtures of cells containing PERV proviruses integrated in different loci but enriched for infectious PERV proviruses. Genomic DNA (gDNA) of these PERV-A or -B infected 293 cells was isolated and used as template in PCR reactions to amplify PERV proviruses in two overlapping halves. These halves were then fused to full-length proviruses using a unique NheI restriction site located within the overlap (see Fig. 1). Several PCR reactions for amplification of 5′ and 3′ halves of each PERV subgroup were run in parallel and kept separate in the subsequent cloning procedure. PERV-specific PCR products of the expected sizes were obtained only from gDNA of PERV-A or -B infected 293 cells, while no signal was detected with gDNA of uninfected 293 cells (data not shown). PERV-A and -B 5′ and 3′ fragments were isolated, subcloned and fused to generate full-length proviruses using the unique restriction site within the overlap between 5′ and 3′ halves. Altogether nine 5′ half clones (five PERV-A, four PERV-B) and eight 3′ half clones (four PERV-A and four PERV-B) were randomly fused to generate nineteen PERV proviruses (nine PERV-A, six PERV-B, four PERV-4A/-B chimera).

Screen for infectious PERV proviruses

To identify infectious proviruses the 19 clones were initially tested for their ability to express Gag proteins by transfection into human 293 cells; 9 days after cell lysates were prepared, analysed by SDS–PAGE and probed with a rabbit polyclonal antibody against PERV capsid protein, kindly provided by Q-One Biotech (Fig. 2A). Of the nineteen PERV proviruses, six expressed Gag proteins (Fig. 2A, lanes 5, 10, 12, 14, 17 and 19) with a pattern identical to that found in 293 cells infected with a biological PERV-B isolate (lane 22). No signal was detected in lysates derived from untransfected or pcDNA-GFP-transfected cells (lanes 20, 21).

To test infectivity, cell-free supernatants of the six Gag-expressing cultures were harvested 30 days after transfection and used to challenge naive 293 cells. Successful transmission of PERV proviruses into target 293 cells was assayed by PCR for PERV-specific sequences (Fig. 2B). A primer pair specific for viral protease sequence amplified a band of the correct size from gDNA of five of the six target cell populations (Fig. 2B, lanes 1, 2, 3, 4, 6) as well as from gDNA of 293 cells infected with biological isolates of PERV-A or -B (lanes 8, 9). No transmission of protease sequence could be detected for provirus 69 (lane 5) and supernatant from pcDNA-GFP-transfected cells (lane 7), while the presence of template gDNA in all reactions was confirmed with primers specific for the human gene glyceraldehyde-3-phosphate dehydrogenase (Fig. 2B, GAPDH). Further PCR analysis with primers specific for PERV-A or -B envelope sequences confirmed clones 17 and 43.
Genome analysis of infectious PERV-A and -B clones

In summary, nine 5’ half clones (5 PERV-A, 4 PERV-B) and eight 3’ half clones (four PERV-A and four PERV-B) were randomly combined to construct nineteen PERV proviruses (nine PERV-A, six PERV-B, four PERV -A/-B chimera), of which five were found to be replication competent. Three 5’ half and five 3’ half clones were used to construct these five infectious proviral constructs, indicating that at least eight out of total fifteen half clones are functional. While PERV-60, which contains a PERV-A env, shares the same 5’ half derived from PERV-A-infected 293 cells with PERV-43 and -53, PERV-17 consists of halves both derived from 293/PERV-B cells. PERV-43 is a chimeric construct derived from PERV-A 5’ half and PERV-B 3’ half. PERV-60 representing PERV-A and PERV-17 representing PERV-B were further studied in detail.

**Genome analysis of infectious PERV- A and -B clones**

To determine their genomic structure, proviruses PERV-17 and -60 were sequenced and compared to three PK-15-derived PERV database sequences. 293-PERV-A(42) and 293-PERV-B(43) are infectious clones from a λ phage library based on 293 cells infected with PK-15 supernatant (Czauderna et al., 2000; Krach et al., 2001). PK15-ERV is a PERV-A cDNA clone from PK-15 cells which is non-infectious due to deletions in pol and at the beginning of the env open reading frame (Akiyoshi et al., 1998). The isolated proviruses PERV-60 and PERV-17 consist of 8714 and 8704 nt, respectively, and are 99% identical to the corresponding sequences displayed by 293-PERV-A(42) and 293-PERV-B(43), respectively.

