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Novel Feline Leukemia Virus Interference Group Based on the *env* Gene

Ariko Miyake,[a] Shinya Watanabe,[a] Takahiro Hiratsuka,[a] Jumpei Ito,[a] Minh Ha Ngo,[a] Isaac Makundi,[a] Junna Kawasaki,[a] Yasuyuki Endo,[b] Hajime Tsujimoto,[c] Kazuo Nishigaki[a]

Laboratory of Molecular Immunology and Infectious Disease, Joint Faculty of Veterinary Medicine, Yamaguchi University, Yamaguchi, Japan[a]; Laboratory of Small Animal Internal Medicine, Joint Faculty of Veterinary Medicine, Kagoshima University, Kagoshima, Japan[b]; Department of Veterinary Internal Medicine, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan[c]

Feline leukemia virus (FeLV) subgroups have emerged in infected cats via the mutation or recombination of the *env* gene of subgroup A FeLV (FeLV-A), the primary virus. We report the isolation and characterization of a novel *env* gene, TG35-2, and report that the TG35-2 pseudotype can be categorized as a novel FeLV subgroup. The TG35-2 envelope protein displays strong sequence identity to FeLV-A Env, suggesting that selection pressure in cats causes novel FeLV subgroups to emerge.

Feline leukemia viruses (FeLVs) are pathogenic retroviruses of domestic cats (1, 2), which are classified into subgroups A (the parent virus), B, C, D, and T based on their interference and *in vitro* host range properties (3, 4, 5, 6, 7, 8). Subgroups B and D arose from the recombination of FeLV-A *env* and the *env* genes of endogenous FeLV or endogenous retroviruses in the genomes of domestic cats (ERV-DCs) (7, 9, 10). Subgroups C and T possibly arose from mutations in FeLV-A *env* (11, 12). The recombination or mutation of *env* often alters the interference and host ranges of FeLVs by affecting their receptor usage (5, 6, 13, 14, 15, 16).

FeLV *env* genes were isolated by PCR from the blood DNA of a 1-year-old castrated male cat, TG35, with a bite injury, stomatitis, loss of appetite, and FeLV infection, although he had been vaccinated with inactivated FeLV (genotype III) (16). Five clones (TG35-1 to -5) were isolated, and we focused on TG35-2, TG35-4, and TG35-5. The *env* sequences of these clones showed strong similarity (Fig. 1), and the viruses clustered phylogenetically with those of genotype I/clade I FeLV, found mainly in Japan (16). The encompassing variable region A (VRA) of TG35-2 Env differs at eight amino acids from those of the TG35-4 and TG35-5 Env proteins. The proline-rich regions of TG35-2 and TG35-4, but not TG35-5, contain an inserted sequence of 25 amino acids (Fig. 1) not found in the cat genome database and of unknown origin.

To identify the FeLV subgroup to which this viral strain belongs, we used an interference assay (16) and generated β-galactosidase (LacZ)-encoding pseudotype viruses expressing TG35-2, TG35-4, or TG35-5 envelope (Env) proteins in GPLac cells (7). Pseudotype viruses TG35-2, -4, and -5 infected uninfected HEK293T cells (Table 1). However, HEK293T cells preinfected with FeLV-A/clone 33 (293T/clone 33 cells) (17) or FeLV-A/Glasgow-1 (293T/Glasgow-1 cells) (9) were infected by pseudotype virus TG35-2, but not by TG35-4 or TG35-5. Neither cell type was infected by FeLV-A/clone 33 or FeLV-A/Glasgow-1. Therefore, only the TG35-4 and TG35-5 viruses interfered with FeLV-A. Neither the TG35-2, TG35-4, nor TG35-5 pseudotype interfered with other subgroups of FeLV, or with retroviruses such as ERV-DC10, a replication-competent feline ERV (7) (Table 1). Therefore, FeLV TG35-4 and TG35-5 belong to the FeLV-A subgroup. However, TG35-2 could not be categorized.

We next constructed a replication-competent virus (33TGE2) containing the TG35-2 *env* gene and the *LTR*, *gag*, and *pol* genes of FeLV-A clone 33 (GeneArt; Thermo Fisher Scientific, Waltham, MA). HEK293T cells were transfected with virus p33TGE2, and productive replication was confirmed by detecting the FeLV p27 antigen. 33TGE2-infected HEK293T cells (293T/33TGE2) were successfully infected with FeLV-A (clone 33), FeLV-A (Glasgow-1), FeLV-B (Gardner-Arnstein) (18), FeLV-C (Sarma) (19), FeLV-D (ON-T) (7), TG35-4, TG35-5, ERV-DC10, and amphotropic murine leukemia virus (MLV) 4070A (16), but not with the TG35-2 pseudotype. However, the pseudotype virus infected 293T/FeLV-A, 293T/FeLV-B, 293T/FeLV-C, 293T/FeLV-D, 293T/ERV-DC10, and 293T/4070A cells (Table 1). FeLV 33TGE2 did not interfere with xenotropic MLV (X-MLV) from 22RV.1 cells (20) or vice versa (the viral infectious titers were 8×10^4 and 2×10^5 infectious units [IU]/ml, respectively) in HEK293T cells, but neither virus infected HEK293T cells already infected with itself. Thus, FeLV 33TGE2 displayed the same interference behavior as the TG35-2 pseudotype.

