VIROLOGY 233, 185–192 (1997)
ARTICLE NO. VY978587

Growth of Lion and Puma Lentiviruses in Domestic Cat Cells and Comparisons with FIV

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Received January 13, 1997; returned to author for revision February 12, 1997; accepted April 9, 1997

Feline immunodeficiency virus (FIV-Fca) is a lentivirus that causes gradual immunological deterioration in domestic cats. Lentiviruses related to FIV have been detected in several nondomestic feline species; the biologic significance of these viruses remains to be defined. To examine the *in vitro* cell tropism of these nondomestic cat lentiviruses, prototypical puma and lion lentiviruses (FIV-Pco and FIV-Ple) were cultured in a variety of feline cell cultures. A domestic cat T lymphoma cell line, 3201, best supported the replication of both FIV-Pco and FIV-Ple. Moreover, FIV-Ple was lytic for these cells. RT-PCR amplification of a conserved *pol* gene region demonstrated species-specific primer homology. Sequence and phylogenetic analyses of this amplification product confirmed the identity of the replicating viruses and classified two previously uncharacterized viruses within predictable lion and puma clades. Sequence analysis of a conserved *pol* region demonstrated homology with previously characterized FIV-Ple and FIV-Pco. Western blot analysis using domestic cat anti-FIV-Fca sera showed that both FIV-Pco and FIV-Ple were antigenically related, to differing degrees, to three serotypes of FIV-Fca. These studies demonstrate that though nondomestic cat lentiviruses differ significantly from FIV-Fca and that a viral-specific protocol may be necessary for sensitive viral detection, these viruses can replicate in cells of domestic cats, suggesting the potential for cross-species transmission. © 1997 Academic Press

INTRODUCTION

Serologic surveys of 27 nondomestic feline species have revealed that a minimum of 17 species display antibodies cross-reactive with FIV-Fca antigens (Barr *et al.*, 1989, 1995; Brown, 1993a, 1993b, 1994; Carpenter *et al.*, 1996; Letcher and O'Conner, 1991; Lutz *et al.*, 1992; Olmsted *et al.*, 1992; Spencer *et al.*, 1992). Further, some populations of endangered feline species demonstrate a high prevalence of seropositivity (Brown *et al.*, 1994; Olmsted *et al.*, 1992). Genetic characterization of puma, lion, and pallas cat isolates has determined that these viruses are distinct from each other and related to the lentivirus of the domestic cat, feline immunodeficiency virus (FIV) (Barr *et al.*, 1995; Brown *et al.*, 1994; Carpenter *et al.*, 1996; Olmsted *et al.*, 1992). The pathogenic nature of these viruses in their natural hosts has not been fully characterized; while one report associated lymphoma in a captive lion with an FIV-like lentivirus, other analyses have not correlated specific disease expression with seropositivity to these (Brown *et al.*, 1994; Olmsted *et al.*, 1992; Poli *et al.*, 1995).

In domestic cats, naturally occurring FIV-Fca infection usually leads to long clinical latency during which a gradual immunological deterioration is marked by manifestations similar to those of HIV-induced AIDS (Pedersen, 1993; Sparger *et al.*, 1989). Five subtypes of FIV-Fca (FIV-A, B, C, D, and E) have been identified thus far based on V3-V5 *env* sequence analysis (Kakinuma *et al.*, 1995; Sodora *et al.*, 1994; Yamada *et al.*, 1995). One of these subtypes, FIV-C, has been identified only in cats from British Columbia, and one FIV-C isolate has shown greater virulence than other FIV-Fca after serial *in vivo* passage in experimental studies (Diehl *et al.*, 1995). Although not supported in the analysis of *pol* genes (Carpenter *et al.*, 1996), serologic surveys of domestic cats and pumas from this area suggest a geographic component in viral associations between strains from these two species (Langelier *et al.*, 1995).

Phylogenetic analyses of FIV *pol* gene sequences from four Felidae species (domestic cat, lion, puma, and leopard) have revealed extensive genetic polymorphism and widespread geographic distribution of divergent FIV-Fca-*pol*-defined phylogenetic clusters or clades (Brown *et al.*, 1994; Carpenter *et al.*, 1996; Olmsted *et al.*, 1992). Large sequence divergence, broad geographic dispersal and nearly complete species monophyly of FIV, particularly in lions, pumas, and leopards, suggest that the FIV-related lentivirus has been endemic within these species for a long period with cross-species transmission being very rare. Nonetheless, a single case of a domestic cat strain of

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FIV infection in a captive puma housed in a zoo in Lima, Peru, has been observed (Carpenter *et al.*, 1996). Sequence analyses of lentivirus *pol* sequences from wild cat species suggest that although divergence is monophyletic within species, the closest relative to any felid species' FIV strain is that from another cat species.

