A rodent or other small animal model for HIV-1 has not been forthcoming, with the principal obstacles being species-specific restriction mechanisms and deficits in HIV-1 dependency factors. Some Carnivora may harbor comparatively fewer impediments. For example, in contrast to mice, the domestic cat genome encodes essential nonreceptor HIV-1 dependency factors. All Feliformia species and at least one Caniformia species also lack a major lentiviral restriction mechanism (TRIM5α/TRIMCyp proteins). Here we investigated cells from two species in another carnivore family, the Mustelidae, for permissiveness to the HIV-1 life cycle. *Mustela putorius furo* (domesticated ferret) primary cells and cell lines did not restrict HIV-1, feline immunodeficiency virus (FIV), equine infectious anemia virus (EIAV), or N-tropic murine leukemia virus (MLV) postentry and supported late HIV-1 life cycle steps comparably to human cells. The ferret TRIM5α gene exon 8, which encodes the B30.2 domain, was found to be pseudogenized. Strikingly, ferret (but not mink) cells engineered to express human HIV-1 entry receptors supported productive spreading replication, amplification, and serial passage of wild-type HIV-1. Nevertheless, produced virions had relatively reduced infectivity and the virus accrued G → A hypermutations, consistent with APOBEC3 protein pressure. Ferret cell-passaged HIV-1 also evolved amino acid changes in the capsid cyclophilin A binding loop. We conclude that the genome of this carnivore can provide essential nonreceptor HIV-1 dependency factors and that ferret APOBEC3 proteins with activity against HIV-1 are likely. Even so, unlike in cat cells, HIV-1 can replicate in ferret cells without *vif* substitution. The virus evolves in this novel nonprimate cell adaptive landscape. We suggest that further characterization of HIV-1 adaptation in ferret cells and delineation of Mustelidae restriction factor repertoires are warranted, with a view to the potential for an HIV-1 animal model.

Exogenous lentiviruses infect vector species in four mammalian orders: Primates, Perissodactyla, Artiodactyla, and Carnivora. Endogenous and now apparently extinct lentiviruses have been identified in several Lagomorpha and lemur genomes (22, 33, 34). Extant lentiviruses exhibit narrow tropisms with no cross-order and highly limited cross-species infection. HIV-1, for example, cannot replicate in a sustained fashion or cause disease in any species besides *Homo sapiens* (3). These impediments have been central considerations for animal model development, and they reflect two complementary issues: viral requirements for specific cellular cofactors and the antiviral activities of species-specific restriction factors such as APOBEC3 proteins, TRIM5 proteins, and tetherin (52, 59, 60, 63). Lentiviruses have evolved counterdefenses to restriction. Impressively, it is now believed that the primary lentiviral accessory genes (*vif, vpu, vpr, vpx, and nef*) are largely devoted to this role (43). Central plus-strand initiation provides an additional defense against APOBEC3G editing of the unduplicated minus strand (28).

Recently, HIV-1 clones that contain only SIV mac *vif* or *vif* and *capsid* sequences were shown to evade macaque TRIM5-alpha and APOBEC3 restrictions (24, 26, 29, 32), and a *vif*-only chimera replicated for up to 6 months in pig-tailed macaques (24). Chronic replication and disease have not yet been observed, but this approach is promising for achieving an HIV-1 animal model, and it highlights the centrality of the known restrictions. In contrast, progress toward transgenic rodent and other common small laboratory animal models for HIV-1 has been confounded not only by multiple specific restrictions but also by complex viral life cycle blocks, particularly to proper particle assembly (5, 9, 17, 45, 64). Such a model would be valuable and informative whether or not macaque HIV-1 models become more fully realized, because of practical limitations intrinsic to research on these nonhuman primates and because of insights that could be gained from observing how the host responds and how HIV-1 evolves as it transitions into a different mammalian order.

