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Molecular Cloning of a Novel Isolate of Feline Immunodeficiency Virus Biologically and Genetically Different from the Original U.S. Isolate

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The Japanese isolate (TM1 strain) of feline immunodeficiency virus (FIV) which replicates in a feline CD4 (fCD4)-positive lymphoblastoid cell line (MYA-1 cells) was molecularly cloned from extrachromosomal closed circular DNA. The restriction map of the clone, termed pFTM 191 complete genome (CG), showed a considerable difference from that of the U.S. isolate (Petaluma strain) of FIV. The sequence homology in the long terminal repeat between the TM1 and Petaluma strain was 82%. The pFTM 191 CG was biologically active after transfection into Crandell feline kidney cells which were permissive for replication of FIV Petaluma. However, the progeny virions could not reinfect fCD4-negative Crandell feline kidney cells but could infect fCD4-positive MYA-1 cells. When a specific-pathogen-free cat was inoculated with the virus derived from the pFTM 191 CG, the cat seroconverted within 8 weeks postinoculation and FIV was reisolated at 4, 8, and 20 weeks postinoculation. These results indicate the infectivity of the pFTM 191 CG in vivo.

A retrovirus which could infect and kill feline T-lymphocytes was isolated from a cat with an immunodeficiency-like syndrome in the United States in 1986 (45). This virus has magnesium-dependent reverse transcriptase and is morphologically similar to the human immunodeficiency virus, the causative agent of AIDS in humans. Therefore, the feline retrovirus was classified as a member of the lentivirus subgroup and was designated feline immunodeficiency virus (FIV). Recently, additional isolates have been reported in both the United Kingdom (18) and Japan (26, 37). Seroepidemiological surveys were also conducted in the United Kingdom (16), the United States (54), Japan (14, 24, 25) Canada (54), and New Zealand (50). These reports reveal that FIV did not arise recently (e.g., as early as 1968 in Japan [14]) and has spread all over the world.

FIV Petaluma, the first isolate whose genome has been analyzed, infects FL74 and LSA-1 cells, which are T-lymphoblastoid cell lines productively infected with feline leukemia virus; primary feline T lymphocytes; and Crandell feline kidney (CRFK) cells (55). In addition, FIV Petaluma was molecularly cloned from the genome of CRFK cells persistently infected with the virus (43, 44, 51). On the other hand, the Japanese isolates (TM1 and TM2 strains) could not grow in CRFK cells (39). Most recently, the second U.S. isolate (PPR strain) of FIV was molecularly cloned from peripheral blood lymphocytes of a cat infected with the virus (46). The PPR strain could not productively infect CRFK cells either (46).

Recently, we established a feline T-lymphoblastoid cell line which is free of exogenous retroviruses. This cell line, designated MYA-1, is highly sensitive for replication of FIV (38). In the present study, we cloned FIV TM1 from infected MYA-1 cells and compared it genetically with FIV Petaluma and FIV PPR. Furthermore, we reconstituted the clone to proviral form and examined the infectivity of the clone in vitro and in vivo.

The TM1 strain (37) from MYA-1 cells and the Petaluma strain (55) from CRFK cells persistently infected with the respective viruses were used in this study. FIV Petaluma-infected CRFK cells (CRFK/Petaluma) and uninfected CRFK cells (7) were maintained as described previously (37). The MYA-1 cells were grown in RPMI 1640 growth medium as described before (38).

The MYA-1 cells which were persistently infected with the FIV TM1 (39) were cocultured with the uninfected MYA-1 cells at a ratio of 1:5. After coculturing for 72 h, the extrachromosomal DNA was extracted by the Hirt method (20). The DNA samples were treated with proteinase K (50 μg/ml) at 37°C for 2 h, extracted with phenol-chloroform-isoamyl alcohol (24:24:1), and precipitated with ethanol.

