Subtle Mutational Changes in the SU Protein of a Natural Feline Leukemia Virus Subgroup A Isolate Alter Disease Spectrum

Chandtip Chandhasin,1,2,3 Patricia N. Coan,4 and Laura S. Levy1,2,3*

Department of Microbiology and Immunology,1 Program in Molecular and Cellular Biology,2 Tulane Cancer Center,3 and Department of Vivarial Science and Research,4 Tulane University Health Sciences Center, New Orleans, Louisiana

Received 20 July 2004/Accepted 22 September 2004

FeLV-945 is a representative isolate of the natural feline leukemia virus (FeLV) variant predominant in non-T-cell malignant, proliferative, and degenerative diseases in a geographic cohort. The FeLV-945 surface glycoprotein (SU) is closely related to natural horizontally transmissible FeLV subgroup A (FeLV-A) but was found to differ from a prototype to a larger extent than the members of FeLV-A differ among themselves. The sequence differences included point mutations restricted largely to the functional domains of SU, i.e., VRA, VRB, and PRR. Despite the sequence differences in these critical domains, measurements of receptor utilization, including host range and superinfection interference, confirmed the assignment of FeLV-945 to subgroup A. Other proviruses isolated from the cohort contained similar sequence hallmarks and were assigned to FeLV subgroup A. A provirus from cat 1046 contained a histidine-to-proline change at SU residue 6 within an SPHQ motif that was previously identified as a critical mediator of fusion events during virus entry. The 1046 pseudotype virus entered cells only in the presence of the soluble cofactor FeLIX provided in trans, but it retained an ecotropic host range even in the presence of FeLIX. The mutational changes in FeLV-945 were shown to confer significant functional differences compared to prototype FeLV-A viruses. The substitution of FeLV-945 envelope gene sequences for FeLV-A/61E sequences conferred a small but statistically significant replicative advantage in some feline cells. Moreover, substitution of the unique FeLV-945 long terminal repeat and envelope gene for those of FeLV-A/61E altered the disease spectrum entirely, from a thymic lymphoma of a T-cell origin to an as yet uncharacterized multicentric lymphoma that did not contain T cells.

Feline leukemia virus (FeLV) is a naturally occurring gammaretrovirus of the domestic cat, and infection with this virus causes degenerative, proliferative, and malignant diseases of a hematologic origin (17). FeLV does not occur in nature as a single genomic species but as a genetically complex family of closely related viruses. Genetic variation in FeLV is generated during virus replication through error-prone reverse transcription and by recombination with endogenous FeLV-related sequences. The consequence of this variation is a genetically diverse virus population that is continuously shaped by selective pressures in vivo and from which variants arise as predomi-

nate species. Natural isolates of FeLV most commonly exhibit sequence variations within the viral long terminal repeat (LTR) or the surface glycoprotein (SU) gene (24, 27). It has been shown that subtle mutational changes accumulate in FeLV SU during infection in vivo, which may alter the biological properties of the virus, such as receptor utilization or affinity, replication kinetics, or pathogenic potential (7, 15, 19, 20, 24, 27, 34). As is typical for gammaretroviruses, the FeLV SU protein comprises an amino-terminal receptor-binding domain (RBD) followed by a proline-rich region (PRR) that mediates conformational changes required for entry. Two variable regions within RBD, designated VRA and VRB, define the specificity for receptor binding (5, 6, 38, 39).

FeLV occurs in nature in four subgroups, designated A, B, C, and T, that are distinguished genetically by sequence differences in SU and functionally by distinct requirements for entry. FeLV subgroup A (FeLV-A) is a weakly pathogenic, ecotropic, horizontally transmissible virus that is thought to represent the predominant agent in cat-to-cat spread in nature. The known isolates of FeLV-A share >97% amino acid sequence identity for SU, which is particularly remarkable since they were isolated over more than a decade from distant geographic locations across the world (11, 24, 27). The FeLV B, C, and T subgroups are thought to arise from FeLV-A de novo through point mutations, insertions, and/or recombination with endogenous FeLV-related sequences during virus replication in infected animals. The FeLV B, C, and T subgroups are associated with distinct disease outcomes in infected animals, which is likely a consequence of their access to distinct target cell populations through differential receptor utilization and perhaps as a result of other functional consequences of SU mutation as well (15, 20, 24, 36). While FeLV-A infection is associated with the induction of thymic lymphomas of a T-cell origin (24, 31), FeLV-B, -C, and -T are specifically associated with lymphomas, anemia, and immunodeficiency disease, respectively (12, 24, 34).

We previously reported the isolation of a natural FeLV variant, termed FeLV-945, as the predominant species in non-T-cell diseases in a temporal and geographic cohort of naturally infected cats. FeLV-945 was originally identified in multicentric lymphomas with an unknown phenotype (2, 23) and was subsequently demonstrated in degenerative and proliferative diseases of myeloid and erythroid origins in the cohort (8). FeLV-945 was shown to have a distinctive sequence in two
respects. First, the FeLV-945 LTR contains a unique sequence motif comprised of a single copy of a transcriptional enhancer followed downstream by a 21-bp sequence that is triplicated in tandem. The 21-bp triplication was shown to contribute an enhancer function to the LTR in a cell type-specific manner and to confer a replicative advantage to the virus in those cells (3, 8, 29). The activity of the 21-bp triplication was recently shown to depend on binding of the c-Myb transcription factor and the consequent recruitment of the coactivator CBP (14). Second, the FeLV-945 SU gene was shown have a distinctive sequence. While clearly of exogenous origin and closely related to FeLV subgroup A, FeLV-945 SU was observed to have a different amino acid sequence from that of a FeLV-A prototype to an extent that was larger than the differences of known FeLV-A isolates among themselves (2). The sequence differences in FeLV-945 SU, which were largely restricted to VRA, VRB, and PRR, were considered remarkable because SU proteins have nearly identical amino acid sequences among natural FeLV-A isolates (11, 24, 27). The present study was designed to examine the possibility that the distinctive SU sequence conferred biological properties that allowed FeLV-945 to predominate and/or to induce non-T-cell disease in the cohort from which it was isolated. Experiments were performed to test the possibility that the mutational changes in FeLV-945 SU may alter receptor utilization, increase growth kinetics, or change the disease spectrum relative to a prototype FeLV-A isolate.