When we examined AH927 feline cells, the TG35-2 pseudotype virus infected AH927 cells infected with FeLV-A, FeLV-B, or FeLV-C, but not 33TGE2-infected AH927 cells (Table 2), as seen with human HEK293T cells (Table 1). To determine whether TG35-2 interferes with FeLV FY981, which uses the THTR1, FLVCR1, and FLVCR2 receptors (21), we constructed a plasmid expressing a chimeric gene-synthesized FY981 *env* gene (FY33) encoding the surface glycoprotein SU of FeLV FY981 and the transmembrane (TM) protein of FeLV clone 33. Pseudotype virus FY981 infected 293T/33TGE2 and AH927/33TGE2 cells, but not FeLV-C-infected cells, so FY981 was newly categorized as FeLV-C (Tables 1 and 2). We then tested whether TG35-2 uses known

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Address correspondence to Kazuo Nishigaki, kaz@yamaguchi-u.ac.jp.

A.M. and S.W. contributed equally to this work.

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                   Signal peptide                    Start of SU and RBD                                  VRA
FeLV-A/clone33     MEGPTHPKPSKDKTLSWDLMILVGVLLRLDVGIASPSPHQIYNVTWVITNVQNNTRANATSMLGTLTDAYPTLYVDLCDLVGNTWEPIVLNPSSVKHG--ARYISSNYGCKTEDRKKGQQ 118
FeLV-A/Glasgow-1   ..S..............N.AF...I.FTI.I.M.N.................T..Q.................H........D.........TN....--...S..K.....T....Q.. 118
FeLV-C/Sarma       ..S...........FP.N.VF...I.FQI.M.M.N.....V...........T.S...........................D.....AP---DPRSW--...S..TH....T....Q.. 115
FeLV  FY981        ..S................VF...I.STI.I.M.N....................Q.................H...............P--RN.RW---.H.S..TH....T....Q.R 115
clone TG35-2       ..............F............G...........................Q.................H................D.RN.P.ETL.H.P........A..R.Q.R 120
clone TG35-4       ..............F............G...........................Q.................H................D..N....--...P...........R.Q.. 118
clone TG35-5       ..............F............G...........................Q.................H................D..N....--...P...........R.Q.. 118

                                                                          VRB
FeLV-A/clone33     SYPFYVCPGHTPSMRPKGTHCGGAQDGFCAAWGCETTGEAWWKPTSSWDYITVKRGTTN--NDCTGRCNPLVLQFTQKGRQASWDGPKMWGLRLYRTGHDPVALFTVSRQVSTITPPQAM 236
FeLV-A/Glasgow-1   T.........A..LG.........................................SSQ.D.S.E.K...............................Y..I.................. 237
FeLV-C/Sarma       T.........A...G....Y....................................SNQ.D.S.K.K..................R..........S.Y..I...S.....M........ 234
FeLV  FY981        T.........A..LG.........................................NSQ.DES.E.K..................R............Y..I.................. 234
clone TG35-2       ..........................................................TQD...E...........P..K.....................I....T............. 240
clone TG35-4       ..........................................................TQI...E...........P..K..................P..I....T............. 238
clone TG35-5       ..........................................................TQD...K...........P..K..........................T............. 238

                               PRR
FeLV-A/clone33     GPNLVLPDQRPPSLQSQIES-------------------------KVATQSPQRNTSSVSGTDTPTTISPKRAGTGDRLIGLIQGTYLALNATNPNKTKDCWLCLVSRPPYYEGVAILGN 331
FeLV-A/Glasgow-1   .........K...R...TG.-------------------------.....R..-TNE.APRSVA...MG...I.......N.V..........D....................I..... 331
FeLV-C/Sarma       .........K...R...TK.-------------------------..T..R..-I...TPRSVASA.MG...I.......N.V..........D....................I.V... 328
FeLV  FY981        .........K...R...TG.-------------------------.....R..-TNE.APRSIA...M....I.......N.V..........D....................I..... 328
clone TG35-2       .................M..KVAPGGEGKGSRGEDSDRASRRIES....R.........P....................S.................................I..... 360
clone TG35-4       .................M..KVAPGGEGKGSRGEDSDRASRRIES....R.........P....................R.................................I..... 358
clone TG35-5       ....................-------------------------....R.........P....................S.................................I..... 333

                              → C domain                                                                                   TM →
FeLV-A/clone33     YSNQTNPPPSCLSTPQHKLTISEVSGQGLCIGTVPKTHQVLCNRTQQGHTGAHYLAAPNGTYWACNTGLTPCISMAVLNWTSDFCVLIELWPRVTYHQPEYVYTHFDKTARSRREPISLT 451
FeLV-A/Glasgow-1   ............................M..........A...K..............................................................A.AV.F........ 451
FeLV-C/Sarma       .......................................A..KK..K..K.T..................................................I.....AV.F........ 448
FeLV  FY981        ...........Q.I.........................A...E..............................................................A.AV.F........ 448
clone TG35-2       ..............L........................A...................................T............................................ 480
clone TG35-4       ..............L........................A................................................................................ 478
clone TG35-5       ..............L........................A................................................................................ 453

