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Infectious Endogenous Retroviruses in Cats and Emergence of Recombinant Viruses

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Endogenous retroviruses (ERVs) comprise a significant percentage of the mammalian genome, and it is poorly understood whether they will remain as inactive genomes or emerge as infectious retroviruses. Although several types of ERVs are present in domestic cats, infectious ERVs have not been demonstrated. Here, we report a previously uncharacterized class of endogenous gammaretroviruses, termed ERV-DCs, that is present and hereditary in the domestic cat genome. We have characterized a subset of ERV-DC proviral clones, which are numbered according to their genomic insertions. One of these, ERV-DC10, located in the q12-q21 region on chromosome C1, is an infectious gammaretrovirus capable of infecting a broad range of cells, including human. Our studies indicate that ERV-DC10 entered the genome of domestic cats in the recent past and appeared to translocate to or reintegrate at a distinct locus as infectious ERV-DC18. Insertional polymorphism analysis revealed that 92 of 244 domestic cats had ERV-DC10 on a homozygous or heterozygous locus. ERV-DC-like sequences were found in primate and rodent genomes, suggesting that these ERVs, and recombinant viruses such as RD-114 and BaEV, originated from an ancestor of ERV-DC. We also found that a novel recombinant virus, feline leukemia virus subgroup D (FeLV-D), was generated by ERV-DC *env* transduction into feline leukemia virus in domestic cats. Our results indicate that ERV-DCs behave as donors and/or acceptors in the generation of infectious, recombinant viruses. The presence of such infectious endogenous retroviruses, which could be harmful or beneficial to the host, may affect veterinary medicine and public health.

Retroviruses are classified as exogenous or endogenous according to their mode of transmission. Endogenous retroviruses (ERVs) are present in all vertebrate genomes and are thought to be the remnants of ancestral germ line infections by exogenous retroviruses. ERVs make up a significant fraction of the mammalian genome (for example, 8 to 10% of the human or mouse genomes) and are transmitted in a Mendelian fashion (4, 18, 42). Most ERVs contain deleterious mutations, large deletions and insertions of repetitive elements that render them unable to make infectious viral particles, but these proviruses often are transcribed and produce functional gene products (4). Domestic cats (*Felis silvestris catus*) are descended from *Felis silvestris lybica*, the Middle Eastern wildcat (13), and are sometimes infected with feline leukemia virus (FeLV), which is a gammaretrovirus that is horizontally transmitted among domestic cats during grooming or through bites (11, 12). The endogenous counterparts of exogenous FeLV, termed enFeLVs, are integrated into the genomes of the *Felis* genus. Primary colonization of the feline germ line by FeLVs is believed to have occurred in an ancestor of the domestic cat after separation from the leopard lineage (2, 11, 31). Like most endogenous retroviruses, enFeLVs are noninfectious and nonpathogenic (4). However, recombination occurs between inherited enFeLVs and exogenous FeLVs (exFeLVs) following infection, which can give rise to new infectious agents that may be pathogenic (11, 29). In addition to enFeLV, other feline ERVs are found in the domestic cat. The RD-114 ERV, which was originally isolated from human rhabdomyosarcoma cells transplanted into fetal kittens (22), is a replication-competent retrovirus which shows a high level of homology to the baboon endogenous retrovirus (BaEV); however, an infectious RD-114 provirus has not been demonstrated in the cat genome. MAC-1, which is a genetically transmitted type C virus of primates, has been reported as a feline noninfectious ERV (5, 39); however, no further studies have been reported. Another class of feline ERVs with high homology to the *pol* region of BaEV and RD-114 has also been described and partially characterized as ECE1 and FcEV (3, 41). In the present study, we have further characterized this later class of feline ERVs, which we term ERV-DCs. Our studies indicate that while most ERV-DCs contain deletions and mutations that render them noninfectious, several ERV-DCs are infectious and mobile in the domestic cat, using reinfection, translocation to another locus, or recombination with an exogenous retrovirus to amplify their progeny. These infectious ERV-DCs can infect a wide range of mammalian cells, including human. Most ERV-DCs are not yet fixed in the cat genome and have the potential to generate recombinant retroviruses which could be transmitted among cats and other species.

MATERIALS AND METHODS

Samples. Blood for peripheral blood mononuclear cell (PBMC) isolation and spleen and muscle tissues from domestic cats and *Prionailurus bengalensis euptilurus* were collected at private veterinary hospitals (26) and the Tsushima Wildlife Conservation Center (TWCC) in Japan.

Expression vectors and cell lines. The *env* genes of FeLV-A/Glasgow-1 (pFGA5) (38), FeLV-B/Gardner-Arnstein (pFGB) (24), FeLV-C/Sarma (pFSC) (33), FeLV-D/Ty26, FeLV-D/ON-T, and ERV-DC10

Received 2 February 2012 Accepted 22 May 2012
Published ahead of print 6 June 2012
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Supplemental material for this article may be found at http://jvi.asm.org/.

doi:10.1128/JVI.00280-12

were PCR amplified with specific primers and cloned into pFUΔss expression plasmid, which was a modified pFUSE-hIgG2-Fc2 vector (Invitrogen, Carlsbad, CA). The nβ-*gal* gene (kindly provided by Masaaki Nakaya, Yokohama City University, Kanagawa, Japan) was introduced into the pMXs-IP vector (17) (kindly provided by Toshio Kitamura, The University of Tokyo, Tokyo, Japan) to produce pMXs-nLIP. HEK293T cells were persistently infected with FeLV-A (clone33) (27), FeLV-B (pFGB), FeLV-C (pFSC), ERV-DC10 (pDC10), or ERV-DC18(pDC18). 293T cells expressing FeLV-D/ON-T were established by transfection with a full-length FeLV-D/ON-T plasmid and then were infected with FeLV-A/clone33 for viral interference assays. An *env*-negative packaging cell line containing a LacZ-coding retroviral vector, designated GPLac, was also established.

RNA isolation and reverse transcription. Total RNA was isolated using a FastPure RNA kit, and cDNA was synthesized with a PrimeScript II first-strand cDNA synthesis kit (TaKaRa, Tokyo, Japan). Viral RNA was isolated from infected cell culture supernatants using the QIAamp viral RNA minikit (Qiagen, Tokyo, Japan).

PCR. Primers used in this study are listed in Table S4 in the supplemental material. Prime Star GXL polymerase (TaKaRa), KOD FX Neo (Toyobo, Japan), and Prime Star HS DNA polymerase (TaKaRa) were used for various cloning procedures. The latter two enzymes were also used to screen for FeLV-D and monitor viral infection. KOD FX Neo and GoTaq (Promega, Madison, WI) were used for genotyping insertional polymorphisms.

Southern blotting. A total of 7.5 μg sample DNA was isolated from PBMCs or tissue samples (34), digested with EcoRI and BamHI, and hybridized to a digoxigenin (DIG)-labeled FeLV-D/ON-T *env* probe generated by PCR with primers Fe-9S and Fe-3R (see Table S4 in the supplemental material). A CSPD luminescent detection kit was used to visualize the bound probe (Roche Molecular Biochemicals, Mannheim, Germany).

Cloning of ERV-DC proviruses. Splenic DNA from a single cat (ON-T) was digested with EcoRI or Sau3AI and ligated to Lamda DASH II vectors (Stratagene, La Jolla, CA). DNA libraries were screened with a DIG-labeled FeLV-D *env* probe. ERV-DC proviruses and flanking sequences were obtained from the NCBI cat genome database; other sequence data were obtained by screening genomic libraries. We PCR amplified full-length ERV-DC and partial ERV-DC proviruses from feline chromosomal DNA using specific primers and cloned amplicons into pCR4 blunt-TOPO (Invitrogen, Carlsbad, CA). Some flanking sequences of ERV-DCs were obtained by gene walking using the Right Walk kit (Bex Co., Ltd., Tokyo, Japan). Preintegration sites and ERV-DC provirus were detected by PCR.

