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Three Distinct Envelope Domains, Variably Present in Subgroup B Feline Leukemia Virus Recombinants, Mediate Pit1 and Pit2 Receptor Recognition

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Subgroup B feline leukemia viruses (FeLV-Bs) evolve from subgroup A FeLV (FeLV-A) by recombining with portions of endogenous FeLV envelope sequences in the cat genome. The replication properties of FeLV-B are distinct from those of FeLV-A; FeLV-B infects many nonfeline cell lines and recognizes the human Pit1 (HuPit1) receptor, whereas FeLV-A infects primarily feline cells, using a distinct but as yet undefined receptor. Here, we demonstrate that some FeLV-Bs can also use human Pit2 (HuPit2) and hamster Pit2 (HaPit2) for entry. By making viruses that contain chimeric surface (SU) envelope proteins from FeLV-A and FeLV-B, and testing their infectivity, we have defined genetic determinants that confer host range and specific receptor recognition. HuPit1 receptor recognition determinants localize to the N-terminal region of the FeLV-B SU, amino acids 83 to 116, encompassing the N-terminal portion of variable region A (VRA). While this 34-amino-acid domain of the FeLV-B VRA is sufficient for infection of some cells (feline, canine, and human), amino acids 146 to 249 of FeLV-B, which include variable region B (VRB), were required for efficient infection in other cell types (hamster, bovine, and rat). Chimeras encoding FeLV-B VRA and VRB also infected cells expressing HaPit2 and HuPit2 receptors more efficiently than chimeras encoding only the VRA of FeLV-B, suggesting that VRB provides a secondary determinant that is both cell and receptor specific. However, viruses containing additional FeLV-B sequences in the C terminus of SU could not recognize HuPit2, implying that there is a determinant beyond VRB that negatively affects HuPit2 interactions. Thus, Pit2 recognition may drive selection for the generation of specific FeLV-B recombinants, offering an explanation for the two major classes of FeLV-B that have been observed in vivo. Furthermore, the finding that some FeLV-Bs can use both Pit1 and Pit2 may explain previous observations that FeLV-B and GALV, which primarily uses Pit1, display nonreciprocal interference on many cell types.

Exogenous feline leukemia viruses (FeLVs) have been classified into three receptor interference groups, subgroups A, B, and C (46, 49). Subgroup A FeLVs (FeLV-As) efficiently infect feline cells but are limited in their ability to infect heterologous cells, whereas subgroup B (FeLV-B) and C (FeLV-C) viruses infect several feline and nonfeline cells lines (21). The FeLV-C determinant for host range has been mapped to the N-terminal 87 to 92 amino acids (aa) of the surface glycoprotein (SU) encoded by the envelope (*env*) gene (6). Presumably, this envelope determinant plays a role in receptor recognition, although this has not specifically been analyzed because the FeLV-C receptor has not been defined. To date, only the human homolog of the FeLV-B receptor, human Pit1 (HuPit1, formerly known as Glvr1), has been identified. HuPit1 is a phosphate symporter protein that functions as a receptor for FeLV-B as well as gibbon ape leukemia virus (GALV) and murine leukemia virus (MuLV) 10A1 (22, 31, 32, 35, 36, 55). The FeLV-B determinants that specifically recognize HuPit1, or the as yet unidentified feline homolog, as the viral receptor have not been defined.

All FeLV-B proviruses characterized to date contain endogenous FeLV (enFeLV) sequences in portions of their genomes, including the envelope gene. FeLV-related endogenous sequences, which are present at multiple copies in the feline genome, are transcribed and translated but do not generate infectious virus (4, 23, 29). Subgroup B FeLVs include viruses that encode variable portions of enFeLV SU, which shares approximately 82% homology with the exogenous FeLV-A parental virus (5). Two major classes of FeLV-B recombination structures have been observed to date; members of one, typified by FeLV-B-Snyder/Theilin, FeLV-B-90Z, and FeLV-EARLE, contain enFeLV through nearly all of SU (5, 34, 38). Members of the other, typified by FeLV-B-Gardner/Arnstein and FeLV-EAGLE, contain enFeLV through only the N-terminal domains of SU (5, 38, 56). The fact that all FeLV-B variants that have been molecularly characterized share N-terminal SU sequences from enFeLV suggests that receptor binding determinants within this region are required for the expanded host range properties of FeLV-B (5). However, the host range and receptor use properties of these FeLV-B classes remain unclear because no comparative studies have been performed with specific molecular clones.

Evolutionarily, FeLVs are most related to MuLVs (9). MuLVs have been categorized into five subgroups: ecotropic MuLV (E-MuLV), which infects only murine cells; xenotropic MuLV (X-MuLV), which infects only nonmurine cells; and amphotropic (A-MuLV) and polytropic (P-MuLV) MuLVs and MuLV-10A1, which infect both murine and nonmurine cells with somewhat distinct specificities (8, 17, 18, 25, 26, 41, 42, 45). The viral host range determinants of several MuLVs have been identified: E-MuLV determinants lie in the N-terminal half of the protein (19, 33, 40, 42). P-MuLV, A-MuLV, X-MuLV, and MuLV-10A1 also rely on N-terminal sequences

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for host range specificity; more specifically, variable region A (VRA) is a primary determinant of host range for these viruses, although additional *env* sequences, including variable region B (VRB), may serve a secondary role in their infectivity or structure (2, 3, 33, 39). Biochemically, VRA encompasses two disulfide bonded loops, and VRB corresponds to a single loop (27, 28). While the viral determinants of MuLV host range have been well defined, no studies have analyzed the domains of MuLV that confer recognition of specific receptors despite the fact that many of these molecules have been identified: E-MuLV recognizes the cationic amino acid transporter CAT (formerly known as Rec1) (1); A-MuLV recognizes the phosphate symporter Pit2, both human (HuPit2, formerly known as Glvr2) and hamster (HaPit2) homologs (31, 57); and MuLV-10A1 recognizes HuPit1, HuPit2, and HaPit2 (32, 55).

FeLVs are also evolutionarily related to GALV (9). While FeLV-B and GALV both use HuPit1 as a viral receptor and display similar host ranges in vitro, these viruses have historically been placed in different interference groups because they display nonreciprocal interference in some cell lines (47, 49). Studies with hybrid HuPit1 and HuPit2 receptors suggest that GALV and FeLV-B rely on different HuPit1 domains for recognition (39). Amino acid comparisons between representative MuLV, FeLV-B, and GALV indicate that they share limited primary sequence conservation: A-MuLV and FeLV-B show 39% amino acid identity in SU; GALV and FeLV-B show 23% amino acid identity in SU (3). These differences may provide a molecular explanation for subtle differences in Pit receptor recognition by these related mammalian type C retroviruses.

We undertook this study to more precisely define FeLV-B host range and receptor recognition determinants. For this purpose, we constructed chimeric viruses between subgroup A and B FeLVs and examined the infection properties of these chimeras. Using these chimeric viruses, we demonstrate that VRA is a primary determinant of FeLV-B host range and receptor interactions. However, there are distinct subdomains of FeLV-B SU that are important for efficient infection of different cells and recognition of different Pit receptors. Our studies also show that FeLV-B can recognize both hamster and human alleles of the Pit2 receptor.

