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.

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 non-domestic 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 research (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-
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 μg/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 × 10⁶ 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 (λ1-8) by proteinase K digestion (50 mM EDTA, pH 8.0, 0.5% SDS, 100 μg/ml proteinase K), phenol:chloroform extraction, and ethanol precipitation. Restriction enzymes (BamHI, EcoRI, HindIII, KpnI, NheI, PstI, SacI, Sall, 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 sequencing kit; Epicentre Technologies). MacVector/Assembly LIGN (International Biotechnologies, Inc., Eastman Kodak Co.), NCBI Blast sequence similarity search (GenBank), and MegAlign (DNASTar, 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 μg/25-cm² flask) from λ or plasmid clones using a commercial calcium phosphate reagent (CellPhase transfection kit; Pharmacia). Supernatants from the transfected cells were harvested daily after transfection and were assayed for reverse transcriptase (RT) activity. Supernatant samples (10 μl/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(ribo)nucleotide (poly(rA):poly(dT)ₙ₋₁₅) as template:primer, and [³H]dNTP. 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’-AGTCCCTGTTCCGGGCGCA-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 λ2. 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'-GCTCTTAAGGCTATG-TCGCA-3', corresponding to bases 3633–3614 of the FIV-Oma proviral genome. A 2.4-kb fragment was amplified from the 3' end of λ2 using a forward primer 5'-TGTCCAGTGTTAGAGTCGGTAG-3', corresponding to bases 7182–7203, and a reverse primer 5'-GTCGAC-GTCGACGGTCCGGGAGGCTATG-3', corresponding to bases 9747–9725 of the FIV-Oma genome. Amplification conditions included a 2-min “heat shock” step at 95°C prior to 30 cycles of amplification (94°C—1 min, 55°C—2 min, 72°C—3 min) and a final cycle of 72°C—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 (λ1–8) 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 Sacl fragment (Fig. 1a, lanes 1, 2, 4, 5, and 7) and a 7.0-kb Psfl 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 32P-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 production 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 λ5 and λ7, both of which had the internal Sacl and Psfl fragments, were chosen for further study. Nucleotide sequence analysis was performed on subclones of the internal 7-kb Psfl 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 λ7 was truncated at the 3' end, while proviral clone λ5 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 λ2 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
putative vif, tat (ORFA 2), and rev ORFs (Kiyomasu et al., 1991; Olmsted et al., 1989; Phillips et al., 1990; Talbott 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 lentiviruses (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.
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 λ2 was full-length and it was not infectious, subclones of λ7 and λ2 were used to construct a...

**TABLE 1**

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<th>Virus</th>
<th>% similarity with FIV-Oma</th>
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<tr>
<td></td>
<td>gag</td>
</tr>
<tr>
<td>FIV-Fca (Petaluma)</td>
<td>66/64</td>
</tr>
<tr>
<td>FIV-Fca (TM2)</td>
<td>63/64</td>
</tr>
<tr>
<td>FIV-Fco (PLV-14)</td>
<td>62/55</td>
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Note: FIV-Fca, domestic cat FIV; FIV-Fco, puma FIV; NA, nucleic acid; AA, amino acid. Calculated using MegAlign (DNASTar, Inc.) sequence analysis software. Gaps in the sequences were given the weight of one residue, regardless of their length.
full-length infectious proviral clone. The 7-kb internal PstI subclone from $\lambda^7$ was flanked with PCR-amplified and restriction enzyme-digested 5' (AflII) and 3' (Eco47III) ends of $\lambda^2$ 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 viros produced did not infect either CrFK cells or feline PBMCs. Cotransfection of pFIV-Oma1 with a subclone containing the env gene from $\lambda^5$ 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 viros were able to infect and spread in the transfected cell culture. Supernatants from the FIV-Oma1/$\lambda^5$ 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 Eco47IIId/NotI fragment of FIV-Oma1 containing the env orf was replaced with the same fragment from $\lambda^5$. This proviral clone, pFIV-Oma3, produced infectious viros 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 viros 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 lentviruses (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 cytopathic 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-PPR 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/ORF3 of other feline lentiviruses. This ORF is not required for replication of domestic cat isolates in CrFK cells and T lymphoblastic 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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