Characterization of nondomestic cat lentiviruses has been limited by difficulty in propagating these viruses *in vitro* as well as lack of virus-specific reagents (Brown *et al.*, 1994; Lutz *et al.*, 1992). The present study was undertaken to more closely examine the growth characteristics and properties of FIV-Ple and FIV-Pco in order to aid further studies of these viruses both *in vitro* and *in vivo.*

MATERIALS AND METHODS

Viruses and cells

Prototypic strains of puma lentivirus (FIV-Pco) were obtained from Dr. Stephen J. O'Brien, Dr. Ken Langelier, or Dr. William D. Hardy. These samples were supplied as a puma PBMC culture supernatant (PLV-14 from a Florida panther, supplied by S.J.O.), heparinized blood samples obtained from free-ranging British Columbia cougars (K.L.), or a 3201 cell line infected with a British Columbian cougar isolate (W.D.H.). The PLV-14 genome has been fully sequenced (Langley *et al.*, 1994); other isolates had not been genetically characterized. A Serengeti lion PBMC supernatant (Ple-458) was also obtained from S.J.O. (Brown *et al.*, 1994). This sample had been shown to be EM-positive for lentivirus particles but had not been genetically characterized. The FIV-Fca (domestic cat FIV) supernatant used for a source of virus for controls in RT assays, antigen-capture ELISA, and RT-PCR was a field strain of FIV-Fca inoculated into a naive cat (FIV-2546, subtype A). Virus isolated from PBMC of this animal was used to infect Crandell feline kidney cells in high-serum medium. Anti-FIV-A, B, and C sera were collected from cats infected with field strains of each subtype; naive serum from an uninfected cat was used as a negative control. Naive cat PBMC were collected from SPF cats. Viably frozen naive puma and lion cells for co-cultivation were supplied by S.J.O. These samples were from seronegative pumas (Pco 187, 188, and 189) or a seronegative captive lion (KD 1724).

Tissue culture

FIV-Ple. RT-positive supernatant from seropositive lion PBMC (Brown *et al.*, 1994) was used to infect 2×10^6 of the following cells: feline lymphoma 3201 cells (Snyder *et al.*, 1978), feline adherent lymph node LNC cell line (Dow and Hoover, 1993), lion PBMC, and cat PBMC. PBMC were collected and isolated per standard protocols and either frozen until use or used immediately (Quackenbush *et al.*, 1990). Cells were propagated and passaged in 20% FCS in standard medium; PBMC were fed with conA- and IL2-supplemented medium throughout the experiment (O'Neil *et al.*, 1995). Supernatant was collected biweekly for 6 weeks for RT assay; cells were split as necessary. PBMC cultures were fed with 1×10^5 naive cells weekly.

FV-Pco. RT-positive supernatant from PLV-14-infected puma PBMC was used to infect 2×10^6 puma PBMC, cat PBMC, or 3201. PBMC from a seropositive cougar from British Columbia were isolated by standard methods, and 1×10^6 cells were co-cultivated in cell-associated viral infectivity studies. Cell isolation and culture methods were identical to those outlined above.

RT assay

Supernatants from cultures were collected biweekly for 3–7 weeks and frozen at −70°. After clarification by centrifugation, a microvolume magnesium-dependent ^{32}P RT assay was performed on samples (Goldstein *et al.*, 1990; Willey *et al.*, 1988). Incorporated label was bound to DEAE-81 paper (Whatman Labsales, Hillsboro, OR) and assayed by autoradiography or by analysis in a beta counter.

Antigen capture ELISA

Supernatants containing FIV-Pco or FIV-Ple with high levels of RT activity were subjected to an FIV-Fca p24 monoclonal antigen capture ELISA as previously described (Dreitz *et al.*, 1995). Supernatant was also tested using a commercially available FIV-Fca antigen detection kit per the manufacturer's directions (Feline Immunodeficiency Virus Antigen Test Kit, IDEXX, Portland, ME).

RT/PCR

RNA was isolated from 200 μl of FIV-Ple or FIV-Pco RT-positive supernatant using a commercially available kit (Qiamp Blood Kit, Qiagen, Inc., Chatsworth, CA). After ethanol precipitation, first-strand cDNA synthesis utilizing random primers (cDNA Cycle Kit, Invitrogen, San Diego, CA) was performed. One-fourth to one-half of the product generated was used immediately in a nonstringent PCR using consensus sequence primers for a conserved region of the *pol* gene as follows (Brown *et al.*, 1994; Olmsted *et al.*, 1992). The FIV-Pco primers were 1258F (2430 bp) 5′GAAGCATTAACAGAAATAGTAG3′; 1260R (3007 bp) 5′GTTCTTGTTGTAATTTATCTTC3′; the FIV-Ple primers were 6635F (2511 bp) 5′CCTATATTTTGCATTAAAAAG3′; 6637R (2944 bp) 5′ACCCCATATGATATCATCC3′; and the FIV-Fca primers were 669F (2403 bp) 5′CAATGGCCATTAACAAATG3′, and 1217R (3118 bp) 5′CCTGCTAATTTTTGCAACTCATT3′. Hot-start PCR was utilized followed by 35 cycles of hybridization at 37°, extension at 72°, and denaturation at 94°. Each reaction contained 1.5 m*M* $MgCl_2$, 150 m*M* dNTPs, 8 U of AmpliTaq DNA Polymerase (Perkin–Elmer Corp., Norwalk, CT) and each primer at 1 m*M.* Products were