FeLV-D screening and cloning. DNA from FeLV-positive PBMCs or tumor tissues was PCR screened using primers Fe-14S and Fe-3R. Gene walking was performed to obtain the flanking DNA sequences of FeLV-D/ON-T provirus using each Fe-14S (first PCR) and Fe-2S (second PCR) primer with each adaptor primer (Right Walk kit; Bex Co., Ltd.). After obtaining one sequence of the preintegration sites, the full-length FeLV-D/ON-T proviral genome was PCR amplified with Prime Star HS (TaKaRa) DNA polymerase and Fe-63S and Fe-73R primers and cloned into pCR4 blunt-TOPO. Partial FeLV-D/Ty26, FeLV-D/ON-C, FeLV/44B, and FeLV-D/re-T sequences were obtained by PCR.

Immunoblotting. Immunoblotting was performed as previously described (40). Primary antibodies were goat anti-RD114 (National Cancer Institute [NCI], Frederick, MD), goat anti-FeLV gp70 (NCI), and mouse anti-beta actin (Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies were horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (GE Healthcare Japan, Tokyo, Japan) or anti-goat IgG antibody (Santa Cruz Biotechnology).

Infection assay. HEK293T cells were transfected with 4 μg ERV-DC10 or ERV-DC18 plasmid DNA using Lipofectamine 2000 (Invitrogen). Viruses in the culture supernatant collected 72 h posttransfection were detected by PCR. Supernatants (300 μl; filtered with 0.22-μm-pore-size filters) from persistently infected HEK293T cells were used to infect Molt-4, K562, HepG2, HeLa, MCF7, THP-1, Vero, Cos7, L929, BHK-21, and NIH 3T3 cells (3.65×10^5 cells/well) in the presence of Polybrene (8 μg/ml). KwDM cells established from dog mammary tumor were also used for infection assay. Cellular DNA was collected, and ERV-DC proviral DNA was detected by PCR using primers Fe-13S and Fe-18R.

Green fluorescent protein (GFP)-containing pMXs-IP vector with or without ERV-DC10 or ERV-DC18 sequences was also transfected into HEK293T cells. At 72 h posttransfection, CRFK, AH927, and G355 cell lines were infected with the HEK293T supernatant in the presence of Polybrene (8 μg/ml). GFP-positive cells were determined by microscopy or flow cytometer.

Viral preparation from feline chromosomal DNA. ERV-DC8, ERV-DC10, ERV-DC14, or ERV-DC18, amplified by PCR with primers Fe-57S and Fe-54R for ERV-DC8, Fe-122S and Fe-38R for ERV-DC10, Fe-58S and Fe-56R for ERV-DC14, or Fe-76S and Fe-81R for ERV-DC18, using feline chromosomal DNA was directly transfected to HEK293T cells, and the filtered supernatant was used to infect fresh HEK293T cells. Viral infection was monitored by detecting proviral ERV-DC *env* and long terminal repeats (LTR) or immunoblot analysis using anti-RD114 antibody. Viral transmission was confirmed by further infection.

Transmission electron microscopy. HEK293T cells persistently infected with ERV-DC10 were washed with Dulbecco's phosphate-buffered saline (D-PBS), fixed with 2% glutaraldehyde at 4°C for at least 2 h, washed with D-PBS (twice for 15 min each), and postfixed with 2% osmium tetroxide for 1.5 h. Cells were dehydrated through a graded ethanol series and propylene oxide and then embedded in EPON 812 for 48 h at 60°C. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with an electron microscope (JEM-1200EX; JEOL, Japan).

Viral interference assays. GPLac cells (3×10^6) were transfected with each *env*-expressing plasmid to produce pseudotype viruses. After culturing with 200 μg/ml zeocin and 1 μg/ml puromycin for >2 weeks, supernatants were collected, filtered through a 0.22-μm filter, and stored at −80°C. Target cells (10^5) were infected with viral supernatant in the presence of Polybrene (2 μg/ml) for 2 days and then stained with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Single-cycle infectivity was titrated by counting blue-stained nuclei using a microscope following incubation with X-Gal. X-Gal-positive cells were considered infectious units.

FISH analysis. PBMC cultures stimulated with concanavalin A were treated with 5-bromo-2′-deoxyuridine for 5.5 h and harvested after colcemid treatment for 0.5 h. Replication-banded chromosomes were obtained by exposure of the chromosome slides to UV light after staining with Hoechst 33258. The pTKDC10L2.8 (2.8 kb of ERV-DC10 flanking sequences amplified by PCR with primers Fe-137S [5′-ATTCCTTAGCT CATGGTCCTTTTCT-3′] and Fe-38R [5′-CACACATGCTCTAGACAC AATACCC-3′]) and pTKDC18L3.4 (3.4 kb of ERV-DC18 flanking sequences amplified by PCR with primers Fe-146S [5′-GTCAATTGGACT GCCTTGAATCTAT-3′] and Fe-138R [5′-AAAGTTCCCAGATAATCC TGATGGT-3′]) plasmid DNAs were labeled with digoxigenin-11-dUTP by nick translation for use as probes. After hybridization, the slides were washed and probe signals were detected with Cy3-labeled anti-digoxigenin. Fluorescence *in situ* hybridization (FISH) images were captured with the CW4000 FISH application program (Leica Microsystem, Wetzlar, Germany).

Phylogenetic and genomic analyses. Nucleotide sequences of ERV-DC regions and amino acid sequences of Pol and Env used in the phylogenetic analyses are summarized in Table S5 in the supplemental material. For these analyses, we generated multiple alignments for each gene using MAFFT (L-INS-i) (15). Amino acid substitution models of LG (19) with gamma-distributed rate variation (+Γ; $\alpha = 1.672557$) and rtREV (8) with gamma-distributed rate variation and inferred proportion of invariable sites (+Γ+I; $\alpha = 0.912332$; invar = 0.000117) were selected for Env and Pol alignments, respectively, using the Akaike information criterion implemented in PROTTEST 3 (6). Phylogenetic trees were con-

