

Proviral Organization and Sequence Analysis of Feline Immunodeficiency Virus Isolated from a Pallas' Cat

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The nucleotide sequence and genomic organization have been determined for a highly cytopathic feline immunodeficiency virus (FIV) isolated from a Pallas' cat. The 9747-bp provirus of this virus, FIV-Oma, has typical lentivirus organization with LTRs, *gag*, *pol*, and *env* open reading frames (ORFs), putative *vif* and *rev* ORFs, and an ORF similar to ORF2/ORFA of domestic cat FIV isolates. Although the FIV-Oma provirus is 300 to 600 bp longer than other FIV proviruses, these additional bases are distributed throughout the genome. Phylogenetic analysis of a conserved region of the *pol* gene suggests that FIV-Oma is more closely related to some of the puma and lion lentiviruses than it is to domestic cat FIV isolates; however, many regions of the genome exhibit extensive nucleotide sequence divergence. None of the eight molecular proviral clones isolated from a genomic library are infectious, but we have constructed an infectious, cytopathic clone of FIV-Oma from subcloned and PCR-amplified fragments of these proviral clones. This clone will be useful for identifying the genetic determinants of FIV-Oma's biological activities. © 1997 Academic Press

INTRODUCTION

Lentiviruses infect many species of domestic and non-domestic cats. Molecular clones of several domestic cat isolates of feline immunodeficiency virus (FIV) have been sequenced (Maki *et al.*, 1992; Miyazawa *et al.*, 1991; Olmsted *et al.*, 1989; Phillips *et al.*, 1990; Talbott *et al.*, 1989); however, the full sequence for only one nondomestic cat lentivirus (isolated from a puma) has been reported (Langley *et al.*, 1994). Nucleotide sequence data for short regions of the *pol* gene have been obtained for lentiviruses infecting additional pumas, several lions, and a Pallas' cat (Barr *et al.*, 1995; Brown *et al.*, 1994; Olmsted *et al.*, 1992). Based on these limited analyses, the various species of cats appear to be infected with their own unique lentiviruses. The degree of nucleotide sequence divergence between the lentiviruses infecting different species of cats is similar to that between human immunodeficiency virus (HIV) and simian immunodeficiency virus isolates (Olmsted *et al.*, 1992). Because FIV infection in cats is a laboratory model for HIV and human AIDS re-

search (Bennett and Hart, 1995; Siebelink *et al.*, 1990), study of the feline lentiviruses provides an opportunity not only for enhancing knowledge of the evolutionary history of lentiviruses, but also for increasing the understanding of the complex virus–host interactions in lentivirus infections which result in immunosuppression.

Infection of Crandell feline kidney (CrFK) cells with a lentivirus isolated from a Pallas' cat, FIV-Oma, results in extensive syncytium formation and lysis of the monolayer (Barr *et al.*, 1995). In contrast, domestic cat FIV isolates either establish noncytolytic infections in CrFK cells or fail to infect these cells (Yamamoto *et al.*, 1988). Although fusogenic activity in CrFK cells by the domestic cat FIV-Petaluma isolate when grown in low serum concentrations has been observed, extensive cell death does not occur (Tozzini *et al.*, 1992). A highly cytopathic Zairian strain of HIV-1, HIV-1 NDK, has biological properties in CEM cells (Hirsch *et al.*, 1992) which are similar to those of the FIV-Oma isolate in CrFK cells. The HIV-1 NDK determinants of cytotoxicity and fusogenic effect have been mapped to different regions of the genome, with its increased cytotoxic effect and rapid replication being associated with the terminal half of the *gag* gene and a large portion of the *pol* gene. The formation of large syncytia has been associated with a combination of the leader and 5' *gag* sequences and the HIV-1 NDK *env* gene (Hirsch *et al.*, 1992). The determinants of the biological activities of FIV-Oma may be similar, thus providing a useful model in which the cytopathic effects of lentiviruses in cultured cells can be analyzed and related to the pathogenic activity of these viruses in the host ani-

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mal. This report details the molecular analysis of the FIV-Oma proviral genome and the construction of an infectious, cytopathic proviral clone as the first steps in exploring the determinants responsible for FIV-Oma cytotoxicity, fusogenic activity, and replicative ability.