Carnivorans comprise over 260 species of placental mammals. They group phylogenetically into two suborders, the Feliformia (Felidae, Hyaenidae, Herpestidae, and others) and the Caniformia (Canidae, Ursidae, Pinnepedia, Mustelidae, etc.). Variants of feline immunodeficiency virus (FIV) currently infect approximately half of Felidae, and FIV-ancestral lentiviruses have been endemic in the *Panthera* (lion) lineage since at least the late Pleistocene and perhaps earlier (4, 53, 68). AIDS very similar to the human syndrome results in one feline species, the domestic cat, in which the virus is pandemic and acquisition occurred relatively recently. Differences in the respective host-lentiviral equilibria of Primates and Carnivorans are also informative and potentially exploitable. For example, considering restriction factors, Feliformia lack functioning antiviral Trim5α or TRIMCyp genes (48), as does at least one Caniformia species, the dog (57). The domestic cat does have an effective APOBEC3 repertoire that restricts HIV-1 (19, 49, 50, 62). However, when FIV Vif was stably expressed in *trans* in a feline cell line (CrFK) that also expressed HIV-1 entry receptors, productive spreading replication was enabled (62). *vif*-chimeric HIV-1 clones that encode FIV Vif in *cis* replicated in such cells, too (62, 73). The most important implication of these results is that,
except for entry receptors, the domestic cat genome can supply the dependency factors needed for HIV replication, which is a fundamental difference from the mouse (9). SIVmac Vif was also effective in mediating feline APOBEC3 evasion, showing for the first time that a Vif could function effectively in a different mammalian order (62). Since corroborated for SIVmac Vif and extended to visna virus Vif as well (38), this is an exception to the general theme of narrow species specificity in evolved retroviral evasions.

Based on these results, we here examined cells of a different carnivore family, Mustelidae (suborder Caniformia). There are good precedents for effective Mustelidae models of human viral diseases. One species, the domesticated ferret, is a favored experimental host for studies of important human RNA virus pathogens (influenza virus, severe acute respiratory syndrome [SARS] coronavirus, and Nipah virus). No Mustelidae antiretroviral restriction factors have been cloned or characterized.

MATERIALS AND METHODS

HIV-1 entry receptor-expressing stable Mustelidae cell lines. Adherent cell lines and T cell lines were maintained in Dulbecco modified Eagle medium (DMEM) and RPMI 1640 medium, respectively, with 10% heat-inactivated fetal calf serum (FCS), penicillin-streptomycin, and l-glutamine. Mpf, a ferret (Mustela putorius furo) brain-derived cell line, and Mv.1.Lu, an American mink (Neovison vison, formerly Mustela vison) fetal lung-derived cell line, were obtained from ATCC. The M. putorius furo lung cell line, PAEpC, was recently derived as described previously (37). Mpf.CD4.X4 cells were derived in two selection steps, with FIV-based lentiviral vectors (55) used consecutively as described previously (62). One vector encoded hCD4 plus neo (G418 resistance), and the second encoded hCXCR4 plus pac (puromycin resistance), with each receptor and resistance gene linked by an intervening internal ribosome entry site (IRES). Cells were selected and maintained in 1 mg/ml G418 and 1 μg/ml puromycin. To establish Mpf.CD4.X4, PAEpC.CD4.X4, and Mv.1.Lu.CD4.X4 cell lines, a single HIV-1-based lentiviral vector derived from TSINcherry (40) was used; the transfer vector has the following elements in series: hCD4-porcinie teschovirus 2A (P2A) peptide-hCXCR4-IRES-pac. Cell surface expression was verified by flow cytometry using mouse anti-hCXCR4 (RD Systems) and anti-hCD4 (BD Biosciences Pharmingen; phycoerythrin and fluorescein isothiocyanate [FITC] conjugated, respectively). Competence for gp120-mediated entry was assayed by infecting with an HIV-1 LAI luciferase reporter virus kindly provided by M. Emerman (54). Luciferase activities were determined by infecting with an HIV-1 luciferase reporter virus, and a time zero p24 sample was collected. Cultures were washed 24 to 36 h later with DMEM five times to remove input virus, and a time zero p24 sample was collected. Cultures were maintained by splitting them 1:5 or 1:10 when confluent, and supernatants were sampled every 2 to 4 days for p24 measurements. Supernatants were filtered (0.45 μm) before passage to uninfected cells.