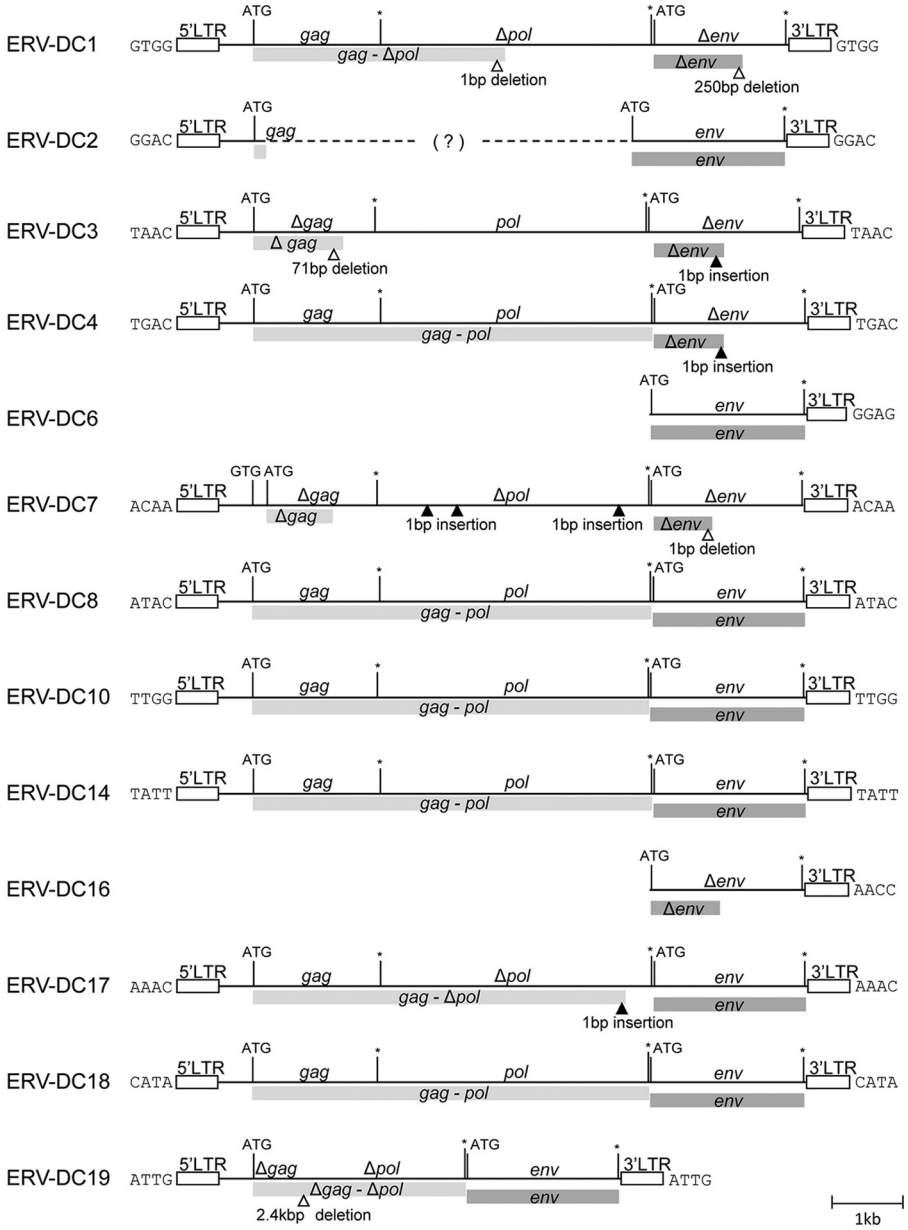


FIG 1 ERV-DC structures. Structures of the genomes of 10 full-length ERV-DC proviruses and three partial ERV-DC proviruses. The *gag*, *pol*, and *env* genes are illustrated together with the 5′ and 3′ LTRs and positions of the *gag* and *env* translational initiation codons (ATG). Asterisks indicate conserved stop codons. Gag and Pol proteins may be synthesized as a large single polypeptide precursor via termination suppression (44). An open triangle indicates a deletion of nucleotides, and a filled triangle indicates an insertion of nucleotides. Flanking 4-bp TSD sequences are shown for each provirus.

structed using the maximum-likelihood method (10) in RAxML v7.2.8 with robustness evaluated by rapid bootstrapping (1,000 times). To identify ERVs we used RetroTector (35) and default parameters for genomes of various species provided by Ensembl (http://www.ensembl.org/). Four types of retrovirus-like sequences in the genome were identified, and we conducted a homology search with BLASTP using the Pol or Env sequences of ERV-DC as a query.

Time estimation of ERV-DC insertion. Because the mutation rate of LTRs in ERV-DCs is unknown, we alternatively utilized a mean divergence rate of noncoding regions in the domestic cat genome (1.2×10^{-8} substitutions/site/year) (20). Genetic distances between LTRs were calculated using the Kimura two-parameter model (16).

Nucleotide sequence accession numbers. The sequences reported here have been deposited in DDBJ/EMBL/GenBank under accession numbers AB673426 to AB673432, AB674572 to AB674577, and AB674439 to AB674452.

RESULTS

Characterization of ERV-DCs. Ten full-length and three partial feline endogenous gammaretroviruses, which were previously partially characterized as ECE1 and FcEV (3, 41), were isolated by genomic library screening and PCR using locus-specific primers. Sequence analysis revealed that four of these proviruses (ERV-DC8, ERV-DC10, ERV-DC14, and ERV-DC18) have intact open reading frames (ORFs) for *gag*, *pol*, and *env* (Fig. 1) and may produce infectious retroviruses. Although ERV-DC10 and ERV-DC18 were isolated from different cats and had different integra-

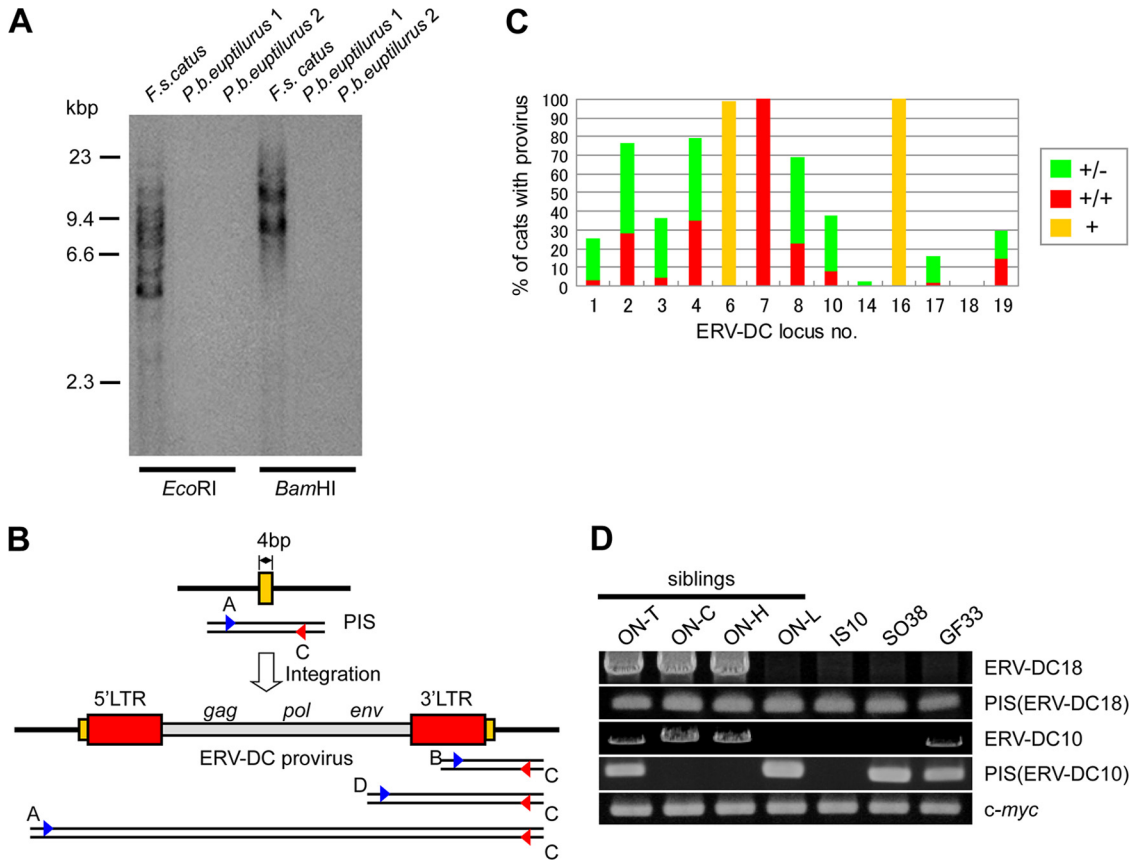


FIG 2 Detection of ERV-DC proviruses in the domestic cat genome. (A) Southern blotting of ERV-DC with an *env* probe derived from FeLV-D/ON-T in chromosomal DNA from the domestic cat (*Felis silvestris catus*) and *Prionailurus bengalensis euptilurus*. (B) PCR for detection of preintegration sites (PISs) and proviruses. Red boxes, viral LTRs; yellow boxes, target site duplications. Primers A to D are shown. The AC primer pair generated a PIS and/or full-length provirus. (C) Insertional polymorphisms of 13 ERV-DCs in 244 domestic cats. Green, provirus was detected on heterozygous loci; red, provirus was detected on homozygous loci; yellow, provirus was detected. (D) ERV-DC18 was only present in 3 of 4 siblings from one family. Each PIS, for full-length ERV-DC10 or ERV-DC18, was detected by primers Fe-122S and Fe-38R for ERV-DC10 or Fe-76S and Fe-81R for ERV-DC18 (see Table S4 in the supplemental material).

tion loci, their ERV-DC nucleotide sequences were almost identical. Only 1 out of 8,863 bp in the primer binding site was different between ERV-DC10 and ERV-DC18. The *gag* gene had a predicted size of 1,644 bp and encoded a putative 547-amino-acid (aa) protein. The *pol* gene had a predicted size of 3,570 bp, and the deduced Pol protein encoded a putative protease, reverse transcriptase, RNaseH, and integrase. It is likely that the Gag and Pol proteins are synthesized as a single large polypeptide precursor via termination suppression. The *env* ORF of ERV-DC10/18 was located in a different reading frame than that of the *gag-pol* gene. The *env* gene had a predicted size of 2,016 bp and encoded a putative 671-aa protein, including a signal peptide, surface (SU) protein, and transmembrane (TM) protein.