MATERIALS AND METHODS

Production and screening of the genomic DNA library

FIV-Oma was isolated in our laboratory as previously described (Barr *et al.*, 1995) and propagated in CrFK cells. Supernatants (1.0 ml/flask containing 10^7 cpm of reverse transcriptase activity) were inoculated onto 150-cm² flasks of CrFK cells at 50% confluency and cultured in the presence of complete CrFK growth medium (MEM with 20% L-15, 4 mM L-glutamine, 1% gentamicin, 5% fetal bovine serum). The CrFK cells were divided (1:5) once after 3 days of cultivation. The cells were monitored daily for syncytium formation. When large syncytia were noted, genomic DNA was isolated from the infected cells using standard lysis, digestion, and phenol:chloroform:isoamyl alcohol extraction procedures (Sambrook *et al.*, 1989). The DNA was precipitated with ethanol, resuspended in TE buffer containing 0.1% SDS and 25 μ g/ml RNase A, incubated 48 hr at 37°, and dialyzed for 24 hr against 50 mM Tris-HCl, 10 mM EDTA, pH 8.0. Following partial digestion with *Sau3A*, 10- to 20-kb fragments of DNA were selected by centrifugation on a 5 to 25% NaCl gradient. The fragments were ligated into λ EMBL3 arms (Stratagene) and packaged using Gigapack II (Stratagene) packaging extract. The extract was plated on P2392 *Escherichia coli*, and approximately 1.5×10^6 plaques were screened for FIV-Oma sequences using a ³²P-labeled FIV-Oma *pol* gene fragment (Barr *et al.*, 1995).

Restriction analysis of λ clones

Bacteriophage from positive plaques were purified and amplified according to the Gigapack II instructions, and bacteriophage preparations were banded on cesium chloride gradients (Sambrook *et al.*, 1989). Following dialysis (0.1 M Tris-HCl, pH 8.0, 0.05 M NaCl, 1 mM MgCl₂), DNA was prepared from the FIV-Oma-positive clones (λ 1–8) by proteinase K digestion (50 mM EDTA, pH 8.0, 0.5% SDS, 100 μ g/ml proteinase K), phenol:chloroform extraction, and ethanol precipitation. Restriction enzymes (*Bam*HI, *Eco*RI, *Hind*III, *Kpn*I, *Nhe*I, *Pst*I, *Sac*I, *Sal*I, and others) were used to digest the positive clones, fragments were separated by agarose gel electrophoresis, and Southern hybridization was performed using a ³²P-labeled FIV-Oma *pol* fragment or ³²P-labeled FIV-Oma viral cDNA as probes (Barr *et al.*, 1995).

Subcloning and sequencing of λ clones

Restriction enzyme fragments of FIV-Oma λ clones were purified from agarose gels using SpinBind DNA

recovery system (FMC Bioproducts) and ligated into pBlueScript II (SK⁻) phagemid cloning vector (Stratagene). Nucleotide sequencing was performed using standard primers to vector sequences and FIV-Oma-specific primers to obtain data for both strands of DNA. The sequencing was performed using automated DNA sequencing (Applied Biosystems, Inc., Model 373A) and conventional chain termination sequencing (Isotherm DNA sequencing kit; Epicentre Technologies). MacVector/AssemblyLIGN (International Biotechnologies, Inc., Eastman Kodak Co.), NCBI Blast sequence similarity search (GenBank), and MegAlign (DNASar, Inc.) programs were used for sequence analysis, assembly, and comparison. The nucleotide sequence for the FIV-Oma proviral genome was deposited into the GenBank sequence database under Accession No. U56928.

Cell transfections and infectivity assays

Crandell feline kidney cells were transfected with DNA (5 to 10 μ g/25-cm² flask) from λ or plasmid clones using a commercial calcium phosphate reagent (CellPfect transfection kit; Pharmacia). Supernatants from the transfected cells were harvested daily after transfection and were assayed for reverse transcriptase (RT) activity. Supernatant samples (10 μ l/reaction) were incubated at 37° for 1 hr in a solution containing 20 mM KCl, 50 mM Tris, pH 7.8, 20 mM MgCl₂ (or 0.6 mM MnCl₂), poly(rA):oligo(dT)₁₂₋₁₈ as template:primer, and [³H]TTP. The remainder of the assay followed the procedure of Heine *et al.* (1980).

Supernatants from transfected cells were inoculated onto uninfected CrFK cells and PBMCs, and the cultures were assayed for RT activity as described above. Cultures were examined daily for visible cytopathic effects.