**MATERIALS AND METHODS**

**Viruses, cell lines, and tissues.** An infectious molecular clone of FeLV-A/61E (28) was obtained from the NIAID AIDS Research and Reference Reagent Program (donated by James I. Mullins). An infectious molecular clone of FeLV-B/Gardner-Arnstein was a gift from James Casey. FEA feline embryonic fibroblasts (a gift from Jennifer Rojko) and D17 canine osteosarcoma cells (ATCC CCL 183) were cultured in Eagle minimal essential medium (MEM) with 10% fetal bovine serum (FBS) and nonessential amino acids. FCS, BM, an adherent bone marrow-derived feline cell line (ATCC CRL-6081) was maintained in Dulbecco modified Eagle medium (DMEM) with 10% FBS. 3201 is a FeLV-negative thymic lymphoma-derived cell line of feline origin (35) and was maintained in 50% Leibovitz L-15 medium–50% RPMI 1640 with 15% FBS. MK2 rhesus monkey kidney cells (ATCC CCL-7.1) were cultured in MEM with 10% FBS. The human K-562 human malignant hematopoietic progenitor cell line (ATCC CCL-243) was maintained in RPMI 1640 with 10% FBS. The 293 human embryonic kidney cell line (ATCC CRL-1573) and its derivative, 293T, were cultured in DMEM with 10% FBS. The 104C1 guinea pig fibroblast cell line (ATCC CRL-1405) was maintained in DMEM with 15% FBS. Diseased tissues from naturally FeLV-infected cats were collected from a single veterinary practice in Pasadena, Calif., over a period of 6 years (a gift from Murray Gardner).

**Construction of infectious recombinant FeLVs.** Recombinant FeLVs were constructed in the backbone of FeLV-A/61E to contain the env gene and/or the LTR of FeLV-945 (2, 23). For construction of the recombinant 61E/945SL provirus, the env gene and LTR of FeLV-945 were substituted for the homologous sequences in FeLV-A/61E between the conserved XhoI site in the proviral genome (163 bp upstream of the env start codon) and a downstream restriction site in the cloning vector. Thus, 61E/945SL contains the 5’ LTR, gag, and a portion of the pol gene of FeLV-A/61E but contains the env gene and 3’ LTR of FeLV-945. The recombinant 61E/945SL contains the LTR of FeLV-945 in the backbone of FeLV-A/61E, beginning at the conserved EcoRV site in the FeLV LTR. The construction of 61E/945 (previously termed 61g/945SL) was described elsewhere (3). The proviral DNA of 61E/945SL or 61E/945SL was introduced into cultured feline cells and was found to establish a productive infection as determined by an enzyme-linked immunosorbent assay (Synbiotics Corp., San Diego, Calif.) for detection of the FeLV p27 Gag antigen in culture supernatants. For verification of the structures of the recombinant FeLVs and confirmation of the stability of the transfected proviruses, reverse transcription-PCR was used to amplify the env gene and LTR from total cellular RNAs of infected cells. Sequence analyses of amplification products confirmed the presence of the predicted viral species.

**Virus replication assays.** FEA cells (2 × 10^5 per well) or FC6.BM cells (8 × 10^4 per well) were deposited in quadruplicate wells in six-well dishes. The next day, plasmid DNA encoding the recombinant FeLV provirus (250 ng) was introduced by lipid-mediated transfection (Lipofectamine Plus reagent; Invitrogen Corp., Carlsbad, Calif.). For 3201 cells, 4 × 10^6 cells were electroporated in triplicate with plasmid DNAs encoding each recombinant FeLV. As a confirmatory approach, 4.5 × 10^5 3201 cells per well were deposited in quadruplicate wells in 24-well dishes in the presence of an infectious virus stock of each recombinant FeLV. Inocula were normalized for equivalent amounts of reverse transcriptase activity. At regular intervals after transfection, electroporation, or infection, culture supernatants were collected for measurements of reverse transcriptase activity as previously described (29). A series of twofold dilutions of each virus suspension was assessed to establish the linearity of the reaction. Data from replicate assays were analyzed statistically by a one-way analysis of variance and the Bonferroni post-test. P values of <0.05 were considered statistically significant.

