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Characterization, Mapping, and Distribution of the Two XMRV Parental Proviruses

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Xenotropic murine leukemia virus-related virus (XMRV) was previously reported to be associated with human prostate cancer and chronic fatigue syndrome. Our groups recently showed that XMRV was created through recombination between two endogenous murine retroviruses, PreXMRV-1 and PreXMRV-2, during the passaging of a prostate tumor xenograft in nude mice. Here, multiple approaches that led to the identification of PreXMRV-2, as well as the distribution of both parental proviruses among different mouse species, are described. The chromosomal loci of both proviruses were determined in the mouse genome, and integration site information was used to analyze the distribution of both proviruses in 48 laboratory mouse strains and 46 wild-derived strains. The strain distributions of PreXMRV-1 and PreXMRV-2 are quite different, the former being found predominantly in Asian mice and the latter in European mice, making it unlikely that the two XMRV ancestors could have recombined independently in the wild to generate an infectious virus. XMRV was not present in any of the mouse strains tested, and among the wild-derived mouse strains analyzed, not a single mouse carried both parental proviruses. Interestingly, PreXMRV-1 and PreXMRV-2 were found together in three laboratory strains, Hsd nude, NU/NU, and C57BR/cd, consistent with previous data that the recombination event that led to the generation of XMRV could have occurred only in the laboratory. The three laboratory strains carried the Xpr1^{n} receptor variant nonpermissive to XMRV and xenotropic murine leukemia virus (X-MLV) infection, suggesting that the xenografted human tumor cells were required for the resulting XMRV recombinant to infect and propagate.

Gammaretroviruses have a broad host range, infecting organisms as diverse as mammals, birds, and reptiles (9). Perhaps the best-studied retroviruses within the gammaretrovirus genus are murine leukemia viruses (MLVs), which have both endogenous and exogenous counterparts. Mouse genomes contain a large number of endogenous MLVs, and different *Mus* subspecies are highly variable in the number of MLV insertions that they harbor.

Endogenous proviruses are carried as part of the genome of the host species and are subject to the same forces of evolution as their host, resulting in the slow accumulation of mutations over time that can eventually render them inactive. However, in some species there are intact endogenous proviruses that can be activated to produce infectious virus, through external stimuli or through recombination with other endogenous or exogenous viruses (31, 33).

XMRV (xenotropic murine leukemia virus-related virus) was first identified as a possible human pathogen in 2006 in a prostate cancer (PC) patient cohort (58). It was later reported in a high percentage of chronic fatigue syndrome (CFS) patients, as well as some healthy subjects, although no causal link between XMRV and any human disease has ever been established (35). In contrast, there are a large number of studies from many laboratories in which either no virus or no association with disease was found in a variety of human populations, despite the use of multiple highly sensitive detection methods (2, 10, 12–14, 17, 18, 21, 22, 26, 27, 44, 45, 47, 53, 59, 60). Strikingly, two recent replication studies by Knox et al. and Shin et al. showed that patients who were previously reported as XMRV positive were, in fact, XMRV negative (29, 49). Furthermore, viral sequences detected in human samples do not show the extent of diversity that would be expected from a human retrovirus that replicates within a population, suggesting that the observed clinical isolates could be explained by laboratory contamination from a single source (23). Finally, XMRV is highly susceptible to the antiviral effects of human cellular restriction factors, making the virus an unlikely candidate to overcome the blocks against its replication in the context of human infection (7, 8, 19, 40, 52). Taken together, these findings challenge the notion that XMRV is a genuine human virus.

The 22R*v1* cell line was derived from a human prostate tumor (CWR22) passaged repeatedly as xenografts in nude mice (50). 22R*v1* cells contain multiple XMRV insertions (28) and produce the virus at very high titers ($\sim 10^9$ to 10^{10} RNA copies/ml; data not shown). The virus produced by these cells differs by only 1 nucleotide (nt) from the consensus XMRV sequence reported in patient samples (28). In a recent study, analysis of genetic material from early and late xenograft samples of the tumor line that was used to generate 22R*v1* cells revealed the absence of XMRV from early samples but its presence in the late samples (39). Moreover, the discovery of two endogenous MLV proviruses, PreXMRV-1 and PreXMRV-2, present in the mouse tissues used to passage the xenografts was reported. A heterozygous progeny virion derived from these proviruses most likely generated XMRV by a unique recombination event (39). Here we describe multiple screening

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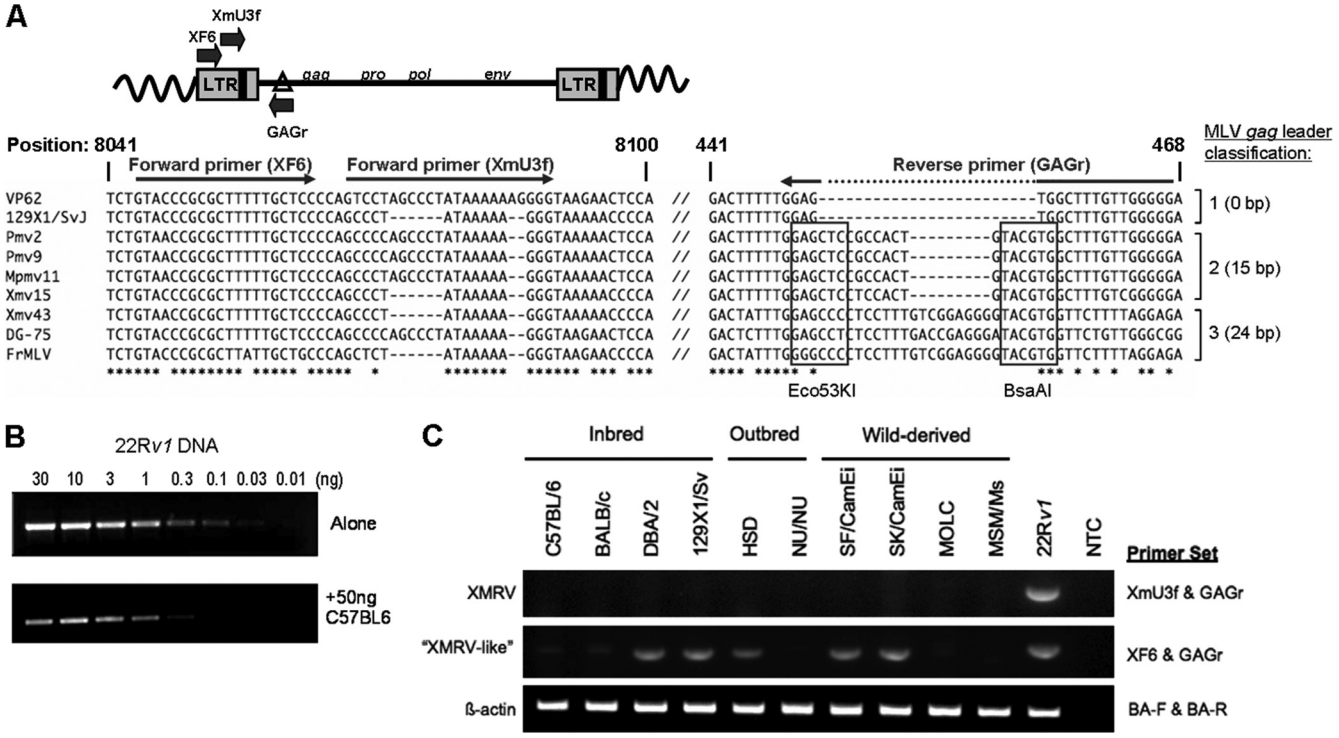