Primer extension analysis

The 5' end of the FIV-Oma viral RNA was determined by primer extension analysis. Polyadenylated mRNA species were isolated from FIV-Oma-infected CrFK cells using the Oligotex Direct mRNA kit (Qiagen). Reverse transcription was performed with Superscript II reverse transcriptase (Gibco BRL) using a 5'-end-radiolabeled primer (5'-AGTCCCTGTTCGGGCGCCA-3') complementary to the primer binding site just downstream of the FIV-Oma LTR. The sequence of the resulting product was compared to that of the full FIV-Oma LTR to identify the transcriptional initiation site.

Phylogenetic analysis

Phylogenetic analysis was performed on nucleotide sequences from the *pol* gene of FIV-Oma and other feline lentiviruses. A region of the RT portion of *pol*, corresponding to bases 2690–3094 in the FIV-Oma proviral genome, was chosen for analysis because data from lion lentivi-

ruses (FIV-Ple) were available only for that region. The sequences were aligned using DNASTar MegAlign and analyzed using the principle of maximum parsimony with PAUP Version 3.1.1 (Swofford, 1990). The corresponding HIV-1 NDK sequence was included as the designated outgroup in the analysis.

Amplification of FIV-Oma sequences

Polymerase chain reaction was performed using commercially available reagents (GeneAmp; Perkin-Elmer Cetus) and standard techniques (Saiki *et al.*, 1988) on FIV-Oma λ 2. The following FIV-Oma-specific primers were used to amplify a 3.6-kb fragment from the 5' end: forward primer 5'-GCGGCCGCTGGGAGGATTGGAGGT-CCT-3', corresponding to bases 1–19 with an added 5' *NotI* site, and reverse primer 5'-GCTCTTAAGGCTATGTCGCA-3', corresponding to bases 3633–3614 of the FIV-Oma proviral genome. A 2.4-kb fragment was amplified from the 3' end of λ 2 using a forward primer 5'-TGCCAGTGTAGAGTCGGTAG-3', corresponding to bases 7182–7203, and a reverse primer 5'-GTCGACTGCTAAGGTCTCCGCCCCGAATC-3', corresponding to bases 9747–9725 of the FIV-Oma genome. Amplification conditions included a 2-min "heat shock" step at 95° prior to 30 cycles of amplification (94°—1 min, 55°—2 min, 72°—3 min) and a final cycle of 72°—10 min in a commercial thermocycler (Perkin-Elmer). The products were cloned into the pCRII vector (TA cloning kit; Invitrogen Corp.) and confirmed by nucleotide sequencing. These fragments were used to reconstruct a full-length infectious proviral clone (see Results).

RESULTS

Isolation of FIV-Oma proviral clones

Eight positive clones (λ 1–8) were isolated from a genomic library of FIV-Oma-infected CrFK cells using an FIV-Oma *pol* gene probe. The clones were analyzed by restriction enzyme digestion and Southern hybridization. Using the *pol* probe, we identified several internal fragments of the FIV-Oma provirus, including a 4.0-kb *SacI* fragment (Fig. 1a, lanes 1, 2, 4, 5, and 7) and a 7.0-kb *PstI* fragment (Fig. 1b, lanes 2, 5, and 7); however, these fragments were not present in several of the clones. The blots were also hybridized with 32 P-labeled FIV-Oma viral cDNA (data not shown) to detect additional viral fragments. Based on the degree of heterogeneity in restriction patterns of the clones, we suspected that several of the clones were less than full length or had aberrant integration patterns.

To determine if any of the proviral clones were infectious, each clone was transfected into CrFK cells. The transfected cells were observed for virion production by assaying for RT activity in the supernatant. Virus produc-

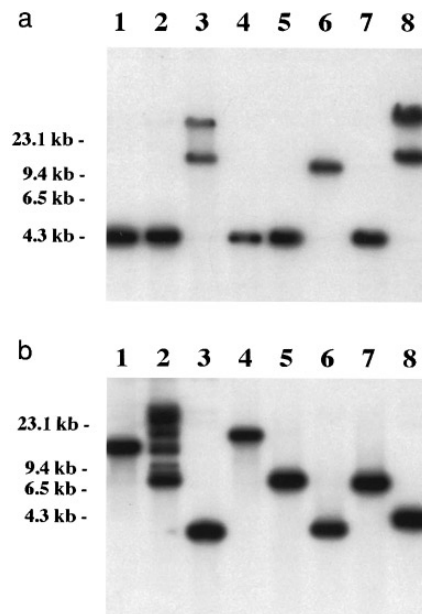


FIG. 1. Southern blots of FIV-Oma proviral clones in λ EMBL3 bacteriophage, digested with (a) *SacI* or (b) *PstI* restriction enzymes and hybridized with a 32 P-labeled *pol* gene fragment. Lane numbers correspond to clone numbers. Locations of molecular weight markers are shown to the left of the blots. Digestion of λ 2 with *PstI* was incomplete (b, lane 2).

tion was not evident in any of the transfected cells at any time for up to 5 weeks after transfection, indicating that none of the eight FIV-Oma λ clones were replication competent and infectious.