MATERIALS AND METHODS

Cell culture studies. Feline fibroblasts (AH927, a gift from W. Nelson-Rees), canine osteosarcoma cells (D17 [43]), murine cells (MDTF [24]; MDTF-HuPit1, MDTF-HuPit2, and MDTF-HaPit2 [13, 55]), hamster cells (E36 [16]), rat cells (NRK [12]), and human cells (HOS [30] and 293T [11]) were maintained in Dulbecco's modified Eagle medium with 10% fetal calf serum. Hamster cells (CHOK1 and glycosylation mutant CHOK1/lec8 [50, 51]) were maintained in minimum essential medium with 10% fetal calf serum. All media were supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin fungicide (0.25 mg/ml), and L-glutamine (2 mM).

Plasmid constructions. For these studies, we used a prototype subgroup A virus, FeLV-61E (10), and a subgroup B virus, FeLV-90Z (5). These plasmids, which represent full-length proviral clones derived from the tissues of infected cats, were used in the construction of all chimeric FeLV envelope plasmids. Envelope sequences were constructed in a subclone of the 3′ half of the FeLV-A-61E genome (3′EE, nucleotides 5318 through the end of the virus [37]). After construction of the chimeric envelope sequences, the 5′ half of FeLV-A-61E from *Eco*RI-*Xho*I was added to the 3′ subclones as described previously (5).

We constructed chimeras in which we exchanged five regions of the SU encompassing VRA and VRB defined previously in MuLV (3) (amino acid numbers [in parentheses] refer to the SU amino acid positions of the FeLV-B-90Z genome [GenBank accession no. L25632]): region 1, the first conserved region (aa 1 to 82); region 2, the N-terminal half of VRA (aa 83 to 116); region 3, the C-terminal half of VRA (aa 117 to 145); region 4, the entire VRB (aa 146 to 249); and region 5, the C terminus of SU (aa 250 to 662). In previous studies, the four-letter names for FeLV clones have signified the origin of the 5′ long terminal repeat (5′LTR)-*gag*, *pol*, SU, and transmembrane (TM)-3′LTR regions, respectively (thus, "EEZZ" represents a clone containing the FeLV-A-61E 5′LTR-*gag* and *pol* and the FeLV-B-90Z SU, TM, and 3′LTR) (5). The chimeric envelopes used in these studies are designated according to the origin of each of these five regions within SU, numbered 1 to 5 as described above. Thus, EEZZ is now defined as EE(Z1-5)Z.

The following restriction endonuclease sites were used in the cloning of chimeric viruses; base numbers (in parentheses) refer to the nucleotide sequence of the FeLV-A-61E genome: *Xho*I (5818) is in *pol*; *Hin*dIII (6542) is in the SU gene (there is a second *Hin*dIII site in the multiple cloning region of the pUC18 vector); *Aoc*I sites (5920 and 6682) are in the *pol* and SU genes, respectively. EE(Z1-4)E was constructed by ligating the 0.7-kb 3′*pol*-5′*env Aoc*I fragment from EEZZ with the 5.6-kb 3′*env*-LTR *Aoc*I fragment of the 3′EE vector.

All other chimeric FeLV plasmids were assembled by cleaving PCR-derived fragments with *Xho*I-*Hin*dIII, which spans 3′*pol* and 5′*env*, and then introducing the purified DNA fragments into a complementary 3′EE plasmid. The precise sequences and locations of all primers used are shown in Fig. 1A, with approximate locations of several envelope primers depicted in Fig. 1B.

To construct EE(Z1-3)E, FeLV-EEZZ was amplified with FeLV-*pol*-1 and FeLV-B-*env*-26 primers to create a novel *Hin*dIII site (which corresponds to the *Hin*dIII present in FeLV-A-61E at position 6542). Amplified DNA fragments were digested with *Xho*I and *Hin*dIII and cloned into a similarly digested 3′EE plasmid.

EE(Z1-2)E, EE(Z2)E, and EE(Z3)E were made by overlap extension PCR (20). Briefly, two separate first round reactions were carried out with FeLV-A-61E as the template for one reaction and FeLV-B or chimeric FeLV-A/B as the template for the other reaction. For each construct, FeLV-*pol*-3 was used as the 5′ primer for the 5′ template and FeLV-*env*-8 was used as the 3′ primer for the 3′ template. These primers were each combined with FeLV-A/B primers that were specific for each chimera: EE(Z1-2)E was made with FeLV-A/B-*env*-46 and -47, using EE(Z1-4)E as a 5′ template and FeLV-A-61E as a 3′ template; EE(Z2)E was constructed with FeLV-A/B-*env*-36 and -37, using EE(Z1-2)E as the 5′ template and FeLV-A-61E as the 3′ template; EEZ(Z3)E was constructed with FeLV-A/B-*env*-36 and -37, using FeLV-A-61E as the 5′ template and EE(Z1-3) as the 3′ template. First-round reactions were carried out with approximately 0.1 pg of template. For second-round overlap extension, first-round product was combined and amplified with additional FeLV primers (FeLV-*pol*-1 and FeLV-*env*-20). In all cases, second-round PCR product was digested with *Xho*I and *Hin*dIII for subsequent cloning.

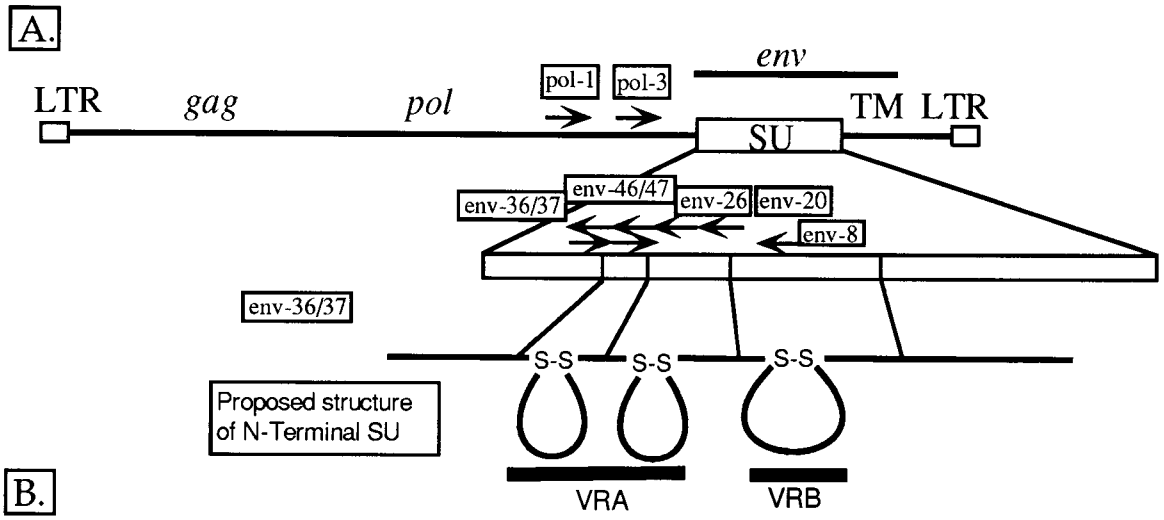
Consistent PCR conditions were used for all reactions (35 rounds of amplification consisting of a denaturing step [94°C, 1 min], a primer-annealing step [40°C, 1 min], and a primer extension step [72°C, 3 min]). For PCR-derived constructs, appropriate regions were verified by nucleotide sequence analysis. The structure of each construct was also verified by restriction fragment analysis.