FIG 1 Screening of mouse DNA for XMRV and XMRV-like elements by PCR. (A) Schematic representation of primers used (upper panel); their exact locations are indicated by arrows (lower panel). Positions are based on the VP62 XMRV genome (EF185282). MLV *gag* leader regions can be divided into three subgroups based on the length polymorphism. Unique restriction sites BsaAI and Eco53KI, which distinguish proviruses in the first group (0 bp; XMRV-like) from the rest (15 or 24 bp), are shown in boxes. (B) Sensitivity and specificity of the PCR assay. Serial dilutions of genomic DNA from 22R*v1* cells were amplified with the XMRV-specific primer set (GAGr and XmU3f) in the presence or absence of 50 ng of C57BL/6 genomic DNA. (C) The absence of XMRV (top row) and presence of an XMRV-like provirus (middle row) in a panel of representative mouse strains. The primer sets used for each screening assay are indicated on the right. Note that the NU/NU and Hsd nude mice are outbred strains; only one representative of each is shown. Analysis of larger numbers of individual mice from these outbred strains is shown in Fig. 5. β-Actin served as amplification control. NTC, no-template control.

approaches that led to the identification of PreXMRV-2, the integration sites of the parental proviruses in the mouse genome, and their distribution among 94 different mouse strains.

MATERIALS AND METHODS

DNA samples. The xenograft samples from the CWR22 prostate tumor were obtained as previously described (39). Some of the mouse DNA samples were kindly provided by the following individuals: *Mus cervicolor popaeus* (J53), *Mus caroli* (J136), *Mus cookii* (J135), and *Mus spicilegus* (Halbturn) (J131) from Christine Kozak (NIAID, Bethesda, MD); *Mus macedonicus* (XBS), *Mus cervicolor cervicolor* (CRV), *Mus musculus bactrianus* (BIR), *Mus famulus* (FAM), *Mus platythrix* (PTX), *Mus spicilegus* (ZRU), and *Mus musculus musculus* (MPB) from François Bonhomme (ISE, Montpellier, France); and C57BR/cd, LPT/Le NZW/Lac, PWK/Ph, and WMP/Pas from Greg Towers (UCL, London, United Kingdom); Harlan Sprague Dawley nude (Hsd nude) and NIH Swiss nude mouse DNAs were obtained from Harlan Laboratories, Indianapolis, IN. *Mus dunni* DNA was prepared from tail fibroblast (MDTF) cells. NCRNU-M DNA was purchased from Taconic Farms. NU/NU DNAs were prepared from spleens purchased from Charles River Laboratories (Wilmington, MA). All other mouse genomic DNA samples were purchased from The Jackson Laboratory (Bar Harbor, ME).

PCR. All PCR analyses were performed using either a Bio-Rad C1000 Thermal cycler or an MJ Research Peltier Thermal Cycler PTC200. For screening experiments and host-virus junction DNA amplification, 50 ng of mouse genomic DNA was used per 25-μl reaction mixture. GAGr (5′-TCCCCCAACAAAGCCACTCCA-3′) is specific for the XMRV *gag* leader deletion, and XmU3f (5′-GTCCTAGCCCTATAAAAAAGGGG-3′) is specific for the 2-nt insertion in the U3 region of the XMRV long terminal repeat (LTR), while XF6 (5′-GTACCCGCGCTTTTTGCTCC-3′), just upstream of XmU3f in the U3 region, would amplify many endogenous MLVs (Fig. 1A). To amplify PreXMRV-2 host-virus junctions, flanking primers for chromosome 12 (upstream, C12-1F, 5′-TGCTGGACAGAAT CTCTGGTCTCT-3′; downstream, C12-4R, 5′-GATACTCAAGTGGTT CCCACCC-3′) were used in combination with internal provirus primers 129-1R (5′-GCGGTTTCGGCGTAAAACCGAAAGCA-3′) and envOUT1 (5′-CTGACCCAACAGTATCACCAACTC-3′), respectively. To amplify PreXMRV-1 host-virus junctions, flanking primers for chromosome 3 upstream, C3-2F (5′-GTAGCCATCAATGAGTTGTGAC-3′), and downstream, C3-4R (5′-GGATCTTCCAGTAGAACTATGTCC-3′), were used with XgR2 (5′-AGAGACAAAGACAAAACGATCGCCGGCC-3′) and XF6 (see above), respectively. The preintegration site of PreXMRV-1 was detected using the flanking primers for Chr.3 upstream, C3-1F (5′-CCACCACATATACGTACACCTTC-3′), and downstream, C3-6R (5′-GCAGTTTCTGGATGGTCATTCC-3′). β-Actin was amplified using the BA-F (5′-AGCCATGTACGTAGCCATCC-3′) and BA-R (5′-CTCTCAGCTGTGGTGGTGAA-3′) primer set, which amplifies both mouse and human β-actin. Intracisternal A-type particles (IAP) were amplified as described previously (38, 43).

For single-genome amplification, 3-fold serial dilutions of mouse genomic DNA were amplified with primer set 303F (5′-GCTAACTAGA TCTGTATCTGGCGG-3′) and 1018R (5′-CTTTATAGAGGGGGTAAG GGCAG-3′) in the first round and then with 419shF (5′-ATCAGTTAAC CTACCCGAGTCG-3′) and 628R (5′-GGTAGTTACGGTCTGTCCCA T-3′) in the second round, 10 reactions per dilution. The dilution at which 3/10 reactions are positive was selected, nested PCR was performed in a

96-well format, and reactions were analyzed by electrophoresis on precast 2% E-gels (Invitrogen). Positive reaction products were digested with BsaAI (NEB); fragments that remained undigested were isolated using a gel purification kit (Qiagen) and sequenced to confirm the presence of the *gag* leader deletion.

For Xpr1 cloning and sequencing, exons 10, 11, 12, and 13, comprising portions of the ECL3 and ECL4 loops, were amplified using the primers as previously cited (64).

Detection of provirus fragments in unblots. Unblot assays were performed as described previously, with slight modifications (55). The probe gL1 for detecting the *gag* leader deletion was 5′-TCGGACTTTTTGGAG TGGCTTTGTTGGGGG-3′. The probe was end labeled with [γ-^{32}P]ATP using polynucleotide kinase (NEB) at 37°C for 1 h, and the labeled probe was cleaned using Micro Bio-Spin chromatography columns (Bio-Rad). Mouse genomic DNA (15 μg) either was directly digested with BsmI or Eco53KI (NEB) or was first obtained by whole-genome amplification from much smaller quantities using the REPLI-g midikit (Qiagen) and phenol extraction. Digested genomic DNA samples were electrophoresed on a 0.9% agarose gel for 20 to 30 h, the gel was dried under vacuum, and DNA was visualized by ethidium bromide (EtBr) staining. The dried gel was denatured for 15 min in 0.5 M NaOH, 1.5 M NaCl; neutralized for 15 min in 1 M Tris-HCl, 1.5 M NaCl, pH 8.0; and hybridized overnight with the radiolabeled probe (7.5×10^6 cpm) in hybridization buffer (5× SSPE [1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA (pH 7.7)] 0.1% SDS, pH 7.4). The hybridized gel was washed (2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% SDS) and subjected to autoradiography.

