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Rmcf2, a Xenotropic Provirus in the Asian Mouse Species *Mus castaneus*, Blocks Infection by Polytropic Mouse Gammaretroviruses

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Cells from the Asian wild mouse species *Mus castaneus* are resistant to infection by the polytropic host range group of mouse gammaretroviruses. Two factors are responsible for this resistance: a defective XPR1 cell surface receptor for polytropic murine leukemia viruses (P-MLVs), and a resistance factor detectable only in interspecies hybrids between *M. castaneus* and mice with an XPR1 variant that permits infection by xenotropic MLVs (X-MLVs) as well as P-MLVs. This second novel virus resistance phenotype has been associated with expression of viral Env glycoprotein; Northern blotting with specific hybridization probes identified a spliced X-MLV *env* message unique to virus-resistant mice. These observations suggest that resistance is due to expression of one or more endogenous X-MLV envelope genes that interfere with infection by exogenous P-MLVs. *M. castaneus* contains multiple X-MLV proviruses, but serial backcrosses reduced this proviral content and permitted identification of a single proviral *env* sequence inherited with resistance. The resistance phenotype and the provirus were mapped to the same site on distal chromosome 18. The provirus was shown to be a full-length provirus highly homologous to previously described X-MLVs. Use of viral pseudotypes confirmed that this resistance gene, termed *Rmcf2*, prevents entry of P-MLVs. *Rmcf2* resembles the virus resistance genes *Fv4* and *Rmcf* in that it produces Env glycoprotein but fails to produce infectious virus; the proviruses associated with all three resistance genes have fatal defects. This type of provirus Env-mediated resistance represents an important defense mechanism in wild mouse populations exposed to endemic infections.

Studies on retroviruses and retrovirus-induced diseases have identified numerous host genes responsible for resistance. Many of these genes interfere directly with virus infection and replication. Some of these cellular blocks to virus replication affect early postentry and preintegration stages of the virus life cycle and include *Fv1*, APOBEC3, and TRIM5 (4). Another class of resistance genes prevent virus entry by affecting the interactions of viruses with their specific cell surface receptors. There are two types of resistance genes that target the receptor-virus interaction. The first type results from polymorphic variations of the cell surface receptors. For the mouse gammaretroviruses, this type of resistance has been described for the ecotropic mouse leukemia viruses (MLVs) and for the nonecotropic xenotropic MLVs (X-MLVs) and polytropic MLVs (P-MLVs). Thus, *Mus dunni* cells are resistant to ecotropic Moloney MLV because they carry a variant of the CAT-1 ecotropic receptor (12). The function of this *M. dunni* receptor is also influenced by glycosylation; resistance to Moloney MLV can be eliminated by inhibitors of glycosylation (11).

The X-MLVs and P-MLVs use the same receptor, XPR1 (also termed SYG1) (3, 41, 45). There are three functionally distinct variants of this receptor in the mouse. The XPR1 variant found in laboratory mice permits infection with P-MLVs but not X-MLVs. The Sxv receptor variant ($Xpr1^{Sxv}$) is found in many wild mouse species and permits efficient infection with P-MLVs and less efficient infection by X-MLVs (24). The third allele of this gene ($Xpr1^{Cas}$) is found in the Asian mouse species *Mus castaneus*, which is resistant to infection with P-MLVs (27). Genetic studies and characterization of the cloned *M. castaneus* receptor have shown that $Xpr1^{Cas}$ lacks receptor function (27, 29).

A second group of receptor-mediated MLV resistance genes function through an interference mechanism. The best-characterized of these genes, *Fv4*, encodes an ecotropic envelope glycoprotein, expression of which is thought to interfere with receptor binding of exogenous ecotropic MLVs (19). This proviral gene also has a mutation in the fusion peptide of its transmembrane domain so that virions that are produced in $Fv4^r$ cells are fusion defective (42). Studies on another gene, the *Rmcf* resistance locus of DBA/2 mice (17), suggest that an analogous interference mechanism may be responsible for *Rmcf*-mediated resistance to the P-MLVs. This suggestion was based on the observation that cells of these mice express a unique cell surface P-MLV Env glycoprotein (1) and are resistant to P-MLV-induced disease (38, 39). We recently identified a provirus linked to the *Rmcf* resistance gene and confirmed that its expression is associated with resistance (22).

Further studies on *M. castaneus* showed that in addition to carrying *Fv4* and a defective XPR1 receptor, this mouse contains an additional factor responsible for resistance to P-MLVs

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(28). The observed correlation between this additional resistance and expression of nonecotropic Env glycoproteins suggested that *M. castaneus* may harbor an *Rmcf*-like resistance gene (28). In the present study, we describe genetic crosses done to characterize this resistance gene, termed *Rmcf2*. We map *Rmcf2* to chromosome 18, we identify and clone a novel wild mouse X-MLV provirus associated with *Rmcf2* resistance, and we show that inheritance of the *Rmcf2* X-MLV provirus blocks infection by P-MLVs.