Virus and LAPSN pseudotype origins, transfection, and infection. The replication of the chimeras was first assessed by transfection of feline AH927 cells, which are susceptible to both subgroup A and B FeLV, and canine D17 cells, which are susceptible to only subgroup B FeLV. These cells were transfected with plasmid DNA by electroporation (400 V, 960-μF capacitance), and viral replication was monitored 3 to 5 weeks later, at which time FeLV p27 Gag (Synbiotics Corporation, San Diego, Calif.) was measured; under conditions used for transfection, p27 Gag can be detected only if virus is amplified by replication in the culture. Virus was also expressed by transiently transfecting 293T cells by the calcium phosphate method (Stratagene mammalian cell transfection kit). The transfection efficiency in these cells is high enough to circumvent the need for amplification through replication. At 40 h after transfection, virus was harvested and used to infect feline AH927 cells, feline T cells (3201 cells), and canine D17 cells.

For quantitative infection studies, we generated FeLV particles that encapsidated retroviral vector RNAs encoding a selectable marker. The vector chosen, LAPSN, is a MuLV-based retroviral vector that encodes the genes for alkaline phosphatase (AP) and neomycin resistance (32). The LAPSN vector, which can be efficiently packaged by FeLV (7), was stably expressed in D17 cells (44). To generate FeLV particles carrying LAPSN RNA, cell-free viral supernatant from each of four FeLV-B-like chimeras [FeLV-EE(Z1-5)Z, -EE(Z1-4)E, -EE(Z1-3)E, and -EE(Z2)E] was used to infect D17-LAPSN cells. Virus was assayed by reverse transcriptase activity. The infectious titer was determined by AP staining of D17 cells. Cells were infected for 36 to 48 h in the presence of Polybrene (4 μg/ml) before fixation and staining for AP activity (15). A focus of two to four AP-staining cells was scored as one infection event in this assay because during 36 to 48 h, the cells have divided once or twice; the infectious titer is reported as the number of AP focus-forming units (FFU)/milliliter. Each infection was repeated at least four times, in duplicate and in two independent experiments. Additional control viruses for these studies were a defective FeLV-A-61E that carries the LAPSN vector, D17-61EΔΨ/LAPSN (7), and a similar defective GALV/LAPSN (31).

RESULTS

FeLV-A and -B host range determinants. To define sequences important for FeLV-A and FeLV-B host range specificity, we constructed chimeras between FeLV-A and FeLV-B in which five specific regions of the SU were exchanged (Fig. 2). EE(Z1-5)Z contains FeLV-B through the entire *env*;



Oligonucleotide	NucleotideSequence	Location
Non-Mutagenic Primers		
FeLV-A/B-*pol*-1	5'-AACCAAGAACCTCGAGCCACGG-3'	5807-5828
FeLV-A/B-*pol*-3	5'-CCTTCTCTGGCTGGGCCGAG-3'	5224-5243
FeLV-A/B-*env*-8	3'-CTCTTCGGAACCTGACAGCT-5'	7297-7317
FeLV-A/B-*env*-20	3'-GAACCACCTTCGGGAGGAGG-5'	6453-6472
Mutagenic Primers	HindIII	
FeLV-B-*env*-26	5'-GGGTCTCCAATAAGCTTCCCCGG-3'	6438-6461
Chimeric Overlap Primers		
FeLV-A/B-*env*-46/47	5'-CCCTTTTATGTCTGCCCCGGAC-3'	6341-6362
FeLV-A/B-*env*-36/37	5'-GACTTATGTGACATAGTAGG-3'	6206-6225

FIG. 1. Diagrammatic description of primers used for PCR construction of SU chimeras. (A) Schematic diagram of the relative locations of these primers in the context of the FeLV genome. The SU-encoding portion of the genome has been expanded, and the putative SU structures that correspond to exchanged regions are shown below (3, 27, 28). (B) Oligonucleotides used in this study, with the location of each primer relative to the nucleotide position in FeLV-A-61E (GenBank M18247). For consistency, FeLV-A-61E was chosen as a reference for all primers, even though, in some cases (e.g., FeLV-*env*-26), the sequence of the primer most closely resembles the sequence of FeLV-B-90Z. The *Hin*dIII site is also indicated above primer FeLV-B-*env*-26, with the altered nucleotides underlined.

EE(Z1-4)E contains FeLV-B through the N-terminal VRA and VRB; EE(Z1-3)E contains FeLV-B through VRA; EE(Z1-2)E contains FeLV-B through the N-terminal VRA domain; EE(Z2)E contains FeLV-B in only the N-terminal VRA domain, not in the first conserved region pre-VRA; EE(Z3)E contains FeLV-B in only the C-terminal VRA domain. The host range of each of these viruses was analyzed in feline and nonfeline cells. Feline AH927 cells are infectable by both FeLV-A and FeLV-B. Feline 3201 T cells can be infected by FeLV-A but not FeLV-B, presumably because there is receptor interference in these cells by an endogenous FeLV-SU protein (29). In contrast, canine D17 can be efficiently infected by FeLV-B but not FeLV-A. Therefore, we defined the FeLV-B determinant as the FeLV-B SU domain that conferred infection of both feline AH927 and canine D17 cells but not feline 3201 cells. We defined the FeLV-A determinant as the SU domain that conferred infection of feline AH927 and 3201 cells but not canine D17 cells. For this purpose, cells were exposed to FeLV and monitored for virus spread by enzyme-linked immunosorbent assay (ELISA) for FeLV p27 Gag. The results of these infection experiments are summarized in Fig. 2. EE(Z1-5)Z, EE(Z1-4)E, EE(Z1-3)E, EE(Z1-2)E, and EE(Z2)E all replicated in D17 and AH927 cells, consistent with FeLV-B host range. The minimal FeLV-B sequence shared by these constructs encompasses aa 83 to 116 encoded by the 5′ subdomain of VRA which contains a putative cysteine loop. EE(Z3)E, which encodes the N-terminal VRA of FeLV-A-61E and the C-terminal VRA from FeLV-B-90Z, replicated only in feline cells, consistent with FeLV-A host range.