FIV-Oma provirus sequence and genomic organization

FIV-Oma λ 5 and λ 7, both of which had the internal *SacI* and *PstI* fragments, were chosen for further study. Nucleotide sequence analysis was performed on subclones of the internal 7-kb *PstI* fragment in pBluescript II (SK⁻) phagemid cloning vector using vector-specific and FIV-Oma-specific primers. Subclones of additional partially overlapping restriction fragments were then sequenced. Based on this analysis, proviral clone λ 7 was truncated at the 3' end, while proviral clone λ 5 was integrated aberrantly (3' *gag*, *pol*, *env*, partial 3' LTR, 5' LTR, 5' *gag*); therefore, data from both clones were required to determine the full-length FIV-Oma provirus sequence. Portions of FIV-Oma clone λ 2 were also sequenced when we determined that it was the only full-length proviral clone containing 5' and 3' long terminal repeats (LTRs) in the correct positions.

Potential regulatory and coding regions in the FIV-Oma provirus were identified by sequence analysis. The genomic organization of the FIV-Oma provirus is typical of other lentiviruses (Narayan and Clements, 1989), with LTRs, *gag*, *pol*, and *env* open reading frames (ORFs) and

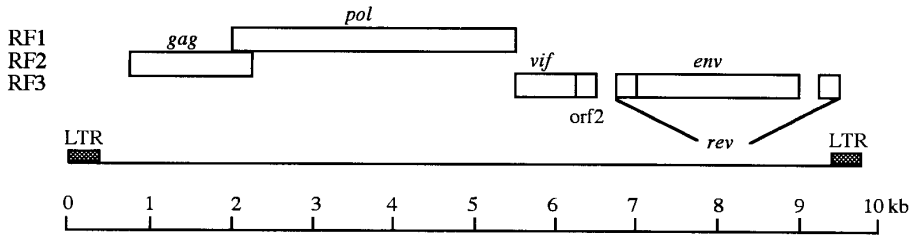


FIG. 2. Proviral genome organization of FIV-Oma. Open boxes represent predicted coding regions. RF, reading frame; LTR, long terminal repeat.

putative *vif*, *tat* (ORFA, ORF 2), and *rev* ORFs (Kiyomasu *et al.*, 1991; Olmsted *et al.*, 1989; Phillips *et al.*, 1990; Talbot *et al.*, 1989; Tomonaga *et al.*, 1992) (Fig. 2). Several additional small ORFs of questionable significance are also present (not shown). The LTR of FIV-Oma was aligned with those of other feline lentiviruses, and potential enhancer and promoter regions were identified (Fig. 3). The first 5 (5'-TGGGA-3') and last 3 (5'-GCA-3') nucleotides of all the feline lentivirus LTRs are conserved, as is the primer binding site located immediately downstream of the LTR. Potential enhancer-binding sequences identified in the FIV-Oma LTR include an AP-1 site, an AP-4 site, and two EBP20 sites, all located upstream of the TATA element. The G residue at position 224 was confirmed to be the 5' end of the viral message using primer-extension analysis of the viral RNA. The polyadenylation site (R/U5 junction) was identified by its position in relationship to the polyadenylation signal (5'-AATAAA-3') and by analogy with the other feline lentivi-

ruses (Langley *et al.*, 1994; Maki *et al.*, 1992; Olmsted *et al.*, 1989).

The Gag polyprotein is encoded by bases 680 to 2173 of the FIV-Oma genome and has a predicted molecular weight of 55.3 kDa. The *gag* gene encodes the matrix (16.7 kDa), capsid (27.3 kDa), and nucleocapsid (11.3 kDa) proteins, based on assignments by sequence analogy with FIV-Fca (Petaluma). The *gag* and *pol* ORFs overlap by 198 bases; two heptanucleotide frameshift signals (5'-GGGAAAG/C-3') are located 147 and 135 bases upstream of the 3' end of the overlap (Fig. 4). A potential pseudoknot structure, similar to that reported for FIV-Fca isolates (Morikawa and Bishop, 1992), is just downstream of the second signal. With -1 frameshifting during translation, a 180-kDa Gag/Pol fusion polyprotein would result. The *pol* gene encodes predicted protease (13.8 kDa), reverse transcriptase (64.7 kDa), dUTPase (14.2 kDa), and integrase (32.2 kDa) proteins, again determined by analogy with other feline lentiviruses.