FeLV-A/clone33     VALMLGGLTVGGIAAGVGTGTKALLETVQFRQLQMAMHTDIQALEESVSALEKSLTSLSEVVLQNRRGLDILFLQGGGLCAALKEECCFYADHTGLVRDSMAKLRERLKQRQQLFDSQQG 571
FeLV-A/Glasgow-1   ...........................A...................I...........................E.......................N.................... 571
FeLV-C/Sarma       ...........................A......I............I...........................E.......................N.................... 568
FeLV  FY981        ...........................A...................I...........................E.......................N.................... 568
clone TG35-2       ........A..................................................................E.......................................N.... 600
clone TG35-4       ...........................................................................E....V..................................N.... 598
clone TG35-5       ...........................................................................E.......................................N.... 573

FeLV-A/clone33     WFEGWFNRSPWFTTLISSIMGPLIILLLILLFGPCVLNRLVQFVKDRISVVQALILTQQYQQIKQYDPDQP 642
FeLV-A/Glasgow-1   .......K..............L...........I..................................R. 642
FeLV-C/Sarma       .......K..............L........L..I.............................Q...S.R. 639
FeLV  FY981        .......K..............L...........I..................................R. 639
clone TG35-2       ..................................I..................................R. 671
clone TG35-4       ..................................I.................................... 669
clone TG35-5       ..................................I.................................... 644
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FIG 1 Receptor-binding domains (RBDs), proline-rich regions (PRRs), C domains, and TM regions of the Env protein are shown for FeLV-A clone 33 (17), FeLV-A Glasgow-1 (9), FeLV-C Sarma (19), FeLV FY981 (21), FeLV TG35-2 (16), TG35-4 (accession number LC029807), and TG35-5 (accession number LC029808). The variable regions, VRA and VRB, are also shown. Dots indicate identical residues, and dashes indicate spaces that were introduced for the amino acid alignment. Boxes indicate the positions of the PCR primers (16). The Env sequences were aligned with the Genetyx program (Genetyx Corporation, Tokyo, Japan).

FeLV receptors, such as THTR1 (15), PIT1 (5), PIT2 (22), FLVCR1 (13, 14), or FLVCR2 (21), for cell entry. The TG35-2 virus infected neither MDTF cells nor MDTF cells expressing any of these receptors (Table 3).

We then determined the minimal changes in the Env protein required to produce the TG35-2 phenotype. When the VRA of TG35-2 was replaced with that of TG35-4, the pseudotyped viruses (chimeras 1 and 2) showed the phenotype of TG35-2, and conversely, when the VRA of TG35-4 was replaced with that of TG35-2, the pseudotyped virus (chimera 3) showed the phenotype of FeLV-A/TG35-4. Furthermore, pseudotyped viruses (mt2,3,4,5 and mt2,3,4) generated by the site-directed mutagenesis of the VRA showed that a substitution of 2 to 3 amino acids in addition to the insertion of threonine and leucine in the VRA of TG35-4 conferred the TG35-2 phenotype (Fig. 2A and B). Therefore, specific amino acids within the VRA are responsible for the TG35-2 and FeLV-A phenotypes.

The host range of TG35-2 was determined using infectious clone 33TGE2 (Fig. 2C). 33TGE2 infected a broad range of cell lines yielding high titers, whereas FeLV-A displayed restricted infection. Neither virus infected mouse, hamster, or guinea pig cells. Therefore, the host range properties of FeLV 33TGE2 and FeLV-A differ.

We have characterized a novel FeLV that does not interfere with known feline retroviruses, including FeLV-D and ERV-DC10. A pseudotype virus expressing TG35-2 Env infected FeLV-A-, FeLV-B-, and FeLV-C-infected cells, and all cells that express the receptors THTR1, PIT1/2, and FLVCR1 (data not shown). TG35-2 did not infect guinea pig cells (104C1), which are permissive for FeLV-C and FY981 (FeLV FY33) (Table 2) (4, 21, 23), suggesting that TG35-2 differs from FeLV-C and FY981. MLV 4070A was used as a positive control because it and some FeLV-B viruses use PIT2 for viral entry (24, 25, 26). The wide host range of TG35-2 is not attributable to its xenotropic nature (27). A pseudotype virus expressing TG35-2 Env infected FeLV-D- or ERV-DC10-infected cells, but the receptors of neither virus have been identified. FeLV-T requires FeLIX for infection (6), but TG35-2 infected cells without FeLIX. Artificial mutation of the FeLV-A

TABLE 1 Receptor interference assay using various pseudotyped FeLVs in HEK293T cells