Integration site mapping. The integration sites for PreXMRV-1 and PreXMRV-2 in the mouse genome were identified using the GenomeWalker kit (Clontech) according to the manufacturer's recommendations, with some modifications. To clone the PreXMRV-2 integration site, DBA/2J genomic DNA was digested with six restriction enzymes separately (NEB). Fragments with overhangs were blunted with the Quick Blunting kit (NEB). Adaptors supplied with the kit were ligated to fragment ends to construct genomic DNA libraries, which were serially diluted to avoid generation of recombinants during PCR. Nested PCR was performed with two sets of adaptor-specific primers (AP1 and AP2) provided with the kit and provirus-specific primers, 129-1R (see above) and 129-2R (5′-GGTCTGTCCCATGATCTCGAGAACAC-3′), on diluted libraries. Fragments from the first round of PCR were also subjected to BsaAI digestion (NEB) to prevent most proviruses without the *gag* leader deletion from being amplified in the second round. A single band was observed after the second PCR, which was cloned into pCR4-TOPO (Invitrogen). Clones were screened by PCR for the presence of a >1.2-kb insert as well as the absence of the restriction site by BsaAI digestion; those that passed both tests were sequenced. Five such clones revealed identical fragments containing the *gag* leader deletion, a complete 5′ LTR, and flanking cellular DNA, which mapped to mouse chromosome 12.

To clone the integration site of PreXMRV-1, C57L/J DNA was subjected to the same process as described for PreXMRV-2. Two of the restriction enzymes chosen were ApaLI and Hpy166II, both of which would digest most C57BL/6 MLVs in the LTR upstream of the provirus primer binding site and prevent their amplification but would leave PreXMRV-1 intact to be amplified. Nested PCR was performed with AP1 and AP2 supplied with the kit and provirus-specific primers XgR1 (5′-GCGACTC AGTCTATCGGATGACTGG-3′) and XgR2 (see above). Fragments were cloned, sequenced, and screened as described for PreXMRV-2. The correct clones with PreXMRV-1 LTR and flanking sequence mapped the provirus to mouse chromosome 3.

Primers were then designed for the flanking region based on the cloned sequence and combined with virus-specific primers to screen for and sequence both proviruses in different strains. Sequencing was performed by the Tufts University Core Facility.

Nucleotide sequence accession numbers. The complete provirus sequences of PreXMRV-1 and PreXMRV-2 plus the flanking regions in the mouse genome have been deposited in GenBank under the accession numbers HE599400 and HE599401, respectively.

RESULTS

Searching mouse genomes for XMRV-like elements. Four different strategies were employed to search for XMRV-like sequences in mouse genomes. In all cases, proviruses that contain stretches of identity to XMRV were detected; however, no XMRV provirus was present in any of the strains tested, as previously reported (39).

XMRV-specific PCR assay. The XMRV genome shows up to ~95% identity with some MLVs, found as both endogenous and exogenous viruses in mice (58). To detect XMRV sequences in mouse DNA, a highly specific PCR assay was designed that selectively amplified the 5′ region of the XMRV genome but not any of the endogenous MLVs in C57BL/6 under the conditions used (25, 39). Due to the sequence similarity between XMRV and endogenous MLVs, primer selection for PCR was restricted to regions of the provirus that were sufficiently divergent. One of the features that distinguish the XMRV genome from most other MLVs is a 24-bp deletion in its *gag* leader region (Fig. 1A). This deletion was initially thought to be specific for XMRV but has recently been detected in the whole-genome shotgun (WGS) sequence of an inbred strain, 129X1/SvJ (GenBank ID, AAHY01591888.1) (11), and later in a few other strains using deep sequencing (23). A reverse primer (GAGr) spanning the *gag* leader deletion was used in combination with a forward primer (XmU3f) that includes a 2-bp insertion unique to the XMRV U3 region (Fig. 1A). In addition, the sensitivity and specificity of the assay were assessed (Fig. 1B).

In the initial screen with the XmU3f-GAGr primer set, XMRV-specific sequences were not detected in any of the 46 wild-derived or 48 laboratory mouse strains tested (39), while the 22R*v1* control cell line was always positive, implying that no endogenous provirus identical to XMRV was present in these strains (Fig. 1C).

The partial U3 region included in the 129X1/SvJ sequence revealed that the XMRV-specific 2-bp insertion was not present in the 129X1/SvJ provirus (Fig. 1A). Moreover, there was a 6-bp deletion in the XmU3f primer binding site, which prevented the detection of this provirus. A different primer was designed (XF6) to amplify the 129X1/SvJ provirus, which yielded a clear band in several strains when used with the GAGr primer (Fig. 1C). The PCR products from all strains were sequenced and confirmed to be identical to each other, differing from the 129X1/SvJ sequence at two positions (157 and 263 of the GenBank entry) where the deposited sequence has an extra C and T nucleotide, respectively.

SGS. To obtain more accurate sequence information and to rule out potential PCR recombination between different proviruses, a single-genome sequencing (SGS) approach was developed. MLV genomes were classified into three groups based on their *gag* leader profiles: those that contain (i) 0 bp (XMRV-like *gag* leader deletion), (ii) 15 bp, or (iii) 24 bp (Fig. 1A). The first group includes all XMRV isolates, as well as the uncharacterized provirus fragments identified by deep sequencing (23), while the second and third groups include all C57BL/6 proviruses and some exogenous MLVs (25). A diagnostic BsaAI restriction site was identified at the junction of the deletion, which would selectively digest a large fraction of the sequences in the last two groups (Fig. 1A). This selection method eliminated most "unwanted" provi-