**Production of retroviral vectors**. Plasmids used for the production of retroviral vectors for use in single-cycle infection assays were obtained from Julie Overbaugh and were described previously (28). 293T cells were seeded at a density of 2 × 10^5 cells per 75-cm² flask 2 days before transfection. The following three plasmids were cotransfected into 293T cells by the calcium phosphate method (mamalian transfection kit; Stratagene, La Jolla, Calif.): (i) 10 μg of pCML-LTR-Δ5 gap-pol, a packaging-defective genome encoding FeLV Gag and Pol proteins; (ii) 10 μg of pTRT4.3/ΔNLgal, a murine leukemia virus-based vector genome encoding β-galactosidase; and (iii) 10 μg of a pDNA3.1/2Eco expression plasmid, into which the env gene of each recombinant FeLV was cloned (922, -1046, -1049, or -1306 was introduced under the control of a cytomegalovirus promoter, or 10 μg of the FBSSal expression vector, into which the envelope coding sequence of FeLV-C/Bar (a gift from Chetan Tailor) was introduced. Cells were washed twice the following day with Hanks’ balanced salt solution, and the medium was replaced with DMEM supplemented with 10% FBS. Viruses containing supernatants were harvested 48 h after transfection, rendered cell-free by passage through a 0.22-μm-pore-size filter, titrated on FEA cells by enumeration by β-galactosidase-expressing cells, and stored at −80°C. For confirmation of the presence of the desired envelope proteins on pseudotyped viruses, particles were pelleted from cell-free virus-containing supernatants by ultracentrifugation at 28,500 × g in a Beckman SW60Ti rotor for 90 min at 4°C. The pellets were resuspended in cold lysis buffer (150 mM NaCl, 1.0% Nonidet P-40, 20 mM Tris [pH 7.0], 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 10 μg of phenylmethylsulfonyl fluoride/ml, 20 μg of aprotinin/ml, 20 μl of sodium orthovanadate/ml) and incubated on ice for 30 min. Total protein concentrations in viral pellets were determined by the Bradford assay (Bio-Rad Laboratories, Hercules, Calif.), and 15 μg of protein was analyzed by Western blotting. Specifically, samples were diluted 1:5 in 1× SDS sample buffer (70% [vol/vol] 0.5 M Tris-HCl, 30% [vol/vol] glycerol, 10% [vol/vol] SDS, 9.3% [wt/vol] β-mercaptoethanol, 12% [wt/vol] bromphenol blue), boiled for 10 min, chilled on ice, subjected to SDS-polyacrylamide gel electrophoresis, and then transferred to nitrocellulose membranes. SU proteins were detected with goat anti-FeLV gp70 (1:100; Custom Monoclonal Antibodies International, Sacramento, Calif.). Subsequent incubation with a bovine anti-goat antibody conjugated to horseradish peroxidase (1:5,000; Southern Biotechnology, Inc.) was performed, and immunoblots were developed by the detection of horseradish peroxidase with ECL Western blotting detection reagents (Amersham Biosciences Corp., Piscataway, N.J.).

**Host range and superinfection interference assays.** For assays of host range, adherent cell lines representative of different animal species were seeded in triplicate in six-well culture dishes at 10^5 cells/well. The following day, cells were challenged at a multiplicity of infection (MOI) of 1.0 with retroviral vectors pseudotyped with various FeLV envelope proteins. Challenges were performed in the presence of hexamethidine bromide (Sigma-Aldrich, St. Louis, Mo.) at 4 μg/ml. After 48 h, the cells were stained histochemically for β-galactosidase activity (in situ β-galactosidase staining kit; Stratagene) as evidence of virus entry. As a confirmatory approach, the adherent cell lines FEA, D-17, MK2, and 104C1 were seeded in triplicate in six-well plates at a density of 2 × 10^5 cells/well (5 × 10^5 for D-17 cells) and were transfected (Lipofectamine Plus reagent; Invitrogen Corp.) with plasmid DNA (1 μg) encoding infectious FeLV-A/61E or 61E/945 provirus or with an empty vector. For K-562 cells, 10^5 cells were electropo-
were generated by transfecting cells with a plasmid encoding the infectious provirus of FeLV-A/61E, 61E/945SL, or FeLV-B/Gardner-Arnstein and allowing the virus infection to spread throughout the culture for several weeks. Uninfected or chronically infected FEA cells were then seeded in triplicate in six-well culture dishes at 10^7 cells/well. On the following day, cells were challenged at an MOI of 0.1 with retroviral vectors pseudotyped with various FeLV envelope proteins. Challenges were conducted in the presence of hexadimethrine bromide (Sigma-Aldrich) at 4 μg/ml. After 48 h, the cells were stained histochemically for β-galactosidase activity (in situ β-galactosidase staining kit; Stratagen) as evidence of virus entry. Some assays were conducted in the presence of a culture supernatant conditioned by the growth of feline 3201 T cells as a source of FeLIX (1). Conditioned medium was added at a 1:1 dilution at the time of pseudotype challenge.

**PCR amplification of FeLV proviral DNA from diseased tissues.** Genomic DNAs were isolated from multicentric lymphomas of cats 922, 1046, and 1049 and from bone marrow for a case of myeloproliferative disorder in cat 1306 (8). FeLV proviral sequences were amplified from genomic DNAs by PCRs using the oligonucleotide primers H18 (5′ AAG GTA GAC GGA GGT GCC GC 3′) and H20 (5′ GAA GGT CGA ACT CTG GTC AAC C 3′), which recognize sequences in pol upstream of the env start codon and sequences in the U5 region of the 3′ LTR that are conserved among exogenous FeLVs (9), respectively. In some cases, primer H18 or H20 was used in combination with an internal env primer designed to recognize regions in which there is little variability among exogenous FeLV isolates. The primers used were 945envv (5′ AAG GTA GAC GGA GGT GCC GC 3′), 945envw (5′ ATG GGT TTT TTG CGG CAT G 3′), 945env1 (5′ TTT GGT CCC ATT GCC TOA G 3′), and TMenv (5′ CCC TAC AGT GAG TCT GCC TCA A C 3′). To ensure a high fidelity for amplification, we performed PCRs by using Phu DNA polymerase-based enzyme mixes (Expand High Fidelity PCR system, from Roche Applied Science, Indianapolis, Ind.; and Herculase Hotstart DNA polymerase, from Stratagen). Amplification was performed in a 50-μl reaction mixture containing 250 ng of genomic DNA, 250 ng of each primer, and a 0.2 mM concentration of each deoxyribonucleoside triphosphate in the Herculase or High Fidelity polymerase reaction buffer provided by the manufacturer. An initial denaturation step at 95°C for 5 min was followed by 35 cycles of denaturation at 94°C for 30 s, 50°C for 30 s, and 72°C for 2 min 30 s, with a final extension for 15 min, in a thermocycler (Robocycler 40; Stratagen). Amplification products were cloned into the TA vector pCR2.1 (Invitrogen Corp.) or pGEM-T Easy (Promega Corp., Madison, Wis.) and were submitted for automated nucleotide sequence analysis. Each genomic DNA sample was subjected to multiple independent amplifications (two to four per sample), and multiple amplification products were submitted for automated sequence analysis (three to five per sample). The complete sequence of the FeLV-945 envelope protein was determined by automated sequence analysis of the molecularly cloned provirus used for the original description of the isolate (23).