MATERIALS AND METHODS

Mice. CAST/EiJ mice (CAST) mice, an inbred line of the Asian wild mouse species *M. castaneus*, were obtained from The Jackson Laboratory, Bar Harbor, ME. NFS/N mice were originally obtained from the Small Animal Section, National Institutes of Health (Bethesda, MD). A congenic strain of NFS/N was developed in our laboratory; NFS/N-*Sxv*/*Sxv* (N/Sxv mice) carry the *Xpr1*^{Sxv} locus of *M. musculus domesticus* (formerly *M. praetextus*) (28). CAST males and females were bred with N/Sxv mice and the F_1 hybrids were mated with N/Sxv mice to produce first-backcross mice (CSS cross). Resistant mice were selected and mated with N/Sxv for eight generations; resistant mice were then brother-sister mated to produce a congenic strain of NFS/N mice carrying both *Xpr*^{SXV} and *Rmcf2*.

Viruses, cells, and virus assays. All viruses were originally obtained from J. Hartley (National Institute of Allergy and Infectious Diseases, Bethesda, MD) and included the P-MLV, Moloney MCF-HIX (13), and the amphotropic MLV 4070A (16).

The susceptibility of individual mice to P-MLV was tested by infecting cultures of tail biopsy tissue prepared as described by Lander and colleagues (26). Briefly, tail tips of 7- to 10-day-old mice were minced and incubated for 45 min with 2.5 ml of collagenase (150 U/ml). Cells from this suspension were cultured in Dulbecco's medium with 10% fetal calf serum and antibiotics. When the cultures reached confluency, the cells were passaged and infected one day later with dilutions of MLV stocks in the presence of Polybrene (4 μg/ml; Aldrich, Milwaukee, WI). After 4 to 5 days, cultures were UV irradiated and overlaid with 6×10^5 mink S^+L^- cells (33). Foci were counted 6 to 7 days later.

Genetic typing. Genomic DNA was prepared by standard methods from liver, from tail biopsies, or from cells cultured from tail biopsies. PCR amplification of simple sequence repeats (SSRs) was carried out as described (10). The SSR markers *D1Mit33*, *D1Mit101*, and *D1Mit116* were used to follow inheritance of the chromosome 1 segment containing *Xpr1* (27), and *D5Mit1* and *D5Mit4* were used as linked markers for *Rmcf* (22). Other SSR markers were selected for a genome scan based on their location at spaced intervals on all chromosomes and for their high degree of polymorphism among the common strains. Additional distal chromosome 18 markers were selected and typed as indicated. PCR products were fractionated on 3% gels of MetaPhor agarose (BioWhittaker Molecular Applications, Rockland, Maine) stained with ethidium bromide. Individual backcross progeny were tested for the genome scan; a pooling strategy was not feasible since amplification was often less efficient with the CAST alleles.

Northern and Southern blotting. DNA extracted from livers of adult mice was digested with various restriction enzymes according to the manufacturers' suggestions, separated on 0.4% agarose gels, and transferred to nylon membranes (Hybond N+, Amersham, Piscataway, NJ). Filters were hybridized with radiolabeled DNA segments.

Total RNA was isolated from different adult mouse tissues with the Total RNA Isolation reagent (QIAGEN, Valencia, Calif.). Approximately 20 μg of total RNA from each tissue was used for Northern blotting. Preparation of RNA samples, electrophoresis, and blotting procedures were as described previously (44).

After transfer, the Northern and Southern filters were hybridized with ^{32}P-labeled probes including the 112-bp X-MLV and the 100-bp P-MLV *env*-specific segments (X*env* and P*env* probes) (32) and a 353-bp segment of β-actin amplified by PCR from human liver DNA and provided by M. S. Lyu (National Cancer Institute, Bethesda, MD). An additional probe represented a 519-bp DNA segment flanking the *Rmcf2* provirus that was amplified from CAST genomic DNA using the following primers: F4, 5′-TCATTACTGCCTCCTGACTGTGG; and B3, 5′-GGTAGCATCTTTCCTCATTGGGAC (Fig. 1A). Hybridization was carried out overnight at 42°C in a hybridization solution containing 50% formamide. The filters were washed for 20 min in 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–2% sodium dodecyl sulfate (SDS) at room temperature and twice for 20 min in 0.2× SSC–2% SDS at 65°C and then exposed to X-ray film (XAR5; Kodak, Rochester, N.Y.) at −70°C.