The extrachromosomal DNA of FIV TM1 digested with various restriction enzymes was hybridized with the DNA probe of FIV Petaluma (Fig. 1). In undigested Hirt supernatant (lane 1), one band of the linear form of DNA of about 9.0 kb and one band of the closed circular form of DNA of about 4.5 kb were observed (52). After digestion with *Bam*HI (lane 2) and *Sac*I (lane 5), only one intensive band of around 9.0 kb was detected. From these data, *Sac*I and *Bam*HI were considered to cut at only one site either within or near the long terminal repeat (LTR) of the closed circular FIV DNA. Therefore, *Sac*I-digested extrachromosomal DNA of about 9 kb was pooled and ligated into a λ ZAP II vector (Stratagene, San Diego, Calif.). The resulting library was plaque hybridized with an FIV TM2 strain *Eco*RI 6-kb fragment (35a) as a probe. Three signal-positive phages were cloned into pBluescript (Stratagene). The only pFTM191 clone obtained proved to be infectious (described below), and it was mapped by using nine restriction enzymes. Only 8 of 24

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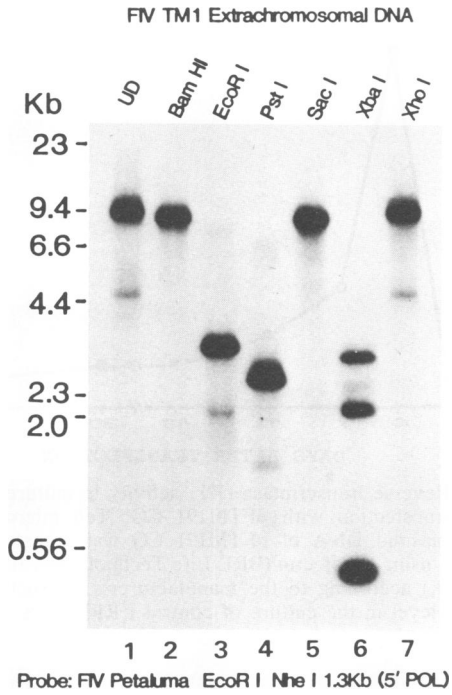

FIG. 1. Detection of unintegrated viral DNA in FIV TM1-infected cell line. Extrachromosomal DNAs of FIV TM1-infected MYA-1 cells were digested with the indicated restriction enzymes and hybridized with a ^{32}P-labeled *Eco*RI-*Nhe*I fragment (1.3 kbp containing 5′ *pol* gene) from p2 FIV Petaluma strain as described previously (43). Results for undigested Hirt supernatant (lane 1) and its digests with *Bam*HI (lane 2), *Eco*RI (lane 3), *Pst*I (lane 4), *Sac*I (lane 5), *Xba*I (lane 6), and *Xho*I (lane 7) are shown.

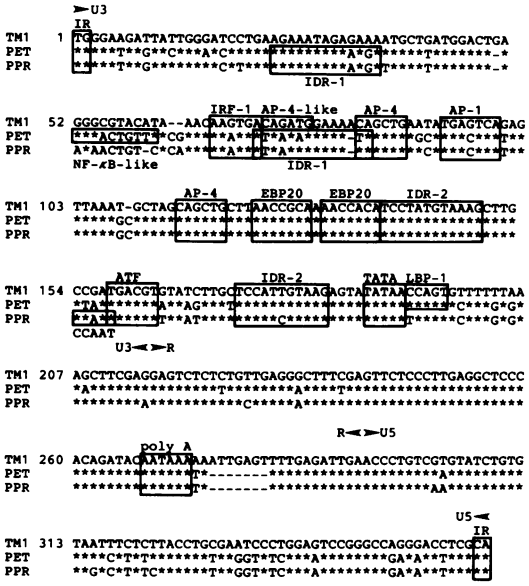

FIG. 3. Nucleotide sequence of the LTR of strain TM1 and comparison with that of strains Petaluma (51) and PPR (46). Only mismatched sequences with the TM1 strain in the Petaluma and PPR strains are indicated. Asterisks mark similar nucleotides; bars represent gaps introduced for optimized alignment. The beginning of the U3, R, and U5 regions are indicated above the sequences. Important structural features are boxed: the inverted repeats (IR), transcription initiation (TATA box), CCAAT box, two sets of imperfect direct repeats (IDR-1 and -2), poly(A) signal (AATAAA), LBP-1 binding site (CCAGT), and the recognition sequences of the enhancer proteins NF-κB-like (GGGACTGTT), IRF-1 (AA[A/G]TGA), AP-1 (TGA[G/C]TCA), AP-4 (CAG[C/A]TG), AP-4-like (CAGATG), EBP-20 (AACC[A/G]CA), and ATF (TGACGT).

restriction sites of TM1 were conserved when compared with the Petaluma strain (Fig. 2).