visualized on a 1% agarose gel using Tris–borate–EDTA buffer and stained in ethidium bromide.

Western blot

Thirty milliliters of RT-positive supernatant was clarified and then ultracentrifuged at 27,000 rpm for 2 hr. The pellet was resuspended in Tris–EDTA–sodium chloride buffer and protein concentration was determined using a commercial kit (Bio-Rad Protein Assay, Bio-Rad Laboratories, Hercules, CA). Western blot assay was performed essentially as previously described (Diehl *et al.,* 1996). Twenty milligrams of protein was run on a 12% polyacrylamide gel and transferred to nitrocellulose using standard conditions. After blocking overnight, each filter was cut into approximately 30 strips. Then 1:100 dilutions of serum from five cats infected with FIV-Fca (two with FIV-B, two with FIV-C, and one with FIV-A) were bound to nitrocellulose. Strips were washed and labeled with goat anti-cat HRP conjugated antibodies.

Genetic analysis

A conserved region of the pol gene from a British Columbian isolate of FIV-Pco and from a Serengeti isolate of FIV-Ple was amplified as described above, though primer 1261R (2990 bp, 5′ATCTTCAGGAGTTTCAAATCCCCA3′) replaced primer 1260R for FIV-Pco amplification (Olmsted *et al.,* 1992). Newly synthesized products with 3′-deoxyadenosine overhang nucleotides were ligated into the vector PCRII, which contained 3′-deoxythymidine sticky ends (Original TA Cloning Kit, Invitrogen). Ligated product was transformed and plated on selective plates. Ten colonies were amplified in minipreps; inserts of appropriate size were detected by gel analysis of *Nsi*I-digested plasmids and blotting using a PLV-14 end-labeled oligonucleotide as described (Sambrook *et al.,* 1989). Sequence analysis was performed on an ABI 373A automated sequencer (Applied Biosystems Inc., Foster City, CA). Phylogenetic analysis was performed using three major algorithms: minimum evolution estimated by neighbor-joining (Saitou and Nei, 1987), maximum parsimony (Swofford, 1993), and maximum likelihood (Felsenstein, 1981). Each of these employs differing optimality criteria, but concordance among the resultant trees is interpreted as support for the true phylogeny. A bootstrap analysis (Felsenstein, 1985) was used in conjunction with neighbor-joining and maximum parsimony. This resampling analysis consisted of 100 iterations and yields proportions of the bootstrapped trees that support each node within the topology. Both neighbor-joining and maximum likelihood analysis used the computer program PHYLIP, Version 3.5 (Felsenstein, 1993) and maximum parsimony utilized the program PAUP, version 3.1.1.

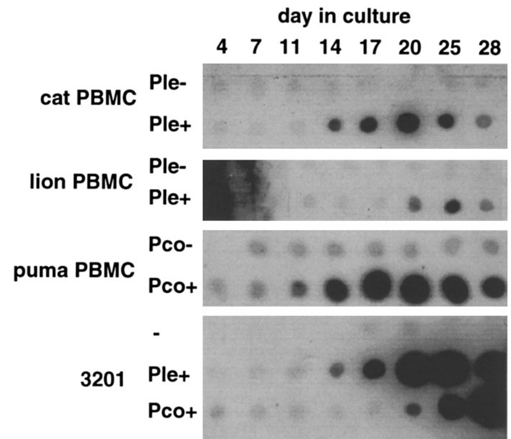

FIG. 1. Magnesium-dependent RT activity in FIV-Ple- and FIV-Pco-infected PBMC and 3201 cells. Ple, lion lentivirus; Pco, puma lentivirus; 3201, feline lymphoma 3201 cells. Cell-free FIV-Pco and cell-associated FIV-Pco were inoculated into cat and puma PBMC and 3201 cells and cultured as described in the text. Supernatant was collected twice a week, clarified, and tested for RT activity. Radiographic exposure denotes positive RT activity.