Hypermutation analysis. Virus particles were pelleted by ultracentrifugation over a sucrose cushion for 2 h at 25,000 rpm. Viral RNA was isolated (RNasey; Qiagen), and reverse transcribed with a Transcriptor first-strand cDNA synthesis kit (Roche). Genomic segments spanning gag-pcr and the 5’ and 3’ long terminal repeat (LTR) and leader were amplified with Phusion Hot Start DNA polymerase. Products were gel purified and cloned (StrataClone Ultra Blunt PCR cloning kit; Stratagene). Eight to 10 independent clones for each virus were sequenced.

Cloning of an Mpf cell cyclophilin A (CypA) cDNA and ferret TRIM5α exon 8 sequences. Degenerate primers were designed from the canine and feline sequences. The forward primer, which contained a hemagglutinin (HA) epitope tag, was FfHuFerCypA (5’-ATATGATGCCAC ATGTACCTAGATYTGTCCACAGTCAGCAATGG-3’). The reverse primer was KpnFfHuFerCypA (5’-CTCTCGTGATATCAGCACTTCTT-3’). Mpf, PAEpC, and Mv.1.Lu TRIM5α exon 8 sequences were isolated using the primers described by McEwan et al. (47, 48), i.e., gex8 fex5, ATCCCTTYYTACGGTACACA, and gex8 fex5, MATGAARAGAYKATATAGCAGAA ACC, where M = A/C, K = G/T, R = G/A, and Y = C/T.

Caspid mutants H87Q and A92T. The H87Q and A92T caspid mutants were constructed in the HIV-1-GFP or NL4-3 backbone by site-directed mutagenesis using the QuickChange Lightning site-directed mutagenesis kit (Agilent). For virus-like particle (VLP) saturation assays, a fixed dose of GFP-encoding vector was coinfected with 4-fold serial dilutions of VSV-G-pseudotyped HIV-1 vector encoding pac. Cyclosporine (CSA; Paddock Laboratories) was obtained from the Mayo Clinic pharmacy and used at 5 μM. GFP-positive cells were counted by fluorescence-activated cell sorting (FACS) 48 after transduction.

Immunoblots. Cells were lysed in RIPA buffer (150 mM NaCl, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 1% NP-40, 150 mM Tris-HCl, pH 8.0) with protease inhibitors (Complete Mini; Boehringer). Protein was quantified with the Bradford assay. Twenty micrograms of lysate was boiled in Laemmli buffer with β-mercaptoethanol for 10 min and subjected to 10% SDS-polyacrylamide gels for immuno blotting. Control supernatants from uninfected cells were collected and processed the same way.