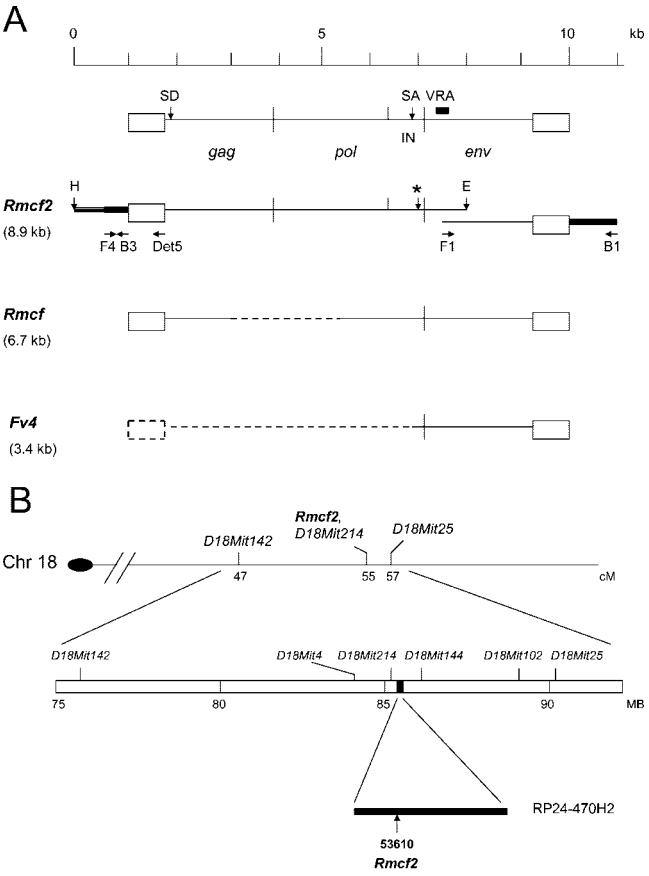

FIG. 1. Chromosomal map location and structure of *Rmcf2*. (A) At the top is shown the general structure of a mouse gammaretrovirus with relative positions for the long terminal repeats, *gag*, *pol*, and *env* genes along with the *pol* IN sequence, the SU *env* VRA region, and the splice acceptor (SA) and splice donor (SD) sites. Below this are the two overlapping clones representing the 3′ and 5′ cell-virus junction fragments of the *Rmcf2* provirus. The 5′ clone is a HindIII (H)-EcoRI (E) segment cloned using an X-MLV-specific probe (X*env*) that overlaps VRA. The first 538 bp of the 5′*Rmcf2* clone is a repeated sequence and is indicated with a thicker line. The thickest line represents sequences contained in BAC clone RP24-470H2. The 3′ clone is a PCR product amplified using a proviral VRA primer (F1) and a reverse primer (B1) based on the RP24-470H2 sequence. Arrows indicate additional primers used to amplify a 5′-flanking sequence (primers F4 and B3) and an *Rmcf2*-specific cell virus junction fragment (F4 and Det5). *Rmcf2* contains a termination codon (*) in IN. At the bottom of the panel are structures for the proviruses associated with resistance genes *Rmcf* and *Fv4*. *Fv4* contains a truncated provirus, and *Rmcf* has a deletion that includes a substantial segment of *gag* and *pol*. Dashed lines indicate the proviral segments missing from *Rmcf* and *Fv4*. (B) The map at the top shows the genetic map location of *Rmcf2* relative to the SSR markers used to position the resistance phenotype. Positions in cM were taken from the Mouse Genome Database (Mouse Genome Informatics web site, Jackson Laboratory [http://www.informatics.jax.org/]). Below this map is the sequence-based map from Ensembl (Mouse Genome Server, Wellcome Trust, Sanger Institute [http://www.ensembl.org/Mus_musculus]). Positions are given in megabases for all SSR markers as well as the BAC clone RP24-470Hz, which contains the integration site for *Rmcf2* at position 53610.

Cloning the *Rmcf2*-associated provirus. Genomic DNA of seventh backcross mice carrying *Rmcf2* was digested to completion with EcoRI and HindIII and

separated on a 0.6% agarose gel. DNA enriched for fragments of about 8 kb was gel purified using a gel extraction kit (QIAGEN, Valencia, CA). The fragments were cloned into the lambda vector pSCREEN-1b(+) (Novagen, Madison, WI) and clones containing the 5′ end of the *Rmcf2* provirus (Fig. 1A) were identified using X*env* as the probe.

PCR was used to amplify the 3′ end of the provirus using a forward primer designed from the VRA region of the sequenced 5′ *Rmcf2* proviral segment (F1: 5′-GACCCAGAACCCGAGATTGGGG-3′) (Fig. 1A). The reverse primer was designed from 3′ cellular flanking sequence (B1: 5′-GTCATTCGCCTGAAAG GGAGGGAT-3′). The PCR was carried out in a GeneAmp PCR system 9700 machine (PE Applied Biosystems, Foster City CA). The reactions were performed for 30 cycles with a 1-min DNA denaturation step at 95°C, a 1-min annealing step at 55°C, and a 4-min extension step at 72°C. A DNA fragment of about 3.5 kb was amplified and cloned into the pCR2.1-TOPO vector (Invitrogen, Purchase, N.Y.).

The presence of the *Rmcf2* provirus was detected in mouse genomic DNA as a 1,265-bp cell-virus junction fragment by PCR using F4 as the forward primer and Det5, 5′-GACCCTCTCAAGGACCAGCGAGAC, as the reverse primer (Fig. 1A). Mice lacking this provirus were identified by the production of a 1,526-bp PCR product using F4 and B1 as primers.

Pseudotype assay. LacZ pseudotyped virus was generated by transfection of human TELCeB6 cells with expression vectors for the MCF247 P-MLV Env, pCRUCM, or the amphotropic 4070A MLV Env, pCRUCA, both of which were provided by J.-L. Battini (Institut Pasteur, Paris) (2). TELCeB6 produces noninfectious viral particles harboring the MFGnlslacZ retroviral vector. Cultured tail fibroblasts from various mice were infected with the LacZ pseudotypes. One day after infection, cells were fixed with 0.4% glutaraldehyde and stained to reveal the presence of β-galactosidase activity using as the substrate 5-bromo-4-chloro-indolyl-β-D-galactosidaside (X-Gal, 2 mg/ml; ICN Biomedicals, Aurora, Ohio).

Nucleotide sequence accession numbers. The sequence of *Rmcf2* and its 3′- and 5′-flanking segments have been deposited in GenBank under accession numbers AY999005.