The LTR and envelope regions of the pFTM191 clone were sequenced and compared with the sequences of FIV Petaluma (51) and FIV PPR (46). The LTRs of the TM1 and Petaluma strains or of the TM1 and PPR strains had 82% nucleic acid identity (Fig. 3). The sequence homology of the

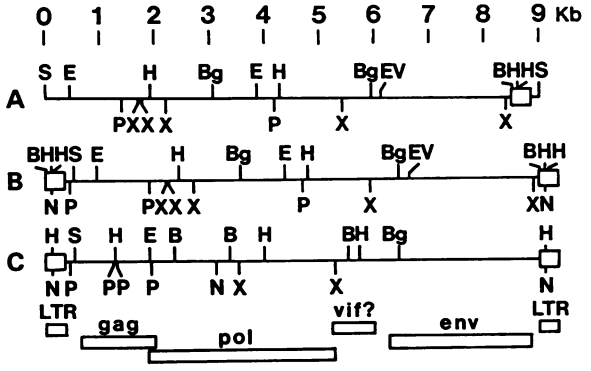

FIG. 2. Comparison of restriction maps of FIV TM1 and FIV Petaluma. (A) Restriction map of the permuted *Sac*I fragment of the pFTM191 clone. (B) Restriction map of a FIV TM1 provirus, deduced from the mapped *Sac*I fragment. (C) Restriction map of FIV Petaluma which was generated from the published nucleotide sequence (51). Abbreviations: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; N, *Nhe*I; P, *Pst*I; S, *Sac*I; X, *Xba*I.

envelope region between strains TM1 and Petaluma was 81% (79 and 85% in the external glycoprotein and transmembrane protein, respectively) at the amino acid level. The LTR of the TM1 strain consisted of 361 bp, and the lengths of its internal domains U3, R, and U5 were estimated to be 215, 78, and 68 bp, respectively. In the U3 region of the LTR, the TATA box (15, 43, 44, 46, 51), 2-bp inverted repeats (43, 44, 46, 51), one set of imperfect direct repeats (IDR-2) (46) and AP-1(TGA[G/C]TCA) (3, 4, 19, 32, 33, 46), IRF-1 (AA[G/A]TGA) (12), ATF (TGACGT) (17, 21, 35, 46), two sets of AP-4 (CAGCTG) (19, 36, 46) binding sites, and an inverse complement sequence (AACC[A/G]CA) of the EBP-20 (TG[T/C]GGTT) binding site (27) were perfectly conserved among the three strains. One additional AP-4-like binding site (CAGATG) was found in the TM1 strain upstream from the first AP-4 binding site. However, one set of IDR (IDR-1) was seen in both the Petaluma and PPR strains (46) but not in the TM1 strain. Further, the NF-κB enhancer element-like sequence (GGGACTGTT) (43, 44, 46, 51) and the CCAAT promoter sequence (9, 40, 46) were identified in the Petaluma and PPR strains, respectively, but these sequences were not found in the TM1 strain. In addition, a putative LBP-1 binding site (CCAGT) (28, 46) was found in TM1. In the R region, the poly(A) signal (AATAAA) (43, 44, 46, 47, 51) was conserved among three strains. Furthermore, although the sequence homology of the FIV TM1 strain with human or simian immunodeficiency virus in the R region cannot be detected, the RNA transcribed between nucleotides 222 and 264 had the potential to form a stem-loop structure similar to