The sequence differences between PERV-60 and 293-PERV-A(42) as well as between PERV-17 and 293-PERV-B(43) are summarized in Table 1. While the LTRs and leader sequences displayed interesting changes, the open reading frame amino acid sequences of PERV-60 and 17 displayed five residue changes, and the deduced envelope sequences two changes found in the genomes of PERV-60 and -17 were conserved in PK15-ERV and were also present in two or more of the five separately isolated molecular PERV clones (data not shown). This suggests that most of these changes reflect the sequence of the template proviruses rather than PCR errors. Limited sequencing of the other three infectious clones proved that they have small number of base changes and therefore are independent clones.

The deduced Gag amino acid sequences of PERV-60, -17 and 293-PERV-A(42) were identical while Gag of 293-PERV-B(43) differed in one residue. The deduced Pol amino acid sequences of PERV-60 and PERV-17 displayed five residue changes, and the deduced envelope sequences two changes each in comparison to 293-PERV-A(42) and 293-PERV-B(43). Finally, PERV-60 displayed a loss of one residue in Pol. However, the majority of these changes were found to be conserved in PK15-ERV (see Table 1) and other PERV database sequences (data not shown).

The LTR and leader sequences of PERV-17 and -60 were highly homologous to those of 293-PERV-A(42) and 293-
Cloning and titration of PERV

PERV-B(43) with 97 and 98% nucleotide identity, respectively. Within U3 of the 5’ LTR PERV-60 contained a deletion of 39 nt compared to 293-PERV-A(42). This sequence motif has previously been shown to act as viral enhancer and to multimerize dynamically upon serial passage of PERV in human cells (Scheef et al., 2001). PERV-17 and -60 contain two such enhancer repeats within their 5’ LTR and three within their 3’ LTR. The 5’ LTR of PERV-60 was also found to contain a base triplet insertion (CGC) located 5 nt downstream of the transcriptional start site at the beginning of R (Czauderna et al., 2000), which is not present in any other PERV sequence and whose function is unknown.

The primer binding sites (PBS) of 293-PERV-A(42) and 293-PERV-B(43) are complementary to the last 18 nt of tRNA_Gly but differ from those of PERV-60 and -17 at one position (G677A, G638A, respectively), as shown in Table 2. However, this change is conserved in other endogenous retroviruses, feline RD114 and yellow baboon (Papio cyno-
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Fig. 3. Transmission of the molecular PERV clones on 293 cells after three serial transfers of supernatant. Supernatants from 293 cells transfected with PERV-17, -43, -53, -60, -71 or pcDNA-GFP were serially transferred three times onto fresh 293 cells every 3 weeks; 8 days after the third transfer, cell lysates were prepared and probed with an anti-PERV capsid antibody.


tRNA\textsubscript{Glu} sequence. The PBS of PERV-17 contains an additional base change (T640G) which is only conserved in a mouse retrovirus-like 3OS genetic element. The effect of this second base change in the PBS of PERV-17 on priming of reverse transcription is unclear but overall the replication efficiency of this virus was comparable to that of the other molecular and biological PERV isolates (data not shown). PBS changes have not been reported for PERV previously. While HIV as well as avian viruses have been shown to be stringent in tRNA primer utilization, MLV, a close relative of PERV seems to have less stringent requirements. Similar to our observations for PERV, endogenous MLVs with altered PBSs have been isolated (Nikbakht et al., 1985; Colicelli & Goff, 1986; Petersen et al., 1991; Yamauchi et al., 1995; Lund et al., 2000). However, subtle base changes in the PBS of MLV have been shown to restore replication in a non-permissive embryonic stem cell line through the disruption of a negative regulatory element in the immediate vicinity of the PBS (Petersen et al., 1991; Yamauchi et al., 1995). While the alterations in the PBS had no effect on the replication efficiency of our PERV clones in 293 cells, it remains to be determined whether this is the case in other cell types. The remainder of the leader sequence of PERV-17 contained three further nucleotide changes and one