RESULTS

Genetic crosses. In a previous study, we showed that hybrids between virus-resistant *M. castaneus* mice and virus-susceptible strains are resistant to infection by P-MLVs (28). Several observations suggested that this resistance resembles that mediated by *Fv4* and *Rmcf*. First, this novel resistance phenotype is dominant, as would be expected for an interfering *env* gene. Second, resistance is unlinked to the *Xpr1* receptor gene and therefore does not represent another variant of this gene. Third, resistance is associated with expression of a nonecotropic Env glycoprotein. Finally, the fact that this resistance is only observed in hybrid mice with the $Xpr1^{Sxv}$ receptor that allows entry by X-MLVs suggests that this resistance might be attributable to X-MLV rather than P-MLV *env* expression. This suggestion is also supported by the proviral content of *M. castaneus* (25). As determined by Southern blotting, this mouse carries only two to three proviral P-MLV *env* copies, but several dozen copies of the X-MLV *env* (Fig. 2). In contrast, most laboratory mouse strains carry many copies of both the P-MLV and X-MLV *env* genes (14, 32).

To determine if this novel resistance is related to a specific provirus, new genetic crosses were done between CAST/EiJ mice and a congenic strain carrying $Xpr1^{Sxv}$, NFS/N-*Sxv*/*Sxv* (N/Sxv). This was done because the original cross in which this resistance was defined was complicated by the fact that the *M. castaneus* mice had been bred to DBA/2 strain mice, and the progeny therefore inherited the additional resistance gene *Rmcf*. In the present study, N/Sxv strain mice were selected because they are susceptible to P-MLVs and X-MLVs and because, unlike most of the common inbred strains, they carry only one proviral X-MLV *env* gene detectable on Southern blots (18) (Fig. 2). First backcross mice, N/Sxv × (N/Sxv × CAST)F1, were typed for virus resistance as shown for a representative litter in Table 1. All mice of this CSS cross were tested in duplicate, and each set of cultures was also infected with amphotropic virus as a positive control. At the virus dilutions used, little or no virus was detected in cultures scored as P-MLV resistant; virus titers were reduced in resistant mice by 2 logs or more. Cells were not tested for X-MLV suscepti-

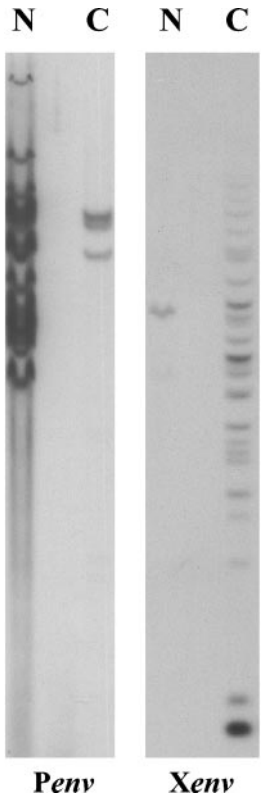

FIG. 2. Southern blot analysis of DNAs from CAST (C) and N/Sxv (N) mice digested with EcoRI and hybridized with probes specific for the X-MLV and P-MLV *env* genes.

TABLE 1. Susceptibility of individual mice from a single litter in the CSS backcross to polytropic and amphotropic MLVs[a]

Mouse[b]	No. of foci		P-MLV resistance	Allele[c]	
	A-MLV	P-MLV		*D1Mit33 (Xpr1)*	*D5Mit1 (Rmcf)*
576-1	168	305	S	NN	CN
576-2	136	4	R	CN	NN
576-3	148	32	R	CN	NN
576-4	164	708	S	NN	NN
576-5	168	8	R	CN	NN
576-6	212	2	R	NN	CN
576-7	256	432	S	CN	CN
576-8	112	452	S	CN	CN

[a] Cultured tail cells from individual mice were infected with MCF MLV (Moloney MCF-HIX) and with 4070A amphotropic MLV and 4 days later were irradiated and overlaid with mink S^+L^- cells. Foci were read 5 to 8 days later. P-MLV titers were used to identify mice as resistant (R) or susceptible (S).

[b] CSS backcross: N/Sxv × (N/Sxv × CAST)F_1.

[c] CAST and N/Sxv mouse alleles of the marker loci are designated C and N, respectively.