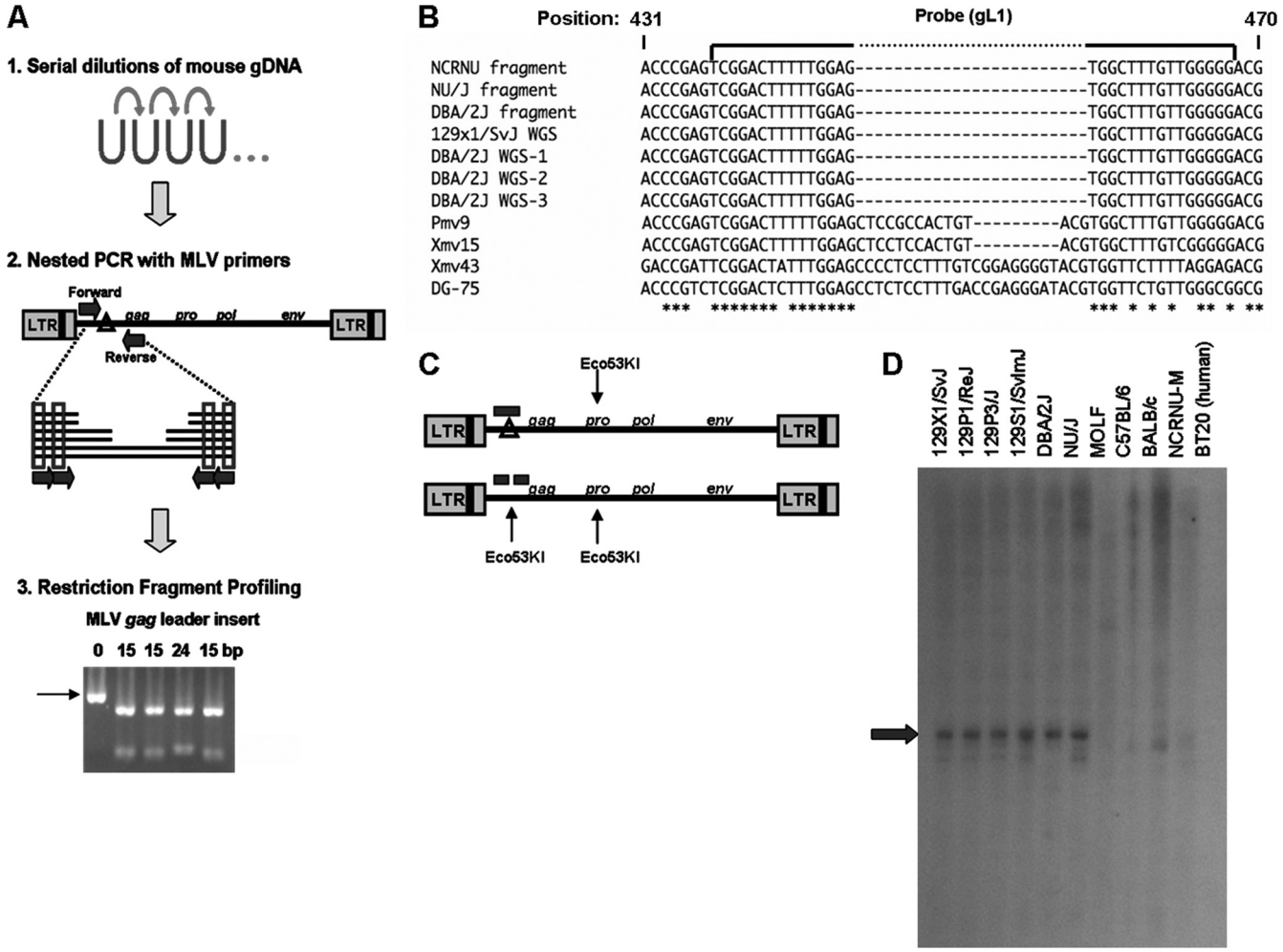

FIG 2 Detection of XMRV-like elements. (A) Outline of the SGS approach. BsaAI digestion profiling (Fig. 1A) distinguishes fragments with the deletion from the rest. (B) Alignment of sequences from three strains positive for the *gag* leader deletion (NU/J, NCRNU, and DBA/2J), the whole-genome shotgun (WGS) sequences from the 129X1/SvJ and DBA/2J genomes in GenBank, and other MLV sequences as a reference. Numbers reflect corresponding positions in the VP62 genome (EF185822). (C) Schematic representation of the Eco53KI sites and the gL1 probe binding site in MLV genomes. (D) Unblots of various mouse genomic DNA samples digested with Eco53KI and hybridized with a radiolabeled probe. A distinct provirus fragment corresponding to PreXMRV-2 was detected in six strains (arrow).

ruses and focused on those with the *gag* leader deletion, plus a few with a polymorphism at the restriction site.

Serial dilutions of genomic DNA were subjected to nested PCR with well-conserved MLV primers upstream and downstream of the *gag* leader deletion (see Materials and Methods). Positive reactions were analyzed by BsaAI digestion. Fragments that remained undigested (i.e., those with the *gag* leader deletion) were analyzed further by sequencing, and the presence of the deletion (or a polymorphism in the restriction site) was confirmed. Using this approach, a total of seven mouse strains were analyzed, three of which contained XMRV-like *gag* leader regions (Fig. 2B), providing further evidence that such elements are present as endogenous viruses in the genomes of several inbred mouse strains.

Detection of provirus fragments in unblots. To investigate the distribution of XMRV-like sequences in mouse genomes and to provide further support for the PCR screening data, genomic hybridization blot assays were performed using the unblotting technique (55). Briefly, genomic DNAs from various mouse strains were digested with a restriction enzyme, run on an agarose gel that was then dried down, and directly hybridized with a *gag* leader-specific radiolabeled probe that crossed the deletion site (see Materials and Methods). The enzyme Eco53KI was chosen since it would selectively digest proviruses lacking the *gag* leader deletion at the probe binding site while leaving proviruses with the deletion intact up to the next downstream restriction site (Fig. 1A and 2C). A single high-intensity band was detected in six out of 10 strains analyzed, in agreement with the genome screening results by PCR (Fig. 2D).

GenBank database searches. To investigate whether other mouse strains besides the reported 129X1/SvJ strain also carried the *gag* leader deletion (11, 23), an extensive search of the NCBI Trace Archives was performed by querying the *gag* leader region of XMRV. This search returned an additional three hits in the DBA/2J genome and one unassigned record with exactly the same sequence (GenBank IDs, ti:1098845661, 1096851092, 1096808503, and 1039132836). None of these sequence files

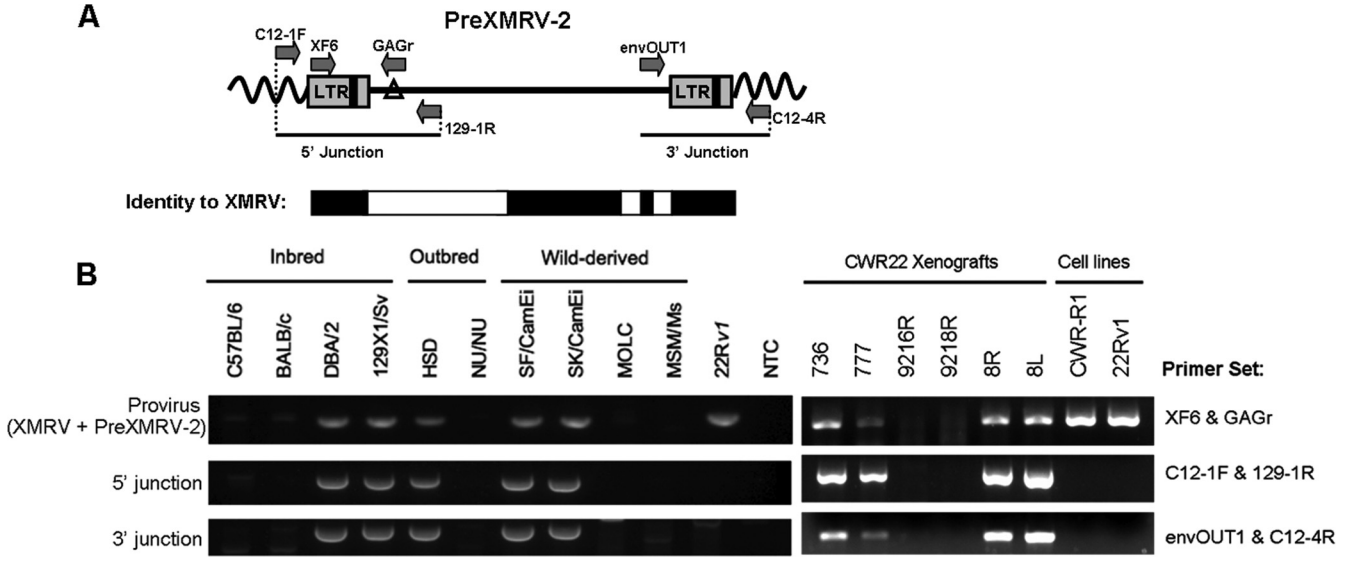

FIG 3 Detection of PreXMRV-2 in mouse strains and CWR22 xenografts. (A) PCR approach to amplify host-virus junctions from genomic DNA. Regions of identity between XMRV and PreXMRV-2 are depicted as white bars; regions of difference are shown as black bars (not drawn to scale). (B) Genomic DNAs from representative mouse strains (left lanes) and early-passage samples of the CWR22 prostate cancer xenograft (right lanes) were analyzed for the presence of PreXMRV-2 (Table 1 has a complete list of mouse strains screened). In the 22R*v1* and CWR-R1 cell lines, the fragments detected by internal provirus primers are XMRV, as neither contains PreXMRV-2. NTC, no-template control.

extended beyond the LTR into the flanking region, providing no information about the integration site. Nevertheless, the presence of this XMRV-like sequence in multiple reads further confirmed our results and those of others (23) that XMRV-like endogenous sequences are indeed present in the genomes of several strains of mice.