Table 1. Sequence comparison of PERV-60 and -17 proviral sequences with 293-PERV-A(42) and 293-PERV-B(43)

<table>
<thead>
<tr>
<th>Nucleotides</th>
<th>PERV-60 vs 293-PERV-A(42)</th>
<th>PERV-17 vs 293-PERV-B(43)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>5' LTR</strong></td>
<td>\textit{Δ}39bp (enhancer), insertion 510ccg511</td>
<td>\textit{C}373T, \textit{C}561T</td>
</tr>
<tr>
<td>PBS</td>
<td>\textit{G}677A</td>
<td>\textit{G}638A, \textit{T}640G</td>
</tr>
<tr>
<td>leader</td>
<td>–</td>
<td>\textit{C}651T, \textit{T}994C, insertion 750gltttcgggtatg752</td>
</tr>
<tr>
<td><strong>3' LTR</strong></td>
<td>\textit{G}8556A</td>
<td>Additional 39 bp enhancer, insertion 8645A8646</td>
</tr>
<tr>
<td><strong>Deduced amino acid sequences</strong></td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>Env</td>
<td>--</td>
<td></td>
</tr>
</tbody>
</table>

* Nucleotide A at position 5131 found only in 293-PERV-A(42) but not in any other PERV database sequence.
Table 2. Sequence analysis of the primer binding site

<table>
<thead>
<tr>
<th>Sequence source</th>
<th>Consensus</th>
<th>tRNA</th>
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<tbody>
<tr>
<td>Hs tRNA&lt;sub&gt;Gly&lt;/sub&gt; minus strand</td>
<td>TGC GTC GGC CGG GAA</td>
<td>Glycine</td>
</tr>
<tr>
<td>293-PERV-A(42)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>293-PERV-B(43)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PK15-ERV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hs tRNA&lt;sub&gt;Gly&lt;/sub&gt;1/2 minus strand</td>
<td>– – A – – – – – – – – – –</td>
<td>Glycine</td>
</tr>
<tr>
<td>PERV-60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RD114</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P&lt;sub&gt;c&lt;/sub&gt;EV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PERV-17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mm VL30</td>
<td>– A – A – G – – – –</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 4. Comparison of focus forming assays by immunostaining and vector transfer. Supernatant of 293 cells persistently infected with PERV-A and expressing the MFG<sub>Gal</sub>lacZ vector genome was titrated on 293 cells in duplicate. After a 3 day incubation one set was stained for capsid expression and the other for lacZ expression. 50 µl of neat virus supernatant or its 6-fold dilution was added to 293 cell cultures in 96-well plates (LacZ staining for 6-fold dilution is not shown). Control stains were performed on uninfected 293 cells.