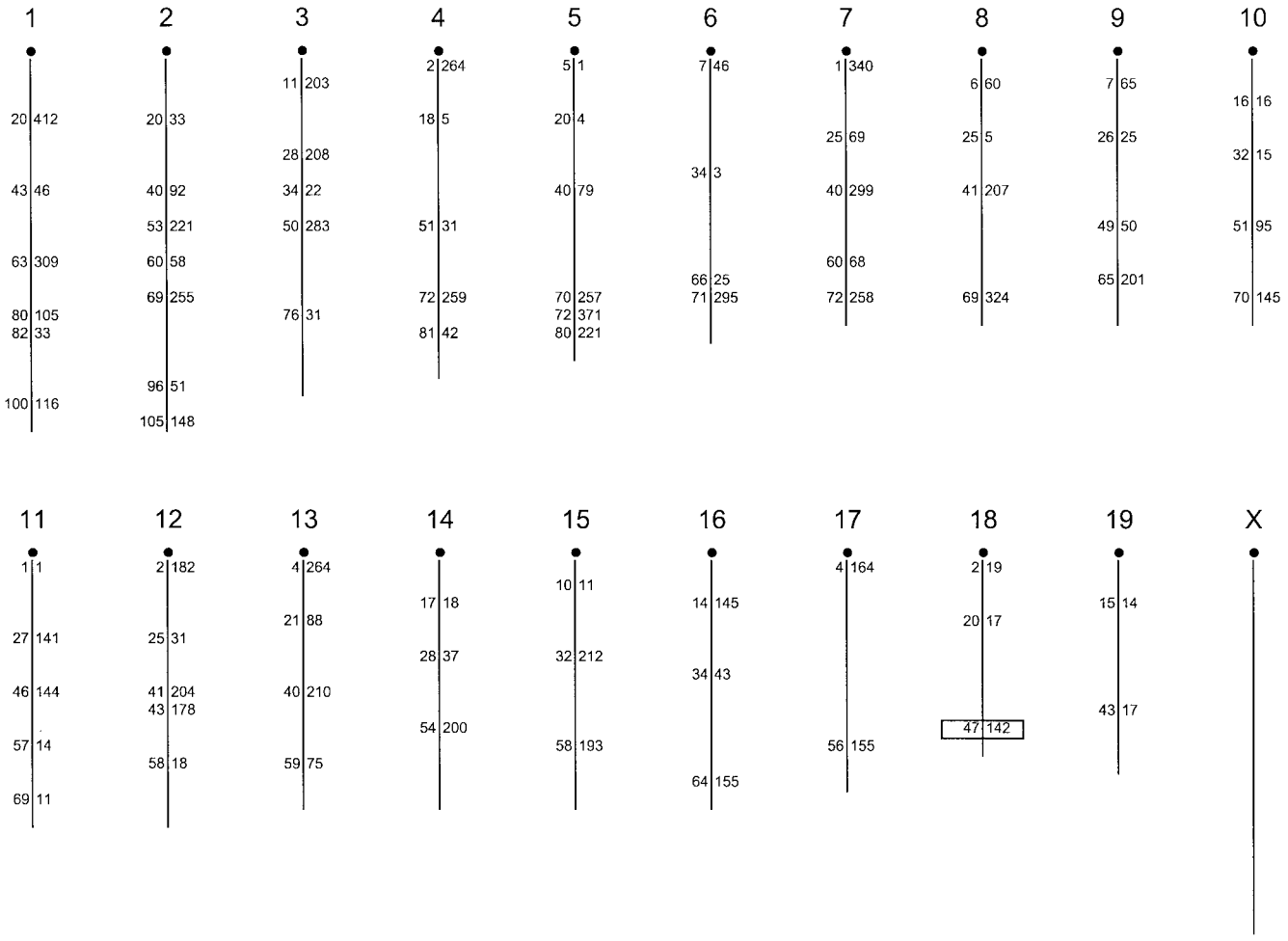

FIG. 3. Chromosomal positions of the 80 SSR markers typed in CSS backcross mice. The number designating each marker (*D18Mit#*) is given to the right of each chromosome stick figure; the number on the left represents the distance of each marker in centimorgans from the centromere. Linkage was detected between *Rmcf2* resistance and the boxed marker *D18Mit142* ($r = 7.3 \pm 2.5$ cM).

bility because the low X-MLV titers expected for the susceptible N/Sxv parental strain would not permit reliable identification of resistance. A total of 51 of the 110 mice tested were resistant to P-MLV infection, consistent with inheritance of a single gene ($\chi^2 = 0.58$, $P < 0.5$). This resistance was not linked to chromosome 1 markers for *Xpr1* (for *D1Mit33*, $r = 38/88 = 0.43$, $\chi^2 = 1.64$, $P = 0.2$) or *Rmcf* (*D5Mit1* $= 24/38 = 0.68$, $\chi^2 = 2.60$, $P < 0.2$) indicating this is a novel gene, which we term *Rmcf2*.

Genome scan. To position *Rmcf2* on the mouse linkage map, we typed a subset of the CSS progeny for 80 polymorphic SSR markers well distributed over the genome (Fig. 3). This initial scan identified linkage to one marker, *D18Mit142*, on distal mouse chromosome 18 with three recombinants in 28 mice ($\chi^2 = 15.75$, $P < 0.001$). The remaining 82 mice were typed for this marker along with other chromosome 18 markers. Gene order and recombinational distances were determined as follows: *D18Mit142*–7.3 ± 2.5–*Rmcf2*, *D18Mit214*–1.8 ± 1.3–*D18Mit25* (Fig. 1B). Closest linkage was observed between *Rmcf2* and *D18Mit214*, with no recombinants in 110 mice, indicating that at the 95% confidence level, these genes are within 2.7 centimorgans (cM). Additional SSR typing of these mice and the progeny of additional backcross generations determined that *Rmcf2* is within an interval bounded by *D18Mit4* and *D18Mit25*.

We examined the available chromosome 18 maps (Ensembl Genome Browser, Mouse Genome Server, Wellcome Trust, Sanger Institute [http://www.ensembl.org/Mus_musculus/]; Mouse Genome Database, Mouse Genome Informatics web site, Jackson Laboratory [http://www.informatics.jax.org/]). This chromosomal region contains no previously identified MLV resistance genes.

Identification of an *Rmcf2*-associated provirus. In order to identify proviral sequences that might be associated with this resistance gene, first backcross CSS mice typed as P-MLV resistant were selected and mated to N/Sxv mice. The progeny of this second cross were tested and resistant mice were mated to N/Sxv mice for the next generation. After eight serial backcrosses, resistant mice were brother-sister mated to generate a congenic line carrying *Rmcf2* and $Xpr1^{Sxv}$ on an NFS/N genetic background.