	U3>	NF-κB	AP-4	
OMA	TGGGAGGATTG..GAGGTCCTAAAGACCCCTCAGATTGTGTATGCTCTTAAACAGAACATGTAAACCTAGGAAAATTA.AAA.ACAAAAATAGCAT			91
PET	TGGGATGAGTATTGGAACCCCTGAAGAAA.T.AGAAAGAA.TGCTTATGGACTGGGACTGTT.TACGAACAAATGATAAA.AGGAAATAGCTC			88
TM2	TGGGAAGATTATTGGGATCCTGAAGAAA.T.AGAGAAAA.TGCTGATGGACTGAGGGC.GCA.CATAACAAGTGACAGATGAAAA.CAGCTC			88
PLV	TGGGAGAG.ATT..AATTT...AGAGGCT...GAAAG.T...TTAGA...AGAACTAGATCTCATATAATTGTTGGCTGTAAGAAAG..A			73
	AP-1 AP-4	AP-4	EBP20 X 2	ATF AP-1
OMA	GTTAAGAAACAGCTGTGTAACCGCAAGCC...TTAACCACAA.CCATATCCGTGCTAAAGTGACGCTTGTCTAGCTAGTATGACTCTTTT			177
PET	AGCAAGACTCAATAGTTAAAGCGCTAGCAGCT.GCTTAACCGCAAAACCATCTCTATGAAA...GCTTGCTATGACGATATAAGTTGTTTC			175
TM2	AATATGAGTCAAGTAAA.TGCTAGCAGCT.GCTTAACCGCAAAACCATCTCTATGAAA...GCTTGCCGATGACGATGATCTTGCTC			174
PLV	AATATAGCTTTAAGAGTGA.CGCAAGTAACCTAGCTTAACCGCAAA.CCGCA...GATAAA...ACCC...ACATCCATATAGAAATGAC			150
	TATAA	U3<>R		
OMA	AAGTTTCCAGTAGAATAGTATATATAGAGAAACCTTTAGTCTGTTCAGGGCCACTTCTTTGGACTTGCAACTAGCTTGTCTAGGGGCTTGCTCCT			270
PET	CATTGTAAGAG.....TATATAA.CCAGTGCTTTGTGAACTTCGAGGAGTCTCTTTGTTGAGGACTTTTGAGTTCTCCCTTGAGGCTCCC			260
TM2	CATTGTAAGAG.....TATATAA.CCAGTGTTTTTTAAAGCTTCGAGGAGTCTCTCTGTTGAGGCTTTTCGAGTTCTCCCTTGAGGCTCCC			259
PLV	AGTAAGAACC.....TTTTAAAGCCTGCAACTTAG.TCTGTAAGGGG.....TCTGTCTCTTAGAGCAGAT			211
	Poly A	R<>U5		
OMA	CTGAAGGGTCCCTCAGGCACAAATAAATTGCTCGTGAGATTT...GAACCTGCCGTGTGTCTGAG...TCTTTTCTTTCTGTGAGGCTCCGGA			356
PET	AC.....AGATACAATAAATATTT...GAGATT...GAACCTGTGAGATCTGTGTAATCTTTT.TTACCTGTGAGGCTCCGGA			334
TM2	AC.....AGATACAATAAATAATTGAGTTTTGAGATTGAACCTGTGCTGTATCTGTGTAATTTCT.CTTACCTGCGAATCCCTGGA			340
PLV	CC.....TCAGGTATGCTTTAATAAAGAGTCTTGAGAGTGAACCTTGGTGGCTACCTGAG...TTTTA.TGTGGGGTTCTGTTGAGG			290
	<U5			
OMA	TTCGGACG.GAG.ACCTTGCA			375
PET	ATCCGGCCGAGAACTTCGCA			355
TM2	GTCCGGCCAGGACCTCGCA			361
PLV	GTCCGGCCAGAACTCTGCA			311

FIG. 3. Alignment of the long terminal repeat regions of feline lentiviruses. Conserved bases at the 5' and 3' ends are boxed, as are the sequences of several enhancer/promoter regions including NF-κB, AP-1, AP-4, EBP20, ATF sites, and the TATA element. Poly A, polyadenylation signal. Vertical lines designate the transcriptional start sites. OMA, FIV-Oma; PET, FIV-Fca (Petaluma isolate, U.S.A.); TM2, FIV-Fca (TM2 isolate, Japan); PLV, FIV-Fco (puma isolate, PLV-14).