Insertional polymorphic distribution of ERV-DC in cats. Multiple bands of ERV-DCs were detected in the healthy domestic cat genome by Southern blot analysis with a specific *env* probe (Fig. 2A), indicating that ERV-DCs have infected cats and are vertically inherited. An insertional polymorphic distribution of ERV-DC was determined among 244 cats in Japan using PCR primers based on the genomic DNA flanking sequences unique to each ERV-DC or with second primers based on the proviral sequence (Fig. 2B). Randomly chosen cats from different genetic backgrounds and geographic origins within Japan were screened for the presence of 10 full-length ERV-DC proviruses and three partial ERV-DC proviruses. All were represented in the domestic cat genome at a frequency of 0.4 to 100% (Fig. 2C; also see Table S1-1 in the supplemental material). We estimated that each cat had 7 to 17 copies of ERV-DC proviruses, with the majority having 9 copies. The ERV-DC10 locus was heterozygous in 29.5% of the cats examined and homozygous in 8.2%. The ERV-DC18 locus was detected in only one cat (cat ON-T) and was heterozygous. When we examined ERV-DC provirus in siblings of ON-T, the ERV-DC18 locus was heterozygous in three of four siblings born to the same mother. Interestingly, these 3 cats also had ERV-DC10 and tested positive for FeLV infection (Fig. 2D; also see Table S1-2). The sequences of ERV-DC18 are almost identical to those of ERV-DC10; therefore, ERV-DC10 appears to have been mobilized as an infectious virus in one of the parents of the three positive siblings, generating provirus ERV-DC18. Although some ERV-DCs have become fixed in the cat genome, others have not (Fig. 2C). These results indicate that ERV-DC proviruses are currently invading domestic cats. Sequence analysis verified that ERV-DC sequences are integrated into the cat genome, and analysis of genomic sequences flanking the proviruses, including identical target site duplication (TSD), identified unequivocally the various ERV-DCs (Fig. 1). Furthermore, 5′ and 3′ LTR sequences

TABLE 1 Properties of ERV-DC 5′ and 3′ LTRs

Provirus	No. of differences between 5′ and 3′ LTRs	Homology between 5′ and 3′ LTRs (%)	Length of LTR (bp)	
			5′	3′
ERV-DC1	0	100	550	550
ERV-DC2	3	99.5	555	555
ERV-DC3	1	99.8	550	550
ERV-DC4	0	100	550	550
ERV-DC7	42	96.7	545	566
ERV-DC8	1	99.8	550	550
ERV-DC10	0	100	551	551
ERV-DC14	1	99.8	551	551
ERV-DC17	4	99.5	551	550
ERV-DC18	0	100	551	551
ERV-DC19	3	99.5	555	555

of each ERV-DC were found to have high sequence identity or were identical to each other (Table 1). There was no evidence of LTR recombination as determined by analyzing the 4-bp identical TSD sequences (Fig. 1) and the phylogenetic tree of LTR sequences (see Fig. 5D). These results suggest that integration of ERV-DC occurred relatively recently in the evolutionary history of cats. Supporting this idea is the absence of ERV-DC proviruses and RD-114 in the Tsushima wild cat *Prionailurus bengalensis euptilurus* (Fig. 2A; also see Fig. S1), which lives in close proximity to domestic cats. The domestic cat is estimated to have separated from the leopard cat and the Tsushima wild cat approximately 6.2 to 9.3 million years ago (MYA) (13, 32). By comparing the nucleotide sequences of the 5′ and 3′ LTRs of ERV-DC7 (Table 1), which is fixed in the cat population (Fig. 2C; also see Table S1-1), we estimated that the oldest time of ERV-DC insertion was 2.8 MYA, assuming an average divergence rate of noncoding regions of domestic cat genomes (20). Thus, we hypothesize that ERV-DC and RD-114 infected domestic cats after separation from the leopard lineage.

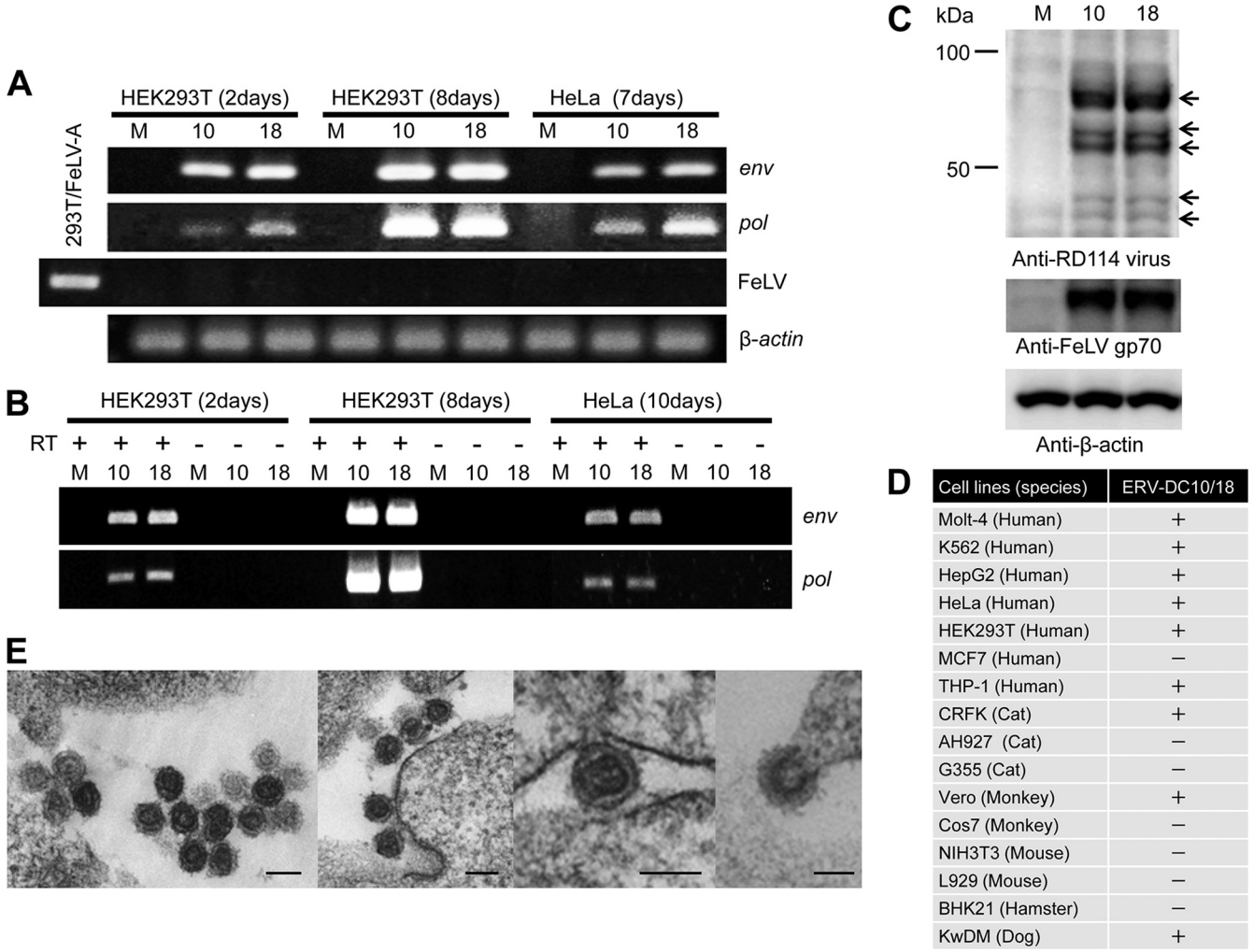
Infectious viruses ERV-DC10 and ERV-DC18. Since ERV-DC8, ERV-DC10, ERV-DC14, and ERV-DC18 have intact *gag/pol/env* reading frames, we carried out studies to determine if they were infectious. We transfected human embryonic kidney 293T (HEK293T) cells with proviral plasmid DNA, and ~72 h later we collected cell-free supernatants (filtered at 0.22 μm) that were used to infect HEK293T and HeLa cells. ERV-DC10 and ERV-DC18 were shown to be infectious by the presence of proviral DNA and viral RNA (Fig. 3A and B), whereas ERV-DC8 and ERV-DC14 were not. Viral Gag and Env proteins were detected by immunoblotting (Fig. 3C). Surprisingly, goat anti-FeLV gp70 detected ERV-DC Env as well as FeLV Env. Human cell lines persistently infected with ERV-DC10 and ERV-DC18 were established. Cell-free supernatants from HEK293T cells infected with ERV-DC10 and ERV-DC18 could infect a number of other human cancer cell lines, such as K562, Molt-4, HepG2, THP-1, dog mammary tumor cells (KwDM), and monkey Vero cells (Fig. 3D; also see Fig. S2 in the supplemental material). The viruses were also able to infect the feline kidney cell line CRFK but not other feline cell lines that can be infected with other feline retroviruses (e.g., AH927 fibroblasts that are readily infected with FeLV or G355 astrocytes that can be infected with RD-114). Transmission electron microscopy of HEK293T and HeLa cells persistently infected with ERV-DC10 revealed 90- to 100-nm particles that were morphologically consistent with a type C retrovirus (Fig. 3E).