At the seventh generation of serial backcrosses to N/Sxv, 16

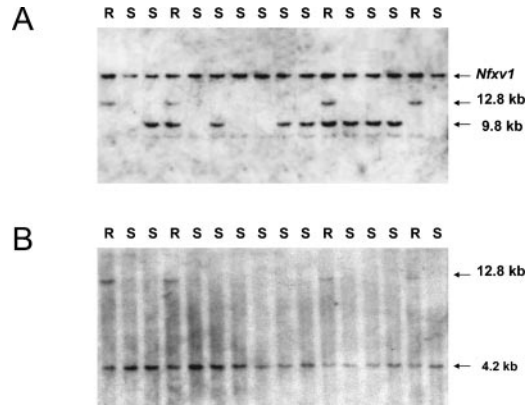

FIG. 4. Southern blot analysis of progeny of the seventh serial backcross generated by mating virus-resistant progeny at successive generations with virus-sensitive N/Sxv mice. DNAs were cleaved with HindIII. Sensitivity and resistance to P-MLV infection are indicated at the top of each lane and were determined by infecting cultured tail cells from individual mice. (A) Hybridization using X*env* as the probe identified the NFS/N X-MLV provirus *Nfxv1* in all mice, along with two segregating CAST proviruses. (B) The panel A filter was stripped and hybridized with a probe representing a 558-bp cellular sequence flanking *Rmcf2* that was amplified with primers F4 and B3. Mice that inherit the *Rmcf2* provirus are identified by a 12.8-kb fragment.

mice from several litters were selected for testing. High-molecular-weight DNA was isolated from livers for Southern blotting, and tail cultures established from the same mice were tested for susceptibility to P-MLV infection. Southern blots showed that all 16 mice contained the single NFS xenotropic provirus *Nfxv1* (18), identified as a HindIII fragment of approximately 20 kb with homology to the X*env* probe (Fig. 4A). Two additional CAST proviruses of 9.8 and 12.8 kb were present in nine and four mice, respectively. Only the four mice with the 12.8-kb fragment were identified as P-MLV resistant.

Another litter of eight mice were tested for virus resistance and duplicate tail cultures were extracted for RNA. Northern blots of these samples hybridized with X*env* revealed a 3.0-kb *env* message in six of these mice (Fig. 5); all six mice were also resistant to virus infection. These results indicate that inheritance of resistance is associated with inheritance of X-MLV *env* sequences on a 12.8-kb HindIII genomic fragment and that resistance is associated with expression of an X-MLV *env* message.

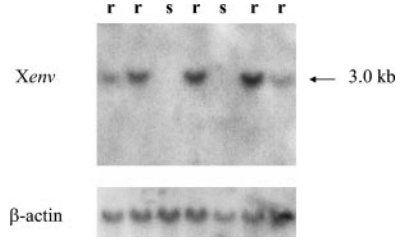

FIG. 5. Northern blot analysis of mice typed for virus resistance. Duplicate tail cultures from individual mice were harvested for RNA and typed for sensitivity/resistance to P-MLV infection. An arrow indicates the 3.0-kb spliced X-MLV *env* message in the upper panel. Virus susceptibility is indicated at the top of each lane.

Cloning and characterization of *Rmcf2*. DNA from one virus-resistant mouse carrying only *Nfxv1* and the 12.8-kb X-MLV of CAST (mouse J499, Fig. 4A, first lane) was selected for genomic DNA library construction. Repeated attempts to clone the gel-purified 12.8-kb HindIII fragment were unsuccessful. Therefore, 5′ and 3′ cell-virus junction fragments were cloned separately.

To obtain the 5′ end, J499 DNA cleaved with HindIII and EcoRI was used to construct a genomic library in pSCREEN-1b; 61 positive clones were identified by hybridization with the X*env* probe. One of these clones (L30-1) was isolated and analyzed in detail. This clone was 8,049 bp, of which 1,190 bp at the 5′ end represented nonviral sequences. The first 538 bp of this flanking sequence is a repeated element with multiple copies in the mouse genome. The remaining 655 bp were identified as a unique sequence contained within bacterial artificial chromosome (BAC) clone RP24-470H2, AC134545.5, bp 52977 to 53610. This BAC has been positioned on distal chromosome 18 at 85.3 to 85.4 Mb with the gene order *D18Mit142–D18Mit214*-RP24-470H2-*D18Mit25* (Fig. 1B). This position is consistent with the chromosome 18 map location of the *Rmcf2* resistance phenotype determined using conventional genetic crosses. The sequenced BAC clone from a C57BL6/J mouse does not have a provirus integration at bp 53610.

A 519-bp segment of the L30-1 5′ flank was used as a hybridization probe to analyze the same 16 mice used to identify the *Rmcf2*-associated X-MLV (Fig. 4B). This probe identified the same 12.8-kb fragment as the X*env* probe in the four resistant mice (Fig. 4A). All 16 mice contained a 4.2-kb fragment reactive with the flanking sequence probe. This 8.6-kb difference in the flanking sequence fragment sizes is consistent with the integration of a full-length provirus in resistant mice.