**Infection and analysis of pathogenesis in vivo.** Specific-pathogen-free pregnant dams were obtained from Liberty Research (Waverly, N.Y.). Within the first 24 h postpartum, neonatal kittens were inoculated intraperitoneally with 5 × 10^5 50% tissue culture infective doses of 61E/945SL (n = 4) or 61E/945LSL (n = 5) in a total volume of 0.5 ml. Kittens remained with the natural queen until weaning at 8 weeks of age, at which time they were separated by sex and inoculated into two animals per cage. At 4 months of age, the animals were housed individually. Blood was collected biweekly and assayed for the presence of the FeLV p27 Gag antigen by an enzyme-linked immunosorbent assay (Symbiotics Corp.). Animals were observed by daily monitoring and biweekly physical examinations for evidence of disease, including progressive weight loss, anorexia, diarrhea, dehydration, pallor, inactivity, or debilitation. Animals evincing symptoms were euthanized by an intravenous barbiturate overdose (Beuthanasia-D; Schering Plough Animal Health, Union, N.J.) and were subjected to a complete necropsy and histopathological examination. High-molecular-weight DNAs were isolated from tumors, and Southern blot analysis was performed as previously described (2). The T-cell receptor β (TCR-β) locus was examined by the use of β-6T5, a 600-bp EcoRI fragment from the mouse TCR-β cDNA (16). Proviral insertion patterns were analyzed by use of a hybridization probe specific for the LTR of exogenous FeLV and containing LTR sequences between EcoRV and HindII restriction sites.

**Nucleotide sequence accession numbers.** The nucleotide sequences obtained in this study were submitted to GenBank (accession numbers AY662447 and AY662449 to AY662461).

**RESULTS**

In previous studies, the FeLV-945 SU gene was shown by sequence analysis to be of exogenous origin and closely related to those of horizontally transmissible FeLVs of subgroup A (FeLV-A). It was striking, however, that FeLV-945 SU differed in sequence from an FeLV-A prototype to a larger extent than the highly conserved FeLV-A isolates differ among themselves (2). In the present study, a sequence analysis of the remainder of FeLV-945 Env revealed the sequence differences to be restricted largely to the VRA, VRB, and PRR domains of SU. Specifically, the FeLV-945 SU protein was shown to be 89, 73, and 85% identical in its predicted amino acid sequence to prototype FeLV-A/61E SU across VRA, VRB, and PRR, respectively (Fig. 1). In contrast, the segments of FeLV-945 SU between variable regions were 94% identical to those of FeLV-A/61E, and the 197 residues of the predicted transmembrane product were 97% identical to those of FeLV-A/61E (Fig. 1). For comparison, the known natural isolates of FeLV subgroup A have nearly identical (>97%) amino acid sequences across the entire SU protein, although they were isolated from different continents over a period of many years (11, 24, 27). Thus, FeLV-945 Env, while closely related to FeLV-A Env, was shown to contain sequence differences localized to regions in SU that were identified as important for determining receptor interactions and viral entry. Considering the potential impact of these mutational changes, it was important first to determine whether FeLV-945 actually functions as a member of FeLV subgroup A. The functional definition of FeLV subgroup A, i.e., entry through the ecotropic FeLV-A receptor, can be established experimentally by measuring host range and superinfection interference as measures of receptor utilization. To perform these studies, we generated retroviral vector particles in which pRT43.2Tnβgal, a murine leukemia virus-based β-galactosidase-encoding genome, was pseudotyped with the envelope protein of FeLV-A/61E or FeLV-945. Recombinant infectious FeLV proviruses were also constructed in which the env gene and LTR of FeLV-945 were substituted for the homologous sequences in FeLV-A/61E or in which only the FeLV-945 LTR was substituted. These recombinant FeLVs were designated 61E/945SL and 61E/945LSL, respectively (Fig. 2).

To assess the host range determined by FeLV-945 SU, we
subtracted from the results; thus, the final result may be 10 to 14 days thereafter, and reverse transcriptase (RT) activity was quantified as previously described (29). The background value for mock-transfected cells was for homologous sequences of FeLV-A/61E.

A/61E. Particle entry was quantified by enumerating this purpose, feline fibroblasts (FEA cells) were chronically infected with FeLV-A/61E or 61E/945SL, and then quantified entry by enumerating

FeLV-945 pseudotypes, were observed to enter cells of feline origin but not those from any other species (Table 1). The indicated cell lines were transfected with plasmid DNA encoding the infectious provirus of FeLV-A/61E or FeLV-945. Pseudotype entry was quantified by enumerating β-galactosidase-expressing cells. The data shown are averages of three replicates (± standard errors of the means).

The indicated cell lines were transfected with plasmid DNA encoding the infectious provirus of FeLV-A/61E or FeLV-945. Culture supernatants were harvested 10 to 14 days thereafter, and reverse transcriptase (RT) activity was quantified as previously described (29). The background value for mock-transfected cells was subtracted from the results; thus, the final result may be <0. The data shown are averages of three replicates (± standard errors of the means).

a ND, not determined.