Vectors and viruses. HIV-1 (HIV-Luc) and HIV-1 (HIV-Luc), the vesicular stomatitis virus G protein (VSV-G)-pseudotyped NL4-3 (EΔ426 and NL4-3; EΔ426 luciferase reporter viruses, have been described previously (40). Replication-competent NL4-3 clones that express the Vif proteins of HIV-1 NL4-3 or SIVmac239 (HIV-1VH and HIV-1VS) from a Vif frame engineered to not overlap integrase are those of Stern et al. (62). TRIP-luc was constructed by exchanging firefly luciferase for gfp in TRIP-green fluorescent protein (GFP), a gift of Pierre Charneau. Replication-competent viruses were produced by transfection of 293T cells with 10 μg plasmid DNA in 75-cm² flasks. Particle normalization utilized reverse transcription (RT) activity or p24 antigen. RT activity was determined using a 3H-based RT assay as described previously (40). p24 antigen was measured using the ZetaPlex enzyme-linked immunosorbsent assay (ELISA) kit. Mean p24 ± SD from duplicate measurements for each sample was calculated. Infectivity per ng of p24 was determined by titration on GHOST cell lines according to the NIH AIDS Research and Reference Reagent Program protocol. For infections with p24-normalized viruses, 3 × 10⁵ entry receptor–complemented cells were infected in six-well plates. The cells were washed 24 to 36 h later with DMEM five times to remove input virus, and a time zero p24 sample was collected. Cultures were maintained by splitting them 1:5 or 1:10 when confluent, and supernatants were sampled every 2 to 4 days for p24 measurements. Supernatants were filtered (0.45 μm) before passage to uninfected cells.

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and then electrophoresed in 12% Tris-HCl gels (Bio-Rad) and transferred over 1 h to Immobilon P membranes (Millipore). The blocked membranes were incubated for 2 h with the primary antibody (Ab) anti-CypA (Santa Cruz rabbit polyclonal 133494) at 1:250 and then washed with Tris-buffered saline–Tween 20 (TBST) three times for 7 min each. Afterward, membranes were incubated for 2 h at room temperature with the secondary Ab, goat anti-rabbit–horseradish peroxidase (HRP) (Calbiochem), at 1:4,000. After being washed with TBST 3 times for 10 min each, membranes were incubated in SuperSignal West Pico chemiluminescent substrate (Pierce) for 1 to 2 min and exposed to film. Human and ferret primary mononuclear cell lysates and supernatants were electrophoresed in 10% Tris-HCl gels (Bio-Rad) and transferred over 1 h to Immobilon P membranes (Millipore). Blocked membranes were incubated overnight with primary anti-p24 (mouse monoclonal, Abcam 9071) at 1:2,000 and then washed with TBST three times for 7 min each. Afterward, membranes were incubated for 2 h at room temperature with the secondary Ab, goat anti-mouse–HRP (Calbiochem). After being washed with TBST 3 times for 10 min each, membranes were incubated in Lumi-lightplus Western blot substrate (Roche) for 1 to 2 min and exposed to film.

**Nucleotide sequence accession number.** The sequence of exon 8 of the ferret TRIM5α gene from ferret (Mpf and FAEpC) cells was deposited in GenBank under accession no. JQ048543.

**RESULTS**

Pseudotyped luciferase (luc) reporter viruses and vectors were used initially to compare human, rodent, and carnivore cell lines (Fig. 1). These included three lines from two Mustelidae species: a recently established ferret (Mustela putorius furo) lung cell line (FtAEpC cells [37]), an M. putorius furo brain cell line (Mpf cells...
and an American mink (*Neovison vison*, formerly *Mustela vison*) fetal lung cell line (Mv.1.Lu cells [27]). In agreement with a previous study that included the latter two (65), we found that all three Mustelidae cell lines as well as dog and cat cells supported equivalent N- and NB-murine leukemia virus (MLV) infection, whereas N-MLV-restricting human HT1080 cells were much less efficiently infected with N-MLV (Fig. 1A). The Mustelidae and two other carnivore lines were also readily susceptible to HIV-1/Δluc reporter virus infection, yielding luciferase activities that equaled or exceeded those of all human cells infected with the same inputs (Fig. 1B and C). Since Δluc is expressed from the nef open reading frame in HIV-1/Δluc (40), this virus demonstrates competence for the following postentry stages: reverse transcription, integration, and Tat/U3-promoted early (Rev-independent) viral gene expression (40). Similar results were observed when primary human PBMC and primary ferret mononuclear cells obtained from spleen, bone marrow, and lymph nodes were compared (Fig. 2A). Furthermore, similarly equivalent transduction was observed in ferret cell lines with a genome-minimized trans-packaged HIV-1 vector in which an internal human cytomegalovirus (CMV) promoter drives expression (Fig. 1D) and with analogously organized single-cycle FIV and equine infectious anemia virus (EIAV) vectors (Fig. 3A and B).