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                1920                      1940                      1960
UCU UUG AAA UGU UUC AAU UGU GGA AAA CCA GGA CAU UUA GCA AGG AAU UGU AGA GCA CCU AGA AAA
Ser Leu Lys Cys Phe Asn Cys Gly Lys Pro Gly His Leu Ala Arg Asn Cys Arg Ala Pro Arg Lys>

                1980                      2000                      2020
UGU AAU AAA UGU GGC AAA GCA GGC CAU AUU GCA ACA GAU UGU UGG GAC AUG CAG GGA AAG CAG CAG
                                     frameshift
Cys Asn Lys Cys Gly Lys Ala Gly His Ile Ala Thr Asp Cys Trp Asp Met Gln Gly Lys Gln Gln>
POL> Met Trp Gln Ser Arg Pro Tyr Cys Asn Arg Leu Leu Gly His Ala Gly Lys Ala Ala>

                2040                      2060                      2080
GGA AAC UGG CAG AAG GGG AGA GCU GCU GCC CCU AUC AAA CAA GUG CAG CAA UUU CAA ACA GCA GUA
frameshift                                potential pseudoknot
Gly Asn Trp Gln Lys Gly Arg Ala Ala Ala Pro Ile Lys Gln Val Gln Gln Phe Gln Thr Ala Val>
Gly Lys Leu Ala Glu Gly Glu Ser Cys Cys Pro Tyr Gln Thr Ser Ala Ala Ile Ser Asn Ser Ser>

2100                      2120                      2140                      2160
UCA ACA ACU CAG AAU CAG CAA CAA UGU CAA UUA AUA CAG CCU UCG GCU CCU CCA AUG GAG UCC CUU
Ser Thr Thr Gln Asn Gln Gln Gln Cys Gln Leu Ile Gln Pro Ser Ala Pro Pro Met Glu Ser Leu>
Ile Asn Asn Ser Glu Ser Ala Thr Met Ser Ile Asn Thr Ala Phe Gly Ser Ser Ser Asn Gly Val Pro>
                                     ↑PR

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FIG. 4. Nucleotide and predicted amino acid sequences of the overlapping region of the *gag* and *pol* genes of FIV-Oma. Two putative frameshift signals and a potential pseudoknot structure are underlined and identified. The predicted amino terminus of the protease (PR) protein is indicated by an arrow.

An ORF which overlaps the last base of the *pol* gene ORF and extends from base 5423 to base 6178 in the FIV-Oma proviral genome is similar in location and size to the *vif* gene shown to be essential for cell-free infectivity in domestic cat FIV isolates (Tomonaga *et al.*, 1992). An ORF corresponding to ORF2/ORFA in the domestic cat FIV isolates is located immediately downstream of *vif*; however, this ORF does not have a standard AUG initiation codon in the FIV-Oma clones. Several additional clones containing this ORF were generated by RT-PCR of FIV-Oma viral messenger RNAs from both CrFK and lymphocyte cultures; none of the clones had an AUG initiation codon.

The *env* ORF and the first exon of the putative *rev* gene apparently share an initiation codon beginning at base 6507 in the proviral sequence; the second *rev* exon begins just downstream of the *env* gene termination (Fig. 2; based on findings of L. Zou, manuscript submitted). The coding sequence for the hydrophobic leader of the surface (SU) envelope protein is located about 1500 bases into the *env* ORF and almost 200 bases downstream of the predicted splice donor site of the first *rev* exon. The SU coding region contains 22 potential glycosylation sites, while the transmembrane protein coding region has 4 potential glycosylation sites and a 27-residue membrane-spanning hydrophobic region.