We developed an alternative method to determine whether ERV-DC proviruses in cat DNA are infectious. Full-length ERV-DC10 or ERV-DC18 proviral DNA from primary cat PBMC was amplified by PCR with locus-specific primers, and the PCR products, without cloning of proviruses, were transfected into HEK293T cells. After 2 to 3 weeks, each filtered supernatant was used to infect HEK293T cells. As a result, ERV-DC10 or ERV-DC18 was shown to persistently produce infectious virus (Fig. 4A and B). Even HEK293T cells transfected with DNA from the AH927 feline cell line, which has a homozygous ERV-DC10 locus, produced infectious virus (Fig. 4A). Virus production from DNA from cats that lacked ERV-DC10 or ERV-DC18 was never seen in cultures (Fig. 4A, B, and C), indicating that this method specifically assesses the activity of ERV-DC10 and ERV-DC18 loci. Using this method, we also found that DNA from cats harboring ERV-DC8 and ERV-DC14 proviruses was unable to produce infectious virus (Fig. 4C), consistent with data obtained using molecular clones of ERV-DC8 and ERV-DC14. These results demonstrate that ERV-DC10 and ERV-DC18 are potentially active, and that their genomes encode infectious viruses.

To define the active locus, we mapped two active loci by FISH analysis: ERV-DC10 mapped to feline chromosome C1q12-21 and ERV-DC18 to feline chromosome D4q14 (Fig. 4D and E). These results indicate that ERV-DC10 and ERV-DC18 loci are distinct from each other, and we speculate that the ERV-DC10 provirus on C1q12-21 mobilized to D4q14. We could not isolate ERV-DC10 viruses when PBMC from ERV-DC10-positive cats were cultured with HEK293T cells. Thus, although infectious ERV-DC10 provirus is present in the genome of domestic cats, PBMC and AH927 cells may not produce infectious viruses or ERV-DC10 may be silenced in these cells.

Phylogenetic analyses of ERV-DCs. Phylogenetic analyses indicated that ERV-DC proviruses were genetically distinct from exFeLVs, enFeLVs, and RD-114 and comprised a separate group of gammaretroviruses (Fig. 5A and B). ERV-DC proviruses obtained in this study were classified into three groups based on their *env* genes (Fig. 5C). This classification was supported by the phylogenetic tree of their LTR sequences (Fig. 5D) and suggests that independent invasion of ERV-DCs occurred in the ancestry of domestic cats.

To examine if ERV-DCs interact with DNA of other species, we extracted ERV-DC-like ERVs from the genome data of 12 different species (see Table S2 in the supplemental material). As a result, 22, 19, 3, and 2 ERV loci were detected in the genomes of the house mouse, brown rat, common chimpanzee, and rhesus macaque, respectively (see Table S3), although no orthologous relationships were found among them. We then conducted phylogenetic analyses of ERV-DCs using these ERV elements with various endogenous or exogenous viruses (Fig. 5A and B). The Pol protein of ERV-DC was most closely related to that of RD-114 and BaEV (Fig. 5A), whereas the Env/TM protein of ERV-DC was more closely related to that of the FeLV family (Fig. 5B). RD-114 is genetically related to BaEV, and both of these viruses are recombinant viruses that contain a type C (gammaretrovirus group) ORF for *gag-pol* and a type D (betaretrovirus group) *env* gene, and supposedly they arose from cross-species transmission (1, 14, 36). The amino acid identity was 98.6% for Pol proteins of ERV-DC18 and RD-114 SC3C and 81.1% for the TM regions of Env proteins



Cell lines (species)	ERV-DC10/18
Molt-4 (Human)	+
K562 (Human)	+
HepG2 (Human)	+
HeLa (Human)	+
HEK293T (Human)	+
MCF7 (Human)	−
THP-1 (Human)	+
CRFK (Cat)	+
AH927 (Cat)	−
G355 (Cat)	−
Vero (Monkey)	+
Cos7 (Monkey)	−
NIH3T3 (Mouse)	−
L929 (Mouse)	−
BHK21 (Hamster)	−
KwDM (Dog)	+

FIG 3 ERV-DC10 and ERV-DC18 are infectious viruses. (A) *env* and *pol* were detected by PCR at 2 and 8 days after infection of HEK293T cells. The supernatants from 8-day cultures were used to infect HeLa cells. M, mock infection; 10, infected with ERV-DC10; 18, infected with ERV-DC18. (B) Viruses collected from supernatants were used to detect *env* and *pol* genes by RT-PCR. (C) ERV-DC10 and ERV-DC18 proteins were detected in HEK293T cells using goat antiserum against disrupted RD-114 virions and anti-FeLV gp70 antibody. Anti-RD114 antibodies recognize many ERV-DC proteins (indicated by arrows) but not ERV-DC Env. (D) Susceptibility of cells to infection by ERV-DC10/ERV-DC18. (E) Transmission electron microscopy of retroviral particles in HEK293T cells persistently infected with ERV-DC10. The bar under each panel represents a 100-nm scale.

of ERV-DC18 and FeLV-A clone 61E (55.8% for the entire protein). In addition, the ERV sequences in the house mouse, brown rat, common chimpanzee, and rhesus macaque genomes were also found to be similar to those of ERV-DCs (Fig. 5A and B). Pol from ERV-DC10/DC18 is most highly related to the Pol of endogenous primate viruses, including chimpanzee, rhesus macaque, and baboon. In contrast, Env/TM from ERV-DC10/18 is closely related to FeLV and rodent retroviruses. These results indicate that ERV-DCs should be classified as a strain of gammaretroviruses, and that ERV-DCs or ERV-DC-related viruses have passed through several species. The lack of ERV-DC-like elements in the genomes of species other than primates and rodents suggests that ERV-DCs have spread through interspecies transmission among rodents, primates, and cats. The interspecies transmission of ERV-DCs may make it possible to generate recombinant viruses, and ERV-DCs could be donor and/or acceptor viruses in domestic cats.