Secondary host range determinants. In addition to efficiently infecting canine D17 cells, FeLV-B replicates in a variety of other nonfeline cells, with murine cells being a notable exception. We compared the host ranges of FeLV-B-like chimeras EE(Z1-5)Z, EE(Z1-4)E, EE(Z1-3)E, and EE(Z2)E to that of FeLV-A-61E in several mammalian cell lines. We generated virus that carried the retroviral vector LAPSN so that infection could be visualized by AP expression after a single round of infection, thus circumventing the need for virus spread (32). The results of these infection studies are summarized in Table 1. Cell lines exhibited one of three types of susceptibility: (i) some cells could be efficiently infected with all of the chimeric viruses (AH927, D17, and HOS); (ii) some were resistant to all chimeric viruses (MDTF); and (iii) some were differentially sensitive to chimeras encoding larger por-

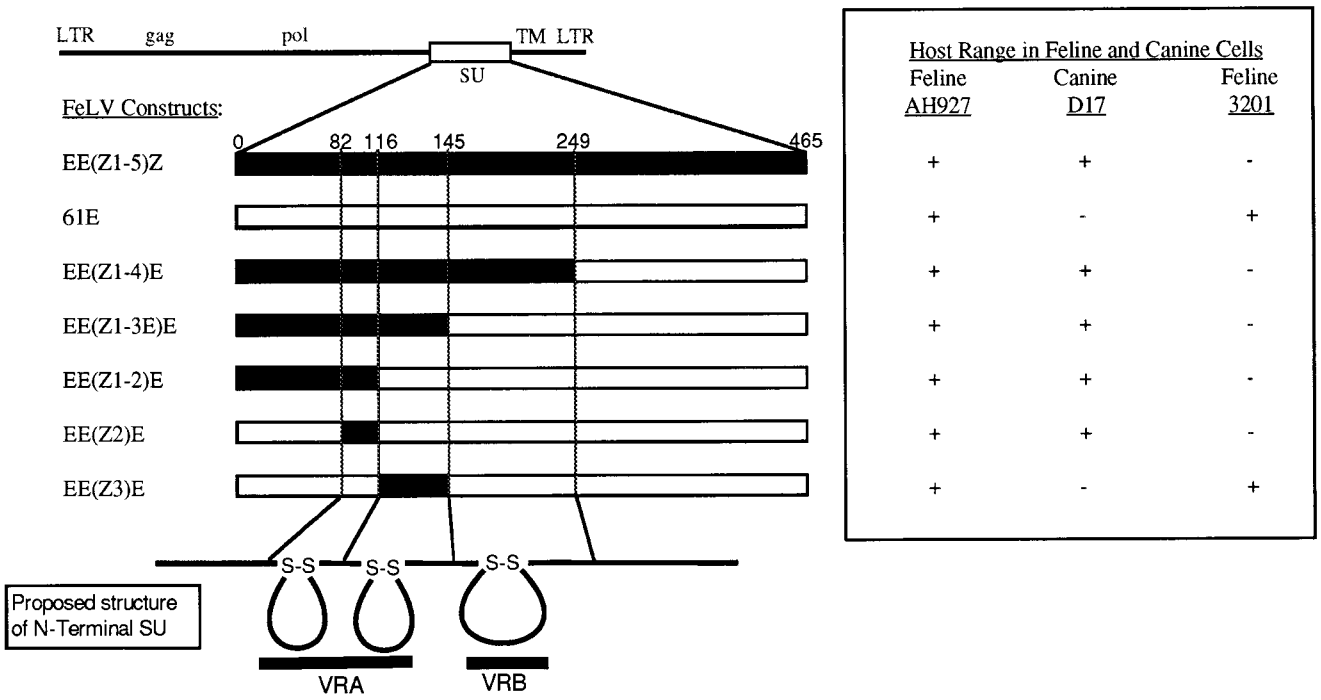


FIG. 2. Schematic representation of chimeric viruses and summary of infection studies in feline and canine cells. For each construct, sequences from the parental FeLV-B-90Z are in black and those from FeLV-A-61E are in white. The SU protein was divided into five regions for this study (approximate exchange junctions are indicated by vertical lines): 1, aa 1 to 82 of FeLV-B-90Z; 2, the N-terminal half of the VRA (aa 83 to 116), 3, the C-terminal half of the VRA (aa 117 to 145); 4, the entire VRB (aa 146 to 249); and 5, the C terminus of SU (aa 250 to 662). A schematic depiction of the N-terminal SU structure, based on that proposed for MuLV VRA and VRB (3), is shown beneath the diagram of the FeLV constructs. The host range properties of the constructs in feline cells (fibroblast AH927 and T-cell 3201) and canine cells (osteosarcoma D17) are indicated at the right. Replication was assessed in three ways, described in Materials and Methods. (i) All constructs were first introduced into AH927 and D17 via electroporation and passaged for 5 weeks. At this time, virus replication was assessed by FeLV p27 Gag ELISA. (ii) Cell-free virus was then transmitted from transfected AH927 [for 61E and EE(Z3)E] or D17 (for all other chimeras) cells onto 3201, AH927, and D17 cells; virus spread was also assessed at 5 weeks postinfection. (iii) Constructs were independently transfected into 293T cells via calcium phosphate transfection, and cell-free virus was transmitted to AH927 and D17 cells. In all cases, virus replication was assayed by p27 Gag ELISA at 3 to 5 weeks posttransfection or postinfection. Similar results were obtained by all of these methods.

tions of FeLV-B, including both VRA and VRB, compared with those containing the VRA alone (NRK, MDBK, CHOK1/lec8, and E36). In this latter group of cells, EE(Z1-5)Z and EE(Z1-4)E, which encode FeLV-B in VRA and VRB, consistently displayed ≥1-log-higher titers (AP units per milliliter) than EE(Z1-3)E and EE(Z2)E, which encode FeLV-B only in VRA. The results of multiple experiments compiled for representative infected cultures (D17, NRK, and MDBK cells) in Fig. 3 demonstrate the reproducibility of the experiment.

FeLV-B receptor recognition determinants. To correlate the determinants for host range specificity with recognition of a specific receptor, we examined receptor use by three FeLV-B-like chimeras: EE(Z1-5)Z, which encodes FeLV-B through the entire *env*, EE(Z1-4)E, which encodes FeLV-B through VRA and VRB, and EE(Z2)E, which encodes FeLV-B only in the N terminus of VRA. Results obtained with cells expressing specific Pit receptors, HuPit1 (35), HuPit2 (57), and HaPit2 (55), are shown in Table 2. Each Pit receptor allele was expressed in

TABLE 1. Host ranges of chimeras in several lines

Cell line	Species	Alkaline phosphatase (FFU/ml)[a]				
		VRA/B chimeras		VRA chimeras		
		EE(Z1-5)Z	EE(Z1-4)E	EE(Z1-3)E	EE(Z2)E	FeLV-A-61E
D17	Canine	170	130	130	170	1
HOS	Human	330	330	100	70	0.3
MDTF	Mouse	<0.003	<0.003	<0.003	<0.003	<0.003
NRK	Rat	30	30	3	3	<0.003
E36	Hamster	2	10	0.3	0.2	<0.003
CHOK1	Hamster	<0.003	<0.003	<0.003	<0.003	<0.003
CHO1/lec8	Hamster	2	3	0.2	0.2	<0.003
MDBK	Bovine	2	7	0.02	0.01	<0.003

[a] Titer following infection with virus/LAPSN normalized to titer observed on feline fibroblasts (AH927 cells). The AH927 cell line was chosen as a standard because it was highly susceptible to infection by all viruses. The titer on AH927 cells for all viruses was approximately 1×10^5 to 5×10^5 FFU/ml and was assigned a value of 100%. Infections were carried out for 2 days so that a focus of two to four cells is scored as one infection event in this assay. Each value represents the mean of at least three separate experiments, each performed in duplicate. Uninfected controls were negative, giving no background (data not shown).