Relationship of FIV-Oma to other lentiviruses

The nucleic acid and amino acid similarities of the *gag*, *pol*, *env*, and *vif* gene regions of FIV-Oma with corresponding regions of FIV-Fca (Petaluma and TM2, domestic cat FIV isolates) and FIV-Fco (puma lentivirus) were

determined. Table 1 summarizes the comparative data for these feline lentiviruses. The nucleotide sequences of a highly conserved region of *pol* were used to construct an evolutionary tree of FIV-Oma and other feline lentiviruses using parsimony analysis (Swofford, 1990). Based on the single most parsimonious tree derived from a branch-and-bound search (Fig. 5), FIV-Oma appears to be most closely related to the puma lentiviruses and one clade of the lion lentiviruses. A very similar relationship is apparent when equine infectious anemia virus is designated as the outgroup (not shown).

Construction of an infectious clone of FIV-Oma

Because only $\lambda 2$ was full-length and it was not infectious, subclones of $\lambda 7$ and $\lambda 2$ were used to construct a

TABLE 1

Nucleic Acid and Amino Acid Sequence Comparisons of the *gag*, *pol*, *env*, and *vif* Genes of FIV-Oma with Those of Other Feline Lentiviruses

Virus	% similarity with FIV-Oma			
	<i>gag</i> (NA/AA)	<i>pol</i> (NA/AA)	<i>env</i> (NA/AA)	<i>vif</i> (NA/AA)
FIV-Fca (Petaluma)	66/64	72/75	44/24	59/54
FIV-Fca (TM2)	63/64	72/72	44/23	60/54
FIV-Fco (PLV-14)	62/55	60/60	53/43	50/36

Note. FIV-Fca, domestic cat FIV; FIV-Fco, puma FIV; NA, nucleic acid; AA, amino acid. Calculated using MegAlign (DNASStar, Inc.) sequence analysis software. Gaps in the sequences were given the weight of one residue, regardless of their length.

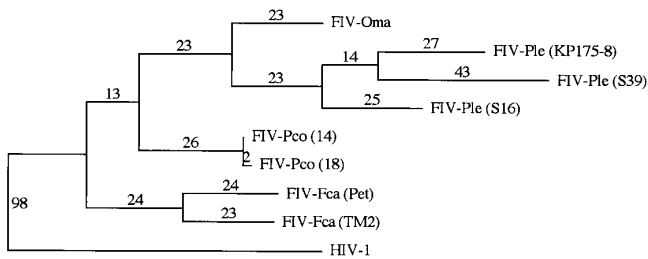


FIG. 5. Single most parsimonious phylogenetic tree for a conserved region of the *pol* gene of FIV-Oma and other feline lentiviruses, prepared using maximum parsimony (PAUP 3.1.1), with HIV-1 the designated outgroup. Tree length = 382; consistency index = 0.73. Branch lengths are representative of the number of nucleotide substitutions (numbers above the branches). Abbreviations: FIV-Oma, Pallas' cat FIV; FIV-Fca (Pet), Petaluma domestic cat FIV, U.S.A.; FIV-Fca (TM2), TM2 domestic cat FIV, Japan; FIV-Pco, puma FIV (number in parentheses represents the animal number); FIV-Ple, lion FIV (number in parentheses represents an animal from each of three clades); HIV-1, NDK strain of HIV-1.

full-length infectious proviral clone. The 7-kb internal *Pst*I subclone from λ 7 was flanked with PCR-amplified and restriction enzyme-digested 5' (*Afl*III) and 3' (*Eco*47III) ends of λ 2 to produce a full-length provirus, pFIV-Oma1, in pBluescript II. Following transfection of this clone into CrFK cells, a short period of particle-associated RT activity was noted; however, the virions produced did not infect either CrFK cells or feline PBMCs. Cotransfection of pFIV-Oma1 with a subclone containing the *env* gene from λ 5 resulted again in a short period of RT activity on Day 3 followed by a second period on Day 13 and exponentially increasing RT activity by Day 15, suggesting that the progeny virions were able to infect and spread in the transfected cell culture. Supernatants from the FIV-Oma1/ λ 5 *env* transfection contained virus which was infectious for new cultures of CrFK cells and feline PBMCs. Because this cotransfection was successful, we concluded that the *env* gene (or the overlapping first exon of *rev*) of FIV-Oma1 was the defective portion. Through a series of subcloning steps, an *Eco*47III/*Nde*I fragment of FIV-Oma1 containing the *env* orf was replaced with the same fragment from λ 5. This proviral clone, pFIV-Oma3, produced infectious virions following transfection into CrFK cells. Cytopathic effects of syncytium formation and vacuolization similar to those described for wild-type FIV-Oma (Barr *et al.*, 1995) were visible by Day 5, and most cells were lysed by Day 11 following transfection. Additionally, progeny virions were infectious and cytopathic for CrFK cells and primary feline PBMCs.