Plasmid DNAs encoding infectious viruses FeLV-A/61E, 61E/945SL, and 61E/945L were introduced by transfection (or electroporation) into three lines of feline cells, i.e., FEA cells (embryonic fibroblasts), FC6.BM cells (adherent bone marrow-derived cells), and 3201 cells (T-lymphoid cells). Virus replication was then quantified by measuring reverse transcriptase activities in culture supernatants at regular intervals thereafter (Fig. 3). The 61E/945SL recombinant virus was observed to replicate significantly more efficiently than FeLV-A/61E in all cell lines examined. These results recapitulate the previous finding (8, 29) that substitution of the triplication-containing FeLV-945 LTR for the LTR of FeLV-A/61E confers a replicative advantage in many cell types. The additional substitution of FeLV-945 Env conferred a small but statistically significant replicative advantage in FEA cells which was evident on days 4, 8, and 12; however, FeLV-945 Env did not confer an additional advantage over that conferred by the FeLV-945 LTR in the other feline cell lines examined (Fig. 3).

FeLV-945 is a representative isolate of the natural variant found to predominate in a geographic cohort of animals with non-T-cell malignant, proliferative, and degenerative disorders. Proviruses isolated directly from diseased tissues were previously reported to contain the distinctive FeLV-945 LTR, characterized by a single transcriptional enhancer followed downstream by a tandemly repeated 21-bp element with two, three, or four copies (2, 8). To determine whether the mutational changes observed with FeLV-945 SU were also characteristic of the isolate in the cohort from which it was identified. Plasmid DNAs encoding infectious viruses FeLV-A/61E, 61E/945SL, and 61E/945L were introduced by transfection into three lines of feline cells, i.e., FEA cells (embryonic fibroblasts), FC6.BM cells (adherent bone marrow-derived cells), and 3201 cells (T-lymphoid cells). Virus replication was then quantified by measuring reverse transcriptase activities in culture supernatants at regular intervals thereafter (Fig. 3). The 61E/945SL recombinant virus was observed to replicate significantly more efficiently than FeLV-A/61E in all cell lines examined. These results recapitulate the previous finding (8, 29) that substitution of the triplication-containing FeLV-945 LTR for the LTR of FeLV-A/61E confers a replicative advantage in many cell types. The additional substitution of FeLV-945 Env conferred a small but statistically significant replicative advantage in FEA cells which was evident on days 4, 8, and 12; however, FeLV-945 Env did not confer an additional advantage over that conferred by the FeLV-945 LTR in the other feline cell lines examined (Fig. 3).

FeLV-945 is a representative isolate of the natural variant found to predominate in a geographic cohort of animals with non-T-cell malignant, proliferative, and degenerative disorders. Proviruses isolated directly from diseased tissues were previously reported to contain the distinctive FeLV-945 LTR, characterized by a single transcriptional enhancer followed downstream by a tandemly repeated 21-bp element with two, three, or four copies (2, 8). To determine whether the mutational changes observed with FeLV-945 SU were also charac-

### Table 1. Host range of pseudotype particles displaying the envelope protein of FeLV-A/61E or FeLV-945

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Species</th>
<th>61E Env (FFU/ml)</th>
<th>945 Env (FFU/ml)</th>
<th>61E Env (cpm/ml)</th>
<th>945 Env (cpm/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEA</td>
<td>Cat</td>
<td>73,000 ± 5,508</td>
<td>86,670 ± 1,667</td>
<td>330,615 ± 51,146</td>
<td>373,808 ± 17,430</td>
</tr>
<tr>
<td>D17</td>
<td>Dog</td>
<td>9 ± 0.7</td>
<td>27,108 ± 2,486</td>
<td>&lt;0</td>
<td>46,604 ± 153</td>
</tr>
<tr>
<td>K-562</td>
<td>Human</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>293</td>
<td>Human</td>
<td>61 ± 7.1</td>
<td>75 ± 7.2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MK2</td>
<td>Monkey</td>
<td>0</td>
<td>0</td>
<td>357 ± 188</td>
<td>113 ± 77</td>
</tr>
<tr>
<td>104Cl</td>
<td>Guinea pig</td>
<td>0</td>
<td>0</td>
<td>774 ± 288</td>
<td>5,683 ± 1,693</td>
</tr>
</tbody>
</table>

a The indicated cell lines were transfected by substituting the envelope gene and/or LTR of FeLV-945 for homologous sequences of FeLV-A/61E.

### Table 2. Superinfection interference by retroviral vectors pseudotyped with the envelope protein of FeLV-A/61E or FeLV-945

<table>
<thead>
<tr>
<th>Infected target cells</th>
<th>Pseudotype entry (FFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>61E Env</td>
</tr>
<tr>
<td>FEA (uninfected)</td>
<td>7,333 ± 555</td>
</tr>
<tr>
<td>FEA (FeLV-A/61E)</td>
<td>5 ± 1.2</td>
</tr>
<tr>
<td>FEA (61E/945SL)</td>
<td>6 ± 0.9</td>
</tr>
</tbody>
</table>

a The indicated target cells were transfected at an MOI of 0.1 with a retroviral vector encoding the pRT43.2TnSgflg genome (38) and pseudotyped with the envelope of FeLV-A/61E or FeLV-945. Pseudotype entry was quantified by enumerating β-galactosidase-expressing cells. The data shown are averages of three replicates (± standard errors of the means).

b Chronically infected FEA cells were generated by transfection of plasmid DNA encoding the infectious provirus of FeLV-A/61E or FeLV-945.