	Titer of pseudotyped virus[a]:										
	FeLV-A						Clone				
Preinfecting virus	Clone 33	Glasgow-1	FeLV-B (GA)	FeLV-C (Sarma)	FeLV (FY981)	FeLV-D (ON-T)	TG35-2	TG35-4	TG35-5	ERV-DC10	Ampho-MLV
None	(4.1 ± 0.4) × 10^3	(4.9 ± 0.3) × 10^3	(2.1 ± 0.0) × 10^5	(7.1 ± 0.6) × 10^3	(1.3 ± 1.0) × 10^3	(4.1 ± 0.2) × 10^4	(1.1 ± 0.1) × 10^4	(1.1 ± 0.6) × 10^3	(2.3 ± 1.3) × 10^3	(1.9 ± 0.0) × 10^3	(8.2 ± 0.4) × 10^4
FeLV-A (clone 33)	0	0	(1.8 ± 0.3) × 10^5	(5.3 ± 0.3) × 10^3	(1.6 ± 1.0) × 10^3	(4.6 ± 0.5) × 10^4	(7.1 ± 0.3) × 10^3	0	0	(2.4 ± 0.2) × 10^3	(1.6 ± 0.1) × 10^5
FeLV-A (Glasgow-1)	0	0	(1.6 ± 0.2) × 10^5	(3.0 ± 0.4) × 10^3	(1.4 ± 0.9) × 10^3	(4.8 ± 0.5) × 10^4	(9.0 ± 1.0) × 10^3	0	0	(1.9 ± 0.1) × 10^3	(1.2 ± 0.0) × 10^5
FeLV-B (GA)	(1.6 ± 0.0) × 10^3	(4.1 ± 1.1) × 10^3	0	(4.3 ± 0.6) × 10^3	(2.9 ± 0.8) × 10^3	(4.2 ± 0.2) × 10^4	(6.6 ± 0.3) × 10^3	(1.1 ± 0.8) × 10^3	(1.6 ± 1.1) × 10^3	(1.1 ± 0.0) × 10^3	(9.8 ± 1.5) × 10^4
FeLV-C (Sarma)	(2.1 ± 0.0) × 10^3	(1.0 ± 0.0) × 10^3	(1.6 ± 0.1) × 10^5	0	0	(4.5 ± 0.4) × 10^4	(1.0 ± 0.1) × 10^4	(8.4 ± 6.3) × 10^2	(1.1 ± 0.8) × 10^3	(1.2 ± 0.1) × 10^3	(1.4 ± 0.1) × 10^5
FeLV-D (33/ON-T)	(2.2 ± 0.2) × 10^3	(5.1 ± 0.7) × 10^3	(1.6 ± 0.2) × 10^5	(3.3 ± 0.4) × 10^3	(1.9 ± 1.3) × 10^3	0	(6.0 ± 0.2) × 10^3	(1.0 ± 0.7) × 10^3	(1.3 ± 0.5) × 10^3	(2.6 ± 0.1) × 10^3	(1.2 ± 0.1) × 10^5
33TGE2	(4.3 ± 0.4) × 10^3	(8.3 ± 1.1) × 10^3	(1.5 ± 0.0) × 10^5	(1.1 ± 0.0) × 10^4	(2.2 ± 1.5) × 10^3	(5.8 ± 0.7) × 10^4	0	(1.0 ± 0.6) × 10^3	(1.4 ± 0.6) × 10^3	(2.4 ± 0.4) × 10^3	(1.2 ± 0.1) × 10^5
ERV-DC10	(2.8 ± 0.1) × 10^3	(6.4 ± 0.8) × 10^3	(1.9 ± 0.1) × 10^5	(6.9 ± 0.7) × 10^3	(2.0 ± 1.2) × 10^3	(3.5 ± 0.2) × 10^4	(1.0 ± 0.1) × 10^4	(1.0 ± 0.5) × 10^3	(1.4 ± 0.5) × 10^3	0	(1.1 ± 0.0) × 10^5
Ampho-MLV	ND	ND	ND	ND	ND	ND	(1.1 ± 0.1) × 10^4	ND	ND	ND	0

[a] The indicated FeLV *env* genes inserted into the pFUΔss expression vector were used to prepare the LacZ pseudotype viruses. GPLac cells, an *env*-negative packaging cell line containing a LacZ-encoding retroviral vector (7), were transfected (with ScreenFect Reagent; Wako, Osaka, Japan) with each *env* expression vector. Supernatants were passed through 0.45-μm filters and used for infection assays. pFUΔss clone 33 (FeLV-A/clone 33 *env*), pFUΔss A5 [FeLV-A/Glasgow-1(pFGA5) *env*], pFUΔss GB (FeLV-B/Gardner-Arnstein *env*), pFUΔss SC (FeLV-C/Sarma *env*), pFUΔss ON-T (FeLV-D/ON-T *env*), pFUΔss DC10 (ERV-DC10 *env*), and pFUΔss 4070A (amphotropic MLV/4070A *env*) have been described previously (7). pFUΔss TG35-2 (TG35-2 *env*), pFUΔss TG35-4 (TG35-4 *env*), pFUΔss TG35-5 (TG35-5 *env*), and pFUΔss FY33 (the SU was derived from FeLV FY981, and the transmembrane [TM] protein was derived from FeLV-A clone 33) were newly constructed in this study. The target cells used were FeLV-A-, FeLV-B-, FeLV-C-, FeLV-D-, FeLV/33TGE2-, ERV-DC10-, and Ampho-MLV (4070A)-infected HEK293T cells. Infectious clone 33TGE2 was constructed from FeLV clone 33 (but contained the TG35-2 *env* gene) and was used to transfect HEK293T cells, thus establishing persistently infected cells. Viral titers were determined as IU per milliliter with X-Gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside) staining, as previously described (7). The data are the means from 3 to 4 independent experiments, with their standard errors. Ampho-MLV, amphotropic MLV; ND, not done.