Evidence for transduction of ERV-DC *env* into FeLV. Recombination events occur in retroviruses by template switching during viral reverse transcription (7). We discovered from the spleen of an FeLV-positive cat (ON-T; Fig. 6A) a recombinant retrovirus, designated FeLV subgroup D (FeLV-D) (strain FeLV-D/ON-T), containing the *env* gene of ERV-DC and the LTR, *gag*, and *pol* sequences from FeLV. Sequence analysis demonstrated that the *env* gene of FeLV-D was derived from ERV-DCs (see Fig. S3A in the supplemental material), and the LTR of FeLV-D was derived from FeLV (see Fig. S3B). The *gag-pol* gene contained an ORF but also some deletions (Fig. 6A), suggesting that the virus is replication defective. To determine if FeLV-D is expressed in the cat, spleen RNA from the cat ON-T was amplified by reverse transcription-PCR (RT-PCR) using FeLV-D-specific primer pairs corresponding to FeLV 5′LTR and ERV-DC *env* sequences. Major bands corresponding to the genomic RNA and spliced *env* mRNA were detected (see Fig. S4) and confirmed by sequencing. To determine if FeLV-D could be isolated from cells cultured from the spleen of cat ON-T, we homogenized the spleen, filtered the sample using a 0.22-μm filter, and used the homogenate to inoculate

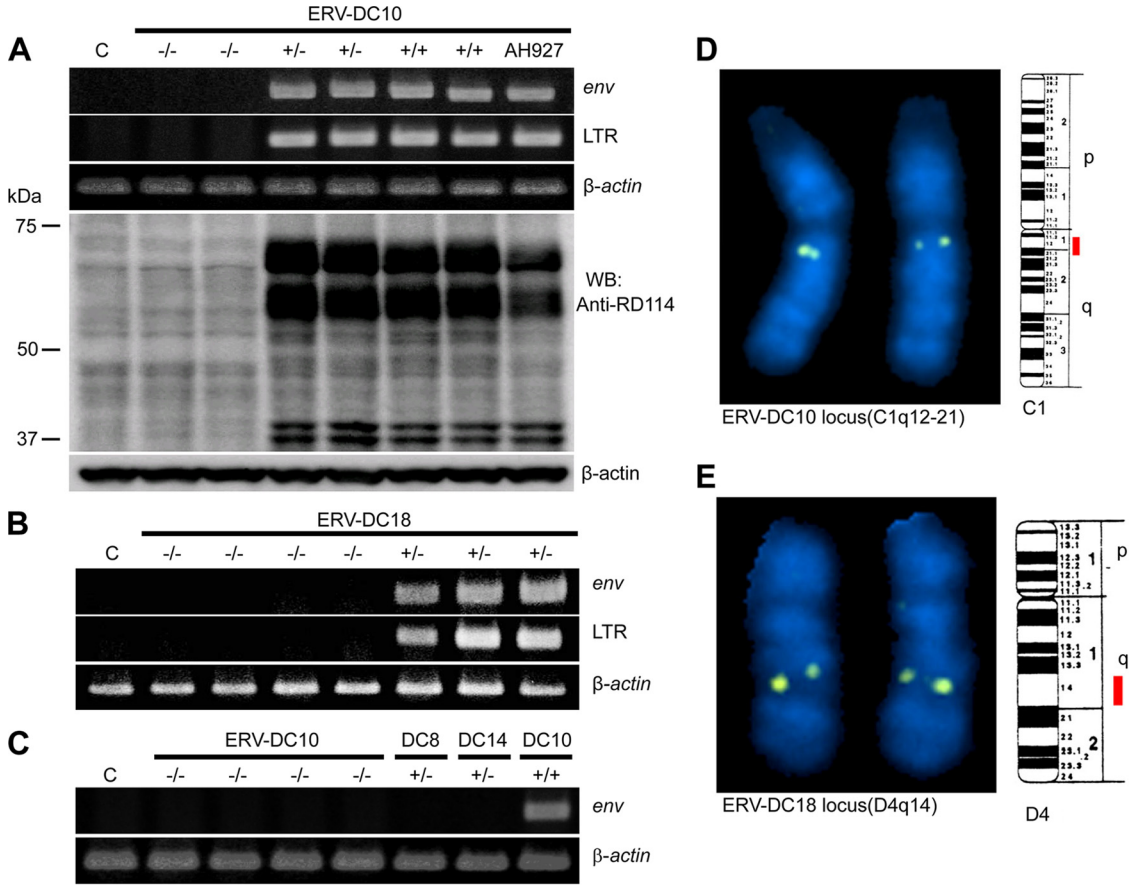


FIG 4 Active loci of ERV-DC10 and ERV-DC18. (A) ERV-DC10, (B) ERV-DC18, or (C) ERV-DC10, -DC8, or -DC14 DNA amplified by PCR with locus-specific primers using feline chromosomal DNA was directly transfected to HEK293T cells, and the filtrated supernatant was used to infect fresh HEK293T cells. Viral infection was monitored by detecting proviral DNAs or viral proteins. Each proviral genotype is indicated as −/− (wild), +/− (heterozygous), and +/+ (homozygous). Independent DNA samples were used in the figures. AH927 is a feline cell line with the ERV-DC10$^{+/+}$ genotype. (D and E) FISH analysis of ERV-DC10 and ERV-DC18 loci on feline chromosomes.

HEK293T cells. FeLV-D, as well as prototype FeLV, was detected at 4 weeks postinoculation by PCR (Fig. 6B). The FeLV-D/ON-T strain was successfully isolated in association with prototype FeLV, and FeLV-D was packaged as a retrovirus. The supernatant from HEK293T cells infected with FeLV-D/ON-T for 6 weeks was used to inoculate fresh HEK293T and HeLa cells, and the virus was successfully transmitted between the cells in culture. We recloned FeLV-D/ON-T (designated FeLV-D/re-T) from the chromosomal DNA of HEK293T cells persistently infected with FeLV-D and verified that the sequences of FeLV-D/re-T and FeLV-D/ON-T are identical (see Fig. S3A). When the DNA from peripheral blood or tumor tissues from 283 other FeLV-positive cats was screened for the presence of FeLV-D, three positive samples were detected (Fig. 6C). One cat (ON-C) was a sibling of ON-T. FeLV-D was molecularly cloned from the three other FeLV-D-positive cats (FeLV-D/ON-C, FeLV-D/Ty26, and FeLV-D/44B) in the region of *env* and 3′LTR (see Fig. S3A, B, and C). A schematic representation of the structures of the *env* genes of the various FeLV-Ds is shown in Fig. 6D. Thus, FeLV-D appears to have been generated by recombination between FeLV and ERV-DC, indicating that ERV-DC can serve as a donor and/or acceptor in the generation of recombinant viruses. The recombination junction between ERV-DC and exFeLV varied among FeLV-D strains, suggesting that FeLV-D was generated *de novo* in each cat. It is not clear whether FeLV-D is an infectious virus by itself or whether it is pathogenic. To determine if the FeLV-D *env* gene plays a role in infection, FeLV-D *env*-expressing pseudoviruses were generated that could transmit β-galactosidase (Fig. 7). The FeLV-D *env*-expressing pseudotype viruses from FeLV-D/ON-T and FeLV-D/Ty26 could infect cells already infected with FeLV-A, -B, or -C or cells persistently infected with ERV-DC10. Therefore, our data show that FeLV-D belongs to a different interference group than ERV-DC10. These results indicate that the receptor usage for both FeLV-D and ERV-DC10 to enter cells is distinct from that of FeLV-A, -B, and -C.