Using primers validated by McEwan et al. to amplify TRIM5α exon 8 from mink, dog, and various feline species genomic DNA (47, 48), we amplified and sequenced exon 8 of the ferret TRIM5α gene from ferret (Mpf and FtAEpC) cells (see above) and found that the Feliformia-specific premature stop codon (48) is lacking, as was previously reported for the dog and mink exons (48, 57). However, multiple other stop codons were present in all reading frames, indicating pseudogenization as in the dog (57). No ferret TRIMCyP transcript could be identified by PCR using primers anchored in the ferret CypA sequence (determined in the present study; see below) and sets of degenerate primers homologous to Carnivora and human exon 2 (data not shown). Late HIV-1 life cycle events were assessed initially by measuring HIV-1 p24 production. Mouse (3T3) cells displayed the previously well-established (9, 17, 46, 64) assembly block to HIV-1, whereas Mustelidae cell lines and primary cells yielded robust HIV-1 p24 production similar to that of human cells (Fig. 1E and Fig. 2B and

**FIG 2** Early and late HIV-1 viral gene expression in primary human and ferret cells. Primary mononuclear cells from human peripheral blood and ferret spleen, bone marrow, and lymph nodes were transduced with increasing doses of VSV-G pseudotyped reporter virus HIV-1/Δluc. (A) Luciferase activity was measured at 5 days posttransduction in cell lysates. (B) Immunoblotting for HIV-1 capsid protein. (C) p24 antigen measured at day 5 posttransduction, in supernatants of the respective cells transduced in panel B.

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C). Taken together, these data indicate that major pre- and postintegration portions of the HIV-1 life cycle are grossly unimpaired in each of the three Mustelidae cell lines tested as well as in primary ferret mononuclear cells derived from spleen, bone marrow, and lymph nodes. There is an absence of TRIM5α/TRIMCyp/Fv1-type postentry blocks to lentiviral and gammaretroviral life cycles, substantial Tat transactivation function, and substantial Rev-mediated protein production, assembly, and particle release in these cells.