TABLE 2 Receptor interference assay using various pseudotype FeLV Env proteins in feline AH927 cells

	Titer of pseudotyped virus[a]:								
	FeLV-A					Clone			
Cell line	Clone 33	Glasgow-1	FeLV-B (GA)	FeLV-C (Sarma)	FeLV (FY981)	TG35-2	TG35-4	TG35-5	Ampho-MLV
AH927	(1.7 ± 0.2) × 10^4	(4.2 ± 0.4) × 10^4	(3.5 ± 0.1) × 10^4	(5.1 ± 0.3) × 10^3	(1.1 ± 0.2) × 10^4	(4.0 ± 0.4) × 10^3	(3.4 ± 0.5) × 10^3	(4.7 ± 0.2) × 10^3	(7.9 ± 0.8) × 10^4
AH927/clone 33	0	0	(8.7 ± 1.6) × 10^4	(6.3 ± 1.6) × 10^3	(1.6 ± 0.1) × 10^4	(5.7 ± 0.7) × 10^3	0	0	(7.7 ± 1.7) × 10^4
AH927/Glasgow-1	0	0	(9.8 ± 0.6) × 10^4	(5.3 ± 0.4) × 10^3	(1.9 ± 0.0) × 10^4	(7.5 ± 0.5) × 10^3	0	0	(6.2 ± 0.7) × 10^4
AH927/GA	(4.5 ± 0.7) × 10^4	(1.4 ± 0.4) × 10^5	0	(9.7 ± 0.9) × 10^3	(2.1 ± 0.2) × 10^4	(9.2 ± 1.3) × 10^3	(1.2 ± 0.1) × 10^4	(1.8 ± 0.2) × 10^4	(1.3 ± 0.1) × 10^5
AH927/Sarma	(3.1 ± 0.2) × 10^4	(1.1 ± 0.1) × 10^5	(8.4 ± 0.9) × 10^4	0	0	(1.1 ± 0.2) × 10^4	(9.3 ± 0.5) × 10^3	(1.2 ± 0.1) × 10^4	(9.0 ± 0.7) × 10^4
AH927/33TGE2	(3.7 ± 0.4) × 10^4	(7.5 ± 0.4) × 10^4	(1.8 ± 1.6) × 10^4	(9.5 ± 1.4) × 10^3	(1.3 ± 0.1) × 10^4	0	(4.8 ± 1.0) × 10^3	(9.8 ± 0.6) × 10^3	(3.1 ± 0.2) × 10^4
104C1	0	0	0	(1.6 ± 0.9) × 10^3	(2.3 ± 1.2) × 10^3	0	ND	ND	(5.5 ± 0.2) × 10^4
CRFK	(1.5 ± 0.1) × 10^4	(4.7 ± 0.6) × 10^4	(9.1 ± 0.4) × 10^4	(2.8 ± 0.4) × 10^3	(3.7 ± 0.4) × 10^4	(1.4 ± 1.2) × 10^3	ND	ND	(9.1 ± 0.5) × 10^4

[a] The target cells used for the viral interference assay were uninfected feline AH927 cells or FeLV-A/clone33-, FeLV-A/Glasgow-1-, FeLV-B/GA-, FeLV-C/Sarma-, or FeLV/33TGE2-infected feline AH927 cells, 104C1 cells (guinea pig), or CRFK cells (feline). Viral titers were determined as IU per milliliter with X-Gal staining as previously described (7). The data are the means from three independent experiments, with their standard errors. ND, not done. Ampho-MLV, amphotropic MLV.

TABLE 3 Receptor usage by FeLVs[c]

Cell line	FeLV-A (clone 33)[a]	FeLV-B (GA)[b]	FeLV (FY981)[a]	Clone TG35-2[a]	33TGE2[b]	Ampho-MLV[a]	Mock
MDTF	−	−	+	−	−	++	−
MDTF/feTHTR-1	++	−	ND	−	−	ND	−
MDTF/fePit-1	−	++	ND	−	−	ND	−
MDTF/fePit-2	−	+	ND	−	−	ND	−
MDTF/hFLVCR1	−	ND	++	−	ND	++	−
MDTF/hFLVCR2	−	ND	++	−	ND	++	−
MDTF/feFLVCR1	−	ND	++	−	ND	++	−

[a] The indicated *env* genes were used to prepare the LacZ pseudotyped viruses.

[b] Replication-competent viruses carrying the LacZ-encoding retroviral reporter were used for infection.

[c] Each retroviral expression plasmid encoding feline THTR1 (feTHTR1), feline PIT1 (fePit-1), feline PIT2 (fePit-2), feline FLVCR1 (feFLVCR1), human FLVCR1 (hFLVCR1), or human FLVCR2 (hFLVCR2) was expressed in MDTF cells under G418 selection. The pooled G418-resistant cells were tested in the viral infection assay using the indicated viruses. Titers were determined from three experiments with X-Gal staining. −, infection titer of 0; +, 1 to 10^3 IU/ml; ++, 10^3 to 10^5 IU/ml. ND, not done. Ampho-MLV, amphotropic MLV.