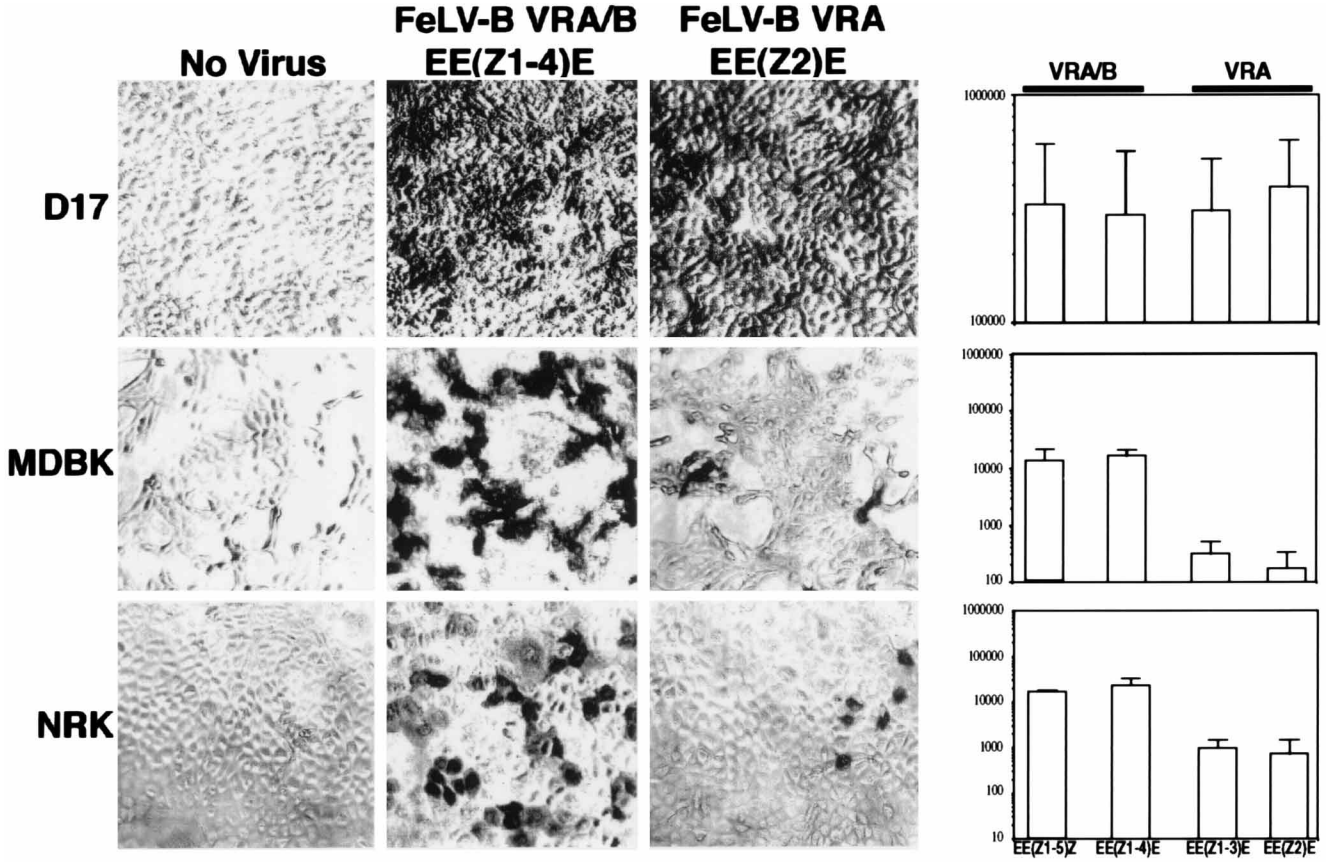


FIG. 3. Infection studies in canine, bovine, and rat cells. The left three-by-three matrix of micrographs shows different cells, indicated to the left, stained for AP following infection with virus/LAPSN pseudotypes (indicated above). Infected cells that appear black express AP, which indicates infection. Viruses tested were EZ(1-4)E, which contains FeLV-B sequences through VRA and VRB, and EE(Z2)E, which contains FeLV-B sequence only in VRA. Cells (canine D17, rat NRK, and bovine MDBK) were plated at 10^4 cells per ml in 24-well dishes and maintained for 12 to 18 h. The same amount of each virus (approximately 10^5 AP-staining units per ml, as assessed on feline fibroblasts [AH927 cells]) was added to each well, in the presence of Polybrene (10 μg/ml), for 36 to 48 h. At this time, cells were fixed, stained, and photographed (objective magnification, ×40). Micrographs were taken with a Nikon inverted microscope and camera. Color slides were digitized by using a Nikon scanner with Adobe Photoshop and converted to grey scale for publication. To the right of the micrographs are corresponding numerical graphs that summarize infection studies with all chimeras EE(Z1-5)Z, EE(Z1-4)E, EE(Z1-3)E, and EE(Z2)E. The number of AP-staining units per milliliter of virus obtained was determined by averaging three infection experiments, each performed in duplicate.

MDTF cells, which are resistant to FeLV-B and GALV infection (reference 13 and Table 1). Cells expressing HuPit1 were infected with equal efficiency by all FeLV-B-like chimeras and the GALV control. Cells expressing HaPit2 were sensitive to infection by both GALV and FeLV-B, although EE(Z1-5)Z and EE(Z1-4)E, which encode FeLV-B sequences in VRA and VRB, consistently display titers that are >1 log higher than those of EE(Z2)E, which only encodes FeLV-B in the N-terminal domains of the VRA. Cells expressing HuPit2 were sensitive to infection by some FeLV-B but not others. EE(Z1-4)E and EE(Z2)E both infected these cells, although EE(Z1-4)E display titers that are >1 log higher than those of EE(Z2)E. In contrast, EE(Z1-5)E, which contains FeLV-B sequences throughout the SU, did not recognize HuPit2. As expected, the GALV control did not infect MDTF-HuPit2 cells. None of the viruses infected MDTF control cells.

DISCUSSION

FeLV-B has an extended host range compared to FeLV-A, which replicates most efficiently in feline cells (21). Previous studies have demonstrated that FeLV-B recognizes the HuPit1 receptor (53). Here, we show that certain FeLV-B recombinants or chimeras can also infect cells expressing either the HuPit2 or HaPit2. To define domains of the viral envelope protein important in receptor recognition, we constructed FeLVs with envelope genes that were chimeric between a prototypic FeLV-A and FeLV-B, exchanging domains that were defined on the basis of related MuLV structural motifs (2, 14, 27, 28). Infection studies with these viruses demonstrated that N-terminal domains of the SU protein encode critical primary and secondary determinants for FeLV-B host range and Pit receptor interactions.