Mapping the integration site of PreXMRV-1 and PreXMRV-2. Although multiple independent lines of evidence implied the presence of XMRV-like elements in various mouse genomes, further analysis required determination of their integration sites. A ligation-mediated PCR approach, described in Materials and Methods, was used to amplify and clone fragments of DBA/2J genomic DNA that, like PreXMRV-2, contained the specific *gag* leader deletion and included the complete 5′ LTR and flanking sequence. Knowledge of the flanking sequence, which mapped to chromosome 12 on the C57BL/6J genome sequence, allowed the design of primer pairs to amplify host-virus junctions from mouse genomic DNA (Fig. 3A).

The integration site of the provirus was confirmed by amplifying both 5′ and 3′ junctions from the DBA/2J genomic DNA, using flanking chromosomal primers and internal provirus primers (Fig. 3A and B). Both junction fragments contained a full-length LTR and the expected flanking region, as confirmed by sequencing, with a four-base "GGAA" target site duplication (TSD). The *gag* leader region included in the 5′ junction fragment was also sequenced again, confirming the presence of the 24-bp deletion. It is important to note that the provirus integration site is found within a rather complex, repetitive, and poorly sequenced region, making it impossible to use PCR to distinguish the preintegration site from the occupied integration site. We previously named this provirus PreXMRV-2 (GenBank ID, FR871850.1), to reflect its role in the generation of XMRV (39).

The integration site of PreXMRV-1 was cloned from C57L/J genomic DNA, using the same approach as that used to clone the integration site of PreXMRV-2, with slight modifications (see Materials and Methods). Sequencing the upstream flanking sequence, which was cloned together with the PreXMRV-1 LTR, revealed that the PreXMRV-1 insertion is found on mouse chromosome 3. Amplification of host-virus junctions from both ends followed by sequencing confirmed that the location indeed corresponded to PreXMRV-1, with a TSD of "GCAG" (Fig. 4A and B).

Strain distribution of PreXMRV-1 and PreXMRV-2 and absence of XMRV. The discovery and characterization of PreXMRV-1 were previously described (39). To determine the distribution of the two XMRV precursors, a panel of mouse DNA samples from 48 laboratory and 46 wild-derived strains were screened for the presence of PreXMRV-1 and PreXMRV-2. As shown in Fig. 1C, no provirus identical to XMRV was present in any of the mouse strains tested (39). In the present analysis, 23 inbred, 2 outbred, and 2 wild-derived strains were positive for PreXMRV-2 (Table 1). Its strain distribution agreed perfectly with the earlier mouse genome screening results with XMRV-like *gag* leader-specific primers (Fig. 3B), consistent with PreXMRV-2 being the only provirus in these strains with the characteristic deletion. PreXMRV-1 was present in 4 inbred, 2 outbred, and 8 wild-derived strains (Table 1). Only one inbred strain (C57BR/cd) and two outbred strains (Hsd nude and NU/NU) contained both proviruses.

PreXMRV-1 seems to be quite rare among inbred strains; to date, it has been detected only in C57L/J, C58/J, C57BR/cd, and NZW/Lac mice. Although PreXMRV-2 is absent from the sequenced C57BL/6 mouse genome, it was present in nearly half of the inbred strains tested, suggesting that it is a common provirus insertion. The two wild-derived strains that harbor PreXMRV-2 belong to *M. m. domesticus* subspecies, SF/CamEi and SK/CamEi (Table 1). *M. m. domesticus* is native to the Near East, Europe, and Africa and later colonized the Americas and Australia as human travel between these continents became common (20). PreXMRV-1 was detected in several strains of Asian mice, *M. m. castaneus* and

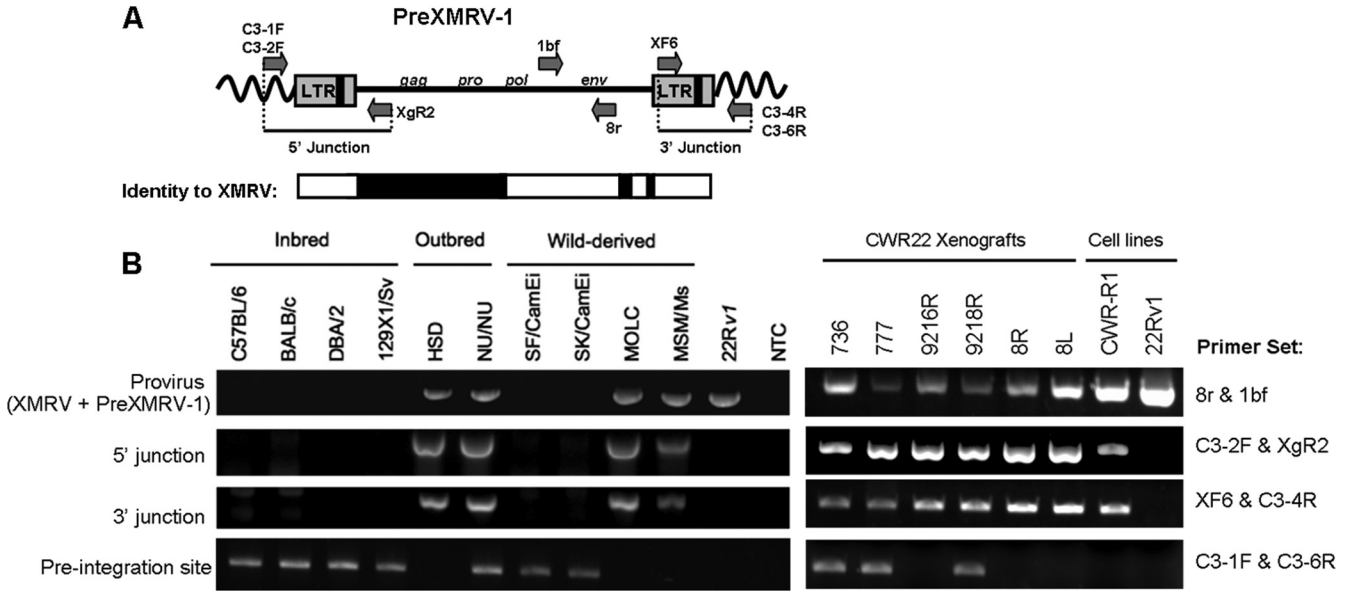

FIG 4 Detection of PreXMRV-1 in mouse strains and CWR22 xenografts. (A) PCR approach to amplify host-virus junctions from genomic DNA. Regions of identity between XMRV and PreXMRV-1 are depicted as white bars; regions of difference are shown as black bars (not drawn to scale). (B) Genomic DNAs from representative mouse strains (left lanes) and early-passage samples of the CWR22 prostate cancer xenograft (right lanes) were analyzed for the presence of PreXMRV-1 or its unoccupied integration site (Table 1 contains a complete list of mouse strains screened). In the 22R*v1* cell line, the fragment detected by internal provirus primers is XMRV. In the CWR-R1 cell line, which is known to contain a small number of cells derived from the mouse host (39), the provirus fragment is from both XMRV and PreXMRV-1, while the detected junction fragments are from PreXMRV-1 alone. NTC, no-template control.

M. m. molossinus, the former being an ancestral species that gave rise to the latter, and also in the CALB/Rk strain thought to be a hybrid between *M. m. domesticus* and *M. m. castaneus* (24).