FIG. 2. Diagrammatic representation of recombinant FeLVs constructed by substituting the envelope gene and/or LTR of FeLV-945 for homologous sequences of FeLV-A/61E.
teristic of the cohort, we obtained proviral DNAs by PCR amplification from three additional cases of multicentric lymphoma (animals 922, 1046, and 1049) and from a case of myeloproliferative disorder (animal 1306). Using oligonucleotide primers representing conserved regions within and flanking the exogenous FeLV env gene, we subjected genomic DNAs from each diseased tissue to multiple independent amplifications. The predominant amplification products were then cloned and sequenced. The VRA, VRB, and PRR regions of SU from each amplification product were compared with those of FeLV-945 and of previously described natural isolates of FeLV-A (Fig. 4A). The results showed that the predominant SU sequences amplified from other animals in the cohort largely shared the distinctive amino acid sequence features of FeLV-945. The sequences were not identical, however, and scattered point mutations were observed within VRA, VRB, and PRR. Examinations of SU sequences outside these regions also revealed scattered amino acid sequence differences, with some of them having potential functional impacts. For example, proviruses isolated from the multicentric lymphoma of cat 1046 contained a histidine-to-proline change at residue 6 in the N terminus of SU (Fig. 4B). The H6P mutation occurred within an SPHQ motif that is highly conserved among gamma-retroviruses. The SPHQ motif, particularly the histidine residue, has been implicated by others as an important determinant for mediating fusion events during virus entry (4, 13, 22, 43). In another case, proviruses isolated from cat 1306, who had a myeloproliferative disease, contained a distinctive cluster of lysine residues in the C-terminal portion of SU. This sequence motif has been previously described for natural FeLV isolates of subgroups B and C and thus may have been derived by recombination with endogenous FeLV-related elements (Fig. 4C).

FIG. 3. Replication kinetics of recombinant viruses containing the LTR of FeLV-945 (61E/945L) or the LTR and the env gene of FeLV-945 (61E/945SL). These recombinants were compared in order to evaluate the impact of FeLV-945 env on virus replication. The replication of recombinant viruses was compared to that of the parental virus, FeLV-A/61E (61E), as a control. Plasmid DNAs containing the indicated FeLV proviruses were introduced into feline FEA cells (A), FC6.BM cells (B), or 3201 cells (C), and culture supernatants were collected at regular intervals thereafter for quantitation of reverse transcriptase activities. The data shown represent the means of three (C) or four (A and B) experiments, expressed as counts per minute of incorporated [3H]TTP per milliliter. Data from replicate assays were analyzed statistically by a one-way analysis of variance and the Bonferroni posttest. In FEA cells, the replication rate of 61E/945SL was significantly higher than that of 61E/945L at days 4, 8, and 12 post-transfection (P < 0.001). The replication kinetics of 61E/945L and 61E/945SL were statistically indistinguishable in other cell lines.

FIG. 4. (A) Comparison of predicted amino acid sequences of VRA, VRB, and PRR domains of SU proteins from previously reported isolates of FeLV-A (11, 37) and from proviruses amplified by PCR from diseased tissues of naturally infected animals. (B) Comparison of predicted amino acid sequence of FeLV-1046 SU to those of previously reported isolates of FeLV-A (11, 37) and FeLV-945. (C) Comparison of predicted amino acid sequence of FeLV-1306 SU to those of previously reported isolates of FeLV-A, -B, and -C (11, 25, 32). Numbers above the sequences correspond to the amino acid positions of the mature FeLV-A/61E Env protein (11). Asterisks indicate amino acid identity.
cohort resembled FeLV-945 but were not identical, their host range and superinfection interference properties were examined. For this purpose, retroviral vector particles were prepared in which the pRT43.2TnIsβgal genome was pseudotyped with the envelope protein from FeLV-A/61E, -945, -922, -1046, -1049, -1306, or FeLV-C/Sarma. Western blot analyses of pseudotype viruses collected from producer cell supernatants showed that all of the SU proteins were efficiently incorporated into virus particles (Fig. 5). For evaluations of the host range, cell lines of feline, canine, human, and guinea pig origin were challenged at an MOI of 1.0 with each pseudotype, and entry was quantified by enumerating β-galactosidase-expressing cells (Table 3). Like FeLV-A/61E, particles pseudotyped with envelope proteins from FeLV-945, -922, and -1049 exhibited an ecotropic host range consistent with assignment to FeLV subgroup A. Similarly, FeLV-1306 pseudotypes exhibited an ecotropic host range, indicating that the distinctive cluster of lysine residues observed near the C terminus of SU (Fig. 4C) did not alter receptor utilization. Particles pseudotyped with the FeLV-1046 envelope protein were unable to enter cells of any origin, including feline cells. This finding is consistent with the previously reported entry defect conferred by mutation of the histidine residue within the highly conserved SPHQ motif at the N terminus of SU (4, 13, 22, 43). Others have shown that wild-type SU proteins or RBD polypeptides supplied in trans are sufficient to compensate for the entry defect of this mutation, provided that the cell surface receptor for the soluble factor is also present (13, 22, 42, 43). For example, isolates of FeLV-T exhibit an H6 mutation and require a soluble cofactor termed FeLIX for entry. FeLIX is encoded by a defective endogenous FeLV-related provirus and contains the RBD of FeLV subgroup B. FeLIX is abundantly secreted by the T-cell targets of FeLV-T infection (1, 15). In the present study, a source of FeLIX was added to the culture medium to determine the effect on entry by 1046 pseudotypes. The results showed that the addition of FeLIX fully restored the infectivity of 1046 pseudotypes in feline cells. It was noteworthy, however, that 1046 pseudotypes retained their ecotropic host range, even in the presence of FeLIX (Table 3).