RESULTS

Table 1 summarizes results of cell culture inoculations with lion and puma lentivirus. Cell-free FIV-Ple was able to infect both naive cat and lion PBMC and 3201 cells. FIV-Ple-infected 3201 cells became lytic at Week 3 or 4 and could not be established as a persistently infected

TABLE 1

In Vitro Growth of FIV-Ple and FIV-Pco[a]

Inoculum	Cat PBMC	Lion PBMC	Puma PBMC	3201	LNC
Cell-free FIV-Ple	++	+	nd	++++[b]	—
Cell-free FIV-Pco	–	nd	–	++++	nd
Cell-associated FIV-Pco	+/–	nd	++	++++	nd

[a] 3201, domestic cat lymphoma cell line; LNC, domestic cat lymph node cell line. (–) <2× background; (+/–) 2–3× background; (+) 3–12× background; (++) 12–60× background; (++++) >100× background. Assessments were made on the basis of three or more consecutive comparisons between inoculated cultures and control RT values. nd, not done.

[b] Lytic infection.

TABLE 2

RT-PCR Amplification Products from Cell Culture Supernatants[a]

Primer	Substrate: FIV	FIV-Ple	FIV-Pco
FIV	715	—	580
FIV-Ple	—	420	—
FIV-Pco	715	—	580

Note. Numbers shown are base pair size estimated by agarose gel electrophoresis; fragment sizes are consistent with expected results.

[a] FIV was propagated in Crandell feline kidney cell culture as described under Materials and Methods. FIV-Ple and FIV-Pco RNAs were isolated from supernatant of infected 3201 cells.

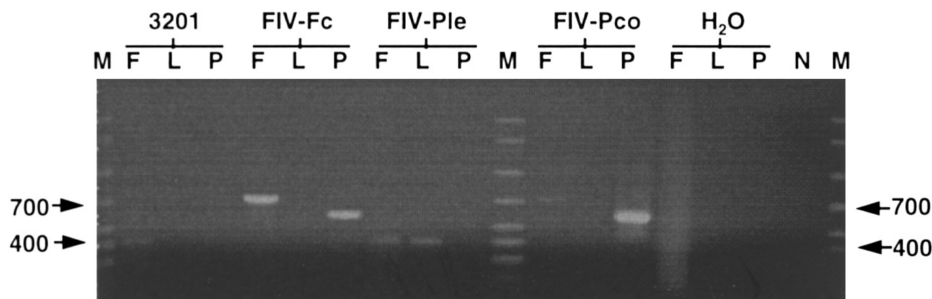

FIG. 2. Feline lentivirus RT-PCR virus-primer specificity. cDNA substrate is indicated in the top row: 3201, uninfected feline lymphoma cell line supernatant cDNA; FIV-Fc, cell free virus cDNA isolated from FIV-Fca persistently infected CrFK cells; FIV-Ple, FIV-Pco, cell-free virus cDNA isolated from 3201-infected cells; H_2O, no cDNA added to reaction. Bottom row indicates primers used in each reaction: M, marker ladder; F, FIV primers; L, FIV-Ple primers; P, FIV-Pco primers; N, no primers added, no cDNA substrate. Primer sequences were based on consensus regions of the *pol* gene for each virus and are described in the text along with methodology. Arrows indicate DNA ladder fragments (in bp).

line. Cell-free PLV-14 could infect only 3201 cells. However, cell-associated British Columbia cougar FIV-Pco could infect puma cells by co-cultivation. Domestic cat PBMC had a low transient RT signal (approximately 1.5× background) after co-cultivation with FIV-Pco PBMC. Figure 1 demonstrates RT activity of FIV-Ple or FIV-Pco in PBMC and 3201.

Supernatant from highly RT-positive 3201 cultures (FIV-Pco or FIV-Ple) was tested several times in two FIV p24-monoclonal antigen capture ELISA assays. Absorbence was below or equivalent to background for samples with RT activity less than 250 times background. Even in extremely concentrated samples (RT activity 250–2000 times background), absorbence was only approximately 30% of the FIV control sample and was not reproducibly detected (data not shown).

Results of RT-PCR are summarized in Table 2 and Fig. 2. FIV-Pco and FIV-Fca *pol* primers could amplify both FIV-Pco and FIV-Fca cDNA. FIV-Ple primers amplified FIV-Ple cDNA only. This establishes that nondomestic cat lentiviruses 3201 infections detected by RT assay can be further characterized by use of nonstringent PCR. A high degree of divergence appears to exist between FIV-Ple and either FIV-Pco or FIV-Fca as evidenced by the inability of the FIV-Ple primers to amplify the other viruses and vice versa.