Interestingly, the only strains that harbor both PreXMRV-1 and PreXMRV-2 are one inbred (C57BR6/cd) and two outbred strains: the Harlan Sprague Dawley (Hsd) nude strain maintained by Harlan Laboratories and the NU/NU strain maintained by Charles River Laboratories (39). Both outbred strains are likely to have been used for passaging the prostate tumor xenografts that later gave rise to the 22R*v1* cell line (39). Because both are outbred strains, the numbers and distributions of specific proviruses may differ among individual mice. Therefore, the distribution of XMRV, PreXMRV-1, and PreXMRV-2 was analyzed in a large panel of Hsd nude mice ($n = 49$) and NIH Swiss mice ($n = 10$) (Fig. 5; Table 2). The NIH Swiss strain was included because it might have contributed to the outbred nude mouse strains (16). Among the Hsd nude mice analyzed, 27% were positive for PreXMRV-1 only, 10% were positive for PreXMRV-2 only, and 53% contained both proviruses, consistent with the null allele frequencies of 0.45 and 0.61, respectively (Fig. 5A and Table 2). Thus, about half of the mice within this outbred colony carried both XMRV parental proviruses (Fig. 5A). This result is consistent with the observation that some, but not all, of the mouse tissues associated with the early tumor xenografts contained both PreXMRV-1 and PreXMRV-2 (Fig. 3B and 4B) (39). NIH Swiss mice were more homogenous; all tested samples contained PreXMRV-2, while none contained PreXMRV-1 (Fig. 5B). The variability of the NU/NU outbred mice ($n = 5$) for the presence of PreXMRV-1 and PreXMRV-2 was shown previously; 2/5 mice contained both PreXMRV-1 and PreXMRV-2 (39). Most importantly, XMRV was not present in any of the 64 outbred samples tested, confirming previous data on its absence from various inbred and wild-derived strains (Fig. 5A and 5B) (39).

The Xpr1 receptor in Hsd nude, NU/NU, C57BR/cd, and CWR22 xenograft mouse DNA is nonpermissive to XMRV entry. Xenotropic MLV (X-MLV) and polytropic MLV (P-MLV) use different variants of the XPR1 receptor (Fig. 6A) to gain entry into cells (63, 64). Many laboratory strains contain the Xpr1^n allele, which does not support X-MLV entry, while P-MLV can use the protein encoded by this variant to infect cells. We determined whether XMRV, which has a xenotropic *env* gene, would have been able to infect the cells of the mice used for passaging the tumor, as well as the three mouse strains found to harbor both parental proviruses. Exon 13 of the Xpr1 gene, corresponding to the ECL4 region, was amplified and sequenced from Hsd nude ($n = 21$) and NU/NU ($n = 5$) mice (Fig. 6B). Every individual mouse tested among these two strains had the Xpr1^n allele, which is nonpermissive to X-MLV entry, suggesting that the XMRV recombinant would not have been able to infect these mice. Exons 10, 11, and 12 of the Xpr1 gene, corresponding to the ECL3 region, were also sequenced from Hsd nude ($n = 4$) and NU/NU ($n = 3$) mice, and all carried the Xpr1^n allele (Fig. 6B). All early xenografts, which contained some host mouse DNA from the original transplant, also carried the nonpermissive Xpr1^n allele (Fig. 6C). The absence of a functional receptor to support X-MLV entry shows that XMRV could not have infected the cells of the host mouse in which it was created and provides further support for the requirement of the transplanted human cells for infection. Lastly, the C57BR/cd mouse, which is positive for both PreXMRV-1 and PreXMRV-2, contained the Xpr1^n allele as well (Fig. 6B). This result is consistent with a previous report by Baliji et al. that showed that C57/BR, a strain related to C57BR/cd, also contains the Xpr1^n allele (5).

DISCUSSION

Recent work identified two endogenous MLV proviruses from the xenografts of the CWR22 human prostate tumor passaged in nude

TABLE 1 Mouse strains tested for the presence of XMRV,[a] PreXMRV-1, and PreXMRV-2

Mouse type and species or subspecies	Strain	Presence of provirus: PreXMRV-1	Presence of provirus: PreXMRV-2	Mouse type and species or subspecies	Strain	Presence of provirus: PreXMRV-1	Presence of provirus: PreXMRV-2
Laboratory ($n = 48$)	129P1/ReJ	−	+	Wild derived ($n = 46$)			
	129P3/J	−	+	*M. m. domesticus*	SF/CamEi	−	+
	129S1/SvlmJ	−	+		SK/CamEi	−	+
	129X1/SvJ	−	+		SK/CamRk	−	−
	A/J	−	−		PERA/Ei	−	−
	AKR/J	−	−		PERC/Ei	−	−
	AKR/J nude	−	−		CALB/Rk[b]	+	−
	B6.129/J	−	−		WSB/Ei	−	−
	B6CByF1/J nude	−	−		BIK	−	−
	BALB/c nude	−	−		ZALENDE/Ei	−	−
	BALB/cByJ	−	−		TIRANO/Ei	−	−
	BALB/cJ	−	−		Poschiavinus	−	−
	BTBR/J	−	+		BFM	−	−
	C3H/HeJ	−	+		WMP/Pas	−	−
	C57BL/6J	−	−	*M. m. castaneus*	CTA	−	−
	C57BR/cdJ	+	+		CASA/Rk	−	−
	C57L/J	+	−		CAST/Ei	+	−
	C58/J	+	−	*M. m. molossinus*	MOLC	+	−
	CBA/J	−	+		MOLD/Rk	+	−
	CByB6F1/J nude	−	−		MOLE/Rk	+	−
	CByJ.Cg/J nude	−	−		MOLF/Ei	+	−
	CE/J	−	−		MOLG/DN	+	−
	CWD/LeJ	−	−		MSM/Ms	+	−
	DBA/1J	−	−		JF1/Ms	−	−
	DBA/2J	−	+	*M. m. musculus*	CZECH/I	−	−
	HRS/J hr/+	−	+		CZECH/II	−	−
	Hsd nude[c]	+/−[d]	+/−		SKIVE/Ei	−	−
	I/LnJ	−	−		MPB	−	−
	LP/J	−	+		PWK/Ph	−	−
	LPT/LeJ	−	+	*M. spretus*	SFM	−	−
	MA/MyJ	−	+		SPRET/Ei	−	−
	NCRNU[c]	−	+/−	*M. spicilegus*	J131	−	−
	NFS/N	−	+		PANCEVO/Ei	−	−
	NIH Swiss[c]	−	+		ZRU	−	−
	NIH-III nude	−	+	*M. caroli*	KAR	−	−
	NU/J	−	+		CAROLI/Ei	−	−
	NU/NU[c]	+/−	+/−		J135	−	−
	NUJM nude	−	+	*M. cookii*	COK	−	−
	NZB/B1NJ	−	−		J136	−	−
	NZW/LacJ	+	−	*M. cervicolor*	CRV	−	−
	P/J	−	−		J53	−	−
	RIIIS/J	−	−	*M. platythrix*	PTX	−	−
	SJL/J	−	+	*M. bactrianus*	BIR	−	−
	SJLSmn.AK nude	−	+	*M. famulus*	FAM	−	−
	SM/J	−	−	*M. macedonicus*	XBS	−	−
	ST/bJ	−	+	*M. dunni*	MDTF	−	−
	STOCK Ces1c nude	−	−	*M. pahari*	*Mus pahari*/Ei	−	−
	SWR/J	−	+				
No. (%) of positives		6 (12.5)	25 (52.1)	No. (%) of positives	8 (17.4)	2 (4.3)	

[a] That XMRV is not found as a single provirus in any laboratory or wild-derived mouse strain was reported elsewhere (39).
[b] CALB/Rk is known to carry haplotypes from *M. m. castaneus* (24).
[c] Outbred strains; individual mice may differ in the number and distribution of proviruses that they harbor (Fig. 5).
[d] +/−, some members of outbred strains are positive, and some are negative (Fig. 5).

mice (39), which led to the widely used 22R*v1* cell line, now known to produce XMRV (28, 50). Early xenograft passages of the tumor did not contain XMRV, whereas the later passages did, implying that XMRV infected the tumor after initial passaging and prior to generation of the 22R*v1* cell line (39). Moreover, the host mouse DNA found associated with the tumor contained both XMRV parental proviruses, strengthening the argument that recombination between these two endogenous MLVs led to the generation of XMRV. Here we described the experimental approaches leading to the identification of PreXMRV-2, the genomic locations of both proviruses, and their distribution among wild-derived and laboratory mouse strains.