The 3′ end of the *Rmcf2* provirus was isolated using PCR. The X*env*-reactive 12.8-kb HindIII fragment was gel purified and used as the template for PCR. The forward primer (F1) was designed using the VRA sequence of the previously cloned 5′ cell-virus junction fragment (Fig. 1A). The reverse primer B1 was designed from single-copy sequences in the BAC clone predicted to be approximately 900 bp from the 3′ end of the proviral insert (positions 54451 to 54472 in RP24-470Hz). A DNA fragment of about 3.5 kb was amplified and then cloned into the pCR2.1-TOPO vector. Nucleotide sequence analysis showed this clone to be 3,331 bp, of which 922 bp at the 3′ end are nonviral sequences. These nonviral sequences were confirmed to be present in BAC clone RP24-470H2.

The *Rmcf2* proviral sequence was determined to be a full-length provirus of 8,950 bp with typical features of mouse gammaretroviruses (9) (Fig. 1B). Closest homology was observed with viruses of the X-MLV host range group; the deduced amino acid sequence of the *Rmcf2 env* showed 98% identity (13 scattered substitutions) with NZB-9-1 (GenBank accession no. K02730) and extensive homology (20 scattered substitutions) with the only fully sequenced X-MLV, DG-75, a virus isolated as a contaminant from the human B-lymphoblastoid DG-75 cell line (35) (GenBank accession no. AF221065). *Rmcf2* also resembles DG-75 in that both use $tRNA^{Thr}$ as a primer for reverse transcription, whereas all other known infectious MLVs use $tRNA^{Pro}$ and most endogenous MLV proviruses have a $tRNA^{Gln}$ sequence (5, 31).

Like other MLVs, the *Rmcf2 gag* and *pro-pol* genes are in the same open reading frame (ORF). In the *gag* ORF, the translated *Rmcf2* capsid sequence has few amino acid substitutions relative to DG-75: matrix (nine differences), p12 (five differences), capsid (one difference) and nucleocapsid (one difference). There was a single amino acid difference between DG-75 and *Rmcf2* in protease and six substitutions in reverse transcriptase. The only obvious fatal abnormality noted in *Rmcf2* was found in the integrase (IN) gene; one of three substitutions generated a termination codon that truncates the IN gene product by 59 amino acid residues. *Rmcf2* has intact splice donor and acceptor sites, and the *env* gene region starts with the sequence ATG. The long terminal repeat contains the polyadenylation signal and a TATA box, but noncanonical CCAAT boxes are found in both *Rmcf2* (CCAAC) and DG-75 (CCACT). The *Rmcf2* long terminal repeat shows most extensive homology with DG-75 and NZB-9-1 among the infectious MLVs, but the *Rmcf2* long terminal repeat also contains the 190-bp insert of unknown functional significance commonly found in endogenous polytropic long terminal repeats, including the *Rmcf* provirus (21, 22).

***Rmcf2* blocks P-MLV virus entry.** The *Rmcf-* and *Fv4*-associated proviruses prevent exogenous MLV infection by interference. To establish that *Rmcf2* also interferes with the virus-receptor interaction, congenic mice carrying *Rmcf2* on the N/Sxv genetic background were mated with virus-susceptible N/Sxv mice, and the F_1 mice were crossed with N/Sxv mice. The progeny of this backcross were tested for susceptibility to P-MLV and also for infectibility with virus pseudotypes in a single-round infection test. The inheritance of the *Rmcf2* provirus was independently scored by PCR using primers F4 and Det5 to identify a 1,265-bp virus-cell junction fragment in *Rmcf2*-positive mice, and primers F4 and B1 to identify a 1,920-bp fragment in *Rmcf2*-negative mice (Fig. 1A). Five of eight mice had the *Rmcf2* provirus (data not shown) and all five of these mice showed resistance to P-MLV infection. All five of these mice also showed reduced susceptibility to P-MLV pseudotypes compared to their *Rmcf2*-negative littermates (Table 2). This result indicates that *Rmcf2* resistance prevents virus entry, consistent with the conclusion that resistance is mediated by provirus mediated interference.

TABLE 2. Susceptibility of individual hybrid mice to polytropic and amphotropic MLVs and to LacZ pseudotypes[a]

Mouse[b]	*Rmcf2*[c]	No. of foci		Log_{10} LacZ titer[d]	
		A-MLV	P-MLV	A-MLV	P-MLV
879-1	CN	50	1	4.1	0.7
879-2	CN	55	0	3.5	1.5
879-3	CN	ND	ND	3.9	1.1
879-4	NN	43	450	3.3	2.9
879-5	NN	50	300	3.96	3.3
879-6	CN	45	0	4.0	0.6
879-7	NN	42	400	3.6	3.2
879-8	CN	ND	0	4.2	1.4

[a] Cultured tail cells from individual mice were infected with P-MLV (Moloney MCF-HIX) and with 4070A amphotropic MLV and 4 days later were irradiated and overlaid with mink S^+L^- cells. Foci were read 5 to 8 days later. ND, not done.

[b] The cross used N/Sxv congenics carrying *Rmcf2*: N/Sxv × (N/Sxv × N/Sxv-*Rmcf2*/*Rmcf2*)F_1.

[c] The presence of *Rmcf2* was scored by PCR using primers F4 and Det5 to identify the cell-virus junction fragment and primers F4 and B1 to identify mice lacking the provirus. CAST and N/Sxv mouse alleles of the marker loci are designated C and N, respectively.

[d] Measured as the number of blue cells in cultures infected with 0.1 ml of pseudotype.