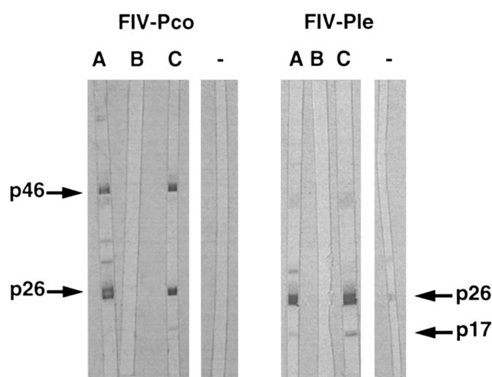

FIG. 3. Recognition of FIV-Pco and FIV-Ple by FIV antisera. (Left) British Columbia cougar FIV-Pco crude proteins were run on a 12% polyacrylamide gel and transferred to nitrocellulose. Then 1:100 dilutions of sera from naive cats (−) or cats chronically infected with FIV-Fca subtype A, B, or C were hybridized to filters; antigen–antibody complexes were visualized as described in the text. (Right) Methodology identical to that described above, but FIV-Ple proteins were bound to nitrocellulose.

Domestic cat FIV-A and FIV-C antisera recognized both FIV-Pco and FIV-Ple antigens, whereas FIV-B antisera did not. FIV-Pco Gag p46 and p26 and FIV-Ple Gag p26 and p17 were recognized by both FIV-A and FIV-C antibodies (Fig. 3). All three cats were seropositive by FIV-Fca ELISA (data not shown). Thus, FIV-Pco and FIV-Ple were antigenically related to FIV-Fca clades to differing degrees.

Pol gene fragments of 394 and 576 base pairs were sequenced from FIV-Ple and FIV-Pco, respectively (Fig. 4). All three phylogenetic methods corroborate the placement of the puma viral sequence (C1) with the sequences taken from pumas from British Columbia. Similarly the L5 sequence (from Ple 458) clusters with high bootstrap support with a strain representing the B clade of FIV from Serengeti lions.

DISCUSSION

These data demonstrate that nondomestic cat lentiviruses are capable of replicating in domestic feline origin cells. The strain of FIV-Ple that was used in these studies was able to replicate in domestic cat PBMC as well as a domestic cat T lymphoma cell line (3201) despite the genetic divergence between FIV-Fca and FIV-Ple demonstrated by RT-PCR and sequence data. Such *in vitro* growth has not been demonstrated previously and suggests that although FIV-Ple is divergent from FIV the viruses are not completely host-specific. Cell-free FIV-Pco established infection only in 3201 and could not infect puma or cat PBMC. In contrast, cell-associated virus isolated from British Columbia cougars could infect puma PBMC as well as 3201. We were not able to demonstrate convincing growth of FIV-Pco in domestic cat PBMC. This is similar to the findings of Olmsted *et al.* (1992).

An FIV p24 Gag-based antigen capture ELISA was not sufficiently sensitive to detect the nondomestic cat