DISCUSSION

This study characterizes 13 endogenous retroviral proviruses in the cat belonging to a class of ERVs, ERV-DC, that has previously been only partially characterized. We show that two of these ERV-DCs, ERV-DC10 and ERV-DC18, are novel feline infectious gammaretroviruses that are inherited on chromosome C1q12-21 and D4q14, respectively. Thus, our study is the first to demonstrate the existence of endogenous, infectious retroviruses in the genome of the domestic cat. Approximately 38% of the cats examined in Japan have heterozygous or homozygous ERV-DC10 loci. Unlike

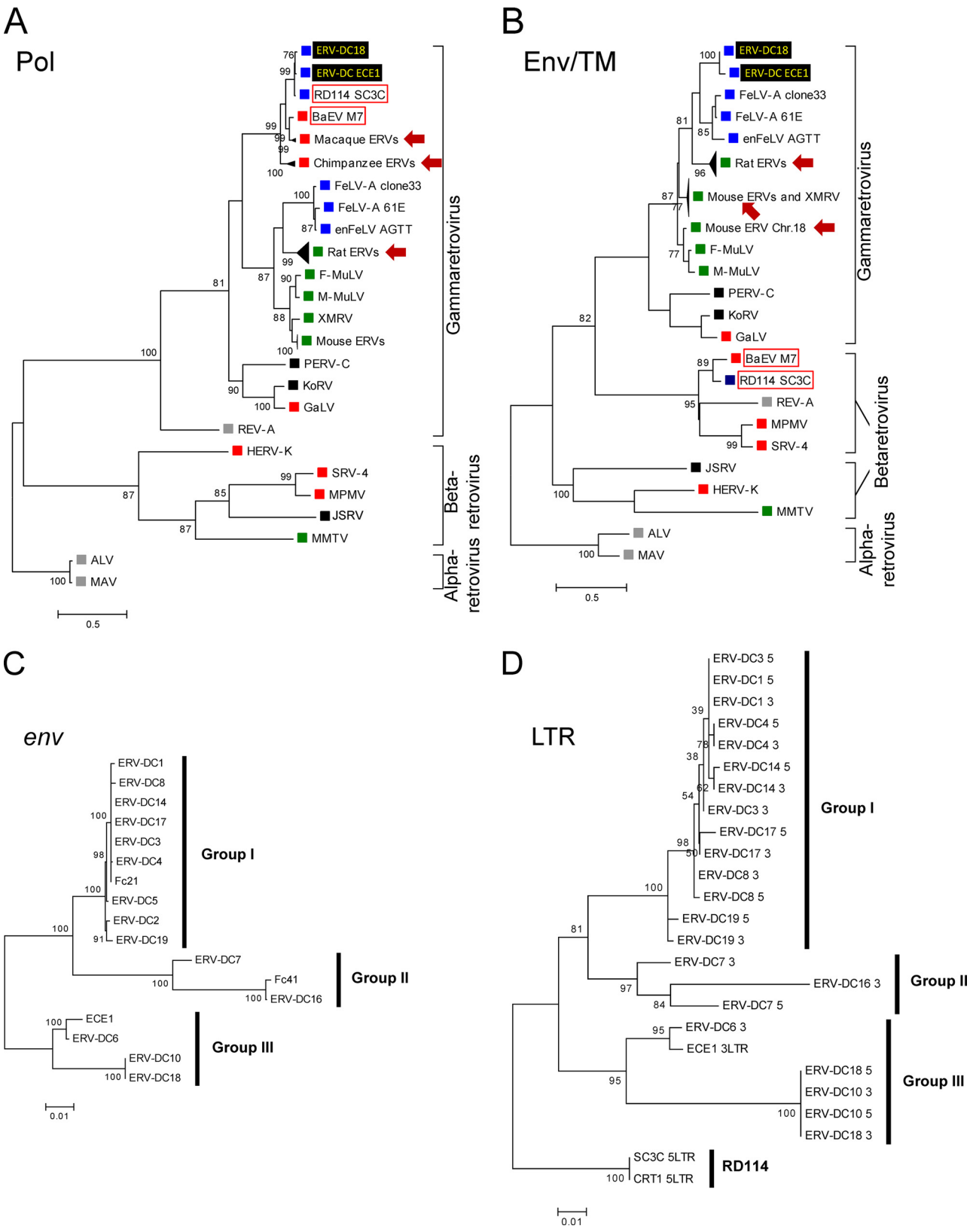


FIG 5 Maximum-likelihood phylogenetic trees of Pol and Env TM proteins. We used amino acid sequences of entire regions of Pol proteins (A) and TM subunits of Env proteins (B) for 46 or 56 endogenous/exogenous viruses (see Table S5 in the supplemental material). A monophyletic subtree of the ERV elements obtained from the same species was compressed into a single branch, as indicated by arrows. Viral origin is indicated as blue (feline), orange (primate), green (rodent), gray (avian), or black (other species). The percent values were determined from 1,000 repeats of fast bootstrapping using RAxML (37) and are indicated at the branch junctions. More than 70 bootstrap values are shown. Maximum-likelihood phylogenic trees of ERV-DC *env* and LTR are indicated in panels C and D. Nucleotide sequences of entire ERV-DC *env* regions (C) or 5′ and 3′ LTRs in ERV-DCs (D) obtained were used. The general time-reversible model with rate heterogeneity among sites (GTR+Γ) was utilized. The percent values were determined from 1,000 repeats of fast bootstrapping using RAxML and are indicated at the branch junctions. Three or 5 indicates 3′ LTR or 5′ LTR. Sequences ECE1 (accession no. X51929), Fc21 (AF155060), Fc41 (AF155061), SC3C (EU030001), and CRT (AB559882) are indicated.