env gene changed its receptor to that of porcine endogenous retrovirus A (PERV-A) (28), but the host range of PERV-A differs from that of TG35-2 (28, 29), and PERV-A isolated from PK15 cells (30) did not interfere with FeLV 33TGE2 in HEK293T cells (data not shown). A mutagenesis analysis confirmed that subtle changes in the VRA altered the interference patterns of the TG35-2 and FeLV-A phenotypes, but some of these Env mutants did not infect AH927 cells (Fig. 2B), although they expressed the

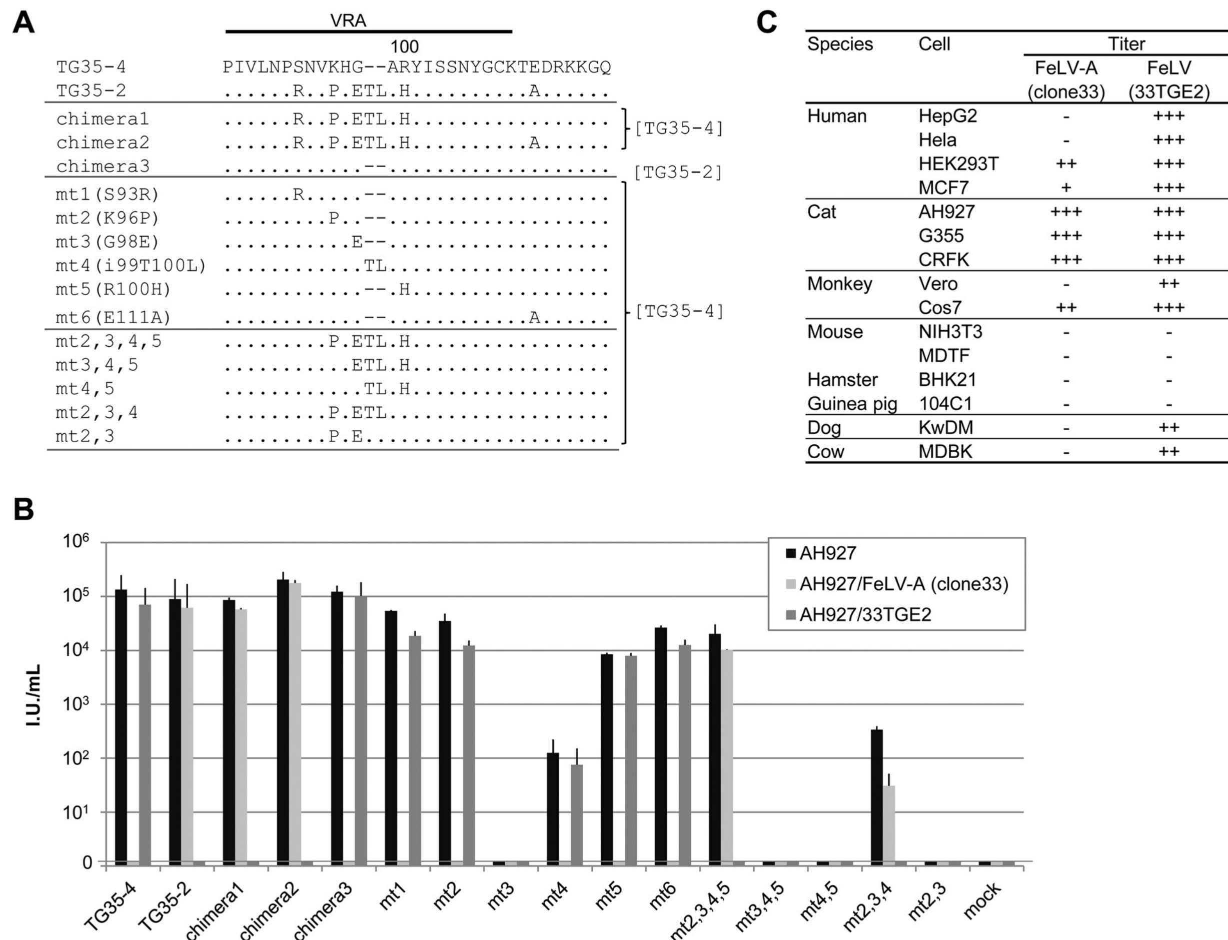

FIG 2 Determination of the amino acids in the Env protein that are required for the TG35-2 phenotype and the host range of FeLV 33TGE2. (A) The indicated mutant FeLV *env* genes, constructed in either the TG35-2 or TG35-4 *env* gene, were generated with site-directed mutagenesis or recombination of the VRA in the pFUΔss vector (7). The *env* sequences other than the VRA, derived from TG35-2 or TG35-4 *env*, are described at right. (B) GPLac cells were transfected with the indicated FeLV *env* genes inserted into the pFUΔss expression vector. Supernatants were passed through 0.45-μm filters and used for infection assays in AH927 cells, AH927/FeLV-A (clone 33) cells, and AH927/33TGE2 cells. Titers were determined with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining (7). The data shown are the averages from several independent experiments, with their standard errors. (C) Replication-competent virus or its LacZ-encoding pseudovirus, produced by the transfection of 293Lac cells (HEK293T cells stably containing a LacZ-encoding retroviral vector) with either FeLV-A clone 33 or the FeLV 33TGE2 plasmid, was used for the infection assay. The indicated target cells were used to determine the host ranges of the viruses. Titers were determined with X-Gal staining. −, zero infection titer; +, 10^1 to 10^3 IU/ml; ++, 10^3 to 10^5 IU/ml; +++, >10^5 IU/ml. The data shown are the averages from three independent experiments.