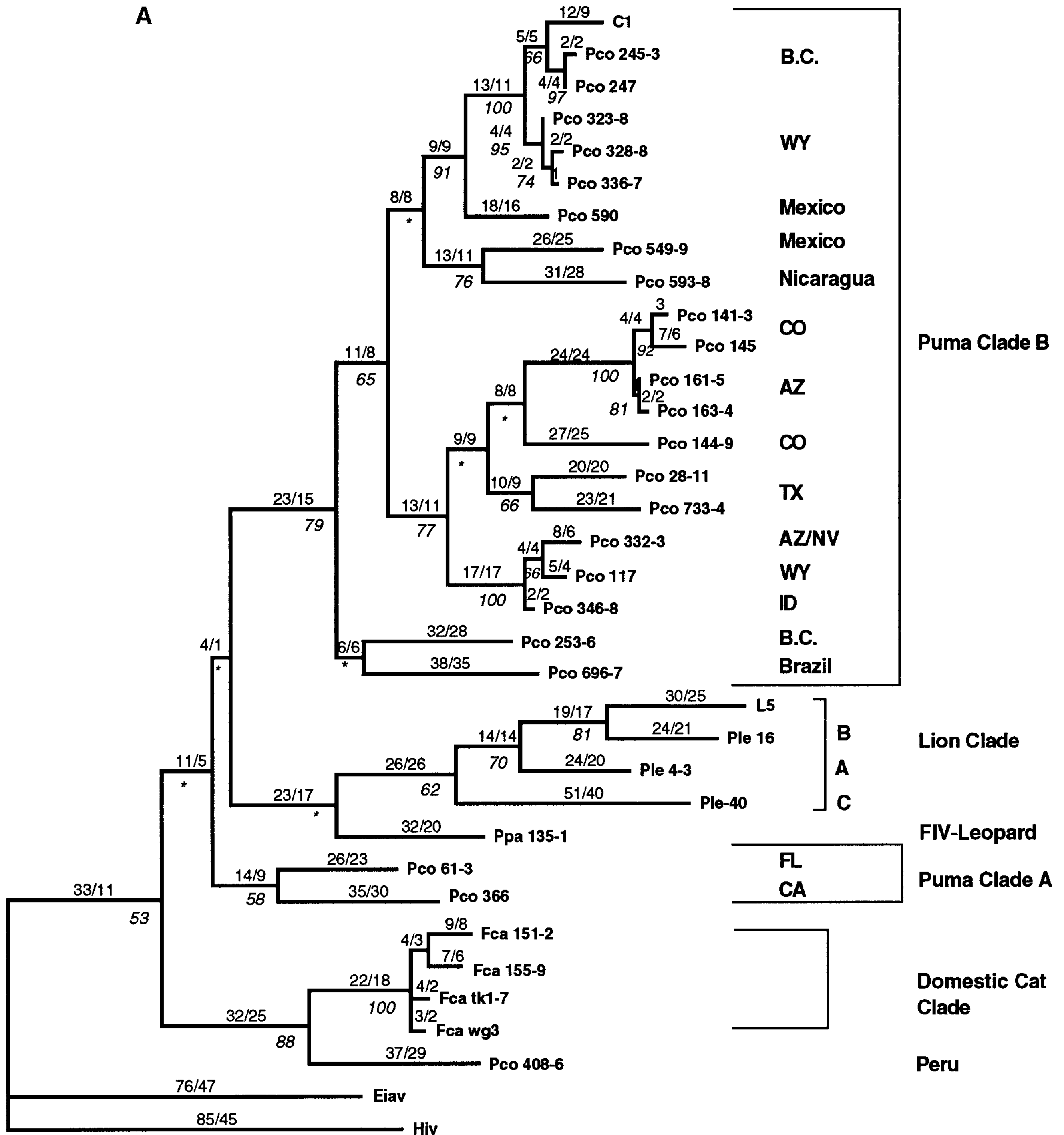

FIG. 4. Phylogenic trees of FIV-Ple 458 isolate (L5) and British Columbia puma isolate (C1) with known FIV strains from domestic cat, leopard, lion, and puma. These isolates were used for tissue culture propagation, RT-PCR, analysis, ELISA, and Western blot studies described in this report. Clade terminology corresponds to that proposed by Carpenter *et al.*, (1996) and Brown *et al.*, (1994). (A) Phylogeny derived from maximum parsimony analysis. Numbers on branches correspond to the number of steps/number of homoplasies. Values in italics are bootstrap proportions in support of the adjacent node based on 100 iterations. (B) Phylogeny based upon minimum evolution estimated by neighbor-joining. Branch lengths estimate the percentage of sequence divergence. Numbers in italics represent bootstrap proportions in support of the adjacent node. (C) Phylogeny derived from maximum likelihood analysis. Numbers on branches represent estimates of the percentage of sequence divergence.

lentiviruses, but a microtiter RT assay was quite efficient for detection. RT-PCR using different primer pairs illustrated that although FIV-Fca and FIV-Pco primers could amplify both FIV-Fca and FIV-Pco, FIV-Ple could be detected only using FIV-Ple-specific primers even under conditions permissive for hybridization of nonhomologous sequences. FIV-Pco amplification with either FIV-Fca or FIV-Ple primers was approximately equivalent.