DISCUSSION

The *Rmcf2* provirus is the third MLV resistance gene in the mouse known to function through an interference mechanism at the cell surface receptor. This xenotropic provirus joins the previously described ecotropic *Fv4* provirus and the polytropic *Rmcf* provirus as resistance genes that protect their hosts from exogenous infection. Like *Rmcf*, *Rmcf2* blocks P-MLV infection at the *Xpr1* receptor, but *Rmcf2*, because it contains an X-MLV provirus, results in resistance only in mice carrying the Sxv allele of this receptor. That is because the Sxv receptor allele, unlike the XPR1 of laboratory mice, permits infection by X-MLVs as well as P-MLVs.

The three mouse proviruses associated with resistance, *Fv4*, *Rmcf* and *Rmcf2*, are structurally very different. While all three proviruses contain an intact *env* with the splice acceptor and a 3′ long terminal repeat, *Rmcf* and *Fv4* both have deletions that remove substantial portions of *pol* and *gag* (19, 22) (Fig. 1b). Also, *Fv4* uses a cellular promoter since it lacks the proviral 5′long terminal repeat. While the *Rmcf2* provirus has no deletions, it is unable to produce the IN gene product because of a stop codon that removes the terminal 59 amino acid residues. As a previous study has shown, deletion of 34 or more amino acids at the carboxy terminus of the MLV IN gene prevents the production of infectious virus (37). Thus, while all three of these resistance genes retain the ability to produce the viral Env glycoprotein, different mutations in each provirus affect the *pol* genes and prevent production of infectious virus while leaving the *env* genes intact.

It is not surprising that *Rmcf2* resistance to P-MLV infection is mediated by an X-MLV provirus. P-MLVs and X-MLVs use the same XPR1 receptor (2, 41, 45). Interference studies have shown that there is cross-interference between the P-MLVs and X-MLVs in cells infectible by both virus types, however, this interference is not reciprocal. The X-MLVs are actually much more efficient than P-MLVs at establishing interference (8, 29). It has been suggested that this difference may be due to the fact that P-MLVs have a lower binding affinity for the XPR1 receptor that fails to result in receptor downregulation (43). This reduced binding affinity may also explain the association between P-MLV infection and pathogenesis, because repeated infections of the same cell may either increase the likelihood of insertional mutagenesis or result in cytopathicity due to the production of high amounts of viral DNA (47). The expression of an endogenous X-MLV Env glycoprotein thus affords effective protection against infection with related viruses and thus confers an obvious survival advantage.

The identification of a third mouse gene that functions through interference suggests that coopting proviral *env* genes may actually be a relatively common defense strategy in populations exposed to endemic infections. Such genes have long been recognized in chickens (36) and cats (30) and have recently been identified in sheep (40). This type of resistance gene is rare in laboratory mouse strains, but interestingly, *Rmcf2* was found along with *Fv4* in the only wild mouse species

known to harbor infectious ecotropic and xenotropic MLVs (7, 25); *M. castaneus* is therefore in need of survival strategies to mitigate the consequences of infection. This mouse is protected from the disease inducing properties of the viruses it carries on three fronts. *Fv4* protects it from ecotropic MLVs and probably originated in this species (20, 21, 25). Protection against nonecotropic MLVs (both X-MLVs and P-MLVs) is provided by a defective *Xpr1* receptor. *Rmcf2* provides a third level of protection to *M. castaneus* mice that acquire a functional XPR1 receptor through breeding or mutation.

It is not clear how widespread *Rmcf2* and the $Xpr1^{Cas}$ variant are in Asian wild mouse populations. *M. castaneus* is one of the major species of commensal mice. Commensal species (the house mouse) diverged from older *Mus* species less than 1 MYA in northern India and subsequently spread west (*M. domesticus*, western Europe), north (*M. musculus*, eastern Europe and northern Asia), and east (*M. castaneus*, Southeast Asia). These species are interfertile and undergo genetic exchanges at the borders of their geographic ranges. *M. musculus* and *M. castaneus* are known to hybridize in China and have formed a hybrid population in Japan (previously classed as the distinct taxonomic group *M. musculus molossinus*) (6, 46). At the western edge of its range, *M. castaneus* is in contact with the poorly characterized but highly diverse populations of south central Asia that have been collectively termed *M. bactrianus* (15).

Of the three distinct virus resistance phenotypes found in *M. castaneus* (*Fv4*, Xpr^{Cas}, and the combination of *Rmcf2* and Xpr^{Sxv}), one, *Fv4*, is clearly widespread in Asian commensal mice; *Fv4* has been found in all *M. castaneus* mice tested, as well as in mice from hybrid zones, and in mice identified as *M. bactrianus* trapped in Pakistan (20). Two observations suggest that the genes conferring resistance to nonecotropic MLVs may also be widespread in Asian commensal mice. First, the receptor allele $Xpr1^{Sxv}$ is present in some commensal mouse populations and predominates in aboriginal species of Asia such as *M. caroli*, *M. cervicolor*, and *M. cookii* (24). These species diverged three to five million years ago, suggesting that Sxv is likely to be the ancestral allele. Second, infectious X-MLVs have been isolated from *M. castaneus* and *M. molossinus* mice (7), and large numbers of X-MLV proviruses are found in *M. castaneus* and in *M. musculus* all the way to the western end of its range in central Europe (25). For mice such as these that are exposed to endemic infection and/or contact with virus-infected populations, survival is dependent on the development of resistance, and studies on *Fv1* suggest that such strongly selected genes are likely to be more widely dispersed than predicted by the observable but limited genetic exchanges (34).

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