Based on these experiments, we proceeded to test directly whether mink or ferret cells could support productive, spreading replication of HIV-1. We derived stable cell lines that express human CD4 and CXCR4 and verified cell surface expression of these proteins by flow cytometry (Fig. 4A). Entry receptor competence was verified by infecting the receptor-complemented cells with HIV-1 LAI-luc (69) (Fig. 4B). The Mustelidae cells were then challenged with HIV-1 NL4-3 (2) and two variants of NL4-3, HIV-1VH and HIV-1VS, and HIV-1Vs was observed in ferret but not human cell lines. Figure 4F shows GHOST cell titrations including HIV-1 first passaged through the ferret cell lines (data not shown). This change, effectively a reversion to wild type, was found at the 5’ end of vif in 8/10 clones and was likely consequent to the duplication of 5’-GGAAAAACAG-3’ (56, 62) at the 5’ end of vif in the input virus, which facilitated recombination during reverse transcription (72) to produce the virus illustrated in Fig. 6B. While G-to-A changes predominated, other kinds of mutations were also seen (Fig. 6A). For example, as previously observed to happen with rat and rabbit APOBEC1 (10, 30), a significant number of plus-strand C-to-T mutations were observed (45, versus 161 G-to-A changes). This outcome might reflect RNA deamination as well (10, 30). The dinucleotide contexts observed for cytidine deamination can vary substantially with different APOBEC proteins (6). Here in ferret cells, it was different from the pattern typically seen with human A3G, where CC and TC (edited minus-strand cytidine underlined) contexts predominate (23, 44). In HIV-1VH(mpfP3), a vif reading frame-preserving closure of the artificial integrase-vif separation arose through deletion of a 62-nucleotide (nt) segment (Fig. 6B and its legend). This change, effectively a reversion to wild type, was found at the 5’ end of vif in 8/10 clones and was likely consequent to the duplication of 5’-GGAAAAACAG-3’ (56, 62) at the 5’ end of vif in the input virus, which facilitated recombination during reverse transcription (72) to produce the virus illustrated in Fig. 6B. Nevertheless, we found that virions produced in these cells were still less infectious per unit of p24 antigen than were virions produced in human cell lines. Figure 4F shows GHOST cell titrations of third-passage viruses from the ferret cell line Mpf.CD4.X4, which we refer to as HIV-1VH(mpfP3) and HIV-1VS(mpfP3). Comparison is made to virus produced in maximally permissive human 293T cells. This producer cell-dependent loss of infectivity in ferret cells suggested that APOBEC3 proteins may be targeting HIV-1. Therefore, we amplified and sequenced long terminal repeat (LTR) and gag/pol-vpr segments of HIV-1VH(mpfP3) and HIV-1VS(mpfP3) genomes as well as from later FtAEpC cell passages (Fig. 6A). A signature of APOBEC3 protein activity, G—A hypermutation, was observed. This genome editing is nevertheless manifestly not lethal since HIV-1 was still amplified exponentially and passed robustly in ferret cell lines. Another finding was a premature stop codon in vpr, a frequent occurrence when HIV-1 is passaged in any cultured cells (51). In HIV-1VH(mpfP3), a vif reading frame-preserving closure of the artificial integrase-vif separation arose through deletion of a 62-nucleotide (nt) segment (Fig. 6B and its legend). This change, effectively a reversion to wild type, was found at the 5’ end of vif in 8/10 clones and was likely consequent to the duplication of 5’-GGAAAAACAG-3’ (56, 62) at the 5’ end of vif in the input virus, which facilitated recombination during reverse transcription (72) to produce the virus illustrated in Fig. 6B.

![FIG 3 FIV and EIAV infection. The indicated cells were transduced with increasing doses of luciferase-encoding single-cycle vectors derived from FIV (A) or EIAV (B). Cell lysates from equal numbers of cells were assayed for activity 72 h later.](http://jvi.asm.org/Downloaded from http://jvi.asm.org.org/jvi.org/Downloaded from October 23, 2017 by guest)
viruses. However, capsid did. Two coding changes arose in the cyclophilin A (CypA) binding loop, which is complexly involved in the viral life cycle, including in TRIM5 protein restriction (41). For HIV-1VH(mpfP3), a T→A change at nt 261 in capsid and, for HIV-1VS(mpfP3), a G→A mutation at nt 274 produced, respectively, H87Q and A92T mutations (Fig. 6C). As the selection of two different mutations in this functionally significant region of capsid after passage in ferret cells was intriguing, we introduced them prospectively, alone and in combination, into HIV-1 NL4-3 reporter viruses and full-length clones. Examined in this genetically defined context, the HIV-1 capsid mutants were found to have moderately increased infectivity in ferret cells compared to wild type (WT) (Fig. 7A).

In the case of replicating virus, capsid mutants replicated to higher peak levels than did wild-type virus in ferret cells (Fig. 7B). We then tested effects of cyclosporine (Fig. 8A through C). In these experiments, the increase in infectivity conferred by the CypA binding loop mutations was again observed in ferret cells and also in owl monkey kidney (OMK) cells (Fig. 8A through C). CsA, which disrupts TRIMCyp restriction (59), produced the well-known dramatic augmenting effect in OMK cells for wild-type NL4-3 and each of the mutants (Fig. 8A). The moderately increased infectivity of H87Q in the absence of CsA in these experiments (Fig. 8A) is consistent with that observed previously in OMK cells (31). In clear contrast, CsA did not boost infectivity in ferret cells for any of the viruses (Fig. 8B and C); rather, a slight inhibitory effect was discernible, particularly for A92T. Since levels of CypA have been reported to play a role in determining HIV-1 infectivity in certain contexts (70), we performed immunoblotting for this protein. CypA was clearly present in the ferret cells, and its levels were also similar to those in human cells (Fig. 8D). A ferret CypA cDNA was isolated by reverse transcriptase PCR (RT-PCR) (Fig. 8E); sequencing and determination of the predicted amino acid sequence did not reveal significant differences in the known HIV-1 capsid-interacting regions (13, 20, 71).