Env protein, as demonstrated with Western blotting (data not shown). Artificial mutation of the FeLV *env* gene may not alter its receptor. However, subtle mutation of the *env* gene may predispose the virus to enhanced replication *in vivo* and subsequent conversion to different FeLV subgroups (31, 32, 33). We also found an intermediary between the *env* genes of FeLV-A and FeLV-B (16). Therefore, the emergence of a novel FeLV subgroup may be mediated by intermediaries arising through several steps, possibly under selection pressure from cats (e.g., by vaccination).

We propose that FeLV-E is a novel interference subgroup of FeLV.

Nucleotide sequence accession numbers. The sequences reported here have been deposited in DDBJ/EMBL/GenBank under accession numbers LC029807 and LC029808.

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REFERENCES

1. **Hisasue M, Nagashima N, Nishigaki K, Fukuzawa I, Ura S, Katae H, Tsuchiya R, Yamada T, Hasegawa A, Tsujimoto H.** 2009. Myelodysplastic syndromes and acute myeloid leukemia in cats infected with feline leukemia virus clone33 containing a unique long terminal repeat. Int J Cancer **124:**1133–1141. http://dx.doi.org/10.1002/ijc.24050.
2. **Hartmann K.** 2011. Clinical aspects of feline immunodeficiency and feline leukemia virus infection. Vet Immunol Immunopathol **143:**190–201. http://dx.doi.org/10.1016/j.vetimm.2011.06.003.
3. **Sarma PS, Log T.** 1973. Subgroup classification of feline leukemia and sarcoma viruses by viral interference and neutralization tests. Virology **54:**160–169. http://dx.doi.org/10.1016/0042-6822(73)90125-6.
4. **Jarrett O, Laird HM, Hay D.** 1973. Determinants of the host range of feline leukaemia viruses. J Gen Virol **20:**169–175. http://dx.doi.org/10.1099/0022-1317-20-2-169.
5. **Takeuchi Y, Vile RG, Simpson G, O'Hara B, Collins MK, Weiss RA.** 1992. Feline leukemia virus subgroup B uses the same cell surface receptor as gibbon ape leukemia virus. J Virol **66:**1219–1222.
6. **Anderson MM, Lauring AS, Burns CC, Overbaugh J.** 2000. Identification of a cellular cofactor required for infection by feline leukemia virus. Science **287:**1828–1830. http://dx.doi.org/10.1126/science.287.5459.1828.
7. **Anai Y, Ochi H, Watanabe S, Nakagawa S, Kawamura M, Gojobori T, Nishigaki K.** 2012. Infectious endogenous retroviruses in cats and emergence of recombinant viruses. J Virol **86:**8634–8644. http://dx.doi.org/10.1128/JVI.00280-12.
8. **Ito J, Watanabe S, Hiratsuka T, Kuse K, Odahara Y, Ochi H, Kawamura M, Nishigaki K.** 2013. Refrex-1, a soluble restriction factor against feline endogenous and exogenous retroviruses. J Virol **87:**12029–12040. http://dx.doi.org/10.1128/JVI.01267-13.
9. **Stewart MA, Warnock M, Wheeler A, Wilkie N, Mullins JI, Onions DE, Neil JC.** 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.
10. **Overbaugh J, Riedel N, Hoover EA, Mullins JI.** 1988. Transduction of endogenous envelope genes by feline leukaemia virus in vitro. Nature **332:**731–734. http://dx.doi.org/10.1038/332731a0.
11. **Donahue PR, Quackenbush SL, Gallo MV, deNoronha CM, Overbaugh J, Hoover EA, Mullins JI.** 1991. Viral genetic determinants of T-cell killing and immunodeficiency disease induction by the feline leukemia virus FeLV-FAIDS. J Virol **65:**4461–4469.
12. **Rigby MA, Rojko JL, Stewart MA, Kociba GJ, Cheney CM, Rezanka LJ, Mathes LE, Hartke JR, Jarrett O, Neil JC.** 1992. Partial dissociation of subgroup C phenotype and in vivo behaviour in feline leukaemia viruses with chimeric envelope genes. J Gen Virol **73:**2839–2847. http://dx.doi.org/10.1099/0022-1317-73-11-2839.
13. **Quigley JG, Burns CC, Anderson MM, Lynch ED, Sabo KM, Overbaugh J, Abkowitz JL.** 2000. Cloning of the cellular receptor for feline leukemia virus subgroup C (FeLV-C), a retrovirus that induces red cell aplasia. Blood **95:**1093–1099.
14. **Tailor CS, Willett BJ, Kabat D.** 1999. A putative cell surface receptor for anemia-inducing feline leukemia virus subgroup C is a member of a transporter superfamily. J Virol **73:**6500–6505.
15. **Mendoza R, Anderson MM, Overbaugh J.** 2006. A putative thiamine transport protein is a receptor for feline leukemia virus subgroup A. J Virol **80:**3378–3385. http://dx.doi.org/10.1128/JVI.80.7.3378-3385.2006.
16. **Watanabe S, Kawamura M, Odahara Y, Anai Y, Ochi H, Nakagawa S, Endo Y, Tsujimoto H, Nishigaki K.** 2013. Phylogenetic and structural diversity in the feline leukemia virus env gene. PLoS One **8:**e61009. http://dx.doi.org/10.1371/journal.pone.0061009.
17. **Nishigaki K, Hanson C, Thompson D, Yugawa T, Hisasue M, Tsujimoto H, Ruscetti S.** 2002. Analysis of the disease potential of a recombinant retrovirus containing Friend murine leukemia virus sequences and a unique long terminal repeat from feline leukemia virus. J Virol **76:**1527–1532. http://dx.doi.org/10.1128/JVI.76.3.1527-1532.2002.
18. **Mullins JI, Casey JW, Nicolson MO, Burck KB, Davidson N.** 1981. Sequence arrangement and biological activity of cloned feline leukemia virus proviruses from a virus-productive human cell line. J Virol **38:**688–703.
19. **Riedel N, Hoover EA, Gasper PW, Nicolson MO, Mullins JI.** 1986. Molecular analysis and pathogenesis of the feline aplastic anemia retrovirus, feline leukemia virus C-Sarma. J Virol **60:**242–250.
20. **Knouf EC, Metzger MJ, Mitchell PS, Arroyo JD, Chevillet JR, Tewari M, Miller AD.** 2009. Multiple integrated copies and high-level production of the human retrovirus XMRV (xenotropic murine leukemia virus-related virus) from 22Rv1 prostate carcinoma cells. J Virol **83:**7353–7356. http://dx.doi.org/10.1128/JVI.00546-09.
21. **Shalev Z, Duffy SP, Adema KW, Prasad R, Hussain N, Willett BJ, Tailor CS.** 2009. Identification of a feline leukemia virus variant that can use THTR1, FLVCR1, and FLVCR2 for infection. J Virol **83:**6706–6716. http://dx.doi.org/10.1128/JVI.02317-08.
22. **Anderson MM, Lauring AS, Robertson S, Dirks C, Overbaugh J.** 2001. Feline Pit2 functions as a receptor for subgroup B feline leukemia viruses. J Virol **75:**10563–10572. http://dx.doi.org/10.1128/JVI.75.22.10563-10572.2001.
23. **Sarma PS, Log T, Jain D, Hill PR, Huebner RJ.** 1975. Differential host range of viruses of feline leukemia-sarcoma complex. Virology **64:**438–446. http://dx.doi.org/10.1016/0042-6822(75)90121-X.
24. **Miller DG, Edwards RH, Miller AD.** 1994. Cloning of the cellular receptor for amphotropic murine retroviruses reveals homology to that for gibbon ape leukemia virus. Proc Natl Acad Sci U S A **91:**78–82. http://dx.doi.org/10.1073/pnas.91.1.78.
25. **van Zeijl M, Johann SV, Closs E, Cunningham J, Eddy R, Shows TB, O'Hara B.** 1994. A human amphotropic retrovirus receptor is a second member of the gibbon ape leukemia virus receptor family. Proc Natl Acad Sci U S A **91:**1168–1172. http://dx.doi.org/10.1073/pnas.91.3.1168.
26. **Boomer S, Eiden M, Burns CC, Overbaugh J.** 1997. Three distinct envelope domains, variably present in subgroup B feline leukemia virus recombinants, mediate Pit1 and Pit2 receptor recognition. J Virol **71:**8116–8123.
27. **Stieler K, Schulz C, Lavanya M, Aepfelbacher M, Stocking C, Fischer N.** 2010. Host range and cellular tropism of the human exogenous gammaretrovirus XMRV. Virology **399:**23–30. http://dx.doi.org/10.1016/j.virol.2009.12.028.
28. **Mazari PM, Linder-Basso D, Sarangi A, Chang Y, Roth MJ.** 2009. Single-round selection yields a unique retroviral envelope utilizing GPR172A as its host receptor. Proc Natl Acad Sci U S A **106:**5848–5853. http://dx.doi.org/10.1073/pnas.0809741106.