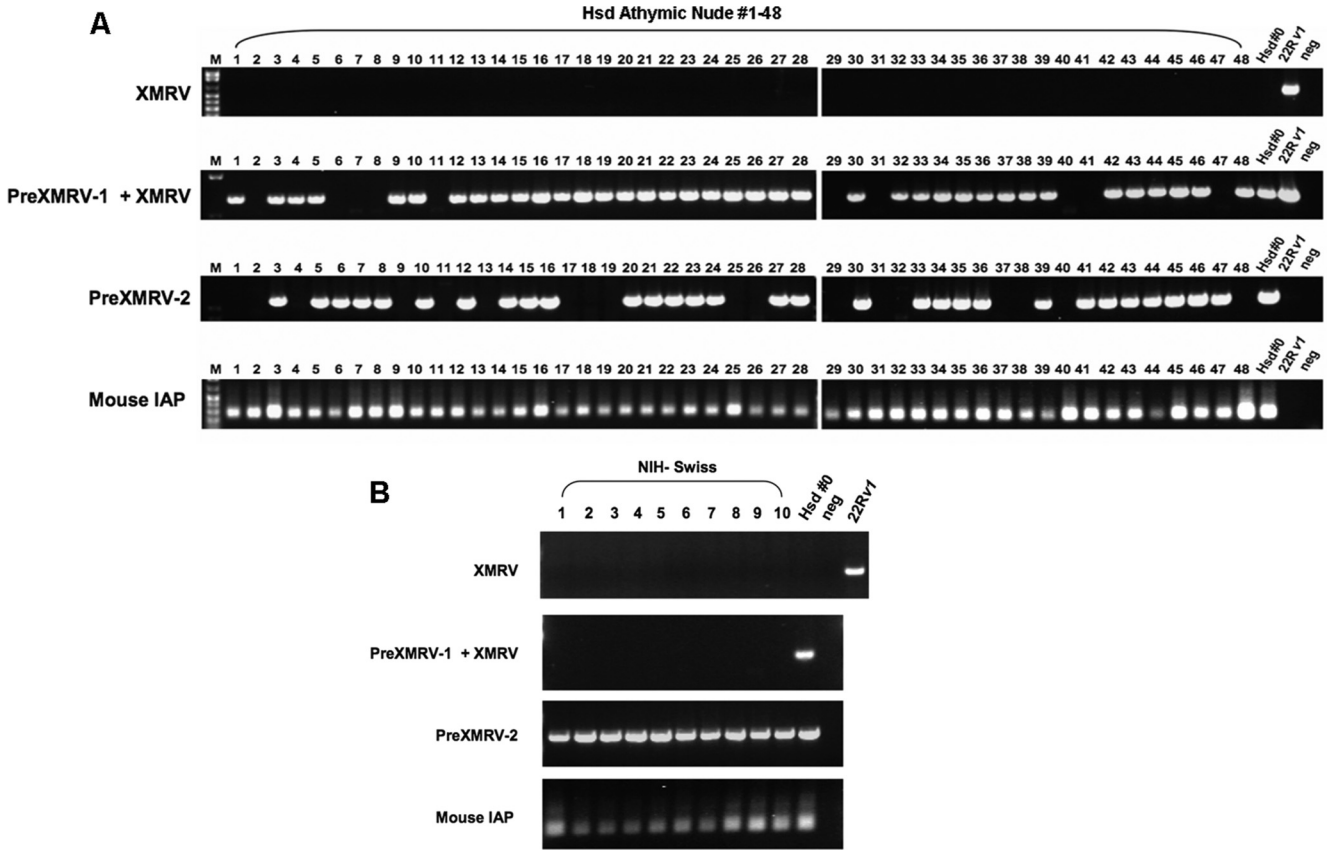

FIG 5 Variability of PreXMRV-1 and PreXMRV-2 and absence of XMRV in outbred mouse strains. Hsd nude mice ($n = 49$) (A) and NIH Swiss mice ($n = 10$) (B) were analyzed for the presence of XMRV, PreXMRV-1, and PreXMRV-2 by PCR as described for Fig. 3 and 4. The primers used for the detection of PreXMRV-1 in this figure also detect XMRV (primer set 8r-1bf [Fig. 4B]). However, the absence of XMRV was confirmed with XMRV-specific primers (top panels), and the presence of PreXMRV-1 was verified by primers flanking the integration site, with identical results (data not shown). Mouse IAP elements served as amplification controls. The Hsd nude sample used in Fig. 1C, 3B, and 4B is labeled as "Hsd #0." Null allele frequencies calculated from these results are shown in Table 2. The presence of PreXMRV-2 but not PreXMRV-1 in NIH Swiss mice is consistent with the detection of the former, but not the latter, in NIH 3T3 cells (36).

The distribution of PreXMRV-1 and PreXMRV-2. As previously reported, XMRV is not present as a naturally occurring single provirus in any mouse genome tested, including ~170 individual mice from 48 laboratory strains and 46 wild-derived strains (Table 1; Fig. 5A and B) (39). The fact that neither provirus is fixed in the subspecies where it occurs suggests that both are of fairly recent origin, having integrated after the origin of these subspecies less than 1 million years ago and prior to the radiation of *M. m. domesticus* in the Americas or the hybridization of *M. m castaneus* and *M. m. musculus* in Asia that gave rise to *M. m. molossinus* (20, 66).

The distribution of PreXMRV-1 and PreXMRV-2 is consistent with previous findings on Xmv and Pmv *env* distribution in wild mice (32). The only mice that we found to harbor both proviruses are the two outbred strains, Hsd nude and NU/NU (39), and one inbred strain, C57BR/cd (Table 1). Inbred mouse strains are largely derived from *M. m. domesticus*, with minor contributions from *M. m. musculus* and *M. m. castaneus*. Interestingly, among inbred mouse strains, the C57BR/cd genome contains some of the greatest *M. m. musculus*-derived content (65). It is particularly noteworthy that the two proviruses found their way into laboratory mice from wild subspecies found on two different continents, Asia (*M. m. molossinus* and *M. m. castaneus*) and Europe (*M. m.*

TABLE 2 Frequency of PreXMRV-1 and PreXMRV-2 in outbred mice

	Frequency in outbred mouse strain:											
	Hsd nude ($n = 49$)				NU/NU ($n = 5$)				NIH Swiss ($n = 10$)			
Provirus	+/+	+/−	−/−	AF[a]	+/+	+/−	−/−	AF[a]	+/+	+/−	−/−	AF[a]
PreXMRV-1	0.31	0.49	0.20	0.45	0.40	0.40	0.20	0.40	0	0	1	1
PreXMRV-2	0.63[b]		0.37	0.61	0.60[b]		0.40	0.63	1[b]		0	<0.05

[a] Frequency of the null (−/−) allele.

[b] Heterozygotes could not be distinguished from homozygotes since repeat regions made it impossible to specifically detect the unoccupied integration site.