FeLV chimeras that displayed a FeLV-B host range and recognized the HuPit1 receptor encoded, minimally, the N-terminal VRA of SU from FeLV-B (aa 83 to 116). Thus, these findings provide experimental support for speculations by ourselves and others that the N-terminal SU of FeLV-B binds the receptor and confers extended host range properties characteristic of this subgroup (5, 29, 38, 48, 52). Our studies also suggest that the N-terminal region of FeLV-A may determine the ecotropic subgroup A host range, although additional chimeras will be needed to fully determine this. These host range-defining sequences are also similar to those demonstrated for FeLV-C, which relies on the N-terminal half of VRA for its unique ability to replicate in guinea pig cells (6). Similarly, studies of MuLV host range have implicated VRA, although

TABLE 2. Receptor use by FeLV-B chimeras

Cells	Alkaline phosphatase (FFU/ml)[a]				
	EE(Z1-5)Z	EE(Z1-4)E	EE(Z2)E	61E	GALV
MDTF expressing:					
HuPit1	100	100	100	<0.003	100
HuPit2	<0.003	6	0.1	<0.003	<0.003
HaPit2	7	10	0.1	<0.003	5
No receptor	<0.003	<0.003	<0.003	<0.003	<0.003
AH927 reference	100	100	100	100	ND

[a] Titer following infection with each virus/LAPSN (FeLV or GALV) normalized to the titer observed on MDTF cells expressing HuPit1 (arbitrarily assigned a value of 100%). This cell line was chosen as a standard because it was highly susceptible to infection by FeLV-B. The actual infectious titer for FeLV chimeras was approximately 1×10^5 to 5×10^5 FFU/ml, and that for GALV was 6×10^4 FFU/ml. Infections with FeLV-A-61E were carried out with up to 5×10^5 FFU/ml, based on infection of feline AH927 cells. Infections were carried out for 2 days. A focus of two to four cells is scored as one infection event in this assay. Each value represents the mean of at least three separate experiments, each performed in duplicate. Uninfected controls were negative (data not shown). ND, not determined.

these analyses have not yet defined a smaller region within VRA that is sufficient to confer in vitro host range.

While VRA appears to be the primary FeLV-B host range determinant, the additional presence of FeLV-B sequences in VRB enhanced infectivity for some nonfeline cells and cells expressing human and hamster Pit2 receptor alleles. Our studies show that the differential infection properties of FeLV-B VRA-VRB and VRA chimeras in MDTF-HaPit2 cells are similar to infection patterns in hamster E36 cells from which HaPit2 was originally isolated. These data may indicate that in E36 hamster cells, FeLV-B recognizes the Pit2 receptor. It is unclear what receptor is recognized by FeLV-B in rat NRK and bovine MDBK cells, where there is also an effect of VRB on viral infectivity. Additional studies using cloned receptors from these cells and/or receptor-specific competitive inhibitors of FeLV-B will be important in clarifying the Pit2 proteins that require FeLV-B VRB determinants for optimal envelope-receptor interactions.

In addition to N-terminal VRA and VRB determinants, a C-terminal SU domain appears to provide a third receptor recognition determinant that is specific for HuPit2. This conclusion is based on the observation that the full-length natural FeLV-B recombinant, FeLV-B-EE(Z1-5)Z, recognizes HaPit2 but not HuPit2. In contrast, the chimera FeLV-B-EE(Z1-4)E recognizes both Pit2 alleles. This differential infectivity may explain why previous groups did not observe FeLV-B infection of cells expressing HuPit2; the FeLV-B used in these studies may have been structurally similar to EEZ(1-5)Z, although it is difficult to assess which FeLV-B recombinant was used because a molecularly uncharacterized isolate was employed (39). It is also possible that these earlier negative findings are attributable to the low infectious titer of FeLV-B used in these experiments because the infectivity of FeLV-B for cells expressing HuPit2 is approximately 3 logs lower than that for cells expressing HuPit1. In any case, it is clear from our data for well-characterized FeLV-B recombinants that some Pit2 homologs can be recognized by FeLV-B.

In the context of the studies presented here, it is perhaps significant that all naturally occurring FeLV-Bs analyzed to date encode N-terminal enFeLV sequences, including VRA and VRB, although only a subset encode enFeLV sequences in the C terminus of SU (5). Our studies suggest that chimeras which encode enFeLV VRA and VRB efficiently recognize both Pit receptors, suggesting that FeLV-B recombinants may be selected for optimal and flexible receptor recognition. The parental virus used for this study, FeLV-90Z, was isolated directly from the tissue of a cat infected with FeLV-A (5). FeLV-90Z and the previously described FeLV-B clone (Snyder-Theilen [34]) encode SU proteins derived almost entirely from enFeLV sequences (5). A similar class of recombinant proviruses, defined as FeLV-B-EARLE, are selected in feline cells infected with a FeLV-A clone (38). A second class of FeLV-B recombinants encode only the N terminus of enFeLV; these include FeLV-B-EAGLE (38), FeLV-B-Gardner/Arnstein (56), and the chimera EEZ(1-4)Z used in this study. While EEZ(1-4)E, which resembles the FeLV-B-EAGLE class, can efficiently infect cells expressing both human Pit1 and Pit2, EEZ(1-5)Z, which resembles the FeLV-B-EARLE class, fails to recognize HuPit2 as a receptor. The presence of VRB in all natural FeLV-B isolates from cats, and in both FeLV-B-EARLE and FeLV-B-EAGLE classes may suggest that Pit2 recognition is an important selection factor in vivo. It will be important to examine this model by using the feline Pit receptor homologs.

Finally, these studies further illustrate the overlap in receptor recognition of feline, murine, and primate type C retrovi-

FIG. 4. Summary of Pit receptor specificity of feline, murine, and primate C-type retroviruses. The schematic depicts receptor use among FeLV-A, three recombinant FeLV-B classes described in this study [FeLV-B-SU, typified by FeLV-EE(Z1-5)Z, FeLV-B-S/T, and FeLV-B-EARLE; FeLV-B VRA/B, typified by FeLV-EE(Z1-4)E, FeLV-B-G/A, and FeLV-B-EAGLE; and FeLV-B VRA, typified by FeLV-EE(Z1-3)E, FeLV-EE(Z1-2)E, and FeLV-EE(Z2)E], GALV, MuLV-10A1, and A-MuLV. The structure of the viral SU is shown schematically to the left. FeLV-A SU sequences are shown in white; FeLV-B sequences are shown in black; MuLV and GALV sequences are shown in different shades of grey, reflecting sequence divergence relative to FeLV. Receptor use by HuPit1, HuPit2, and HaPit2 is summarized in the matrix to the right. *, new data presented in this study.

ruses, which share limited primary sequence identity (summarized in Fig. 4). Our novel finding that FeLV-B uses both human and hamster Pit1 and Pit2 receptors may explain why GALV and FeLV-B have historically shown nonreciprocal interference patterns in certain cell types (47, 49, 54). Taken together, these studies also provide evidence for an evolutionary relationship among these viruses that is reflected not in sequence identity but rather in function.