To complete the analysis with respect to the capsid mutants, VLP saturation experiments were performed. A dose-dependent, clear VLP saturation effect was observed in rhesus FrHK4 cells as anticipated (8, 16), but no such effects occurred in ferret cells (Fig. 9A to D).

DISCUSSION

The results of this study show that, unlike the mouse genome (9), the ferret genome can supply the nonreceptor dependency factors needed for productive, spreading HIV-1 replication. Moreover, in contrast to cells of another carnivore that share this property (62,
73), vif gene substitution was not needed and wild-type HIV-1 was capable of replication and serial passage. As in feline cells, G→A hypermutation and producer cell-dependent infectivity reductions were observed, but in both ferret cell lines they did not prevent productive viral replication. The selection of capsid mutations is also strong corroborative evidence that continuous viral replication occurred. Whether the HIV-1 or SIVmac Vif protein produces partial APOBEC3 mitigation in ferret cells, or might be evolved by repeated passage to acquire it, deserves further specific analysis. The absence of postentry capsid-targeting defenses against N-MLV and lentiviruses is consistent with the apparent lack of an intact Trim5α or TRIMCyp gene in this species. We observed similarly robust completion of early and late events in primary ferret cells (Fig. 2). We were not able to complement primary ferret mononuclear cells with HIV-1 receptors, and lymphoid-lineage cell lines are not yet available. Therefore, the extent to which specific relevant primary cell types (CD4+ T cells) in ferrets in vivo express all needed nonreceptor dependency factors and/or might manifest additional restrictions will be a worthy subject for further study.

The capability to repeatedly passage a primate lentivirus through the novel adaptive environment of a nonprimate cell allows experimental selection for continued viral evolution. So far, after three passages, we have observed selection of two capsid CypA binding loop mutations, H87Q and A92T. We found that these confer moderately increased infectivity in ferret cells and

FIG 5 Productive HIV-1 replication in ferret cells. (A and B) Mpf.CD4.X4, cells were infected with HIV-1VH and HIV-1VS, and virus produced was serially passaged 5 additional times. Input inocula were 1 ng p24. (C) Mpf.CD4.X4, cells were infected with 10 ng p24 of HIV-1 NL4-3, and virus produced was serially passaged 5 additional times. (D and E) Third-passage HIV-1 HIV-1VH(mppP3) and HIV-1VH(mppP3) viruses from Mpf.CD4.X4, replication experiments were serially passaged 4 times on FAEpC.CD4.X4 cells. The passage numbers above the curves reflect the total passages on both ferret lines, while the numbers in the symbol keys refer to the number of passages on FAEpC.CD4.X4 cells. (F) Infectivity determined by titration on GHOST cells.