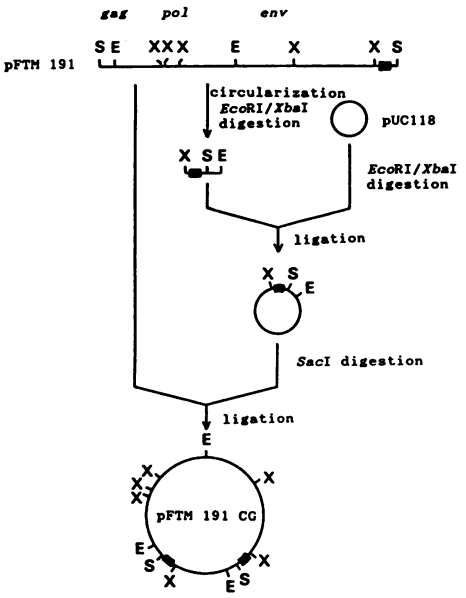

FIG. 4. Construction of a full-length molecular clone of FIV TM1 from pFTM191. Closed box represents the LTR region. The *Sac*I (S) fragment from the pFTM191 clone was circularized and digested with *Eco*RI (E) and *Xba*I (X). An approximately 0.6-kb fragment containing the LTR region was cloned into pUC118, followed by insertion of the *Sac*I fragment from pFTM191, and was designated as pFTM191 CG.

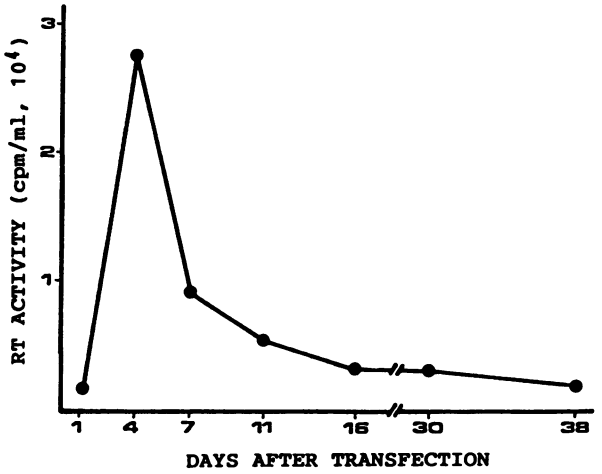

FIG. 5. Reverse transcriptase (RT) activity in culture supernatant after transfection with pFTM191 CG. Ten micrograms of uncleaved plasmid DNA of pFTM191 CG was transfected into CRFK cells, using Lipofectin (BRL Life Technologies, Inc., Gaithersburg, Md.) according to the manufacturer's instructions. The background level in the culture of control CRFK cells was 2,300 cpm/ml.

the *trans*-activation-responsive (*tar*) region which was seen in human (8, 11, 48) and simian (13, 53) immunodeficiency viruses. Outside the LTR, the polypurine tract (43, 44, 46, 51) immediately upstream of the LTR and the primer binding site (43, 44, 46, 51) were identified 3 bases downstream from the LTR.

To test the biological activity of the clones obtained, one full genomic clone from the cDNA library was reconstituted to produce a proviral form (one LTR on both sides of its genome) (Fig. 4) and designated as the pFTM191 complete genome (CG). The pFTM191 CG was transfected into CRFK cells and monitored by Mg^{2+}-dependent reverse transcriptase activity [RTA(Mg)] (42) and indirect immunofluorescence assay (37). The RTA(Mg) was detectable in the culture supernatant of the CRFK cells transfected with the pFTM191 CG (Fig. 5) and FIV antigen was detected in the CRFK cells by indirect immunofluorescence assay. When the culture supernatant from the transfected CRFK cells, 4 days posttransfection, was further transferred to MYA-1 and CRFK cells, RTA(Mg) was detected 7 days after infection in the culture supernatant of MYA-1 cells (2.6×10^5 cpm/ml), but not in that of CRFK cells. Further, FIV antigen was detected in the MYA-1 cells but not in CRFK cells. After several passages of the transfected CRFK cells, the RTA(Mg) in the culture supernatant became undetectable without showing any cytopathic effects.