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REFERENCES

1. **Albritton, L. M., L. Tseng, D. Scadden, and J. M. Cunningham.** 1989. A putative murine ecotropic retrovirus receptor gene encodes a multiple membrane-spanning protein and confers susceptibility to virus infection. Cell **57:**659–666.
2. **Battini, J., O. Danos, and J. M. Heard.** 1995. Receptor-binding domain of murine leukemia virus envelope glycoproteins. J. Virol. **69:**713–719.
3. **Battini, J., J. M. Heard, and O. Danos.** 1992. Receptor choice determinants in the envelope glycoproteins of amphotropic, xenotropic, and polytropic murine leukemia viruses. J. Virol. **66:**1468–1475.
4. **Benveniste, R. E., C. J. Sherr, and G. J. Todaro.** 1975. Evolution of type C viral genes: origin of feline leukemia virus. Science **190:**886–888.
5. **Boomer, S., P. Gasper, L. R. Whalen, and J. Overbaugh.** 1994. Isolation of a novel subgroup B feline leukemia virus from a cat infected with FeLV-A. Virology **204:**805–810.
6. **Brojatsch, J., B. S. Krystal, G. A. Viglianti, R. Khiroya, E. A. Hoover, and J. I. Mullins.** 1992. Feline leukemia virus subgroup C phenotype evolves through distinct alterations near the N terminus of the envelope surface glycoprotein. Proc. Natl. Acad. Sci. USA **89:**8457–8461.
7. **Burns, C. C., M. Moser, J. Banks, J. P. Alderete, and J. Overbaugh.** 1996. Identification and deletion of sequences required for feline leukemia virus RNA packaging and construction of a high-titer feline leukemia virus packaging cell line. Virology **222:**14–20.
8. **Cloyd, M. J.** 1983. Characterization of target cells for MCF viruses in AKR mice. Cell **32:**217–225.
9. **Coffin, J.** 1992. Structure and classification of retroviruses, p. 19–49. *In* J. A. Levy (ed.), The Retroviridae, vol. 1. Plenum Press, New York, N.Y.
10. **Donahue, P. R., E. A. Hoover, G. A. Beltz, N. Riedel, V. M. Hirsh, J. Overbaugh, and J. I. Mullins.** 1988. Strong sequence conservation among horizontally transmissible, minimally pathogenic feline leukemia viruses. J. Virol. **62:**722–731.
11. **DuBridge, R. B., P. Tang, H. C. Hsia, P. Leong, J. H. Miller, and M. P. Calos.** 1987. Analysis of mutation in human cells by using an Epstein-Barr virus shuttle system. Mol. Cell. Biol. **7:**379–387.
12. **Duc-Ngugen, H., E. N. Rosenblum, and R. F. Zeigel.** 1966. Persistent infection of a rat kidney cell line with Rauscher murine leukemia virus. J. Bacteriol. **92:**1133–1140.
13. **Eiden, M. V., K. B. Farrell, and C. A. Wilson.** 1996. Substitution of a single amino acid residue is sufficient to allow the human amphotropic murine leukemia virus receptor to also function as a gibbon ape leukemia virus receptor. J. Virol. **70:**1080–1085.
14. **Elder, J. H., and J. I. Mullins.** 1983. Nucleotide sequence of the envelope gene of Gardner-Arnstein feline leukemia virus B reveals unique sequence homologies with a murine mink cell focus-forming virus. J. Virol. **46:**871–880.
15. **Fields-Berry, S. C., A. L. Halliday, and C. L. Cepko.** 1992. A recombinant retrovirus encoding alkaline phosphatase confirms clonal boundary assignment in lineage analysis of murine retina. Proc. Natl. Acad. Sci. USA **89:**693–697.
16. **Gillin, F. D., D. J. Roufa, A. L. Beaudet, and C. T. Caskey.** 1972. 8-Azaguanine resistance in mammalian cells. I. Hypoxanthine-guanine phosphoribosyltransferase. Genetics **72:**239–252.
17. **Hartley, J. W., and W. P. Rowe.** 1976. Naturally occurring murine leukemia viruses in wild mice: characterization of a new "amphotropic" class. J. Virol. **19:**19–25.
18. **Hartley, J. W., N. K. Wolford, L. J. Old, and W. P. Rowe.** 1977. A new class of murine leukemia virus associated with development of spontaneous lymphomas. Proc. Natl. Acad. Sci. USA **74:**789–792.
19. **Heard, J. M., and O. Danos.** 1991. An amino-terminal fragment of the Friend murine leukemia virus envelope glycoprotein binds the ecotropic receptor. J. Virol. **65:**4026–4032.
20. **Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease.** 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77:**51–59.
21. **Jarrett, O., H. M. Laird, and D. Hay.** 1973. Determinants of host range of feline leukemia viruses. J. Gen. Virol. **20:**169–175.
22. **Kavanaugh, M. P., D. G. Miller, W. Zhang, W. Law, S. L. Kozak, D. Kabat, and A. D. Miller.** 1994. Cell-surface receptors for gibbon ape leukemia virus and amphotropic murine retrovirus are inducible sodium-dependent phosphate symporters. Proc. Natl. Acad. Sci. USA **91:**7071–7075.
23. **Kumar, D. V., B. T. Berry, and P. Roy-Burman.** 1989. Nucleotide sequence and distinctive characteristics of the env gene of endogenous feline leukemia provirus. J. Virol. **63:**2379–2384.
24. **Lander, M. R., and S. K. Chattopadhyay.** 1984. A *Mus dunni* cell line that lacks sequences closely related to endogenous murine leukemia viruses and can be infected by ecotropic, amphotropic, xenotropic, and mink cell focus-forming viruses. J. Virol. **52:**695–698.
25. **Levy, J. A.** 1974. Autoimmunity and neoplasia. The possible role of C-type viruses. Am. J. Clin. Pathol. **62:**258–280.
26. **Levy, J. A.** 1978. Xenotropic type C viruses. Curr. Top. Microbiol. Immunol. **79:**111–213.
27. **Linder, M., D. Linder, J. Hahnen, H. Schott, and S. Stirm.** 1992. Localization of the intrachain disulfide bonds of the envelope glycoprotein 71 from Friend murine leukemia virus. Eur. J. Biochem. **203:**65–73.
28. **Linder, M., V. Wenzel, D. Linder, and S. Stirm.** 1994. Structural elements in glycoprotein 70 from polytropic Friend mink cell focus-inducing virus and glycoprotein 71 from ecotropic Friend murine leukemia virus, as defined by disulfide-bonding pattern and limited proteolysis. J. Virol. **68:**5133–5141.
29. **MacDougall, A. S., A. Terry, T. Tzavaras, C. Cheney, J. Royko, and J. C. Neil.** 1994. Defective endogenous proviruses are expressed in feline lymphoid cells: evidence for a role in natural resistance to subgroup B feline leukemia viruses. J. Virol. **68:**2151–2160.
30. **McAllister, R. M., M. B. Gardner, A. E. Greene, C. Bradt, W. W. Nichols, and B. H. Landing.** 1971. Cultivation *in vitro* of cells derived from a human osteosarcoma. Cancer **27:**397–402.
31. **Miller, D. G., R. H. Edwards, and A. D. Miller.** 1994. Cloning of the cellular receptor for amphotropic murine retroviruses reveals homology to that for gibbon ape leukemia virus. Proc. Natl. Acad. Sci. USA **91:**78–82.
32. **Miller, D. G., and A. D. Miller.** 1994. A family of retroviruses that utilize related phosphate transporters for cell entry. J. Virol. **68:**8270–8276.
33. **Morgan, R. A., O. Nussbaum, D. D. Muenchau, L. Shu, L. Couture, and F. Anderson.** 1993. Analysis of the functional and host range-determining regions of the murine ecotropic and amphotropic retrovirus envelope proteins. J. Virol. **67:**4712–4721.
34. **Nunberg, J. H., M. E. Williams, and M. A. Innis.** 1984. Nucleotide sequences of the envelope genes of two isolates of feline leukemia virus subgroup B. J. Virol. **49:**629–632.
35. **O'Hara, B., S. V. Johann, H. P. Klinger, D. G. Blair, H. Rubinson, K. J. Dunne, P. Sass, S. M. Vitek, and T. Robins.** 1990. Characterization of a human gene conferring sensitivity to infection by gibbon ape leukemia virus. Cell Growth Differ. **1:**119–127.
36. **Olah, Z., C. Lehel, W. B. Anderson, M. V. Eiden, and C. A. Wilson.** 1994. The cellular receptor for gibbon ape leukemia virus is a novel high affinity sodium-dependent phosphate transporter. J. Biol. Chem. **269:**25426–25431.
37. **Overbaugh, J., P. R. Donahue, S. L. Quackenbush, E. A. Hoover, and J. I. Mullins.** 1988. Molecular cloning of a feline leukemia virus that induces fatal immunodeficiency disease in cats. Science **239:**906–910.
38. **Overbaugh, J., N. Reidel, E. A. Hoover, and J. I. Mullins.** 1988. Transduction of endogenous envelope genes by feline leukaemia virus *in vitro*. Nature **332:**731–734.
39. **Pedersen, L., S. V. Johann, M. V. Zeijl, F. S. Pedersen, and B. O'Hara.** 1995. Chimeras of receptors for gibbon ape leukemia virus/feline leukemia virus B and amphotropic murine leukemia virus reveal different modes of receptor recognition by retrovirus. J. Virol. **69:**2401–2405.
40. **Peredo, C., L. O'Reilly, K. Gray, and M. J. Roth.** 1996. Characterization of chimeras between the ecotropic Moloney murine leukemia virus and the amphotropic 4070A envelope proteins. J. Virol. **70:**3142–3152.
41. **Rasheed, S., M. B. Gardner, and E. Chan.** 1976. Amphotropic host range of naturally occurring wild mouse leukemia viruses. J. Virol. **19:**13–18.
42. **Rein, A.** 1980. Interference grouping of murine leukemia viruses: a distinct receptor for the MCF-recombinant virus in mouse cells. Virology **20:**251–257.
43. **Riggs, J. L., R. M. McAllister, and E. H. Lennette.** 1974. Immunofluorescent studies of RD-114 virus replication in cell culture. J. Gen. Virol. **25:**21–29.
44. **Rohn, J. L., M. S. Moser, S. R. Gwynn, D. N. Baldwin, and J. Overbaugh.** Evolution of a novel, syncytium-inducing and cytopathic feline leukemia virus variant *in vivo*. Submitted for publication.
45. **Sarma, P. S., M. P. Cheong, J. M. Hartley, and R. J. Heubner.** 1967. A viral interference test for mouse leukemia viruses. Virology **33:**180–184.