DISCUSSION

The organization of the FIV-Oma proviral genome is similar to that described for domestic cat and puma FIV isolates, although its length of 9747 bp makes it slightly larger than the 9100 to 9468 bp reported for other feline

lentiviruses (Maki *et al.*, 1992; Miyazawa *et al.*, 1991; Olmsted *et al.*, 1989; Phillips *et al.*, 1990; Talbott *et al.*, 1989). The additional sequences are scattered throughout the genome rather than concentrated in one region. Based on this preliminary analysis of the FIV-Oma genome, we anticipate that the differences in biological activity between FIV-Oma and domestic cat isolates are due to differing products or activities of similar genes rather than the presence of an additional gene. By constructing chimeric clones of the infectious, cytopathic molecular clone, pFIV-Oma3, and the minimally cytopathic domestic cat FIV clones, pFIV-PPR and pFIV-14, we will be able to identify the determinants of FIV fusogenic and cytolytic effects in CrFK cells.

Although all the feline lentiviruses examined thus far have several putative regulatory elements present in their LTRs, these motifs are not uniformly conserved. Sparger *et al.* (1992) have demonstrated a decrease in basal activity of FIV LTR constructs derived from FIV-pF34, a molecular clone of FIV-Fca (Petaluma), following mutation of the first AP-4 site, the AP-1 site, or the ATF site. However, the AP-1 site of some domestic cat isolates is not required for virus replication in feline lymphocytes or in cats (Miyazawa *et al.*, 1993; Bigornia *et al.*, 1996). The ATF site appears to play a more important role in replication of FIV-pPPR both in primary cultures of feline lymphocytes and macrophages and in cats (Bigornia *et al.*, 1996). The FIV-Oma LTR lacks the ATF site but has an AP-1 site located approximately the same distance from the TATA box position which may function in a similar manner.

In FIV-Oma, the small ORF which immediately follows the putative *vif* gene corresponds to ORF2/ORFA of other feline lentiviruses. This ORF is not required for replication of domestic cat isolates in CrFK cells and T lymphoblastoid cell lines, but appears to be more crucial for efficient replication in primary feline lymphocytes (Tomonaga *et al.*, 1993; Waters *et al.*, 1996). If this finding holds true for FIV-Oma, then the ORF may be expressed despite the lack of an AUG initiation codon because the virus replicates well in primary feline lymphocytes. We suspect that the CUG codon corresponding to proviral bases 6195–6197 may provide an alternative site for translational initiation, similar to the situation described for equine infectious anemia virus *tat* initiation in its bicistronic *tat/rev* gene (Carroll and Derse, 1993). This CUG codon is in a favorable context for translation and is located just downstream of the corresponding start site of ORF2 in domestic cat FIV isolates.

Analysis of the relationship of FIV-Oma to other lentiviruses adds a small piece of information to the puzzle of lentivirus evolution. In this analysis, FIV-Oma appears to represent a feline lentivirus of nondomestic cats which is almost equally diverged from its lion and puma counterparts. However, FIV from additional Pallas' cats must be isolated and examined to determine if FIV-Oma repre-

sents a unique Pallas' cat lentivirus. Unfortunately, the scarcity of these cats in captivity and the difficulty of sampling them in the wild may prevent a thorough analysis. Other investigators have hypothesized that feline lentiviruses have been infecting and evolving in the separate species of cats for many years (Langley *et al.*, 1994; Olmsted *et al.*, 1992). It is possible that the divergence of lentiviruses in cats parallels the divergence of the various species of cats themselves; alternatively, cross-species transmission may have occurred at various times during the evolutionary history of felids. In order to distinguish between these two scenarios, sequence data must be analyzed for FIV isolates from additional species of cats and from more cats within each species.

The isolation of predominantly noninfectious proviral clones from genomic libraries has been reported for visna virus (Andresson *et al.*, 1993; Staskus *et al.*, 1991). If, as in visna virus-infected cells, aberrant proviruses are the predominant species in FIV-Oma-infected CrFK cells, then the likelihood of isolating an infectious provirus from a single genomic library is diminished. Fortunately, our attempts to reconstruct an infectious, cytopathic clone proved to be successful. The phenotype of FIV-Oma3 in CrFK cells is very similar to that of wild-type FIV-Oma, thus we have constructed a fairly representative clone which will be very useful for investigation of the genetic determinants of host cell tropism, cytopathicity, and replicative ability of lentiviruses.

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