29. **Takeuchi Y, Patience C, Magre S, Weiss RA, Banerjee PT, Le Tissier P, Stoye JP.** 1998. Host range and interference studies of three classes of pig endogenous retrovirus. J Virol **72:**9986–9991.
30. **Patience C, Takeuchi Y, Weiss RA.** 1997. Infection of human cells by an endogenous retrovirus of pigs. Nat Med **3:**282–286. http://dx.doi.org/10.1038/nm0397-282.
31. **Stewart H, Adema KW, McMonagle EL, Hosie MJ, Willett BJ.** 2012. Identification of novel subgroup A variants with enhanced receptor binding and replicative capacity in primary isolates of anaemogenic strains of feline leukaemia virus. Retrovirology **9:**48. http://dx.doi.org/10.1186/1742-4690-9-48.
32. **Rohn JL, Linenberger ML, Hoover EA, Overbaugh J.** 1994. Evolution of feline leukemia virus variant genomes with insertions, deletions, and defective envelope genes in infected cats with tumors. J Virol **68:**2458–2467.
33. **Rohn JL, Moser MS, Gwynn SR, Baldwin DN, Overbaugh J.** 1998. In vivo evolution of a novel, syncytium-inducing and cytopathic feline leukemia virus variant. J Virol **72:**2686–2696.