46. **Sarma, P. S., and T. Log.** 1973. Subgroup classification of feline leukemia and sarcoma viruses by viral interference and neutralization tests. Virology **54:**160–169.
47. **Schnitzer, T. J., R. A. Weiss, and J. Zavada.** 1977. Pseudotypes of vesicular stomatitis virus with the envelope properties of mammalian and primate retroviruses. J. Virol. **23:**449–454.
48. **Sheets, R. L., R. Pandey, V. Klement, C. K. Grant, and P. Roy-Burman.** 1992. Biologically selected recombinants between feline leukemia virus (FeLV) subgroup A and an endogenous FeLV element. Virology **176:**58–69.
49. **Sommerfelt, M. A., and R. A. Weiss.** 1990. Receptor interference groups of 20 retroviruses plating on human cells. Virology **176:**58–69.
50. **Stanley, P.** 1981. Selection of specific wheat germ agglutinin-resistant (Wga^R) phenotypes from Chinese hamster ovary cell populations containing numerous lec^R genotypes. Mol. Cell. Biol. **1:**687–696.
51. **Stanley, P., and L. Siminovitch.** 1977. Complementation between mutants of CHO cells resistant to a variety of plant lectins. Somatic Cell Genet. **3:**391–405.
52. **Stewart, M. A., M. Warnock, A. Wheeler, N. Wilkie, J. I. Mullins, D. E. Onions, and J. C. Neil.** 1986. Nucleotide sequences of a feline leukemia virus subgroup A envelope gene and long terminal repeat and evidence for the recombinational origin of subgroup B viruses. J. Virol. **58:**825–834.
53. **Takeuchi, Y., R. G. Vile, G. Simpson, B. O'Hara, M. K. Collins, and R. A. Weiss.** 1992. Feline leukemia virus subgroup B uses the same cell surface receptor as gibbon ape leukemia virus. J. Virol. **66:**1219–1223.
54. **Weiss, R.** 1984. Experimental biology and assay of RNA tumor viruses, p. 209–260. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), RNA tumor viruses, vol. 1. Molecular biology of tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
55. **Wilson, C. A., K. B. Farrell, and M. V. Eiden.** 1994. Properties of a unique form of the murine amphotropic leukemia virus receptor expressed on hamster cells. J. Virol. **68:**7697–7703.
56. **Wunsch, M., A. S. Schultz, W. Koch, R. Friedrich, and G. Hunsmann.** 1983. Sequence analysis of Gardner-Arnstein feline leukaemia virus envelope gene reveals common structural properties of mammalian retroviral envelope genes. EMBO J. **2:**2239–2246.
57. **Zeijl, M. V., S. V. Johann, E. Cross, J. Cunningham, R. Eddy, T. B. Shows, and B. O'Hara.** 1994. A human amphotropic virus receptor is a second member of the gibbon ape leukemia virus receptor family. Proc. Natl. Acad. Sci. USA **91:**1168–1172.