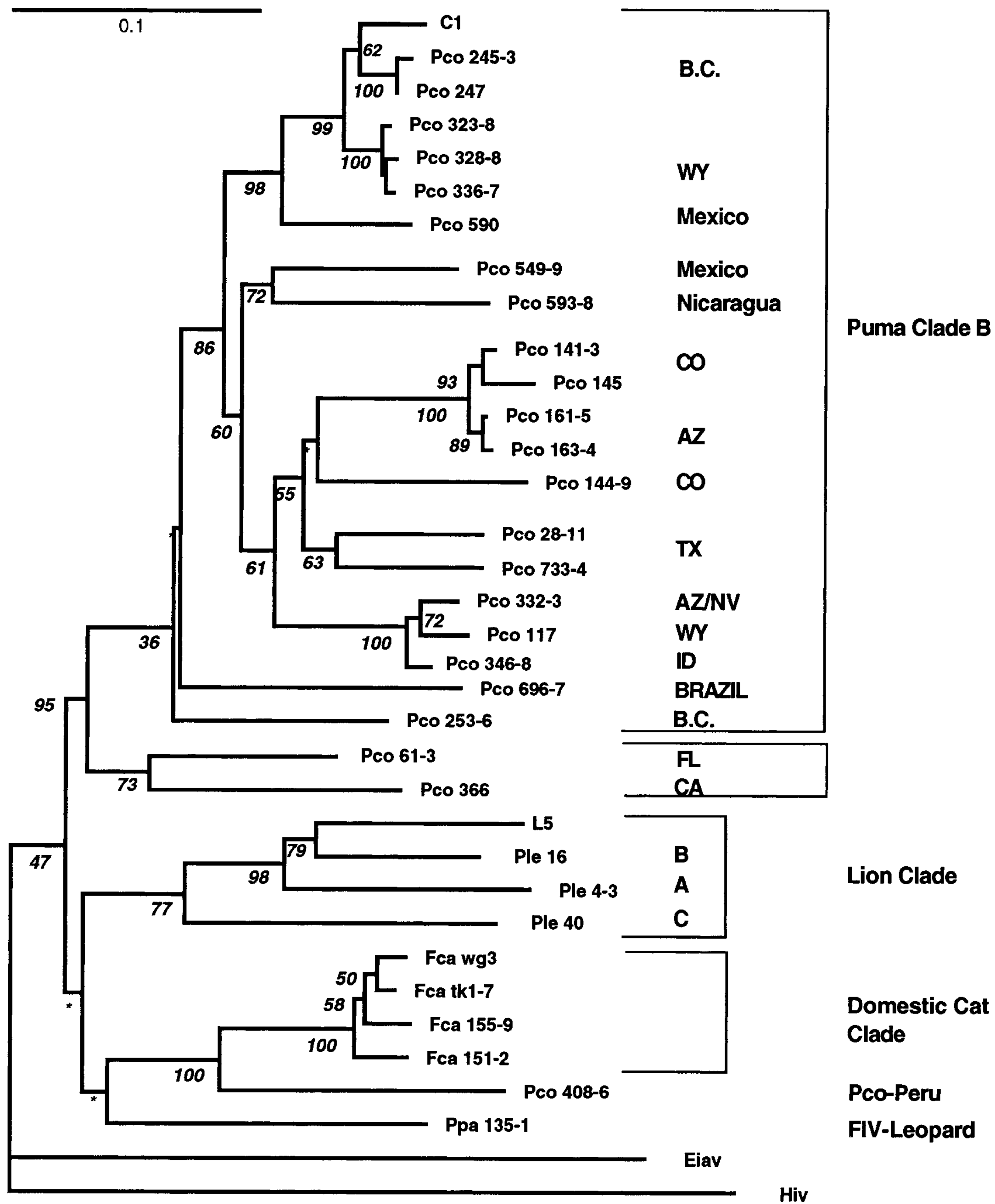

FIG. 4—*Continued*

This methodology illustrates a potential amplification/detection technique which could be used to characterize previously unamplified nondomestic cat lentiviruses.

Sequence analysis confirmed that the uncharacterized nondomestic cat lentiviruses used for these studies fell within the expected lentiviral classifications. The FIV-Ple strain used was classified as a B clade virus. This is thought to be the ancestral clade among three defined FIV-Ple groups and has been identified in other Serengeti FIV-Ple isolates as well as FIV-Ple from lions inhabiting Ngorongoro Crater of Tanzania (Brown *et al.,* 1994). Though the DNA sequence homology between this isolate (FIV-Ple 458) and analogous *pol* regions of FIV-Fca was only 70–73%, it grew readily in cat-origin cells *in vitro.* Examination of other regions of this FIV-Ple genome may help explain its tropism for domestic cat cells. Serologic data have suggested that FIV-Pco originating from British Columbia, Canada, might be more highly related to FIV-Fca from cats in that area than to other FIVs (Langelier *et al.,* 1995). Analysis of the highly conserved *pol* region of one such puma virus isolate clearly showed it to be more related to FIV-Pco than to FIV-Fca. Further