FIG 6 Sequencing of HIV-1VH(mppP3) and HIV-1VH(mppP3) viruses isolated from passage 3 of HIV-1VH and HIV-1VH on the Mpf.CD4.X4, cell line. (A) Sequencing of 290,607 nt (8 to 10 clones per segment) was performed, revealing 161 G→A changes. (B) Recombinant HIV-1VH found in 8/10 clones. Capital letters indicate original mutations used to separate the integrase and vif frames. (C) Capsid mutations selected in the unstructured CypA binding loop of HIV-1 CA. H87Q and A92T were present in 10 of 10 and 9 of 9 sequenced clones, respectively.
that disruption of CypA interaction with CsA only slightly affects this (Fig. 7 and 8). The reasons that these mutations arose are not clear because of the ambiguities that persist about the roles that CypA plays in retroviral life cycles, but they may represent optimal fitness of the CypA binding loop in the presence of CypA but in the absence of any functionally antiviral TRIM5 protein. CypA, which is highly conserved between mammals, is a peptidyl-prolyl isomerase that binds lentiviral capsids. It catalyzes the cis/trans isomerization of the G89-P90 peptide bond in the HIV-1 capsid protein (11). CypA has distinctive and at times opposite context-dependent effects (18, 20, 42). The protein promotes infectivity in human cells (25, 61, 66) but is in contrast necessary for Trim5α restriction in rhesus macaque and African green monkey cells (7). While multiple possibilities have been proposed for the role of CypA in the viral life cycle, a unifying mechanistic explanation remains elusive. It has been hypothesized that this peptidyl-prolyl isomerase protects HIV-1 from human cell restriction by competing with Trim5α restriction in rhesus macaque and African green monkey cells (7). A92T has not been previously reported, but a charge-adding mutation at this residue, A92E, is known to arise when HIV-1 is passaged in HeLa cells in the presence of CsA, thus conferring CsA resistance and in some cell lines actually conferring CsA dependence on the virus (1, 12); this phenomenon was later shown to reflect high levels of CypA in HeLa cells (70). Our experiments make it clear that CypA is present at substantial levels in the Mus-

FIG 7 Infection of ferret cells with WT, H87Q, A92T, and H87Q/A92T NL4-3 clones. (A) HIV-1.rep reporter viruses. The number of GFP-positive cells was determined by FACS at 48 h. The upper and lower graphs show the results of two independent experiments with different vector preparations. Student’s 2-tailed \( t \) test was used to determine \( P \) values for comparisons of the titers of WT virus with each of the capsid mutants. All calculated \( P \) values were < 0.05 except for the comparison of WT and H87Q/A92T mutants in FtAEpC cells (asterisk, upper right plot; \( P = 0.07 \)). (B) Full-length WT and mutant viral clone challenges of Mpf.CD4.X4, cells.
telidae cell lines that we used (Fig. 8D) and also that its amino acid sequence is conserved versus human CypA in the hydrophobic regions known to form the retroviral capsid-interacting domain (13) (Fig. 8E). Thus, these mutations that developed in ferret cells may represent HIV-1 adaptation to a situation where CypA is present but there is no Trim5 protein pressure.

In contrast to ferret cells, we did not observe spreading HIV-1 replication in the mink (Mv.1.Lu.CD4.X4) cell line. This result,
which was verified in repeated experiments, is at variance with a prior report (35). As was discussed previously (62), it may be possible to reconcile these differences by considering that a single round of provirus generation and p24 production occurred in the experiments of Koito et al. (35).

Our results add to emerging evidence that cells of carnivore species appear in general to harbor relatively few restrictions to HIV-1 replication, and prominent among these are APOBEC3-mediated restrictions. Reference 19 provides a recent review of carnivore cell restrictions. We also conclude that, like the domestic cat and unlike the mouse, the ferret genome encodes the major nonreceptor dependency factors for this primate lentivirus. There are robust precedents for modeling human RNA pathogens in the ferret. A ferret genome sequencing project is nearing completion (http://www.broadinstitute.org/scientific-community/science/projects/mammals-models/ferret-genome-project/).

We suggest that further characterization of HIV-1 adaptation in ferret cells and delineation of Mustelidae restriction factor gene repertoires are warranted, with a view to several possible benefits. These include potentials for developing an eventual HIV-1 animal model, for gaining basic insights into mechanisms of species-specific retroviral restriction, and for exploring the extent to which HIV-1 will evolve when confronted with the novel adaptive landscape of a nonprimate cell.

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