FIV proteins in MYA-1 cells infected with the parental TM1 strain (MYA-1/TM1) or FIV from pFTM191 CG (MYA-1/191) and those in CRFK cells infected with FIV Petaluma were analyzed by immunoprecipitation followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with the plasma from a FIV-seropositive cat. Proteins with molecular weights (MWs) of 130,000, 24,000, and 17,000 were identified in MYA-1/TM1 and MYA-1/191 cells, while those with MWs of 170,000, 130,000, 100,000, 24,000, and 17,000 were identified in CRFK/Petaluma cells (Fig. 6). After ultracentrifugation, these proteins were similarly identified in the virus fractions from the culture fluids of cells infected with the three respective viruses. However, a very intense band of 100-kilodalton (100K) protein was identified only in the fluid fraction of CRFK/Petaluma cells.

To test the infectivity of pFTM191 CG in vivo, a specific-pathogen-free cat was inoculated with FIV derived from pFTM191 CG. FIV was isolated from the cat at 4, 8, and 20 weeks after inoculation. Seroconversion was observed within 8 weeks after inoculation when examined by immunoblot analysis. The seroconversion was also confirmed by enzyme-linked immunosorbent assay, using *gag* protein expressed in *Escherichia coli* (14a). However, the antibody responses against the FIV from pFTM191 CG were weaker than those against the parental TM1 strain (data not shown).

Genomic heterogeneity of human (1, 2, 6, 56) and simian (30, 34) immunodeficiency virus genomes has been reported. The restriction map of the TM1 strain was considerably different from that of the Petaluma strain. The percent homology between TM1 and Petaluma strains in the LTR and envelope region was 82 and 81%, respectively. These observations suggest that the genomic heterogeneity is also present among FIV strains, the extent of which is estimated to be about 20% at the nucleotide level. Further studies are required to obtain more information on the extent of the genomic heterogeneity among the different strains of FIV.

In the LTR region, we observed the NF-κB-like binding site only in the Petaluma strain. Whether the NF-κB-like binding site is functional is unknown; however, this difference might explain the lower cytotoxicity of the TM1 strain as compared with the Petaluma strain (29, 38, 39). On the other hand, the TM1, Petaluma, and PPR strains have potential AP-4 and AP-1 binding sites. These binding sites were also identified in the LTR of the visna virus (19) and caprine arthritis and encephalitis virus (19). In particular, these binding sites in the visna virus are significant in the basal activity of the LTR and in viral *trans*-activation (19).