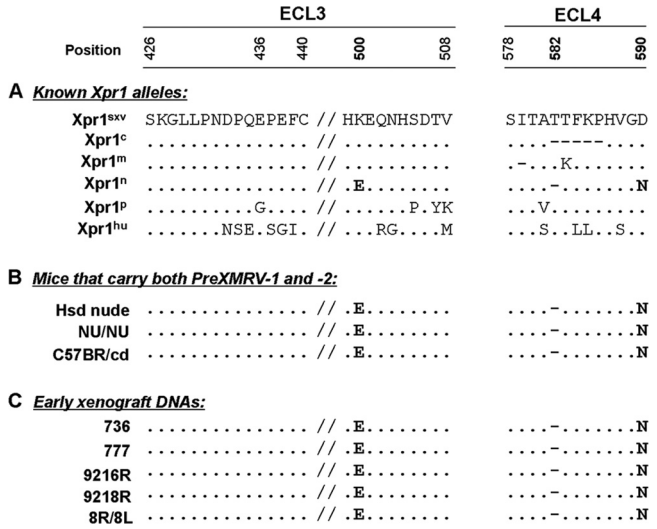

FIG 6 Presence of the Xpr1^n receptor allele in xenografts, outbred Hsd nude and NU/NU strains, and inbred C57BR/cd strains. (A) Known variants of the XPR1 receptor are shown, with the amino acid residues in the ECL3 and ECL4 loops responsible for the inability of the XPR1^n allele to bind xenotropic MLV Env highlighted in bold (figure modified from reference 64). (B) ECL3 and ECL4 loops of the XPR1 receptor for Hsd nude, NU/NU, and C57BR/cd carry the Xpr1^n allele, which does not support entry by a xenotropic MLV Env (30). (C) Early xenografts containing either PreXMRV-1 alone or the two proviruses together (39) carry the Xpr1^n allele in the mouse tissues found associated with the xenografts.

domesticus), and are, therefore, very unlikely to have been present in the same mouse until their recent crossbreeding to create the fancy and laboratory mouse strains (61). In fact, not a single wild mouse strain was found to carry both PreXMRV-1 and PreXMRV-2, making it extremely unlikely that the two parents would be present in the same mouse in the wild, recombine, and cross species to infect humans.

Of the three strains that do carry both PreXMRV proviruses, two (Hsd nude and NU/NU) were most likely to have been used for the passaging of the CWR22 xenograft at the time that it became infected, although precise records were unavailable (37). We had previously shown the presence of both proviruses in xenografts obtained prior to 1996, at frequencies attributable to a low level of mouse DNA derived from the nude mouse host. In the case of PreXMRV-1, the flanking sequence was unknown, and the prior identification relied on internal provirus primers, some of which also recognized XMRV. Here, we confirmed the prior conclusion, using flanking sequence primers, finding that all the early xenograft samples were positive for the PreXMRV-1 provirus and that some, but not all, were positive for PreXMRV-2 (Fig. 3B and 4B). Furthermore, 3/7 of the samples were heterozygous for PreXMRV-1, as revealed by positive PCR signal with flanking primers alone (Fig. 4B). These results are consistent with the conclusion that either of the outbred nude mouse strains, Hsd nude and NU/NU, both of which are polymorphic at the two loci (Fig. 5A) (39), was a host for the CWR22 xenografts. Whatever the host strain, they clearly show that multiple passages off the tumor were through mice that contained both proviruses, providing ample opportunity for recombination to create XMRV.

The presence, in a number of mouse strains, of a provirus carrying the characteristic 24-bp *gag* leader deletion has been previously noted (11). Since the strain distribution of that provirus by PCR using *gag* leader-specific primers is in perfect agreement with that of PreXMRV-2, we conclude that this is the only provirus in these strains with the characteristic deletion. Proviruses with the *gag* leader deletion have also been detected in four inbred strains by deep sequencing (23). Although those fragments most likely correspond to PreXMRV-2, the short length of the deep sequence reads (167 bp) precludes a firm conclusion.

Recombination between PreXMRV-1 and PreXMRV-2. The generation of recombinant viruses with similar crossover patterns can occur multiple times, reflecting selection for advantageous properties such as receptor usage, expression in target cells, evasion of host restriction, and repair of defects in one or the other parental virus, resulting in a selective advantage to the recombinant virus. In the present case, PreXMRV-1 has obvious defects in *gag* and *pol* that were repaired by the corresponding regions in PreXMRV-2. It is worth emphasizing that the probability of generating the same XMRV recombinant with the same crossovers independently more than once is extremely small (39). In a hypothetical situation where by an extremely remote chance such an event did occur, the recombinant (XMRV) would be unable to reinfect the mouse target cells and spread, since the Hsd nude, the NU/NU, and the individual mice used to passage the xenografts carry the Xpr1^n receptor variant (Fig. 6B and C), which is nonpermissive to XMRV infection (63, 64). The presence of the grafted human tumor tissue therefore gave the recombinant the opportunity to infect and propagate. The fact that multiple passages of the CWR22 xenograft took place in mice carrying both proviruses before the recombinant appeared suggests that the event must have been quite rare, possibly reflecting low levels of expression of the parental proviruses.

The biological properties of the parental proviruses are under investigation. As noted, PreXMRV-1 cannot be infectious due to mutations in *gag* and *pol* (39). Preliminary experiments using the cloned full-length PreXMRV-2 provirus to transfect XMRV-susceptible cells suggest that it is also not infectious (data not shown). It is not known whether PreXMRV-1 and PreXMRV-2 are expressed in mouse cells that harbor both proviruses. Further experiments are required to elucidate the infectious properties of potential recombinants that may arise between PreXMRV-1 and PreXMRV-2.

Detection of MLV-like sequences. A previous study testing human samples for the presence of XMRV reported the detection of Pmv/Mpmv sequences from the blood of CFS patients, as well as some healthy controls, by PCR (34). However, several instances of contamination of human tissues or laboratory reagents with very similar sequences have also been reported (29, 38, 43, 46, 49, 57). These results raise concerns about the potential for contamination by mouse DNA, since the only detection method relied on PCR and some of the detected provirus fragments were identical to known Pmvs found in mouse genomes.

It is important to point out that most published "XMRV-specific" PCR assays will in fact detect sequences that are identical between either PreXMRV-1 or -2 and XMRV and would yield a false-positive result if confronted with mouse DNA containing one or the other of these proviruses. Only PCR assays that rely on primers flanking one of the crossover points can be considered to be truly XMRV specific (39). In fact, of all the "XMRV-specific" PCR assays published to date, almost none are truly specific, since the primer sets would detect either PreXMRV-1 or PreXMRV-2

(4, 13, 17, 34, 35, 48, 53, 58). To our knowledge, the only exception is the primer set used by Hohn et al., where the first round of nested PCR spans a crossover site between PreXMRV-1 and PreXMRV-2 in the *gag* leader region (21).

PreXMRV-1 and PreXMRV-2 represent yet another addition to the growing list of endogenous MLV proviruses of mice. Their significance stems from the recombination between the two that ultimately led to the generation of XMRV during the passaging of a human prostate tumor as xenografts in mice. To our knowledge, no other MLV, recombinant or otherwise, acquired by human tissues upon transplantation into mice has ever received this much attention, even though such retroviral acquisitions by heterologous cells occur frequently during passage of human cells in mice or other species and have been known for more than 30 years (1, 6, 15, 56, 62, 67). Even more frequent is unintentional infection of cell lines with retroviruses in the laboratory, often going unnoticed for many years (3, 23, 28, 41, 42, 51, 54). Extreme measures are therefore necessary to avoid false positives, including routine screening of cell lines used in the laboratory, use of highly sensitive assays for detecting mouse DNA contamination as well as potential cross-contamination of assays with viruses released from cell lines, and finally, employing multiple independent assays to support findings, especially when made by PCR alone.

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