The superinfection interference properties of the SU proteins were measured by the use of feline cells infected with 61E/945SL. Specifically, uninfected feline fibroblasts or fibroblasts infected with 61E/945SL were challenged with each pseudotype virus (Fig. 6). As observed previously (Table 2), pseudotypes bearing the envelope of FeLV-945 were able to enter uninfected cells but were blocked from entry into cells expressing a homologous envelope. Particles pseudotyped with the envelopes of FeLV-922, -1049, and -1306 were similarly blocked from entry into cells expressing the envelope of FeLV-945, thus confirming their assignment as members of FeLV subgroup A. In contrast, FeLV-C/Sarma was observed to enter uninfected and infected cells with equal efficiencies, which is a reflection of distinct receptor utilization (30, 40). As observed previously (Table 3), FeLV-1046 pseudotypes were able to enter uninfected cells efficiently only when FeLIX was supplied in trans. Notably, however, there was an absence of superinfection interference when infected cells were challenged with 1046 pseudotypes in the presence of FeLIX (Fig. 6). This finding suggests that FeLIX facilitates 1046 entry through a receptor distinct from the ecotropic FeLV-A receptor used by FeLV-945. In the case of FeLV-T, FeLIX facilitates entry through the widely expressed Pit1 phosphate transporter (1, 21). In the case of the FeLIX-mediated entry of 1046, the restricted ecotropic host range (Table 3) appeared inconsistent with the utilization of Pit1 because of its wide range of expres-

![FIG. 5. Western blot analysis of FeLV SU proteins on pseudotype virus particles harvested from supernatants of producer 293T cells. Virus particles were pelleted from culture supernatants by ultracentrifugation, fractionated by SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes. Envelope SU proteins were detected with goat anti-FeLV gp70 followed by incubation with a bovine anti-goat antibody conjugated to horseradish peroxidase. Molecular mass markers (in kilodaltons) are indicated.](http://jvi.asm.org/)

<table>
<thead>
<tr>
<th>Env variants</th>
<th>Pseudotype entry (FFU/ml)* into indicated cell lines</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FEA (cat)</td>
</tr>
<tr>
<td>61E</td>
<td>73,000 (+5,508)</td>
</tr>
<tr>
<td>945</td>
<td>95,000 (+5,500)</td>
</tr>
<tr>
<td>922</td>
<td>81,000 (+8,500)</td>
</tr>
<tr>
<td>1049</td>
<td>75,000 (+6,500)</td>
</tr>
<tr>
<td>1306</td>
<td>66,000 (+1,150)</td>
</tr>
<tr>
<td>1046</td>
<td>0</td>
</tr>
<tr>
<td>1046 plus/FeLIX</td>
<td>82,000 (+3,500)</td>
</tr>
<tr>
<td>FeLV-C/Sarma</td>
<td>68,000 (+4,500)</td>
</tr>
</tbody>
</table>

* The indicated cell lines were challenged at an MOI of 1.0 with particles containing the pRT43.2TnIsβgal genome (38) and pseudotyped with the indicated FeLV envelope proteins. Pseudotype entry was quantified by enumerating β-galactosidase-expressing cells. The data shown are averages of duplicate assays (± standard errors of the means).

* Source of FeLV envelope proteins expressed on pseudotype virions.

* Culture medium harvested from 3201 cells was added at a 1:1 dilution at the time of challenge as a source of FeLIX (1).
sion. Indeed, Pit1, which was previously identified as the FeLV-B receptor, confers the expanded host range characteristic of FeLV-B (6, 41). To evaluate whether FeLIX facilitates 1046 entry through the Pit1 receptor, we determined the ability of FeLV-B infection to interfere with FeLIX-mediated 1046 entry. Specifically, feline fibroblasts infected with FeLV-B/ Gardner-Arnstein were challenged with FeLV-A/61E pseudotypes as a control or with 1046 pseudotypes in the presence of FeLIX. The results showed that while FeLV-A/ 61E was able to enter FeLV-B-infected cells, FeLIX-mediated 1046 entry was inhibited by 2 orders of magnitude (Fig. 6). Thus, FeLV-B infection interferes significantly with 1046 entry, implicating the involvement of the Pit1 receptor.

A natural or experimental infection with FeLV subgroup A is typically associated with the induction of a thymic lymphoma of T-cell origin after a prolonged latency of 1 to 3 years (9, 10, 31, 33). It was striking that FeLV-945, while a member of FeLV-A, differed from an FeLV-A prototype to a greater extent than the members of FeLV-A differ among themselves. For this reason, it was remarkable to observe that the sequence of FeLV-945 SU, while closely related to FeLV-A, differed from an FeLV-A prototype to a greater extent than the members of FeLV-A differ among themselves. Indeed, Pit1, which was previously identified as the FeLV-B receptor, confers the expanded host range characteristic of FeLV-B (6, 41). To evaluate whether FeLIX facilitates 1046 entry through the Pit1 receptor, we determined the ability of FeLV-B infection to interfere with FeLIX-mediated 1046 entry. Specifically, feline fibroblasts infected with FeLV-B/ Gardner-Arnstein were challenged with FeLV-A/61E pseudotypes as a control or with 1046 pseudotypes in the presence of FeLIX. The results showed that while FeLV-A/61E was able to enter FeLV-B-infected cells, FeLIX-mediated 1046 entry was inhibited by 2 orders of magnitude (Fig. 6). Thus, FeLV-B infection interferes significantly with 1046 entry, implicating the involvement of the Pit1 receptor.

A natural or experimental infection with FeLV subgroup A is typically associated with the induction of a thymic lymphoma of T-cell origin after a prolonged latency of 1 to 3 years (9, 10, 31, 33). It was striking that FeLV-945, while a member of FeLV-A, differed from an FeLV-A prototype to a greater extent than the members of FeLV-A differ among themselves. For this reason, it was remarkable to observe that the sequence of FeLV-945 SU, while closely related to FeLV-A, differed from an FeLV-A prototype to a greater extent than the members of FeLV-A differ among themselves.