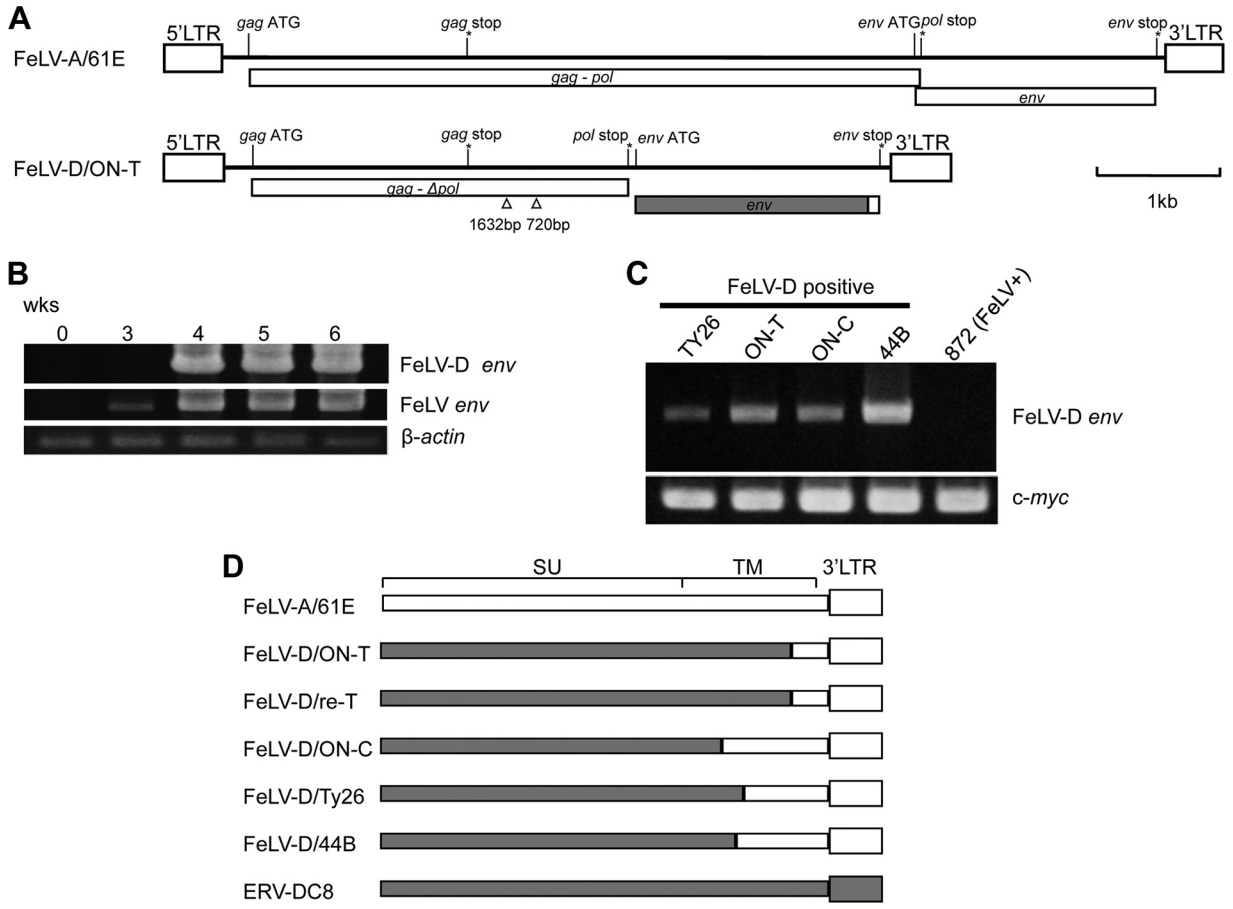


FIG 6 Transduction of ERV-DC *env* into FeLV. (A) Schematic representation of the structure of FeLV-D provirus integrated in spleen DNA of cat ON-T and FeLV-A/61E. White indicates FeLV sequences, and gray indicates ERV-DC sequences. (B) Isolation of FeLV-D in HEK293T cells inoculated with spleen extracts from cat ON-T. FeLV-D- and FeLV *env*-specific PCR was carried out. (C) A total of 283 DNA samples were screened for the presence of FeLV-D, and four positive samples were detected. (D) Structure of FeLV-D *env*. Recombination pattern of each FeLV-D *env* is indicated. White indicates FeLV sequences, and gray indicates ERV-DC sequences.

ERV-DC7, which is fixed in the cat genome but contains deleterious mutations that make it noninfectious, ERV-DC10 has not yet been fixed in the cat genome. It is not known whether ERV-DC10 will eventually disappear or become fixed in cats. Although the open reading frames of ERV-DC8 and ERV-DC14 are intact, they are unable to make infectious viral particles, most likely due to point mutations. It is unclear whether ERV-DCs are harmful or beneficial to the host. However, previous studies have indicated that the presence of other ERVs in vertebrates is associated with protection against infection of related exogenous retroviruses as well as with the generation of pathogenic viruses or tumors (9, 25, 28). Our studies further indicate that ERV-DC10 was not able to produce infectious particles when it was present in some cell types, including feline PBMCs (ERV-DC10$^{+/+}$ genotype) and the feline fibroblast cell line AH927 (ERV-DC10$^{+/+}$), suggesting that the ERV-DC10 promoter can be modified in a tissue-specific manner, similar to the endogenous human ERV syncytin (23), as expression of retroviral genes can be controlled by transcriptional modification (43). Moreover, an infectious provirus not being expressed may be related to its methylation status in the cell, a common mechanism by which endogenous retroviruses are silenced (21). Further investigation will be necessary to understand the gene sequences in ERV-DCs controlling expression and viral production.

Endogenous retroviral sequences have been known to exist in the domestic cat, but until now they have not been identified as encoding infectious viruses. Although RD-114, which was generated by xenotransplantation, is described as an infectious feline ERV (22), an infectious RD-114 provirus has never been detected in the genome of domestic cats. Recent work shows that novel, infectious mouse retroviruses can be generated through *in vivo* passage of human cells in rodents (30). It is therefore possible to generate an infectious ERV through *in vivo* passage in other species.

Our phylogenetic analyses revealed that the *pol* gene of ERV-DCs has high similarity to those of BaEV, RD-114, and primate ERVs, whereas the *env/TM* gene of ERV-DCs is related to those of FeLV, murine leukemia virus, and rodent ERVs. The facts that infectious ERV-DCs are present in domestic cats and that ERV-DC-like elements are detected in cats, rodents, and primates suggest that ERV-DCs have been transmitted to primates or rodents, or vice versa, becoming part of the DNA of the infected species.

FeLV-D was detected and characterized in this study as a recombinant virus generated by transduction of the ERV-DC *env*

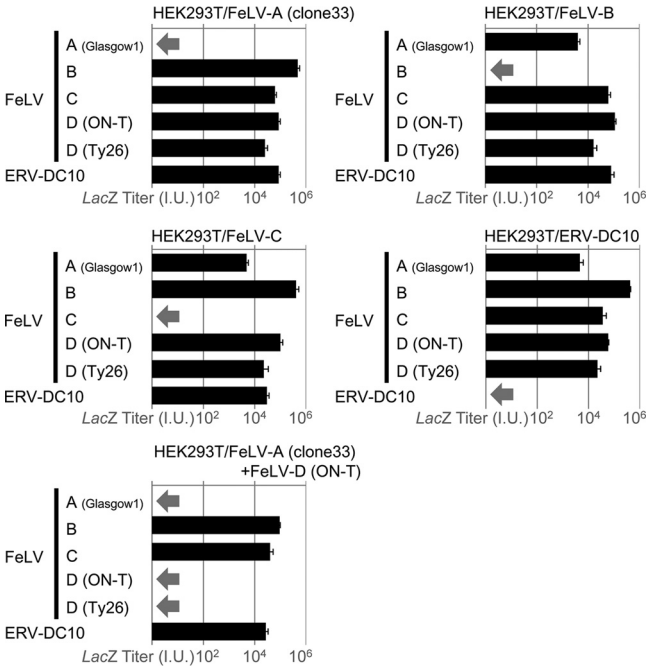


FIG 7 Infection assay of *lacZ* pseudotype virus. FeLV-A/Glasgow-1 *env*, FeLV-B/Gardner-Arnstein *env*, FeLV-C/Sarma *env*, FeLV-D/ON-T *env*, FeLV-D/Ty26 *env*, and ERV-DC10 *env* were used for each pseudotype virus preparation. HEK293T cells infected with FeLV-A/clone33, FeLV-B/Gardner-Arnstein, FeLV-C/Sarma, ERV-DC10, or both FeLV-A/clone33 and FeLV-D/ON-T were used as target cells for an interference assay. X-Gal-positive cells were counted as infectious units (I.U.). Arrow indicates viral interference.

gene into FeLV. This discovery supports the idea that ERV-DCs act as donor and/or acceptor viruses to generate new recombinant viruses. We showed that FeLV-D belongs to a different interference group from that of ERV-DC10, consistent with the fact that ERV-DC10 belongs to ERV-DC group III, whereas the *env* genes in all 4 FeLV-D strains are similar to ERV-DC group I. ERVs belonging to groups I and III may use distinct cell surface receptors, and it will be important to characterize these receptors and assess infectivity of the ERV-DC recombinant viruses. ERV-DC is present in the cat genome with different copy numbers and insertional polymorphisms. Reinfection or infection with ERV-DCs may vary among individual cats based on whether the host genes permit or restrict infection. ERV-DCs are present in domestic cats but not in the Japanese Tsushima leopard cat, *Prionailurus bengalensis euptilurus*. Based on an estimation of viral integration time and ERV-DC LTR sequences, ERV-DCs infected domestic cats within the past 2.8 million years, after separation from the leopard lineage. It will be interesting to determine whether ERV-DC proviruses have played a role in the evolution of cats or in the generation of polymorphisms in the cat genome.

In conclusion, our findings provide evidence for the existence of infectious ERV-DCs in the domestic cat. Because these ERV-DCs are currently being transmitted among cats, and perhaps other species, as infectious ERVs or as recombinant viruses containing ERV-DC sequences, future studies should investigate their evolution and assess the consequences of their presence for the health of domestic cats and other species.

ACKNOWLEDGMENTS

We thank T. Kitamura and M. Nakaya for providing packaging cells and expression plasmids. We also thank N. Oishi (Oishi Veterinary Hospital) and H. Yamamoto (Tsushima Wildlife Conservation Center) for providing blood and tissue samples. We are grateful to Sandra Ruscetti (National Cancer Institute, Frederick) and Kenji Baba (Yamaguchi University) for helpful discussions of the manuscript.

This study was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (K.N.).

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