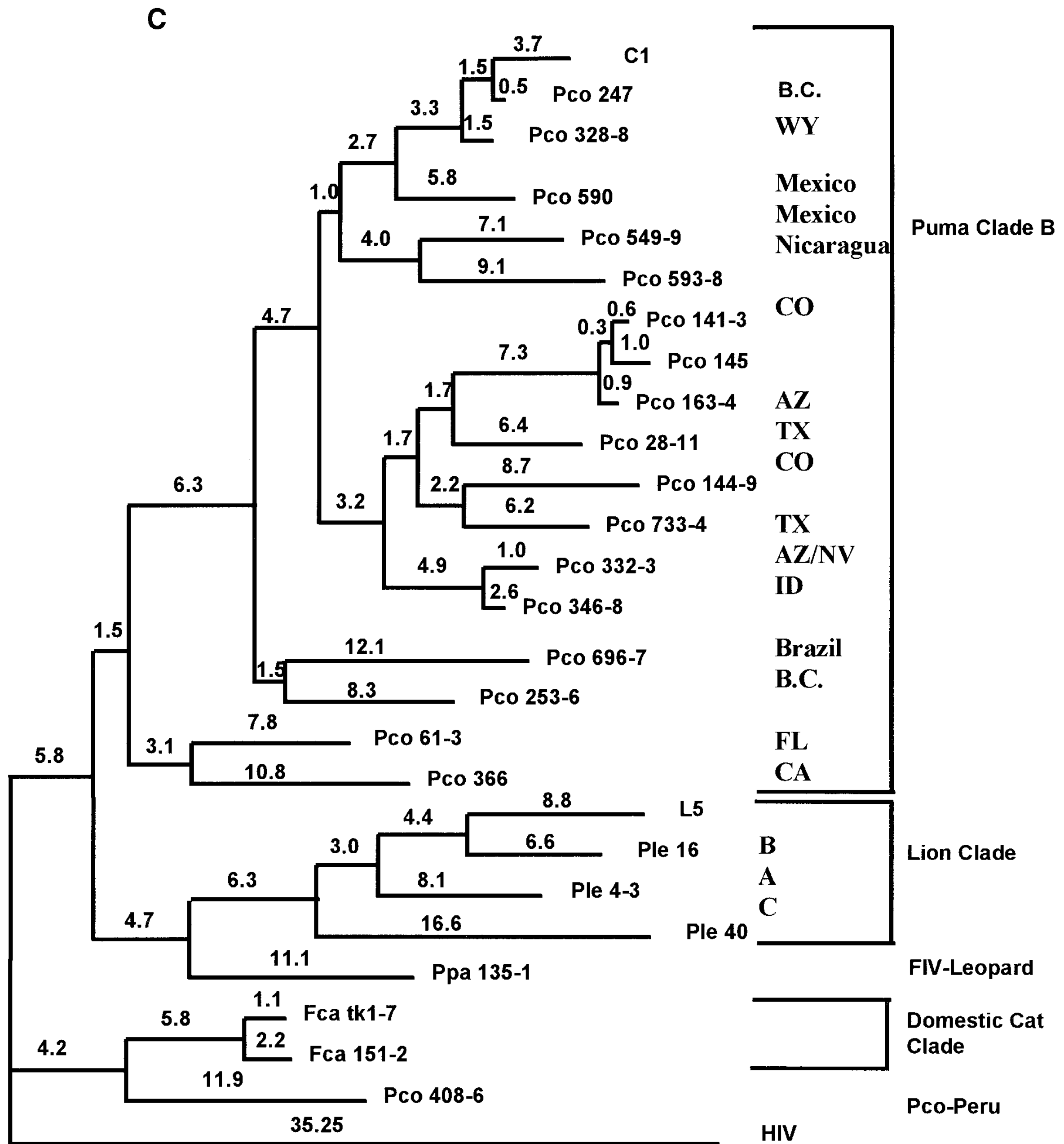

FIG. 4—*Continued*

genomic analysis of *gag* regions might reveal a basis for this pattern of seroreactivity.

Western blot analysis demonstrated that domestic cat FIV-Fca antibodies can cross-react with FIV-Ple and FIV-Pco antigens but have differential cross-reactivity with respect to subtype of FIV-Fca. Sequence analysis of one full-length FIV-Pco (Langley *et al.*, 1994) has shown that the *gag* region has 55% amino acid sequence homology with FIV-14, a subtype A virus. FIV-B is thought to be the oldest FIV-Fca subtype and may exhibit the highest degree of host adaptation (Sodora *et al.*, 1994). Since the FIV-Fca subtype is based on env V3 DNA sequence and since only a few sera were tested, it remains uncertain whether the trend in recognition pattern demonstrated in this study is representative of all seroreactivities between nondomestic cat lentiviruses and FIV-Fca.

Some conclusions which can be drawn from this study and the implications for diagnosis of FIV-Pco and FIV-Ple in animals are as follows: (1) FIV-Fca reagents may not provide sufficient sensitivity for detection of nondomestic cat lentiviruses, particularly when FIV-Fca monoclonal antibodies are being used to detect FIV-Pco or FIV-Ple viral determinants. (2) Antibodies from animals infected with FIV-Pco or FIV-Ple do not cross-react with all FIV-Fca subtypes. (3) Consensus nondomestic cat lentiviruses primers cannot be used to identify all nondomestic cat lentiviruses by PCR, even when permissive annealing conditions are used. (4) Culture amplification

followed by PCR is a more sensitive means of detecting nondomestic cat lentiviruses than PCR alone. (5) Nondomestic cat lentiviruses can replicate in domestic cat cells, suggesting the possibility of cross-species transmission.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the Colorado State University College of Veterinary Medicine and Biomedical Sciences. We thank Ms. Candace Mathiason-DuBard for advice during assay development.

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