The natural isolates of the horizontally transmissible FeLV subgroup A share a remarkable degree of sequence identity, although they were isolated from distant geographic locations over a period of many years. For example, members of FeLV-A share >97% amino acid identity across the SU protein (11, 24, 27). For this reason, it was remarkable to observe that the sequence of FeLV-945 SU, while closely related to FeLV-A, differed from an FeLV-A prototype to a greater extent than the members of FeLV-A differ among themselves. The sequence differences included point mutations restricted largely to VRA, VRB, and PRR, which are domains of SU that
were previously shown to play essential roles in receptor recognition and viral entry (Fig. 1) (5, 6, 38, 39). Despite the sequence differences in these critical domains, measurements of receptor utilization, including host range and superinfection interference, confirmed the assignment of FeLV-945 to subgroup A (Tables 1 and 2). Other proviruses isolated from the cohort contained similar sequence hallmarks in SU (Fig. 4A) and were similarly assigned through measures of receptor utilization to FeLV subgroup A (Table 3; Fig. 6). Proviruses from cat 1306 contained a cluster of lysine residues near the C-terminal portion of SU which is distinctive of FeLV subgroups B and C (Fig. 4C), although the presence of this motif did not alter receptor utilization by FeLV-1306 SU (Table 3; Fig. 6).

Proviruses from cat 1046 contained a histidine-to-proline change at SU residue 6 (Fig. 4B) within an SPHQ motif that was previously identified as a critical mediator of fusion events during virus entry (4, 13, 22, 43). Consistent with the previously described entry defect conferred by the H6 mutation, 1046 pseudotypes were able to enter cells only in the presence of a soluble RBD-containing polypeptide supplied in trans (Table 3). In this case, the soluble RBD function was provided by FeLIX, the product of a defective endogenous FeLV-related provirus that is abundantly secreted by feline lymphoid cells (1, 15). FeLIX contains the RBD of FeLV subgroup B and thereby facilitates entry through the widely expressed phosphate transporter Pit1. The broad expression of Pit1 accounts for the extended host range of FeLV-B (1, 18, 26, 41). Superinfection interference assays using FeLV-B-infected cells implicated the Pit1 receptor in FeLIX-mediated 1046 entry (Fig. 6). Surprisingly, however, 1046 pseudotypes retained the ecotropic host range, even in the presence of FeLIX (Table 3); thus, FeLIX was not able to facilitate 1046 entry through the Pit1 receptor expressed on the cells of other species. These observations suggest that mutational changes in 1046 may affect the interaction with FeLIX and/or the receptor. Alternatively, 1046 entry may require surface interactions in addition to those mediated by FeLIX, perhaps involving surface molecules present only on feline cells. With the exception of 1046, viruses bearing all other SU proteins isolated from the cohort were sensitive to superinfection interference established by a virus expressing the FeLV-945 envelope protein, consistent with their common assignment to FeLV subgroup A (Fig. 6).

Despite its ecotropic host range and common assignment to FeLV-A, the mutational changes in FeLV-945 SU were shown to confer distinctive pathogenic properties compared to FeLV-A/61E. Specifically, when the FeLV-945 LTR was substituted for that of FeLV-A/61E, the resulting 61E/945L recombinant virus induced thymic lymphomas of T-cell origin containing multiple clonally integrated proviruses, which is typical of FeLV-A infections (Fig. 7A) (9, 33). In contrast, when the unique FeLV-945 env gene and LTR were substituted for those of FeLV-A/61E, the resulting 61E/945SL recombinant virus did not induce T-cell lymphomas of the thymus but rapidly induced multicentric tumors that excluded the thymus. The tumors did not contain T cells (Fig. 7B) and in this respect resembled the multicentric lymphomas from which FeLV-945 was originally identified (2, 23). Although the 61E/945SL recombinant virus contained the entire env gene of FeLV-945,
the env genes of FeLV-945 and FeLV-A/61E are nearly identical outside SU (Fig. 1); thus, the change in disease spectrum can most likely be attributed to FeLV-945 SU. In the present study, FeLV-945 SU was expressed in the 61E/945SL recombinant in the context of the FeLV-945 LTR. A direct analysis of the influence of FeLV-945 SU alone would require the use of a recombinant in which only that gene was substituted into FeLV-A/61E. The mechanism by which FeLV-945 SU might influence the induction of multicentric lymphomas is not yet known. The 61E/945SL recombinant was observed to have a small but statistically significant replicative advantage in FEA cells (Fig. 3) and thus may have a similar advantage in some infected targets in vivo. Other possibilities that are as yet untested are also being considered. One possibility is that receptor interactions, e.g., binding affinity, may be altered by the mutational changes in FeLV-945 SU. An increased binding affinity, for example, might permit the efficient infection of a population of target cells with a low receptor density. Another possibility is that the nucleotide sequence differences rather than the amino acid changes in the FeLV-945 SU gene are important, perhaps by affecting the generation of envelope recombinant viruses during infection. Overall, while a detailed phenotypic characterization of the multicentric tumors induced by 61E/945SL is ongoing, the present report indicates that the relatively subtle mutational changes in FeLV-945 SU are responsible for a dramatic shift in disease spectrum in vivo in the natural host.

ACKNOWLEDGMENTS

This work was supported by PHS grant CA83823 from the National Cancer Institute and by Development Funds of the Tulane Cancer Center. C.C. was supported in part by a grant from the Cancer Association of Greater New Orleans.

REFERENCES

15. Sugai, J., M. Elder, M. M. Anderson, N. Van Hoeven, C. D. Meiering, and