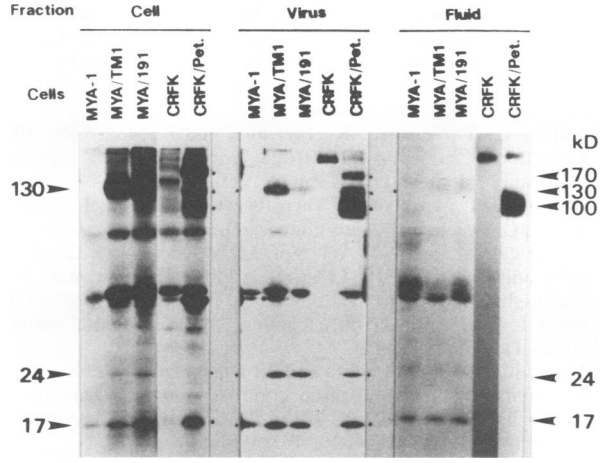

FIG. 6. Immunoprecipitation of the cell, virus, and fluid fractions of FIV-infected cell cultures. FIV-infected and uninfected cells were labeled for 16 h with 50 μCi of L-[^{35}S]cysteine (1,065 Ci/mmol; New England Nuclear, Boston, Mass.) per ml in RPMI 1640 medium containing one-tenth the normal concentration of cystine and 5% fetal calf serum. The cell fraction was prepared by low-speed centrifugation. The resulting supernatant was further centrifuged at 35,000 rpm for 1 h in an SW50.1 rotor to separate the fractions of the virus particles and the soluble proteins as described previously (22). The cell and virus lysates in lysis buffer (0.5% Nonidet P-40, sodium deoxycholate, 0.05 M Tris hydrochloride [pH 7.2], 0.1 M NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) were immunoprecipitated with the plasma of cat MM which was infected with FIV TM1 (37) (indirect immunofluorescence assay titer, 1:256) as described previously (23). Immune complexes were then precipitated with protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden) with constant shaking for 15 min. The FIV proteins in the immunoprecipitates were analyzed by SDS-PAGE (separation gel, 10 to 15% linear gradient polyacrylamide gel; stacking gel, 4% gel) by the method of Laemmli (31). The fluorogram was prepared as described previously (5). Abbreviations: MYA-1, uninfected MYA-1 cells; MYA/TM1, FIV TM1-infected MYA-1 cells; MYA/191, MYA-1 cells infected with FIV derived from pFTM191 CG; CRFK, uninfected CRFK cells; CRFK/Pet., FIV Petaluma-infected CRFK cells.

Therefore, the replication control strategy of FIV is considered to be similar to that of the visna and caprine arthritis and encephalitis viruses. The ATF, EBP-20, and IRF-1 (a consensus hexamer sequence seen in beta interferon gene enhancer) binding sites were conserved among the three FIV strains; therefore, these enhancer sequences were probably functional. Besides the binding sites mentioned above, the LBP-1 binding site and CCAAT promoter element were found in the U3 region of either one or two of the FIV strains compared in this study; however, their functions are unknown.

Previously, we reported that the FIV Petaluma but not FIV TM1 could infect CRFK cells (32). Although the pFTM191 CG obtained here could replicate in the CRFK cells after transfection, the virion derived from the pFTM191 CG could not reinfect CRFK cells but could infect MYA-1 cells. These data suggested that there is no postentry block to replication in the CRFK cells and the receptor for FIV TM1 is absent on CRFK but present on MYA-1 cells. Flow cytometric analysis revealed that the MYA-1 cells were positive for feline CD4 (fCD4) and negative for feline CD8 (fCD8) antigens and that the CRFK and FL74 cells were negative for both fCD4 and fCD8 antigens (39a). So far, the FIV TM1 strain could infect only fCD4-positive cells. Whether the target of the TM1 strain is restricted to the fCD4-positive cells and whether fCD4 is the receptor for the TM1 strain are now under investigation. The host cell ranges of the TM1 and PPR strains resemble each other; however, the TM1 strain seemed to be equally distant from both the PPR and Petaluma strains and the PPR strain is closer to the Petaluma strain than to the TM1 strain in terms of genomic homology. The differences in host cell range might be due to a slight alteration in the envelope region of the virus. Various types of FIVs which have different cell tropisms might be present in vivo.

Immunoprecipitation analysis revealed that the proteins of FIV derived from pFTM191 CG were indistinguishable from the parental isolate of FIV TM1. The MWs of the envelope protein of FIV Petaluma were reported to be 130,000, 110,000, and 40,000 by O'Connor et al. (41) or 130,000 and 40,000 by Steinman et al. (49) in the virus-infected CRFK cells; 140,000, 100,000, and 36,000 by Olmsted et al. (44) in the infectious clone-infected CRFK cells; and 160,000, 120,000, and 43,000 by Egberink et al. (10) in the virus-infected lymphocytes. The MWs of the envelope proteins reported by Olmsted et al. (44) are in good agreement with the calculated MWs of the sequence data of FIV Petaluma cloned from the virus-infected CRFK cells. The MWs of the external envelope protein of the TM1 and Petaluma strains used in this study were considered to be 130,000. The properties of the 100K protein of FIV Petaluma are unknown; however, the protein might be related to the 130K protein.

The specific-pathogen-free cat inoculated with the virus derived from pFTM191 CG seroconverted within 8 weeks postinfection, and the virus was reisolated at 4, 8, and 20 weeks postinfection. These data suggested that pFTM191 CG is infectious in vivo. However, the antibody response against the clone was relatively weak. Because the cloning source of the DNA of the TM1 was the MYA-1 cells persistently infected with FIV TM1, the pFTM191 CG clone might be of lower cytotoxicity.

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