Quantitative analysis of infectivity of molecular and biological PERV isolates

To compare the replication efficiency of molecular and biological PERV isolates, an in situ focus forming assay by immunostaining was established by modifying a previously established method for HIV titration (Simmons et al., 1995). Immunoperoxidase staining for PERV antigens of the same principle has been reported previously (Krach et al., 2000; Stephan et al., 2001; Tacke et al., 2001). 293 cells infected by PERV were screened for expression of Gag proteins 2 or 3 days after infection using our polyclonal antibody raised against a recombinant PERV-A capsid protein in combination with alkaline phosphatase-labelled secondary antibodies. Cells infected by MLV-A and GALV were also clearly stained due to cross-reaction of the primary anti-PERV antibody with their capsid proteins. Pseudotype assays using MLV-based vectors have been used to quantify virus entry and integration by us and others (Qari et al., 2001; Takeuchi et al., 1998; Wilson et al., 2000). To compare these two focus forming assays by immunostaining and vector transfer, we introduced the MFG<sub>Gal</sub>lacZ vector genome into 293 cells persistently infected with PERV isolates as well as MLV-A and GALV. Supernatants of cell populations, more than 99% of which expressed both capsid and LacZ proteins and therefore were supposed to produce both replication competent virus and lacZ vector particles, were obtained and titrated on 293 cells in duplicate. One series was stained for capsid expression and the other for LacZ after a 3 day incubation. Examples of the two detection methods are shown in Fig. 4 and a representative set of results of three repeated experiments is shown in Table 3.
indeed one PERV envelope recombinant has already been shown to alter tropism in the past. Up to now very few infectious clones of PERV have been analysed and assessed for infectivity of wild-type PERV may have been underestimated. MLV-reporter vectors should be interpreted carefully as the sensitivity between the two focus forming assays was only a 2- to 5-fold difference for PERV. For MLV and GALV, however, the difference in infectivity between PERV-A, -B, and other gammaretrovirus isolates was significantly higher than those of the PERV clones irrespective of the focus forming assay used (Table 3). Furthermore, the titres of PERV-A isolates repeatedly were more than two magnitudes higher than those of PERV-B clones, suggesting that PERV-A is more infectious for 293 cells than PERV-B. Finally, we noted a significant difference in the titres obtained for PERV by the two focus forming assays. While the PERV-A titres obtained by lacZ vector transfer were in the range previously reported (about 10^3 IU/ml) (Takeuchi et al., 1998; Wilson et al., 2000), the immunostain revealed up to 8.6 x 10^3 IU/ml, suggesting that this detection method is more sensitive. We calculated the difference in sensitivity between immunostaining and lacZ transfer to be as much as 40-fold for PERV. For MLV and GALV, however, the difference in sensitivity between the two focus forming assays was only between 2- and 5-fold, suggesting that these viruses transfer lacZ vectors more efficiently than PERV. Thus, in retrospect, PERV infectivity and host-range studies assessed by transfer of MLV-reporter vectors should be interpreted carefully as the infectivity of wild-type PERV may have been underestimated. The PERV immunostaining assay described here retains the advantage of being a short-term assay while providing a more sensitive and accurate measurement of PERV infection.

Endogenous retroviruses are known to evolve rapidly and only minor genetic variations within retroviral genomes have been shown to alter tropism in the past. Up to now very few infectious clones of PERV have been analysed and assessed for their ability to evolve by mutation or recombination, and indeed one PERV envelope recombinant has already been reported (Wilson et al., 2000). In order to understand PERV replication and its associated risks for humans in xenotransplantation it will be important to isolate various different PERV genomes from different sources to assess their infectivity as well as their ability to mutate or recombine into novel forms with altered biological properties. We isolated PERV-A and -B proviruses with the same infectious properties as their parent isolates by a PCR-based method. These clones will be useful as standard clones, whilst this cloning method, in combination with the sensitive and quantitative immunocytological detection method described here, will be useful to address these issues.

### Table 3. PERV titration on 293 cells by two focus forming assays

Representative titres of three independent experiments are shown.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Immunostaining*</th>
<th>lacZ†</th>
<th>Immunostaining/lacZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>PERV-60</td>
<td>86000</td>
<td>2100</td>
<td>41</td>
</tr>
<tr>
<td>PERV-A</td>
<td>63000</td>
<td>2100</td>
<td>29</td>
</tr>
<tr>
<td>PERV-17</td>
<td>210</td>
<td>25</td>
<td>8.2</td>
</tr>
<tr>
<td>PERV-B</td>
<td>550</td>
<td>30</td>
<td>18</td>
</tr>
<tr>
<td>MLV-A</td>
<td>680000</td>
<td>290000</td>
<td>2.3</td>
</tr>
<tr>
<td>GALV</td>
<td>14000000</td>
<td>2700000</td>
<td>5.2</td>
</tr>
</tbody>
</table>

* Detection of infection with a cross-reactive anti-PERV capsid antibody. † lacZ rescue.

This work was supported by the Medical Research Council. We thank Jonathan Stoye for helpful discussions, Dimitrios Stefanidis for technical assistance and David Onions (Q-One-Biotech) for the donation of anti-PERV antibody.

### References


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